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Cloning of the complete coding region for human protein phosphatase inhibitor 2 using the two hybrid system and expression of inhibitor 2 in *E. coli*

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Abstract

The yeast two hybrid system has been employed to identify cDNAs encoding proteins which interact with the γ_1 isoform of human protein phosphatase 1. Here we report the isolation of a cDNA encoding human protein phosphatase inhibitor 2. The deduced human sequence of 205 amino acids shows 92% identity to inhibitor 2 from rabbit. Human inhibitor 2 was expressed in *E. coli* and purified to homogeneity. The expressed human protein inhibited both native and bacterially expressed PP1, with the same K_i (1 nM) as inhibitor 2 purified from skeletal muscle. A gene or pseudogene for inhibitor 2 may be present near the major histocompatibility complex on chromosome 6.

Key words: Inhibitor 2; Protein phosphatase; Two hybrid system; cDNA sequence; Human

1. Introduction

The reversible phosphorylation of proteins is a major mechanism by which eukaryotic cellular functions are controlled, the regulation of protein phosphatases being an integral part of this control mechanism [1,2]. Specific targetting subunits have been identified which bind PP1 catalytic subunits (PP1C) to particular subcellular locations and modulate their activity. In skeletal muscle, a 124 kDa targetting subunit associates with PP1C to form a heterodimer referred to as PP1G which binds to glycogen with high affinity and enhances dephosphorylation of glycogen-bound PP1 substrates, such as glycogen synthase and phosphorylase kinase, but not non-glycogen-associated substrates such as myosin P-light chain (reviewed in [3,4]). Phosphorylation of the G subunit in response to insulin enhances the activity of PP1G towards glycogen-bound substrates [5] while phosphorylation of the G subunit in response to adrenaline causes dissociation of PP1C from the G subunit [6]. Interestingly, the G subunit also associates with the sarcoplasmic reticulum of striated muscle [7,8]. Thus, a single targetting subunit can localise the catalytic subunit to at least two target loci.

Other targetting subunits interact with PP1C. In smooth muscle, PP1C associates with a 130 kDa and a 20 kDa subunit to form PP1M [9]. This heterotrimer has enhanced activity towards phosphorylated smooth muscle myosin, but reduced activity towards phosphorylase, compared with PP1C. Striated muscle also contains a PP1M complex which has enhanced activity (relative to PP1C) towards skeletal muscle myosin [10]. A 16–18 kDa protein, termed NIPP-1, forms an inhibitory complex with nuclear PP1C in mammalian cells, the inhibition being relieved by phosphorylation [11], while in fission yeast, the 30 kDa protein product of the *sds22* gene is a nuclear protein which may modulate the activity of PP1C towards nuclear substrates [12].

In addition to its interaction with targetting subunits, PP1C binds to two cytosolic proteins, inhibitor 1 (I-1) and inhibitor 2 (I-2), which completely abolish its activity at nanomolar concentrations [1,13]. These small thermostable proteins sequester free PP1C into heterodimeric complexes, and thus prevent unwanted dephosphorylation events. In addition, I-2 has been shown to act like a molecular chaperone, being required to fold three bacterially expressed isoforms of PP1 into a conformation with the same properties as the native enzyme [14].

Since PP1C comprises a major portion of cellular phosphatase activity and dephosphorylates many phosphoproteins in vitro, it is likely that other targetting subunits exist for this enzyme. In an attempt to isolate such proteins, we have utilised the yeast two hybrid sys-

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tem [15,16,17]. This method employs the yeast GAL4 transcription factor which can be dissected into autonomous DNA-binding and transcription-activating domains. When two fusion proteins, each containing a separate half of the GAL4 protein, are brought together via interaction of their heterologous proteins, an active GAL4 transcription complex is reconstituted. The latter then activates a suitable reporter gene which allows a screen to be performed. This and similar systems have been used successfully by several groups to confirm suspected protein–protein interactions [18,19] or to identify novel protein–protein interactions using libraries of fusion proteins [16,17,20]. Here we describe the use of PP1γ_1 cDNA [21] in the two hybrid system to isolate a cDNA encoding the human protein phosphatase inhibitor 2. We present the complete coding sequence of the human I-2 cDNA together with purification and characterisation of I-2 protein expressed in *E. coli*.

2. Materials and methods

2.1. Bacterial and yeast strains

E. coli strain DH5α (*supE44* ΔlacU169(φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) were the transformation recipient for all plasmid constructions except during the expression of I-2 in *E. coli*, when *E. coli* strain BL21(DE3) pLysS (Novagen) was used. The *S. cerevisiae* host used in the yeast two hybrid system was Y190 [16,17]. This strain contains a mutated *CYH* gene, resulting in resistance to cycloheximide at 2.5 μg/ml.

2.2. Media and materials

E. coli were grown on Luria Broth (LB) plates or in liquid media which was LB or terrific broth (TB) supplemented as necessary with chloramphenicol (25 μg/ml) and/or ampicillin (50–100 μg/ml). Yeast were grown on YPD, YAPD or synthetic complete (SC) medium lacking appropriate amino acids [22]. For library screening in yeast, 3-aminotriazole (3-AT; Sigma) was included at 40 mM final concentration in SC minus tryptophan (Trp), leucine (Leu) and histidine (His). For detection of β-galactosidase activity, yeast were grown on SC-X plates lacking His, Leu and Trp. SC-X plates contain SC medium as above plus: 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 75 mM KOH, 0.8 mM $MgSO_4$, 2 μM $Fe_2(SO_4)_3$ (pH adjusted to 7.0 with 1 M KOH) and 0.1 mg/ml X-Gal added immediately before pouring plates (L.H. Johnston, NIMR, London; personal communication). Restriction endonucleases were purchased from Boehringer or Amersham, Taq polymerase from Promega, T4 DNA ligase from Amersham and Zymolyase from ICN-Flow. dNTPs and ATP were purchased from Pharmacia and [α-^{35}S]dATP and [γ-^{32}P]ATP from Amersham. Oligonucleotides were synthesised by Alastair I. Murchie (University of Dundee) on an Applied Biosystems model 394 DNA synthesizer. The anti-HA antibody (ref: PO(B10 59367) used for immunoblotting was obtained from the Berkeley Antibody Co. Bacterially expressed PP1γ_1 was purified as in [14]. Native PP1 and PP2A catalytic subunits were purified from rabbit skeletal muscle by Dr. Carol MacKintosh and Dr. Don Schelling, respectively [27].

2.3. Construction of the pASPP1γ_1 plasmid

The coding region of the cloned human PP1γ_1 cDNA [21] was amplified by PCR as detailed previously [14]. This product was digested with *Nde*I and *Bam*HI restriction endonucleases and cloned into the *Nde*I-*Bam*HI sites of plasmid pAS2 [17].

2.4. Extraction of proteins from yeast and Western blotting

Crude cell extracts containing soluble proteins were prepared from yeast cells by a modification of [23]. 2.5 ml cultures were grown to an A_{600} of 0.5 in YPD or selective media, harvested by centrifugation, washed in 100 μl of 100 mM Tris-HCl (pH 8.5), 10 mM EDTA, 10 mM β-mercaptoethanol, then resuspended in 100 μl of lysis buffer (100 mM Tris-HCl (pH 7.0), 2 mM EDTA, 7 mM β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine). 100 μl of glass beads (500 μm diameter) were added and the tubes vortexed for 10 min at 4°C. The lysate was collected by puncturing the bottom of the tube with a hot 23-gauge needle, placing the tube inside another 1.5 ml microfuge tube and centrifuging in a bench-top centrifuge at 2,000×*g* for 3 min. The extract in the bottom tube was centrifuged for 1 min at 13,000 rpm. The supernatant was frozen in liquid nitrogen and stored at −70°C.

Protein extracts were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue R-250. Blotting onto nitrocellulose (BA 85, Schleicher and Schuell) was performed using a Transblot apparatus (Bio-Rad) according to the manufacturer's instructions. Non-specific protein binding was reduced by incubation in TSTM (5% Marvel dried milk powder in 100 mM Tris-HCl (pH 7.5), 0.9% NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature. The blot was incubated for 1 h in a 1/1000 dilution of anti-HA antibody in TSTM, then developed using the Vectastain alkaline phosphatase system (Vector laboratories).

2.5. Library screening

Yeast strain Y190 containing plasmid pASPP1γ_1 was grown in 100 ml of SC minus Trp medium at 28°C until a density of 1×10^7 cell/ml was obtained. This culture was diluted 5 times into YAPD (total volume 500 ml) and regrown until the cell density was again 1×10^7/ml. Transformation with 50 μg of human peripheral B lymphocyte cDNA library in plasmid pACT [16] was performed according to [24]. Equal portions of the transformation mix were spread onto 300 mm plates containing SC minus Trp, Leu, His plus 40 mM 3-AT and incubated at 28°C. Portions of the transformation mix were also spread on SC minus Leu and SC minus Leu, Trp plates to determine transformation frequency. Colonies that grew were replated on SC medium minus Trp, Leu, His plus 40 mM 3-AT, then tested for β-galactosidase activity by plating on SC-X plates. Colonies that turned blue after incubation for up to 2–3 days were analysed further.

2.6. Recovery of plasmids from yeast and sequence analysis

Total yeast nucleic acid was prepared according to [22] except that RNase was not added. The nucleic acid was then used to transform electrocompetent *E. coli* by electroporation using an 'Easyject plus' (Equibio) electroporator according to the manufacturer's instructions. Transformants were plated on LB plates containing ampicillin and plasmid DNA was prepared from single colonies [25]. Plasmid DNA was digested with restriction endonuclease *Bgl*II and fragments separated by agarose gel electrophoresis. Plasmids not generating DNA fragments characteristic of the pASpp1γ_1 construct digested with *Bgl*II were analysed further.

DNA sequence analysis was performed using either [α-^{35}S]dATP and Sequenase enzyme according to the manufacturer's instructions (US Biochemicals) or an Applied Biosystems 373A automated DNA sequencer, again according to the manufacturer's instructions, using Taq dye terminator cycle sequencing.

2.7. Expression of I-2 in E. coli

The open reading frame region of the human I-2 cDNA sequence was amplified by PCR using the two oligonucleotides 02999 (5′-GCGC-CATATGGCGGCCTCGACGGCCTC-3′) and 02939 (5′-ACTTTG-TAAGAGCTACCAC-3′). Oligonucleotide 02999 creates an *Nde*I site (underlined in oligonucleotide sequence) at the initiating methionine codon (in italics) and the priming site for oligonucleotide 02939 resides in the 3′ non-coding region of the cDNA downstream of a unique *Cla*I site at position +790 with respect to the A of the initiating methionine codon. Digestion of the PCR product with restriction endonucleases *Nde*I and *Cla*I allowed the complete I-2 coding sequence to be cloned into the *Nde*I-*Cla*I sites of the plasmid pT7–7 [26] to generate plasmid pT7-I2. After verifying that the sequence of the pT7-I2 construct was correct, the plasmid was transformed into *E. coli* strain BL21(DE3)pLysS and transformants selected on LB plates containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). A single colony was inoculated into 5 ml LB containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) and grown overnight at 37°C. This was then diluted 1:100 into 2 litres of the same culture medium and grown

to an A_{595} of 0.6 prior to addition of IPTG to a final concentration of 0.4 mM. After continued incubation at 37°C for 3 h, the bacteria were harvested by centrifugation and resuspended in 40 ml of buffer A (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine). The suspension was incubated in a boiling water bath for 15 min with stirring, cooled on ice, then insoluble material removed by centrifugation at 25,000×g for 15 min. The supernatant was sonicated for 1 min using a Jencons probe sonicator (model GE 50) to shear chromosomal DNA, incubated in a boiling water bath for 5 min, cooled and centrifuged at 45,000×g for 20 min. The supernatant was loaded onto a HiLoad 16/10 Q-Sepharose column (Pharmacia) which was developed with a 400 ml 0–500 mM linear NaCl gradient. The peak fractions of I-2 which eluted at 280 mM NaCl were pooled and the buffer was exchanged for 20 mM NH_4 acetate (pH 5), 0.1 mM EGTA, 1 mM DTT using an Amicon 8050 stirred cell with a YM10 filter. After incubating in a boiling water bath for 5 min then cooling, the sample was loaded onto a Mono Q column (Pharmacia) and developed with a 40 ml 0–250 mM linear NaCl gradient. The peak fraction of I-2 which eluted at 170 mM NaCl was collected and stored at −20°C.

2.8. Protein phosphatase and inhibitor 2 assays

^{32}P-labelled rabbit skeletal muscle glycogen phosphorylase was prepared by phosphorylation with phosphorylase kinase to a stoichiometry of 1 mol phosphate per mol subunit [27]. The specific activity of the [γ-^{32}P]ATP used for all phosphorylations was 10^6 cpm/nmol. Phosphatase assays were performed in the absence of divalent cations [27] using the above substrate. One unit of phosphatase activity is that amount of enzyme which catalyses the release of 1 μmol [^{32}P]phosphate/min from phosphorylase *a*. Inhibitor 2 was assayed as in [28]. One unit of I-2 was that amount which inhibits the dephosphorylation of 0.01 mU of PP1 by 50% in the standard 30 μl assay.

3. Results

3.1. Isolation of the human inhibitor 2 cDNA using the yeast two hybrid system

After cloning the complete open reading frame of human PP1γ_1 into the pAS2 plasmid, expression of the GAL4 DNA-binding domain-PP1γ_1 fusion protein in *S. cerevisiae* strain Y190 was verified by immunoblotting using the anti-HA antibody, which recognises an epitope attached to the GAL4 DNA binding domain. A band of the expected molecular mass (50 kDa) was seen only in protein extracts from yeast cells containing the pASPP1γ_1 plasmid (data not shown). This construct did not activate transcription from the UAS_G (the DNA sequence which binds GAL4) as detected by lack of growth of Y190 cells containing the plasmid on SC minus His medium containing 40 mM 3-AT. In order to isolate proteins capable of interacting with PP1γ_1, yeast cells containing the pASPP1γ_1 construct were transformed with DNA from a pACT library containing human peripheral B lymphocyte cDNA sequences fused to the GAL4 transactivation domain. After growth on the selection medium for 12 days, ten colonies were obtained, representing 0.005% of the cells transformed. These

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CCAATGGCGGCCTCGACGGCCTCGCACCGG CCCATCAAGGGGATCTTGAAGAACAAGACC TCTACGACTTCCTCTATGGTGGCGTCGGCC    87
   (M) A  A  S  T  A  S  H  R  P  I  K  G  I  L  K  N  K  T  S  T  T  S  S  M  V  A  S  A      28
    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  S  .  .  .  R  .  .  .  .

GAACAGCCCCGCGGGAATGTCGACGAGGAG CTGAGCAAAAAATCCCAGAAGTGGGATGAA ATGAACATCTTGGCGACGTATCATCCAGCA   177
 E  Q  P  R  G  N  V  D  E  E  L  S  K  K  S  Q  K  W  D  E  M  N  I  L  A  T  Y  H  P  A     58
 .  .  .  .  .  S  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

GACAAAGACTATGGTTTAATGAAAATAGAT GAACCAAGCACTCCTTACCATAGTATGATG GGGGATGATGAAGATGCCTGTAGTGACACC   267
 D  K  D  Y  G  L  M  K  I  D  E  P  S  T  P  Y  H  S  M  M  G  D  D  E  D  A  C  S  D  T     88
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  I  .  .  .  D  .  .  Y  .  .  .

GAGGCCACTGAAGCCATGGCGCCAGACATC TTAGCCAGGAAATTAGCTGCAGCTGAAGGC TTGGAGCCAAAGTATCGGATTCAGGAACAA   357
 E  A  T  E  A  M  A  P  D  I  L  A  R  K  L  A  A  A  E  G  L  E  P  K  Y  R  I  Q  E  Q     118
 .  T  .  .  .  .  T  .  .  T  .  .  K  .  .  .  .  .  .  .  S  .  .  .  .  .  .  R  .  .

GAAAGCAGTGGAGAGGAGGATAGTGACCTC TCACCTGAAGAACGAGAAAAAAAAGCGACAA TTTGAAATGAAAAGGAAGCTTCACTACAAT   447
 E  S  S  G  E  E  D  S  D  L  S  P  E  E  R  E  K  K  R  Q  F  E  M  K  R  K  L  H  Y  N     148
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

GAAGGACTCAATATCAAACTAGCCAGACAA TTAATTTCAAAAGACCTACATGATGATGAT GAAGATGAAGAAATGTTAGAGACTGCAGAT   537
 E  G  L  N  I  K  L  A  R  Q  L  I  S  K  D  L  H  D  D  D  E  D  E  E  M  L  E  T  A  D     178
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  E  .  .  .  .  .  S  .  .  .  .

GGAGAAAGCATGAATACGGAAGAATCAAAT CAAGGATCTACTCCAAGTGACCAACAGCAA AACAAATTACGAAGTTCATAGACGAGATTT   627
 G  E  S  M  N  T  E  E  S  N  Q  G  S  T  P  S  D  Q  Q  Q  N  K  L  R  S  S  *              204
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  R  .  .  .  S  Q  .  .  *

GTTCAACACTGCAATTGTTTGTTAGATGTA AACCCTGTGACTATAGTACGTTGCTTCTTG TTCTTCACAATTCATGACTTAAGTACCAAA   717
ATGCATACCAGTTATTATATATTGCCAAGA ATTAAATGATAAACTTAGAGACTGATTAGA CTGAAAATGCCTAATCGATATATATATTCT   807
TGTGCCTAGTACTTTACCACAAATACAGTG TAATATCATCAGTCCAAAACTGCATTACTT TTGTAAAAACACTGGTTAATTTGTATAAGA   897
TATTATAGAGCTTTTTATGCTTTAGAAGTT AAACAATATCTTTGGGGGGGAACTAATTTA TTTTCATCACTTGAAATGTGGTAGCTCTTA   987
CAAAGTTTATTGATTTGATTTTTTAAAAA TCAAAAGCCAATTGAACAACAGGATATATA GACTGATAAATATTTAGGCTGAATAGTATT  1077
TTAACACTTGTCTTCAACTTGATTTGTCTG TTTAATTGAAAAGAATTATAAGAGTTACTG TTGCATTTTCTGACCTACTATTTTTAAAAT  1167
TCCTGTTGAGTTTCTTTGTGTTTACAAGGA AAGGACTGAACTTTTTCTCATCAAAACTAG CTTTTTTCCCCACAAATAAATTATCAGGTT  1257
AAACTTTC(A)n                                                                                   1265
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Fig. 1. cDNA and encoded protein sequence of human I-2. The amino acid sequence of rabbit I-2 is shown below the human sequence. Identities with the human sequence are indicated by periods. Numbering within the cDNA is with respect to the first nucleotide of the initiation codon. Numbering of the amino acids is from the Ala following the initiating Met,which is cleaved from the mature protein, and corresponds with that used in [29]. The polyadenylation signal within the human I-2 cDNA is underlined.

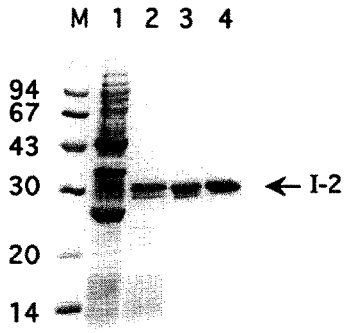

Fig. 2. Purification of human inhibitor 2 from *E. coli* induced to express the protein for 3 h. Samples of protein from various stages of the purification procedure were separated on a 12.5% SDS-PAGE and visualised by staining with Coomassie blue. Tracks are: (1) 7 μg of total *E. coli* protein; (2) 3 μg of protein from boiled *E. coli* extract; (3) 1 μg of protein from Q-Sepharose fraction containing peak I-2 activity; (4) 1 μg of protein from Mono-Q fraction containing peak I-2 activity. Markers are in kDa. Human I-2 protein is indicated by an arrow.

colonies were taken through another round of selection on medium containing 3-AT, then plated onto SC-X plates to determine whether they possessed β-galactosidase activity. Seven of the original ten colonies showed a blue colour, indicating that the β-galactosidase gene was being transcribed and translated in these transformants (data not shown). pACT plasmids were recovered from these clones and their inserts sequenced. One clone was found to encode a protein with very high similarity to the rabbit skeletal muscle inhibitor 2 protein [29]; a specific and potent inhibitor of PP1 activity [30].

3.2. Sequence of the human inhibitor-2 cDNA

Fig. 1 shows the complete coding and 3′ non-coding sequence of the human I-2 cDNA. The predicted human I-2 protein contains 205 amino acids and has a calculated molecular mass of 23,015 Da. Fig. 1 also shows a comparison of the predicted human and rabbit [29,31,32] I-2 protein sequences. The two proteins share 92% sequence identity, which rises to 95% sequence similarity if conservative changes are included.

3.3. Isolation and properties of human inhibitor 2 expressed in E. coli

In order to express human I-2 protein in bacteria the complete coding sequence was cloned into the *E. coli* expression vector pT7–7 as described in the materials and methods section. Induction of cultures containing the expression vector resulted in production of a protein with an apparent molecular mass of 31 kDa on SDS-PAGE (Fig. 2). Although this is larger than that predicted from the sequence, it agrees well with the estimated size of rabbit I-2 on SDS-PAGE [29] which is also known to migrate aberrantly in this electrophoretic system. Human I-2 is detectable in boiled extracts 30 min after the start of induction and is present in maximal amounts at 3 h, when it constitutes approximately 2% of total *E. coli* protein.

The I-2 protein was purified from cultures of *E. coli* induced for 3 h with a final yield of 20% (Fig. 2, Table 1). The purification procedure takes advantage of the known properties of the I-2 protein. The first heating step removes over 90% of contaminating proteins without loss of I-2. The two column steps exploit the low number of histidine residues in I-2. Hence, at markedly different pH values, the protein elutes from anion exchange columns at similar salt concentrations. This allows contaminating proteins to be separated from the major peak of activity. The I-2 protein purified by this method consisted essentially of a single product migrating at 31 kDa on SDS-PAGE, although a minor product of slightly smaller size was also seen (Fig. 2). N-terminal sequencing of the protein preparation confirmed that the major protein present was I-2 and that this protein lacked the N-terminal methionine residue. This analysis also showed that the minor contaminant is a degradation product of I-2, which initiated 13 amino acids after the N-terminal methionine (data not shown).

The purified human I-2 was assayed for its ability to inhibit type 1 and type 2A protein phosphatases in the standard phosphorylase phosphatase assay [27]. It inhibited the bacterially expressed PPIγ_1 catalytic subunit [21] as well as native PP1C purified from rabbit skeletal muscle [27] with an IC_{50} of 1–2 nM, and had no effect on the activity of PP2A catalytic subunit, in accordance with the properties of I-2 protein purified from rabbit skeletal muscle [28] (Fig. 3).

4. Discussion

We have used the yeast two hybrid system [16] to identify novel proteins which interact with a human protein phosphatase 1 catalytic subunit, PP1γ_1 [21]. One such protein was found to be 92% identical in sequence to the rabbit skeletal muscle inhibitor 2 [29], and therefore presumed to be the human homologue. This was confirmed by expression of the human I-2 in *E. coli*, followed by examination of its properties. Since I-2 was previously known to interact with PP1 (reviewed in [1]), our studies demonstrate that the yeast two hybrid system

Table 1
Purification of human I-2 from *E. coli* expressing the full-length protein

	Volume (ml)	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Yield (%)	Purification (-fold)
Culture	2000	148	8×10^6	5.4×10^4	100	1
Boiled *s/n*	40	11.2	8.4×10^6	7.5×10^5	105	14
Q-Sepharose	24	2.9	4×10^6	1.4×10^6	51	26
Mono-Q	1.5	0.57	1.5×10^6	2.6×10^6	19	48

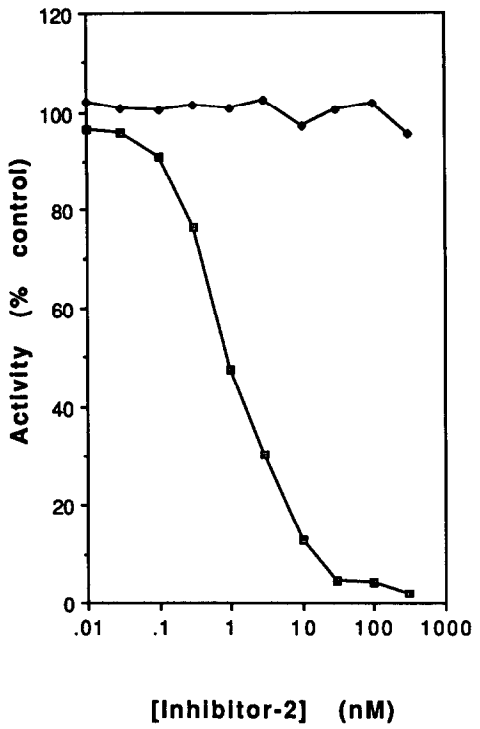

Fig. 3. Effect of bacterially expressed human I-2 on the activity of PP1 (□) and PP2A (●) catalytic subunits purified from skeletal muscle.

is applicable to the isolation of PP1C-associated proteins. In addition, recent studies looking for proteins interacting with retinoblastoma protein identified an interaction between a putative PP1C isoform using the same system [16].

The sequence of human I-2, deduced from the cDNA is one amino acid longer than the rabbit sequence encoded by the cDNA isolated by Zhang et al. [32]; the extra amino acid being a serine residue at the C-terminus. However, fast atom bombardment mass spectrometry of I-2 peptides predicted a C-terminal serine in the rabbit protein [31] additional to the sequence originally determined by the Edman method [29]. N-Terminal sequencing of the bacterially expressed human inhibitor 2 revealed that the initiating methionine residue is removed in *E. coli*. This may also occur in human cells, since rabbit I-2 purified from skeletal muscle lacks an initiating methionine encoded by the rabbit cDNA [29,32]. Although the human I-2 sequence determined here is from B lymphocytes, it is unlikely to be different in other tissues, since I-2 has been shown to be immunologically identical in a variety of rabbit tissues [33]. The overall identity of the human and rabbit I-2 sequences is 92%, and this high conservation of sequence between mammalian species might be expected, since human and rabbit PP1α catalytic subunit sequences are identical [34] as are their PP1β sequences [35].

It has been known for several years that inhibitor 2 can be phosphorylated by glycogen synthase kinase 3 (GSK3) on Thr-72 and by casein kinase II (CKII) on Ser-86, Ser-120 and Ser-121 [36], but it has only recently been demonstrated that one function of phosphorylation of Thr-72 allows newly synthesized PP1 to adopt its native conformation [14]. The phosphorylation of Ser-86 by CKII appears to increase the rate of phosphorylation of Thr-72 by GSK3 [37]. As would be expected Thr-72 and the serine residues at 86, 120 and 121, are conserved between the rabbit and human proteins.

A search of the GenEMBL data base with the human I-2 cDNA sequence showed that 0.12 kb of the 3'-untranslated region of human I-2 (nucleotides 1115–1235) exhibits 90% identity to a section near one end of a genomic DNA sequence containing the class II major histocompatibility (MHC) complex on the short arm of chromosome 6 [38]. Since this fragment just precedes the polyA tail in the I-2 cDNA, it is unlikely that its presence in the cDNA is an artefact. The region was not known to be transcribed in the MHC complex. However, the occurrence of only 90% identity between the cDNA and the genomic DNA indicates that a pseudogene or second gene for I-2 is more likely to be present near the MHC complex, rather than the gene encoding the I-2 sequence identified here.

Expression of human I-2 in *E. coli* enabled isolation of >0.5 mg of the pure protein from 2 litres of bacterial culture. The pure human I-2 protein inhibited PP1C activity in the standard phosphorylase phosphatase assay with an IC_{50} of 1 nM, while having no inhibitory effect on the PP2A catalytic subunit. Thus the expressed human I-2 protein purified from *E. coli* behaved identically to I-2 protein purified from rabbit skeletal muscle. The availability of large amounts of pure I-2 protein will allow further characterisation of the properties of this protein, including its interaction with PP1C. It can also provide a valuable source of I-2 for general use in protein phosphatase assays.

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