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EXPRESSION OF RAT PROTEIN PHOSPHATASE 2C (IA) IN *Escherichia coli*

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A cDNA containing the entire coding sequence of rat type 2C (IA) protein phosphatase was expressed in *Escherichia coli*. An extract of bacterial cells harboring the recombinant plasmid contained a major (M_r = 41,000 - 43,000) and a minor (M_r = 30,000) protein band; both of these reacted with an anti-type 2C protein phosphatase serum. The size of the major protein band agrees well with that of the 2C phosphatase conceptualized from the cognate cDNA. A Mg^{2+}-dependent protein phosphatase activity was detected in extracts containing the recombinant protein, but not in host cell extracts. Based on these results, it is concluded that the isolated cDNA clone encodes a functional type 2C protein phosphatase.

The importance of protein phosphatases in the phosphorylation/dephosphorylation cycle has been well documented (1). The catalytic subunits of the major protein phosphatase in mammalian cells have been divided into two classes, type 1 and type 2, based mainly on their sensitivity to two thermostable proteins, inhibitors-1 and -2 (1,2). The catalytic subunits of type 2 protein phosphatase have been further divided into three classes (2A, 2B and 2C) on the basis of divalent cation requirements (1,3). Those four catalytic subunits appear to be products of separate genes (4-15). Type 2C protein phosphatase has been further subdivided into two isotypes ($2C_1$ and $2C_2$) on the basis of partial amino acid sequences of the rabbit skeletal muscle enzyme (16).

Following the initial identification and purification of a type 2C protein phosphatase and two types of holoenzyme of type 2A protein phosphatase as phosphatases IA, IB and II, respectively, from cytosolic fraction of rat liver (17-22), we undertook a molecular genetic approach to investigate the role of these protein phosphatases in the regulation of cellular function. We have recently characterized a rat kidney cDNA (pST-11) by hybridization with synthetic oligonucleotide probes corresponding to amino acid sequence segments of

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the purified rat type 2C protein phosphatase (15). Judging from the similarity between the amino acid sequences deduced from pST-11 and those of the peptides from the purified rabbit skeletal muscle proteins, we predicted that our cDNA encodes the $2C_1$ isozyme. To confirm this prediction as well as to conduct further studies with this enzyme, we now demonstrate that our putative 2C phosphatase cDNA encodes a type 2C protein phosphatase when expressed in bacteria.

MATERIALS AND METHODS

Materials: Reagents for immunoblotting and plasmid pTTQ18 were purchased from Radiochemical Centre, Amersham, England. Plasmid pKH451 was a generous gift from Dr. M. Takao, Tohoku University, Japan. *E. coli* strain JM48 was a generous gift from Dr. T. Horinouchi, Kyushu University, Japan. Calf thymus whole histone (type II-A, Sigma, St. Louis, MO, USA) was treated with cyclic AMP-dependent protein kinase in the presence of [γ-^{32}P]ATP to yield the phosphorus-32 labeled phosphohistone substrate (18).

Preparation of crude extract of E. coli: *E. coli* strain JM109 transformed with plasmids pKHP2C or pKHP2CR was grown with shaking in LB medium containing ampicillin (50 µg/ml) for 3.5 h at 37°C after which isopropylthiogalactoside (IPTG) was added to a final concentration of 2 mM and the culture incubated for a further 4 h. The preparation of bacterial cell crude extract was preformed essentially as described by Marston (23). Briefly, the bacterial cells were collecetd by centrifugation and incubated in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 150 µM phenylmethylsulfonyl fluoride and lysozyme (1 mg/ml) for 20 min at 25°C. The suspension was cooled on ice, sonicated with two 15 sec bursts (28 KHz) using a Model UR-20P sonicator (Tomy Seiko, Tokyo, Japan) and centrifuged (10,000 x g for 10 min at 4°C). The supernatant fluid was stored at -70°C until use.

Assay of protein phosphatase activity: Histone phosphatase activity was assayed by the release of [^{32}P]phosphate from [^{32}P]phosphohistone essentially as described previously (18). The standard incubation mixture contained 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, 60 µM [^{32}P]phosphohistone (based on alkaline-labile ^{32}P) and enzyme. Magnesium chloride (5 mM) was added as indicated. Other procedures have been described previously (18). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 nmol phosphate in one min at 30°C.

Preparation of anti-type 2C protein phosphatase antiserum: The anti-type 2C protein phosphatase serum was prepared essentially as described (24). Purified rat liver type 2C protein phosphatase (5 µg in 0.1 ml) was mixed with 0.1 ml of Freund's complete adjuvant and injected subcutaneously into a mouse (Balb/C, female) four times at intervals of 12 days. Blood was collected by heart puncture ten days after the final injection and the serum prepared. A ten-fold dilution of serum inhibited the activity of a purified rat liver type 2C protein phosphatase by 40%, whereas this same serum did not affect type $2A_1$ protein phosphatase activity. A major band migrating with a 48,000 dalton standard and minor band migrating more slowly than a 100,000 dalton standard were detected when rat liver extracts were immunoblotted using a 50-fold dilution of the antisera. The larger protein was also detected as a minor contaminant in the immunogen preparation.

Immunoblotting: Immunoblotting was performed by separating the proteins in extracts of *E. coli* or rat liver by NaDodSO$_4$ - polyacrylamide slab gel electrophoresis in an 8% gel with a 4% stacking gel followed by blotting onto a nitrocellulose filter and staining of the filter with antibody (25).

RESULTS AND DISCUSSION

Construction of plasmids harboring cDNA pST-11: A plasmid for expression of the type $2C_1$ phosphatase cDNA in *E. coli* was constructed as outlined in

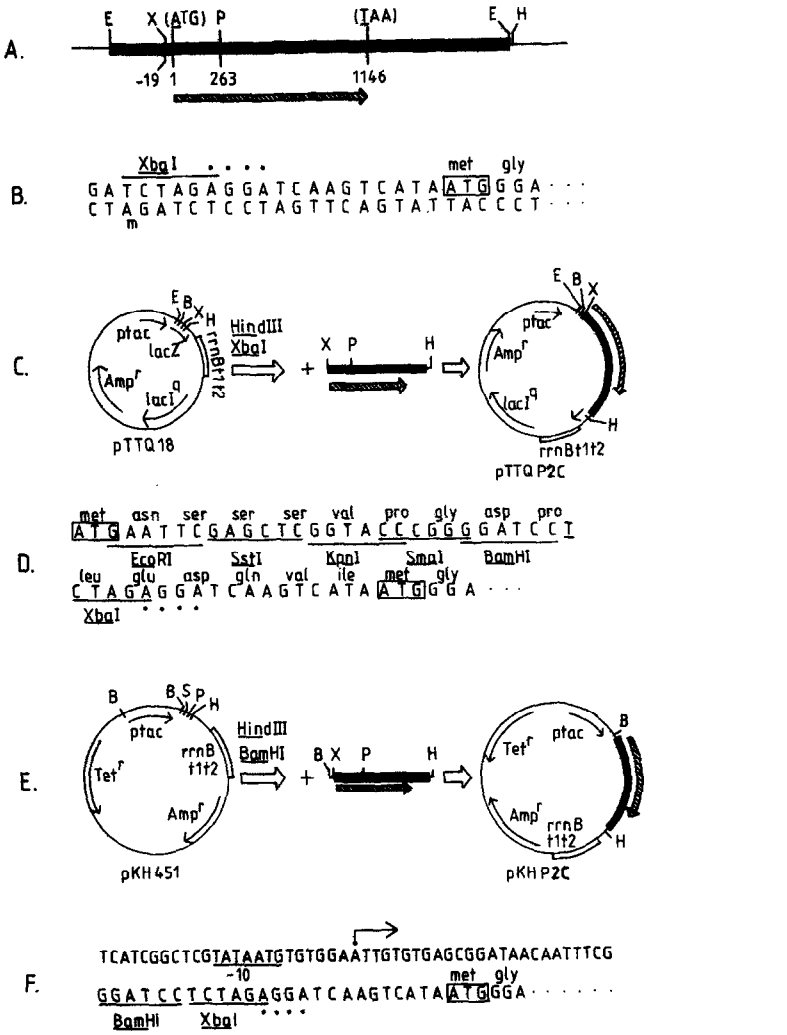

Fig. 1. Plasmid construction for expression of the cDNA in *E. coli* cells. A. Map of the cDNA in pST-11. Closed bar indicates the rat cDNA fragment, arrow indicates the open reading frame. Abbreviations for restriction enzymes used in this figure are, E: *Eco* RI, X, *Xba* I, P: *Pst* I, H: *Hind* III, B: *Bam* HI. B. Nucleotide sequence at the 5' region of the translational initiation codon. The ultimate adenine in the *Xba* I site is methylated. The sequence, AGGA, is dotted. C. The *Xba* I - *Hind* III fragment of pST-11 was first introduced into the same sites of an expression vector pTTQ18 to generate pTTQP2C. D. The sequences at the multi-cloning site after the introduction of the pST-11 cDNA into pTTQ18. E. Introduction of the *Bam* HI -*Hind* III fragment from pTTQP2C into an expression vector pKH451 to generate pKHP2C. Plasmid pKHP2CR contains the *tac* promoter in the opposite direction. F. Nucleotide sequences at the 5' region of the translation initiation codon of pKHP2C. The putative -10 sequence, transcriptional initiation site (depicted by arrow) and SD sequence are shown.

Fig. 1. In the 2.35 kbp *Eco* RI fragment of pST-11 there exists a *Xba* I site at -19 bp relative to the putative translation initiation codon ATG (Fig. 1 A and D). Since the ultimate adenine of the *Xba* I recognition sequence (tctaga) was apparently methylated and thus refractory to digestion, the plasmid was passed through a *dam*$^-$ *E. coli* strain (strain JM48), after which the *Xba* I-*Eco*

RI fragment was isolated from the plasmid. This fragment was subcloned into pTTQ18 to yield pTTQP2C (Fig. 1C). The plasmid pTTQP2C was then digested with *Bam* HI and *Hin*d III and the resulting 2 kbp *Bam* HI-*Hin*d III fragment was introduced into the expression vector pKH451 that had been digested with the same enzymes. Finally, a *Bam* HI fragment containing the *tac* promoter was inserted in the normal (pKHP2C) or in the reverse orientation (pKHP2CR). The sequence upstream of the translational initiation codon of pKHP2C is shown in Fig. 1F. In this plasmid it was expected that the AGGA sequence at -10 bp (to the initiation codon) functioned as a Shine-Dalgarno sequence. Plasmids were introduced in *E. coli* strain JM109. No difference in growth rate was observed between the *E. coli* cells transformed by either pKHP2C or pKHP2CR regardless of the presence or absence of induction by IPTG.

Immunoblotting of protein(s) produced in E. coli cells: We next determined whether a immunoreactive 2C phosphatase was produced in bacteria containing the recombinant plasmids. As shown in Fig. 2, a diffuse band (M_r = 41,000 - 43,000) and a sharp, but fainter, band (M_r = 30,000) were observed when the proteins of *E. coli* cell extracts transformed with pKHP2C were reacted with the anti-type 2C protein phosphatase serum. No bands were visible when preimmune serum was used (data not shown). Since the transformation of *E. coli* by pKHP2CR generated neither band (lanes 3 and 4), it was concluded that the major protein (M_r = 41,000 - 43,000) was derived from the open reading frame of pST-11. The observed size of the major protein agrees well with the molecular weight of the protein deduced from pST-11 (15). We presume that the minor protein (M_r = 30,000) is a proteolytic degradation product of the major form. Perhaps the diffuseness of the major protein band

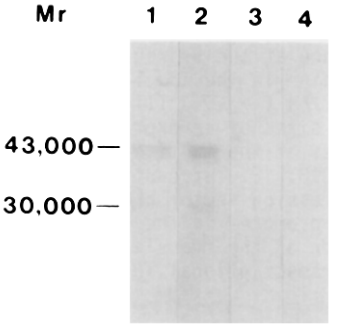

Fig. 2. Immunoblotting of protein(s) produced in *E. coli* cells. *E. coli* cells harboring pKHP2C (lanes 1 and 2) or pKHP2CR (lanes 3 and 4) were incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2 mM IPTG. The cell extracts were then prepared as described in the text. A $NaDodSO_4$-polyacrylamide slab gel electrophoresis was performed with a portion (3.2 μg protein) of each extract. After transfer to nitrocellulose, the proteins were probed with the anti-type 2C protein phosphatase antiserum (60-fold dilution) and the immunoreactive material was detected with alkaline phosphatase-conjugating second antibody.

may also be due to the limited proteolysis of the primary product (predicted MW = 43,000 daltons).

Protein phosphatase activity of extracts of E. coli cells transformed with recombinant plasmids: We next determined whether the crude extract of *E. coli* cells harboring the recombinant plasmids contained protein phosphatase activity. Little activity was found ($\leq 1 \times 10^{-4}$ units/mg protein) in the cell extracts, transformed with vector alone regardless of the presence or absence of Mg^{2+} in the assay mixture (data not shown). The extracts of *E. coli* transformed by pKHP2CR also showed little protein phosphatase activity (Table 1), and addition of IPTG did not increase enzyme activity of these extracts. Extracts of *E. coli* harboring pKHP2C showed Mg^{2+} dependent protein phosphatase activity, and this activity was increased with IPTG treatment (Table 1). The Mg^{2+} dependency of the enzyme activity adds further credence to our identification of the induced protein as type 2C protein phosphatase since this cation is an absolute requirement for the enzyme activity (21). The relatively low level of induction by IPTG (40-50%) may be attributed to the fact that the 5'-untranslated region derived from the original cDNA was not optimal for the function of the *tac* promoter in our construct.

We have shown for the first time the expression of a cDNA in *E. coli* cells generating an active type 2C protein phosphatase. The isolation of full length cDNA clones of the catalytic subunits of type 1 and 2A protein phosphatases and a cDNA clone encoding a portion of type 2B protein phosphatase have already been reported (4-14). However, the expression of any of those cDNAs has yet to be performed and thus their identification must be regarded as tentative. The 2C phosphatase protein produced in *E. coli* cells will be a useful tool for a further understanding of the function of type 2C protein phosphatase.

Table 1. Protein Phosphatase Activity of *E. coli* Extracts from Cells Harboring Recombinant Plasmids

	experiment 1		experiment 2	
	- Mg^{2+}	+ Mg^{2+}	- Mg^{2+}	+ Mg^{2+}
	units enzyme activity/mg protein x 10^{-4}			
pKHP2C, - IPTG	0.3	6.0	0.3	3.9
pKHP2C, + IPTG	0.1	8.4	0.4	5.9
pKHP2CR, - IPTG	0.1	0	0.2	0.1
pKHP2CR, + IPTG	0.1	0.1	0.1	0.2

Extracts of *E. coli* cells transformed by pKHP2C or pKHP2CR and incubated at 37°C for 4 h with or without 2 mM IPTG were assayed for protein phosphatase activity in the presence or absence of 5 mM $MgCl_2$.

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