Purification and characterization of echinoderm casein kinase II

Regulation by protein kinase C

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Casein kinase II (CKII) is one of several protein kinases that become activated before germinal-vesicle breakdown in maturing sea-star oocytes. Echinoderm CKII was purified over 11000-fold with a recovery of ~10% by sequential fractionation of the oocyte cytosol on tyrosine-agarose, heparin-agarose, casein-agarose and MonoQ. The purified enzyme contained 45, 38 and 28 kDa polypeptides, which corresponded to its α , α' and β subunits respectively. The β -subunit was autophosphorylated on one major tryptic peptide on serine residues, whereas the α' -subunit incorporated phosphate into at least two tryptic peptides primarily on threonine residues. Western-blotting analysis of sea-star oocyte extracts with two different anti-peptide antibodies that recognized conserved regions of the α -subunit indicated that the protein levels of the α - and α' -subunits of CKII were unchanged during oocyte maturation. The purified CKII was partly inactivated (by 25%) by preincubation with protein-serine/threonine phosphatase 2A, but protein-tyrosine phosphatases had no effect. The β -subunit of CKII was phosphorylated on a serine residue(s) up to 0.54 mol of P/mol of β -subunit by purified protein kinase C, and this correlated with a 1.5-fold enhancement of its phosphotransferase activity with phosvitin as a substrate. CKII was not a substrate for the maturation-activated myelin basic protein kinase p44^{mpk} from sea-star oocytes, nor for cyclic-AMP-dependent protein kinase. These studies point to possible regulation of CKII by protein phosphorylation.

INTRODUCTION

Casein kinase II (CKII) is a ubiquitous multifunctional protein-serine/threonine kinase that is distributed in the cytosolic, nuclear, mitochondrial and membranous fractions of eukaryotic cells (for reviews, see Krebs *et al.*, 1988; Pinna, 1990; Tuazon & Traugh, 1991). CKII purified from various sources is usually a tetrameric complex (~130 kDa) with an $\alpha_2\beta_2$, $\alpha'\beta_2$ or $\alpha_2\beta_2$ structure. It may exist also as a free monomeric α (~43 kDa) or α' (~38 kDa) subunit (Sternbach & Kuntzel, 1987; Gounaris *et al.*, 1987). cDNA sequence analysis of the α and α' subunits from budding yeast (Chen-Wu *et al.*, 1988; Padmanabha *et al.*, 1990) and chicken (Maridor *et al.*, 1991) established that they are encoded by distinct but highly related genes, which feature homology with the catalytic domains of other protein-serine/threonine kinases. Recently, recombinant α -subunits of CKII from *Caenorhabditis elegans* (Hu & Rubin, 1990) and *Drosophila* (Lin *et al.*, 1991) expressed in *Escherichia coli* were shown to feature most of the characteristics of the tetrameric form, including catalytic activity with either ATP or GTP and an extreme sensitivity to inhibition by heparin (50% inhibition at ~0.1–0.3 μ g/ml). However, the presence of the β -subunit (~28 kDa) appears to stimulate the phosphotransferase activity of the α -subunit towards casein by greater than 10-fold, and it is required to mediate the stimulatory effects of polyamines and highly basic polypeptides such as polylysine on CKII activity. Although the kinase normally undergoes a stoichiometric autophosphorylation reaction on the β -subunit, polylysine can act to inhibit this and stimulate phosphorylation on the α -subunit (Traugh *et al.*, 1990).

CKII phosphorylates a wide variety of proteins *in vitro*.

However, it may play an especially important role in the physiological regulation of nuclear proteins that are implicated in oncogenic transformation. These putative target substrates for CKII include Fos (Carroll *et al.*, 1988), Myb (Luscher *et al.*, 1990), Myc (Luscher *et al.*, 1989), p53 tumour suppressor protein (Meek *et al.*, 1990), adenovirus E1A protein (Carroll *et al.*, 1988), papillomavirus E7 protein (Fitzlaff *et al.*, 1989; Barbosa *et al.*, 1990) and SV40 large T antigen (Grasser *et al.*, 1988; Krebs *et al.*, 1988). Studies with synthetic peptide substrates have indicated that an acidic residue or a prior-phosphorylated serine residue at the third position from the C-terminal side of the target phosphorylatable residue is necessary and sufficient for recognition by CKII (Kuenzel *et al.*, 1987; Marchiori *et al.*, 1988; Meggio *et al.*, 1988; Litchfield *et al.*, 1990). Since CKII is already highly active in the absence of any established secondary messengers such as cyclic nucleotides, Ca^{2+} and diacylglycerols, it would seem that it is the substrates of CKII that must be 'activated', i.e. by prior serine phosphorylation by other protein kinases, rather than CKII itself. However, several studies have documented modest activations of CKII activity in response to growth-factor treatments of cells (Sommercorn *et al.*, 1987; Klarlund & Czech, 1988; Carroll & Marshak, 1989; Ackerman & Osheroff, 1989). The mechanisms by which these stimulations of CKII activity are achieved are obscure, although direct phosphorylation of the enzyme has been implicated. On the one hand, in the case of the epidermal-growth-factor-induced stimulation of CKII activity in human cervical carcinoma A431 cells, this was correlated with increased phosphorylation of the kinase on its β -subunit, and treatment of the activated CKII with alkaline phosphatase was accompanied by a loss of the growth-factor-associated stimulation (Ackerman *et al.*, 1990). On the

Abbreviations used: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CKII, casein kinase II; GVBD, germinal-vesicle breakdown; NBT, Nitro Blue Tetrazolium; p34^{cdc2}, 34 kDa protein kinase encoded by the *Schizosaccharomyces pombe* cell division control-2 gene and its functional homologues in other species; p44^{mpk}, 44 kDa meiosis-activated protein kinase; PKC, Ca^{2+} -activated phospholipid-dependent protein kinase; TBS, Tris-buffered saline; TTBS, TBS containing 0.05% Tween 20.

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other hand, dephosphorylation of rat liver CKII with type 1 and 2A protein-serine/threonine phosphatases was reported to be associated with a 2–3-fold enhancement of its phosphotransferase activity (Agostinis *et al.*, 1987).

In addition to a putative role in signalling from cell-surface growth-factor receptors, CKII has been implicated in the control of cell-cycle progression. In human W138 lung fibroblasts recruited from quiescence with serum, CKII activity was stimulated in a cyclic manner before S phase and again at the time of DNA synthesis (Carroll & Marshak, 1989). These oscillations of CKII activity were independent of protein synthesis *de novo* and might have reflected post-translational modification of the enzyme. During the meiotic maturation of amphibian oocytes in response to progesterone, CKII may also play a regulatory function. A rise in CKII activity in the ribonucleoprotein particles of *Rana temporaria* oocytes occurred about 7 h after exposure of the cells to progesterone, and this was unaffected by the protein-synthesis inhibitor cycloheximide (Kandror *et al.*, 1989). CKII is also modestly activated near the time of progesterone-induced germinal-vesicle breakdown (GVBD) in maturing *Xenopus laevis* oocytes (Cicirelli *et al.*, 1988). Micro-injection studies with purified CKII into *X. laevis* oocytes indicated that the kinase inhibited progesterone-induced maturation, but slightly accelerated the rate of maturation elicited by co-injection with the frog protein-serine/threonine kinase complex composed of p34^{cdc2} and cyclin B, which by-passes earlier steps in the maturation process (Mulner-Lorillon *et al.*, 1988). Maturation of frog oocytes in response to progesterone requires new protein synthesis, whereas p34^{cdc2}/cyclin B-induced maturation does not. Completion of meiotic maturation of sea-star oocytes in response to the hormone 1-methyladenine is also independent of protein synthesis *de novo*. We have recently found that CKII is one of several protein kinases that become activated near the time of GVBD in maturing sea-star oocytes (Pelech *et al.*, 1990, 1991). Herein, we describe the first purification and characterization of CKII from an echinoderm and explore the mechanisms responsible for its activation.

EXPERIMENTAL

Materials

Sea-stars (*Pisaster ochraceus*) were collected from the local beaches surrounding the University of British Columbia (U.B.C.) and Stanley Park in Vancouver. Synthetic peptides based on the N-terminus (SAARVYTDVNAHKPDEYWDYEN-GGC; α -CKII-NT) and an internal sequence (DQLVRIAKVLGT-DQLFRIFRALGT-GGC; α -CKII/cdc2) of *Drosophila* CKII α subunit were kindly provided by Dr. Ian Clark-Lewis (The Biomedical Research Centre), coupled to keyhole-limpet haemocyanin, and used to raise anti-peptide antibodies in rabbits. Protein phosphatases 2A (rabbit skeletal muscle), 1B (human placenta) and CD45 (human spleen) were a gift from Dr. Nicholas Tonks (Cold Spring Harbor). Purified protein kinase C (PKC; a mixture of the α -, β - and γ -isoforms) from rat brain was donated by Dr. Bruce Allen (Faculty of Pharmaceutical Sciences, U.B.C.) (Allen & Katz, 1991). p44^{mapk} was purified to homogeneity from maturing sea-star oocytes as described by Sanghera *et al.*, (1990). Cyclic-AMP-dependent protein kinase, casein (type III), diolein, phosphatidylserine, 1-methyladenine, tyrosine-agarose, heparin-agarose, casein-agarose, Nitro Blue Tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), heparin, spermine, poly(Gly: Tyr; 4:1), myelin basic protein, histones (type IIA, IIB and IIIS), phosvitin, benzamidine, phenylmethanesulphonyl fluoride and trypsin were purchased from Sigma. Okadaic acid was bought from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Electrophoresis reagents, prestained marker proteins and alkaline-phosphatase-conjugated goat anti-mouse IgG were from Bio-Rad Laboratories. [γ -³²P]ATP and the mouse anti-phosphotyrosine monoclonal antibody PY-20 were from I.C.N. (Montreal, Canada).

Preparation of cytosol from maturing sea-star oocytes

Pisaster ochraceus oocytes were induced to undergo maturation as described previously (Sanghera *et al.*, 1990). Immature oocytes and 1-methyladenine-treated oocytes that had completed GVBD were collected by centrifugation at 1000 g for 5 min and then homogenized in buffer A (20 mM-Mops, pH 7.2, 125 mM-glycerol β -phosphate, 12 mM-EDTA, 2 mM-sodium orthovanadate, 1 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-benzamidine) with a Waring blender at top speed for 20 s. The homogenate (30%, v/v) was centrifuged at 10000 g for 10 min, followed by ultracentrifugation of the post-mitochondrial supernatant at 150000 g for 30 min. The supernatant (cytosol) was subsequently stored at -70 °C until required.

Purification of CKII

Our purification procedure for the isolation of CKII was adapted from the protocol of Mulner-Lorillon *et al.* (1988). A 200 ml portion of GVBD-positive sea-star oocyte cytosol was thawed, with stirring on ice, with an equal volume of buffer B (20 mM-Tris/HCl, pH 7.5, 1 mM-dithiothreitol, 2 mM-EDTA) containing 1.6 M-(NH₄)₂SO₄. The extract was stirred over ice for 10 min and then centrifuged at 10000 g for 10 min at 4 °C. The supernatant was removed and loaded on to a tyrosine-agarose column (1.5 cm $\times$ 10 cm) that had been equilibrated with buffer B containing 0.8 M-(NH₄)₂SO₄. The column was developed with a 200 ml linear decreasing gradient from 0.8 M to 0 (NH₄)₂SO₄ in buffer B. Tyrosine-agarose (5 ml) fractions with peak CKII activity [corresponding to 0.3–0.2 M-(NH₄)₂SO₄] were pooled and diluted 1:3 with buffer B before loading on to a heparin-agarose column (1.5 cm $\times$ 10 cm). After washing with buffer B, the heparin-agarose column was developed with a 250 ml linear increasing gradient from 0 to 1 M-NaCl in buffer B. Heparin-agarose fractions (4 ml) with peak CKII activity (corresponding to 0.65–0.85 M-NaCl) were pooled and diluted 1:6 with buffer B before loading on to a casein-agarose column (1.5 cm $\times$ 5 cm). After washing this column with buffer B, CKII was eluted with a 200 ml linear increasing gradient from 0 to 1 M-NaCl in buffer B. Casein-agarose fractions (2 ml) with peak CKII activity (corresponding to 0.4–0.6 M-NaCl) were pooled and diluted 1:4 with buffer B before loading on to a MonoQ column (1 ml bed volume). The MonoQ column was developed with a 20 ml linear increasing gradient from 0 to 1 M-NaCl in buffer B, and 250 μ l fractions were collected. CKII was eluted from MonoQ with 0.3–0.4 M-NaCl.

CKII activity and protein assays

CKII activity in the extracts was assayed for 5–15 min at 30 °C in a final volume of 25 μ l with 5 mg of partially dephosphorylated casein/ml, 5 mM-MgCl₂, 12 mM-Mops, pH 7.2, and 50 μ M-[γ -³²P]ATP (2000 c.p.m./pmol). The reaction was terminated by spotting 20 μ l on to a 1.5 cm² piece of Whatman P81 phosphocellulose paper. After the P81 papers were washed extensively with 1% (w/v) H₃PO₄, they were transferred into 6 ml plastic vials containing 0.5 ml of Ecolume (I.C.N.) scintillation fluid and the radioactivity was measured in a Wallac (L.K.B.) scintillation counter.

Protein was determined by the method of Bradford (1976), with BSA as a standard (A_{280}^{obs} 6.5).

Fig. 1. Fractionation of echinoderm CKII activity through various purification steps

Extracts from mature sea-star oocytes were sequentially fractionated over tyrosine-agarose (a), heparin-agarose (b), casein-agarose (c) and MonoQ (d) as described in the Experimental section. At each purification step, the casein phosphotransferase activity (●) and A_{595} after addition of Bradford reagent (○), were determined.

Electrophoresis

SDS/PAGE was performed on 1.5 mm-thick gels, with acrylamide at 11% (w/v) in the separating gel and 4% (w/v) in the stacking gel, in the buffer system described by Laemmli (1970). Samples were boiled for 5 min in the presence of SDS/PAGE sample buffer (125 mM-Tris/HCl, pH 6.8, 4% SDS, 0.01% Bromophenol Blue, 10% mercaptoethanol and 20% glycerol) and electrophoresed for ~17 h at 10 mA. The separating gels

were silver-stained by the method of Merrill *et al.* (1981). For autoradiography, gels were exposed to Kodak XAR-5 film at room temperature.

Two-dimensional tryptic-phosphopeptide-map analysis of the autophosphorylated CKII was performed by dissecting the radio-labelled CKII band from SDS/PAGE gels and digestion with tosylphenylalanylchloromethane-treated trypsin in 1 ml of 50 mM- NH_4HCO_3 , pH 8.0 at 37 °C. After 24 h, the samples were dried under a vacuum (Speed Vac), washed with $4 \times 200 \mu\text{l}$ of water and re-dried, and then resuspended in $5 \mu\text{l}$ of electrophoresis buffer (1% NH_4HCO_3 , pH 8.9). The sample was spotted in the middle of a cellulose t.l.c. plate 1.5 cm from the bottom. Electrophoresis was performed in the long dimension at 600 V for 40 min. After drying, the plate was run in the second dimension in butanol/pyridine/acetic acid/water (13:10:2:8, by vol.).

Phosphoamino acid analysis of the autophosphorylated CKII was performed by digesting the radio-labelled CKII band in $300 \mu\text{l}$ of constant-boiling HCl at 105 °C for 1 h. The acid-hydrolysed sample was dried under vacuum (Speed Vac), washed with water, re-dried, and then resuspended in $5 \mu\text{l}$ of electrophoresis buffer, containing pyridine/acetic acid/water (1:10:189, by vol.). Phosphoserine, phosphothreonine and phosphotyrosine standards ($1 \mu\text{g}$ each) were electrophoresed alongside the sample. Electrophoresis was performed for 45–60 min at 1000 V with cooling.

Immunological studies

Cytosolic extracts prepared from sea-star oocytes (5 ml of packed cells per sample), which had been incubated at 15 °C with 2 μM -1-methyladenine for various times, were subjected to MonoQ chromatography, and the CKII was step-eluted with 0.6 M-NaCl in buffer B. Each MonoQ sample (~0.5 mg of protein) containing CKII activity was subjected to SDS/PAGE. After electrophoresis, the separating gel was soaked in transfer buffer (25 mM-Tris, 192 mM-glycine, 20% methanol) for 5 min, sandwiched with a nitrocellulose membrane, and then the proteins were transferred for 3 h at 300 mA. Subsequently, the nitrocellulose membrane was blocked with Tris-buffered saline (TBS; 150 mM-NaCl, 20 mM-Tris/HCl, pH 7.5) containing 3% gelatin for 2 h at room temperature. The membrane was washed twice with TBS containing 0.05% Tween 20 (TTBS) for 5 min before incubation with CKII-specific anti-peptide antibodies (in 1% skim milk/TTBS; 1:500 dilution) overnight at room temperature. Next day, the membrane was washed twice with TTBS before incubation with the second antibody (goat anti-rabbit IgG coupled to alkaline phosphatase in 1% skim milk/TTBS; 1:3000 dilution) for 2 h at room temperature. The membrane was rinsed twice with TTBS, followed by one wash with TBS before incubation with BCIP/NBT colour-development solution (mixture of 3% NBT in 1 ml of 70% dimethylformamide and 1.5% of BCIP in 1 ml of 100% dimethylformamide before adding to 100 ml of 0.1 M- NaHCO_3 /10 mM- MgCl_2 , pH 9.8). The colour reaction was terminated after 5–15 min by rinsing the membrane in a large volume of water.

Incubation of CKII with protein kinases and protein phosphatases

Cross-phosphorylation of CKII with various kinases was examined by incubation of purified CKII (1 μg) with 1 μg of the purified test kinases (PKC, p44^{mapk} and the catalytic subunit of cyclic-AMP-dependent protein kinase), 50 μM -[$\gamma\text{-}^{32}\text{P}$]ATP (10000 c.p.m./pmol), 5 mM- MgCl_2 and 12 mM-Mops, pH 7.2 in a final volume of 100 μl at 30 °C. The PKC reactions also included 8 μg of diolein/ml, 50 μg of phosphatidylserine/ml and 0.5 mM- CaCl_2 . Reactions were terminated by addition of an

Table 1. Purification of CKII

The values are the means from three different preparations.

Sample	Volume (ml)	Total Enzyme activity (pmol/min per ml)	Total protein (mg)	Specific activity (pmol/min per mg)	Recovery (%)	Purification (fold)
Homogenate	120	3760	3300	1.14	100	1
Tyrosine-agarose	68	1788	16.2	110	47	96
Heparin-agarose	75	984	0.30	3280	26	2877
Casein-agarose	27	675	0.11	6136	18	5382
MonoQ	3.8	388	0.03	12933	10.3	11344

equal volume of SDS/PAGE sample buffer. After the samples were boiled for 4 min at 100 °C, they were subjected to SDS/PAGE, and autoradiography of the gel was performed. For some experiments, after incubation with PKC a 10 µl sample was removed and assayed for phosphoinositide phosphotransferase activity with 4 µM-staurosporine in a final volume of 25 µl as described above.

To assess the effects of pretreatment of CKII with phosphatases, purified CKII (1 µg) was incubated in a final volume of 100 µl with 5 mM-MgCl₂ and protein phosphatase 2A (50 units), 1B (30 units) or CD45 (30 units) for various time intervals with occasional shaking at 30 °C. The control incubations also contained 4 µM-okadaic acid to inhibit phosphatase 2A or 1 mM-sodium orthovanadate to inhibit phosphatase 1B and CD45 during the preincubation. Subsequently the phosphatases were blocked with the appropriate inhibitor, and 10 µl samples were assayed for CKII activity.

RESULTS

Purification of CKII

Recently we tentatively identified CKII as the major casein kinase in cytosolic extracts from maturing sea-star oocytes (Pelech *et al.*, 1991). To confirm this assignment, we undertook the purification of this casein kinase to near homogeneity. When sea-star oocyte cytosol was adjusted to 0.8 M-(NH₄)₂SO₄, CKII remained soluble. However, this step afforded removal of ~ 20 % of the contaminating protein after low-speed centrifugation to pellet the precipitated material. Furthermore, it raised the ionic strength of the supernatant fraction sufficiently to permit binding of CKII to a tyrosine-agarose column (Fig. 1a). Most of the cytosolic protein was not retained by the hydrophobic resin under these conditions. CKII activity was eluted from the tyrosine-agarose column when the (NH₄)₂SO₄ concentration was decreased to ~ 0.25 M, and the ionic strength of these pooled fractions was further decreased by dilution before application to a heparin-agarose column (Fig. 1b). CKII activity was released from the heparin-agarose with ~ 0.75 M-NaCl, at a substantially higher salt concentration than for most of the contaminating protein. The ionic strength of the fractions that contained the peak of CKII activity was decreased by dilution, and they were loaded next on to a casein-agarose column, from which CKII was later eluted with ~ 0.5 M-NaCl (Fig. 1c). After dilution, this material was applied to a MonoQ column, and CKII was released with ~ 0.35 M-NaCl (Fig. 1d). The MonoQ fractions of CKII could be stored at -70 °C for over 2 months, with less than a 20 % loss of casein phosphotransferase activity.

The averaged results from three separate purifications of CKII are provided in Table 1. Beginning with 3.3 g of cytosolic protein, ~ 30 µg of the enzyme was purified over 11000-fold, with a final

Fig. 2. SDS/PAGE analysis of purified sea-star CKII

The peak tube of casein kinase activity after MonoQ chromatography was subjected to SDS/PAGE in a 11%-polyacrylamide separating gel and silver-stained (lane 1). Migrations of the low-molecular-mass standards phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa) are shown in lane 2.

recovery of ~ 10 % of the starting casein kinase activity. The tyrosine-agarose and heparin-agarose chromatography steps provided the most substantial purifications, and together they facilitated removal of greater than 99.9 % of the contaminating cytosolic proteins with a ~ 26 % recovery of casein phosphotransferase activity (Table 1).

The purity of the peak MonoQ fractions of CKII activity was analysed by SDS/PAGE and silver-staining of the gel (Fig. 2). At least three distinct proteins, of ~ 45 kDa, ~ 38 kDa and ~ 28 kDa, which were consistent with the sizes of the α , α' and β subunits of CKII purified from other species, were detected in the final purified preparation of the enzyme from sea-star oocytes. A portion of the α - and α' -subunits may have undergone further processing by phosphorylation or partial proteolysis, since additional similarly sized proteins could be discerned near the 45 kDa and 38 kDa bands. Such heterogeneity of the α - and α' -subunits has been described previously for CKII from other organisms (Meggio *et al.*, 1986; Qi *et al.*, 1986). Purified CKII

Fig. 3. Autophosphorylation of echinoderm CKII

CKII was subjected to autophosphorylation and SDS/PAGE as described in the Experimental section. The SDS/PAGE gel was silver-stained, and an autoradiogram of the gel is shown in lane 1 of panel (a). The migration of molecular-mass marker proteins is shown in lane 2. The α' - (38 kDa) (b) and β - (28 kDa) (c) subunits of CKII were excised from the gel, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping. In a separate experiment (d), the α' - (lane 2) and β - (lane 3) subunits of CKII were excised from the SDS/PAGE gel, digested with HCl, and then subjected to phosphoamino acid analysis.

behaved as a ~ 140 kDa species on Superose 12 gel-filtration resin in the presence of 200 mM-NaCl (results not shown). The amount of α' -subunit appeared to be greater than that of the α -subunit in all of our preparations (Fig. 2). These data were consistent with tetrameric structures for CKII, where the $\alpha'_2\beta_2$ arrangement probably predominated over the $\alpha\alpha'\beta_2$ form.

Kinetic characterization of purified CKII

The substrate specificity of purified CKII was evaluated. At 1 mg/ml, casein was the most efficient substrate tested, although 1 mg of phosvitin/ml was phosphorylated at $\sim 80\%$ of the rate of casein. At 1 mg/ml, myelin basic protein was phosphorylated relatively poorly, at $\sim 5\%$ of the rate of casein. There was poor phosphorylation (i.e. 2% or less than the rate of casein phosphorylation) of the various histones, protamine and 40 S ribosomes by CKII. Thus the protein substrate specificity of sea-star CKII closely resembles that of its homologues in other species (e.g. Gounaris *et al.*, 1987).

The true K_m values of purified CKII for casein and ATP were estimated from Lineweaver-Burk-plot analysis (results not shown). The K_m for ATP was 60 μ M with 200 μ M-casein, and the K_m for casein was 50 μ M with 100 μ M-ATP. These K_m values were slightly higher than those estimated for CKII from rat liver (Delpech *et al.*, 1986), *Drosophila melanogaster* (Glover *et al.*, 1983) and *C. elegans* (Hu & Rubin, 1990).

The dose-responses of various compounds that are diagnostic for their effects on CKII activity were also determined with the purified enzyme. Heparin produced a 50% decrease in CKII activity at a concentration of ~ 2 μ g/ml (results not shown). This was about a 10–100-fold higher concentration of heparin than that required to produce the equivalent inhibition of CKII activity from other species (Glover *et al.*, 1983; Delpech *et al.*, 1986; Hu & Rubin, 1990). Poly(Glu:Tyr; 4:1) was also a potent inhibitor, and at 75 nM it elicited 50% inhibition of CKII activity (results not shown). Spermine stimulated CKII activity over 2-

fold above the starting levels. Maximum activation was achieved with 5 mM-spermine, and half maximal stimulation was produced with 1.5 mM of this polyamine (results not shown).

Autophosphorylation of CKII

Purified CKII readily underwent autophosphorylation upon incubation with Mg -[γ - ^{32}P]ATP (Fig. 3a). Most of the radiolabel was incorporated in the 28 kDa β -subunit, with only marginal phosphorylation of the 38 kDa α' -subunit. Negligible phosphorylation of the 45 kDa α -subunit was detected in autoradiograms of the autophosphorylated CKII after SDS/PAGE. Tryptic-phosphopeptide mapping of the excised α' - and β -subunit bands from the SDS/PAGE gel revealed one major autophosphorylation site and weaker phosphorylation at at least one additional site for each of these subunits (Figs. 3b and 3c). Phosphoamino acid analysis showed that the major tryptic ^{32}P -labelled peptide derived from the α' -subunit was phosphorylated on threonine (Fig. 3d, lane 2). Conversely, the β -subunit was autophosphorylated primarily on serine (Fig. 3d, lane 3).

Investigation of the mechanism of activation of CKII during oocyte maturation

We have previously described the activation (up to 3-fold) of a cytosolic CKII-like enzyme before the onset of GVBD in maturing sea-star oocytes after MonoQ fractionation (see Fig. 4 in Pelech *et al.*, 1991). The results from the present study strongly supported its identification as CKII. Since the previous report was published, we have noted that this maturation-associated stimulation of CKII activity can be variable, but it has been reproducibly detected with different preparations of oocytes (results not shown).

We considered the possibility that the enhanced CKII activity during sea-star oocyte maturation might arise from an increase in the amount of enzyme protein. To explore this further, affinity-purified anti-peptide antibodies raised in rabbits against

an *N*-terminal sequence and an internal sequence (partially shared by p34^{cdc2}) predicted in the α -subunit of *D. melanogaster* CKII were used for immunoblotting analysis of the sea-star homologue (Fig. 4). Both of these antibodies permitted detection of the α - and α' -subunits of purified CKII (Fig. 4a). Cytosolic extracts from oocytes prepared before and after addition of 1-methyladenine were fractionated on MonoQ, and the peaks of CKII activity that were eluted with 0.7 M-NaCl for each time point were subjected to SDS/PAGE and Western blotting with these antibodies. The α - and α' -subunits were both detected with the two different anti-peptide antibodies, but the levels of these proteins were apparently unchanged during the course of oocyte maturation (Fig. 4b), despite a 2-fold activation of CKII activity 60 min after addition of 1-methyladenine to the cells (results not shown). Furthermore, there was no immunological evidence for proteolytic processing of either subunit to lower-molecular-mass forms during oocyte maturation.

Although there were no apparent changes in the migration rates of the α - and α' -subunits of SDS/PAGE gels (Fig. 4b), as might be expected from altered phosphorylation of CKII, control of its enzymic activity by this form of reversible covalent modification remained an attractive possibility. Immunoblotting studies with the anti-phosphotyrosine monoclonal PY-20 antibody and CKII samples processed in the same manner as for Fig. 4(b) failed to reveal any tyrosine phosphorylation of CKII associated with oocyte maturation (results not shown). Purified CKII was also not immunoreactive with the PY-20 antibody. Therefore it was not surprising that preincubation of purified CKII with the protein-tyrosine phosphatases CD45 (Fig. 5a) and 1B (results not shown) had no effect on the phosphotransferase activity of the kinase (Fig. 5a). However after 40 min at 30 °C, the protein-serine/threonine phosphatase 2A was found to cause a marginal decrease in CKII activity by 25% as compared with a control in which the phosphatase was inhibited by okadaic acid (Fig. 5b). Further incubation of CKII with higher concentrations of phosphatase 2A or for longer time intervals did not cause a greater decrease in CKII activity (results not shown).

Phosphorylation of CKII with other protein kinases

In view of the autophosphorylation of CKII and its sensitivity to partial inhibition by a protein-serine/threonine phosphatase, we were prompted to test for phosphorylation of CKII by other protein-serine/threonine kinases. The activated form of p44^{mapk}, also purified from maturing sea-star oocytes, and the free catalytic subunit of cyclic-AMP-dependent protein kinase failed to increase the phosphorylation state of CKII (results not shown). However, purified PKC from rat brain, which was a mixture of the α , β and γ isoforms, clearly stimulated the incorporation of [³²P]P_i into the 28 kDa β -subunit of CKII on a serine residue(s) (Fig. 6). Via autophosphorylation, CKII incorporated ~0.1 mol of P/mol of β -subunit after 60 min incubation at 30 °C (Fig. 6b). The stoichiometry of PKC-induced phosphorylation of the β -subunit was much higher, at ~0.54 mol of P/mol of β -subunit after the same time interval at the same temperature (Fig. 6b). PKC also appeared to phosphorylate the α' -subunit of CKII, although this was more easily discerned in experiments that are not shown here. The α' -subunit phosphorylation on SDS/polyacrylamide gels was partially obscured by the autophosphorylation of the 45 kDa proteolytically derived catalytic fragment of PKC.

To evaluate whether prior protein phosphatase pretreatment of CKII would allow an enhancement of the PKC-induced phosphorylation of the β -subunit, purified CKII was incubated with 50 units of protein phosphatase 2A for 15 min at 30 °C before incubation with PKC in the presence of 4 μ M-okadaic

Fig. 4. Immunoblotting of CKII from sea-star oocyte cytosol

Purified CKII was immunoblotted with affinity-purified polyclonal anti-peptide antibodies raised against sequences based on the *N*-terminus of *Drosophila* CKII (CKII-NT) (a, lane 1) and a region that is highly conserved in CKII and p34^{cdc2} (CKII/cdc2) (a, lane 2). Cytosolic extracts from sea-star oocytes treated for 0–90 min with 1-methyladenine were fractionated on a MonoQ column as described in the Experimental section. The peak of CKII activity from each time point was subjected to SDS/PAGE, transferred to nitrocellulose, and then probed separately with anti-CKII-NT antibody (b) and anti-CKII/cdc2 antibody (c). Migrations of the pre-stained low-molecular-mass standards BSA (80 kDa), ovalbumin (50 kDa) and carbonic anhydrase (33 kDa), soybean trypsin inhibitor (28 kDa) and lysozyme (19 kDa) are indicated on the right side of each panel.

Fig. 5. Effect of protein phosphatases 2A and CD45 on echinoderm CKII activity

Purified CKII (2 μ g) was incubated, in a reaction volume of 100 μ l, with 30 units of protein-tyrosine phosphatase CD45 (a) and 50 units of protein-serine/threonine phosphatase 2A (b) for various time intervals at 30 °C. At each time point, a 10 μ l sample was removed and assayed for casein phosphotransferase activity in the presence of 4 mM-sodium vanadate (for CD45) and 4 μ M-okadaic acid (for phosphatase 2A) (●). Controls contained phosphatase inhibitors at the start of incubations of CKII with the phosphatases (○). Similar results were obtained in six independent experiments.

Fig. 6. Phosphorylation of CKII with PKC

Purified sea-star CKII ($\sim 1 \mu\text{g}$) was incubated in a final volume of $70 \mu\text{l}$ with rat brain PKC ($\sim 1 \mu\text{g}$) and $250 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 30°C in the absence or presence of phosphatidylserine and diolein (PS/DAG) and Ca^{2+} as indicated above panel (a). In some instances (denoted by '+H'), the PKC or CKII was preincubated at 60°C for 5 min. The incubations were terminated with SDS/PAGE sample buffer, and the samples were subjected to SDS/PAGE and autoradiography. Panel (b) shows the stoichiometry of phosphorylation of the β -subunit in the presence of $4 \mu\text{M}$ okadaic acid as a function of time in the absence (i.e. autophosphorylation; ○) or presence of PKC [with (△) or without (●) pretreatment of CKII with 50 units of phosphatase 2A for 20 min at 30°C]. In the experiments shown here, a less active preparation of CKII was used to decrease the contribution of autophosphorylation. Similar results were obtained in six independent experiments. (c) The β -subunit of CKII from lane 6 (a) was excised and subjected to phosphoamino acid analysis (lane 2). Migrations of free phosphate and phosphoamino acid standards are indicated in lane 1.

Fig. 7. Activation of CKII by PKC

Purified sea-star CKII ($\sim 1 \mu\text{g}$) was incubated with PKC ($\sim 1 \mu\text{g}$) for 3 min at 30°C in the presence of unlabelled ATP, phosphatidylserine, diolein and Ca^{2+} . Subsequently, the phosvitin phosphotransferase activity was assayed upon addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and continuation of the reaction for 1 min. Values are the means $\pm$ S.E.M. from five different experiments performed on separate days. Student *t*-test analysis indicated that the difference between phosvitin phosphotransferase activity for 'CKII only' and 'CKII + PKC' was statistically significant, with $P < 0.005$.

acid. This prior phosphatase treatment of CKII did not facilitate an increased incorporation of radiolabel from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into CKII by PKC (Fig. 6b). Furthermore, after phosphorylation of CKII by PKC, the ^{32}P was not significantly released from the β -subunit by subsequent phosphatase 2A treatment for 20 min at 30°C in the presence of the PKC inhibitor staurosporine ($5 \mu\text{M}$) (results not shown). Therefore it would appear that the PKC

phosphorylation site on CKII β -subunit was not recognized for dephosphorylation by phosphatase 2A.

The consequence of phosphorylation of CKII by PKC was difficult to assess, owing to the ability of both kinases to utilize casein as a substrate. However, studies with phosvitin, an alternative substrate for CKII, indicated that prior phosphorylation of CKII by PKC produced a modest ~ 1.5 -fold activation of the kinase within 2 min of preincubation (Fig. 7). The extent of CKII activation by PKC was not enhanced by prior treatment of CKII with phosphatase 2A in three separate experiments by the protocol outlined above (results not shown). Treatment with phosphatase 2A led to a 25% decline in the phosvitin phosphotransferase activity of CKII before and after phosphorylation of CKII by PKC (Fig. 5b, and results not shown).

DISCUSSION

Herein, we have described the first purification of CKII from an echinoderm source. The simple four-column-step procedure yielded essentially homogeneous enzyme. Its properties were extremely reminiscent of CKII isolated from other species. This was somewhat expected, in view of the greater than 97% identity in primary structures of the α -subunits from humans and chicken, and the complete amino acid identities in their β -subunits (Meisner *et al.*, 1989; Jakobi *et al.*, 1989; Maridor *et al.*, 1991). This implies that findings from the study of sea-star CKII may be of general significance.

Meggio & Pinna (1984) have estimated that rat CKII incorporates up to 2 mol of phosphate per mol of β -subunit via an intramolecular autophosphorylation reaction. Although the autophosphorylation of CKII on its β -subunit had been well documented, at the time that our study was conducted surprisingly little had been established about its precise location. Sea-star CKII autophosphorylated predominantly on a serine

residue in the β -subunit (Fig. 3), as has been noted for human CKII (Pyerin *et al.*, 1986; Litchfield *et al.*, 1991). Analysis of the predicted primary structure of the β -subunits of CKII from human (Jakobi *et al.*, 1989), chicken (Maridor *et al.*, 1991) and *D. melanogaster* (Saxena *et al.*, 1987) sources reveals only two serine residues that lie within a predicted optimal phosphorylation-site consensus sequence for recognition for CKII. These are Ser-2 and Ser-3, which are found to be completely conserved where the β -subunit amino acid sequence has been deduced. A recent study of human CKII by Litchfield *et al.* (1991) established that the major autophosphorylation site(s) is located at the *N*-terminus of the β -subunit. More formal proof via direct protein sequencing that Ser-2 and Ser-3 are the actual autophosphorylation sites is difficult, because the amino group of Met-1 is modified in human (Litchfield *et al.*, 1991), bovine (Takio *et al.*, 1987) and *D. melanogaster* (Padmanabha & Glover, 1987) CKII.

The regulatory significance of the β -subunit autophosphorylation is unclear. Szopa & Rose (1986) noted that autophosphorylated CKII binds more poorly to DNA and exhibits a decreased endonuclease activity. Delpech *et al.* (1986) found that irreversible phosphorylation of CKII with adenosine 5'-[γ -thio]-triphosphate led to a 40% increase in its phosphotransferase activity. Others (Damuni & Reed, 1988) have been unable to measure any changes in CKII activity with β -subunit autophosphorylation.

Sea-star CKII also autophosphorylated on its α' -subunit, mostly on threonine residues, but not on its α -subunit (Fig. 4). Some autophosphorylation of the α -subunit has been described for human CKII (Pyerin *et al.*, 1986; Litchfield *et al.*, 1991). There are five potential CKII phosphorylation sites that are highly conserved in both the α - and α' -subunits of CKII from various organisms. In the human α -subunit sequence, these correspond to Thr-60, Thr-96, Thr-127, Thr-129 and Thr-314 (Meisner *et al.*, 1989). Of these, Thr-129 is the most optimal.

One of the most novel findings of this study was demonstration of CKII serine phosphorylation on its β -subunit up to 0.54 mol of P/mol of β -subunit by purified PKC (Fig. 6). This was correlated with a mean 1.44-fold enhancement of CKII's phosphotransferase activity with phosvitin (Fig. 7). This supports the hypothesis that the ~2-fold stimulation of CKII activity during sea-star oocyte maturation might be mediated via phosphorylation of the kinase. Immunoblotting studies with two different CKII peptide antibodies established that the levels of the α - and α' -subunits of CKII were not altered up to 90 min after resumption of meiotic maturation with 1-methyladenine (Figs. 4b and 4c), which implicated regulation by post-translational mechanisms. Furthermore, CKII that was purified from maturing oocytes could be partially inactivated by incubation with a protein-serine/threonine phosphatase (Fig. 5b). However, if CKII is indeed phosphorylated and activated by a protein kinase before GVBD, this kinase is unlikely to be PKC. There is no evidence for stimulation of PKC activity during sea-star oocyte maturation, and activation of PKC in immature oocytes with phorbol esters actually blocks 1-methyladenine-induced maturation (Meijer *et al.*, 1987).

Ackerman *et al.* (1990) have detected nearly equivalent levels of serine and threonine phosphorylation of the β -subunit of CKII from [32 P]phosphate-labelled human A431 cells, although this could not be confirmed by Litchfield *et al.* (1991), who detected only phosphoserine. However, in the Litchfield *et al.* (1991) study, it was also shown that Ser-209 of the β -subunit was phosphorylated in human A431 cells, and this site could be phosphorylated by active p34^{cdc2} immunoprecipitated from these cells. Mulner-Lorillon *et al.* (1990) have previously described threonine phosphorylation of the β -subunit of *Xenopus* CKII by sea-star p34^{cdc2}, and this was associated with a 1.4–5-fold increase

in the measured CKII activity. Since the primary structure of *Xenopus* CKII has not yet been reported, it is unclear if the amino acid corresponding to human Ser-209 is a threonine residue in the frog. It is possible that p34^{cdc2} might be the physiological protein kinase that activates CKII in maturing sea-star oocytes, since the time courses of 1-methyladenine-induced activation of both kinases are similar (Pelech *et al.*, 1991). In the present study, we have ruled out p44^{mapk} as the kinase that phosphorylates CKII in maturing sea-star oocytes (results not shown). The ability of p34^{cdc2} and other meiosis-activated protein kinases to modulate CKII activity should be further explored.

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REFERENCES

- Ackerman, P. & Osheroff, N. (1989) *J. Biol. Chem.* **264**, 11958–11965
- Ackerman, P., Glover, C. V. C. & Osheroff, N. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 821–825
- Agostinis, P., Goris, J., Pinna, L. A. & Merlevede, W. (1987) *Biochem. J.* **248**, 785–789
- Allen, B. G. & Katz, S. (1991) *Biochemistry* **30**, 4334–4343
- Barbosa, M. S., Edmonds, C., Fisher, C., Schiller, J. T., Lowy, D. R. & Vousden, K. H. (1990) *EMBO J.* **9**, 153–160
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Carroll, D. & Marshak, D. R. (1989) *J. Biol. Chem.* **264**, 7345–7348
- Carroll, D., Santoro, N. & Marshak, D. R. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53**, 91–95
- Chen-Wu, J. L.-P., Padmanabha, R. & Glover, C. V. C. (1988) *Mol. Cell. Biol.* **8**, 4981–4990
- Cicirelli, M. F., Pelech, S. L. & Krebs, E. G. (1988) *J. Biol. Chem.* **264**, 7345–7348
- Damuni, Z. & Reed, L. J. (1988) *Arch. Biochem. Biophys.* **262**, 574–584
- Delpech, M., Levy-Favartier, F., Moisand, F. & Kruh, J. (1986) *Eur. J. Biochem.* **160**, 333–341
- Firzlaff, J. M., Galloway, D. M., Eisenman, R. N. & Luscher, B. (1989) *New Biologist* **1**, 44–53
- Glover, C. V. C., Shelton, E. R. & Brutlag, D. L. (1983) *J. Biol. Chem.* **258**, 3258–3265
- Gounaris, A., Trangas, T. T. & Tsiapalis, C. M. (1987) *Arch. Biochem. Biophys.* **259**, 473–480
- Grasser, F. A., Scheidtmann, K. H., Tuazon, P. T., Traugh, J. A. & Walter, G. (1988) *Virology* **165**, 13–22
- Hu, E. & Rubin, C. S. (1990) *J. Biol. Chem.* **265**, 20609–20615
- Jakobi, R., Voss, H. & Pyerin, W. (1989) *Eur. J. Biochem.* **183**, 227–233
- Kandror, K. V., Benumov, A. O. & Stepanov, A. S. (1989) *Eur. J. Biochem.* **180**, 441–448
- Klarlund, J. K. & Czech, M. P. (1988) *J. Biol. Chem.* **263**, 15872–15875
- Krebs, E. G., Eisenman, R. N., Kuenzel, E. A., Litchfield, D. W., Lozeman, F. J., Luscher, B. & Sommercorn, J. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53**, 77–84
- Kuenzel, E. A., Mulligan, J. A., Sommercorn, J. & Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 9136–9140
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–684
- Lin, W.-J., Tuazon, P. T. & Traugh, J. A. (1991) *J. Biol. Chem.* **266**, 5664–5669
- Litchfield, D. W., Arendt, A., Lozeman, F. J., Krebs, E. G., Hargrave, P. A. & Palczewski, K. (1990) *FEBS Lett.* **261**, 117–120
- Litchfield, D. W., Lozeman, F. J., Cicirelli, M. F., Harrylock, M., Ericsson, L. H., Piening, C. J. & Krebs, E. G. (1991) *J. Biol. Chem.* **266**, 20380–20389
- Luscher, B., Kuenzel, E. A., Krebs, E. G. & Eisenman, R. N. (1989) *EMBO J.* **8**, 1111–1119
- Luscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G. & Eisenman, R. N. (1990) *Nature (London)* **344**, 517–522
- Marchiori, F., Meggio, F., Marin, O., Borin, G., Calderan, A., Ruzza, P. & Pinna, L. A. (1988) *Biochim. Biophys. Acta* **971**, 332–338
- Maridor, G., Park, W., Krek, W. & Nigg, E. A. (1991) *J. Biol. Chem.* **266**, 2362–2368

- Meek, D. W., Simon, S., Kikkawa, U. & Eckhart, W. (1990) *EMBO J.* **9**, 3253–3260
- Meggio, F. & Pinna, L. A. (1984) *Eur. J. Biochem.* **145**, 593–599
- Meggio, F., Grankowski, N., Kudlicki, W., Szyska, R., Gasior, E. & Pinna, L. A. (1986) *Eur. J. Biochem.* **159**, 31–38
- Meggio, F., Perich, J. W., Johns, R. B. & Pinna, L. A. (1988) *FEBS Lett.* **237**, 225–228
- Meijer, L., Pelech, S. L. & Krebs, E. G. (1987) *Biochemistry* **26**, 7968–7974
- Meisner, H., Heller-Harrison, R., Buxton, J. & Czech, M. P. (1989) *Biochemistry* **28**, 4072–4076
- Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437
- Mulner-Lorillon, O., Marot, J., Cayla, X., Pouhle, R. & Belle, R. (1988) *Eur. J. Biochem.* **171**, 107–117
- Mulner-Lorillon, O., Cormier, P., Labbe, J.-C., Doree, M., Poulhe, R., Osborne, H. & Belle, R. (1990) *Eur. J. Biochem.* **193**, 529–534
- Padmanabha, R. & Glover, C. V. C. (1987) *J. Biol. Chem.* **262**, 1829–1835
- Padmanabha, R., Chen-Wu, J. L.-P., Hanna, D. E. & Glover, C. V. C. (1990) *Mol. Cell. Biol.* **8**, 4089–4099
- Pelech, S. L., Sanghera, J. S. & Daya-Makin, M. (1990) *Biochem. Cell Biol.* **68**, 1297–1330
- Pelech, S. L., Sanghera, J. S., Paddon, H. B., Quayle, K. & Brownsey, R. (1991) *Biochem. J.* **274**, 759–767
- Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054**, 267–284
- Pyerin, W., Burow, E., Michaely, K., Kubler, D. & Kinzel, V. (1986) *Biol. Chem. Hoppe-Seyler* **368**, 215–227
- Qi, S.-L., Yukioka, M., Morisawa, S. & Inoue, A. (1986) *FEBS Lett.* **203**, 104–108
- Sanghera, J. S., Paddon, H. B., Bader, S. A. & Pelech, S. L. (1990) *J. Biol. Chem.* **265**, 52–57
- Saxena, A., Padmanabha, R. & Glover, C. V. C. (1987) *Mol. Cell Biol.* **7**, 3409–3417
- Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8834–8838
- Sternbach, H. & Kuntzel, H. (1987) *Biochemistry* **26**, 4207–4212
- Szopa, J. & Rose, K. M. (1986) *Biol. Chem. Hoppe-Seyler* **367**, Suppl. 185
- Takio, K., Kuenzel, E. A., Walsh, K. A. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4851–4855
- Traugh, J. A., Lin, W.-J., Takada-Axelrod, F. & Tuazon, P. T. (1990) *Adv. Second Messenger Phosphoprotein Res.* **24**, 224–229
- Tuazon, P. T. & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res.* **23**, 123–164

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