The cloning expression and tissue distribution of human PP2Cβ

Anna E. Marley*, Adam Kline, Garry Crabtree, Jane E. Sullivan, Raj K. Beri

Cardiovascular, Musculoskeletal and Metabolism Department, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Received 1 June 1998

Abstract We have cloned a novel PP2Cβ isoform from a human liver cDNA library which codes for a protein homologous to other mammalian PP2Cβs at the N-terminus but with an extended C-terminus that is unique amongst the PP2Cs. The protein expressed in *E. coli* is indistinguishable from human recombinant PP2Cα in its cation dependence and insensitivity to okadaic acid. Northern blot analysis of PP2Cβ along with that of PP2Cα shows that human PP2Cs are widely expressed and are most abundant in heart and skeletal muscle.

Key words: Protein phosphatase 2C; Protein phosphorylation

1. Introduction

Protein phosphorylation/dephosphorylation is one of the main regulatory systems of cellular function. The major protein serine/threonine phosphatases in eukaryotic cells have been divided into four groups: PP1, PP2A, PP2B and PP2C [1]. PP2C is distinguished from the other groups of phosphatases by its absolute requirement for Mg^{2+} (or Mn^{2+}) and its insensitivity to the tumour promoter okadaic acid [2].

PP2C is a monomeric, cytosolic enzyme and two highly conserved isoforms (α and β) have been found in rodents [3]. The human PP2Cα isoform has been characterised [4] and recently a novel isoform PP2Cδ has been described [5]. Here we report a novel human PP2Cβ isoform PP2CβX that has a high degree of similarity to rodent PP2Cβ at the N-terminus but with a unique extended C-terminus.

2. Materials and methods

2.1. Cloning

A 1.2 kb probe encoding the open reading frame of rat PP2Cβ (P35815) was generated by PCR and used to screen a UniZap human liver library (Stratagene). DNA probe was prepared using random prime labelling (Pharmacia). Filters were hybridised overnight at 55°C in 50% formamide, 4×SSPE, 20×Denhardts and 2 μg/ml salmon sperm DNA, with a probe concentration of 22 ng/ml. Filters were washed in 2×SSC for 30 min at room temperature, 0.5×SSC for 30 min at room temperature and 0.5×SSC for 30 min at 65°C. The filters were exposed to film overnight at −70°C with intensifying screens. A single positive clone (βXa) was rescued using R408 helper phage following the manufacturers instructions. Since this clone was incomplete, the 5′ end of the gene was amplified from the same library using a vector sense primer combined with an antisense oligo from the 5′ end of the clone described above. A PCR product (βXb) overlapped βXa but was also 5′ incomplete. The remaining 40 base pairs of the 5′ end were generated by PCR amplification from a human liver GT10 library (Clontech) using a vector sense oligo and antisense oligo based on the sequence of βXa/b.

2.2. Expression and analysis of phosphatase activity

A full length cDNA based on the combined sequence of βa/b/c was generated by amplification from HepG2 cell cDNA using HiFi polymerase (Boehringer Mannheim). The oligos were tagged with a 5′ *Nde*I site (GGGAATTCCATATGGGTGCATTTTTGGAT) and 3′ *Xho*I site which also introduced a C-terminal 6 histidine tag to allow purification on a nickel column (CCGCTCGAGTCAGTGGTGGTGGTGGTGGTGTATTTTTTCACCACTCAT). The PCR product was cloned into the TA cloning vector pGEM (Promega) digested with *Nde*I/*Xho*I and cloned into the *E. coli* expression vector pET21a (Novagen). Recombinant plasmids transformed into DH5α cells (Gibco/BRL) were selected by ampicillin resistance and the sequence checked for PCR errors. Plasmid DNA from an error free clone was used to transform BL21(DE3) cells (Novagen). A culture was grown to an OD_{550} of 0.6 and induced with 1 mM IPTG for 2 h at 37°C along with a colony transformed with pET21a alone. Cells were harvested by centrifugation and a 1 ml sample was resuspended in buffer (50 mM Tris, 5 mM EDTA, pH 8), sonicated and the supernatant removed. The insoluble material was resuspended in 1 ml of the above buffer and 50 μl each of the soluble and insoluble material were run on an SDS reducing polyacrylamide gel. The gel was Coomassie stained to assess protein levels. The remainder of the cell pellet was purified on histidine binding resin (Novagen) following the manufacturers instructions. Purified protein was reconstituted in phosphatase assay buffer (50 mM EDTA, 0.5 mM DTT, 10 mM $MnCl_2$) and assayed with para-nitrophenyl phosphate as substrate as described previously [4]. K_m for activity with this substrate was determined using Enzfitter analysis.

2.3. Northern analysis

A commercial Northern blot containing human poly(A)+ RNA from a range of normal human tissues (Clontech) was used for the analysis. Probes corresponding to the unique C-termini of PP2CβX and PP2Cα were generated by PCR and were prepared by random prime labelling (Pharmacia). Filters were pre-hybridised at 65°C for 15 min and hybridised for 2.5 h at 65°C in rapid hybridisation buffer (Amersham). Filters were washed in 2×SSC/0.05% SDS at room temperature and then exposed to film overnight with intensifying screens at −70°C.

3. Results and discussion

3.1. Cloning and protein analysis

The cDNA sequence of PP2CβX (Fig. 1) was compiled from three sequences generated from human liver libraries as described in Section 2 giving an open reading frame of 1.44 kb. A full length cDNA was amplified from HepG2 cells using primers based on this composite clone and the sequence was verified by sequence analysis. As can be seen from the comparison of this sequence with other mammalian PP2Cβs (Fig. 2) the protein is similar to other β isoforms up to the divergence point, glycine 378 (*), but has an extended C-terminal region. This unique region of the sequence does not share homology with any other known protein as determined by BLAST analysis. The protein is 55 kDa in size and is expressed in *E. coli* as a partially soluble protein which puri-

*Corresponding author. Fax: +44 (1625) 583074.

Abbreviations: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s)

The sequence reported in this paper has been deposited in the EMBL database (accession no. AJ005801).

PII: S0014-5793(98)00708-X

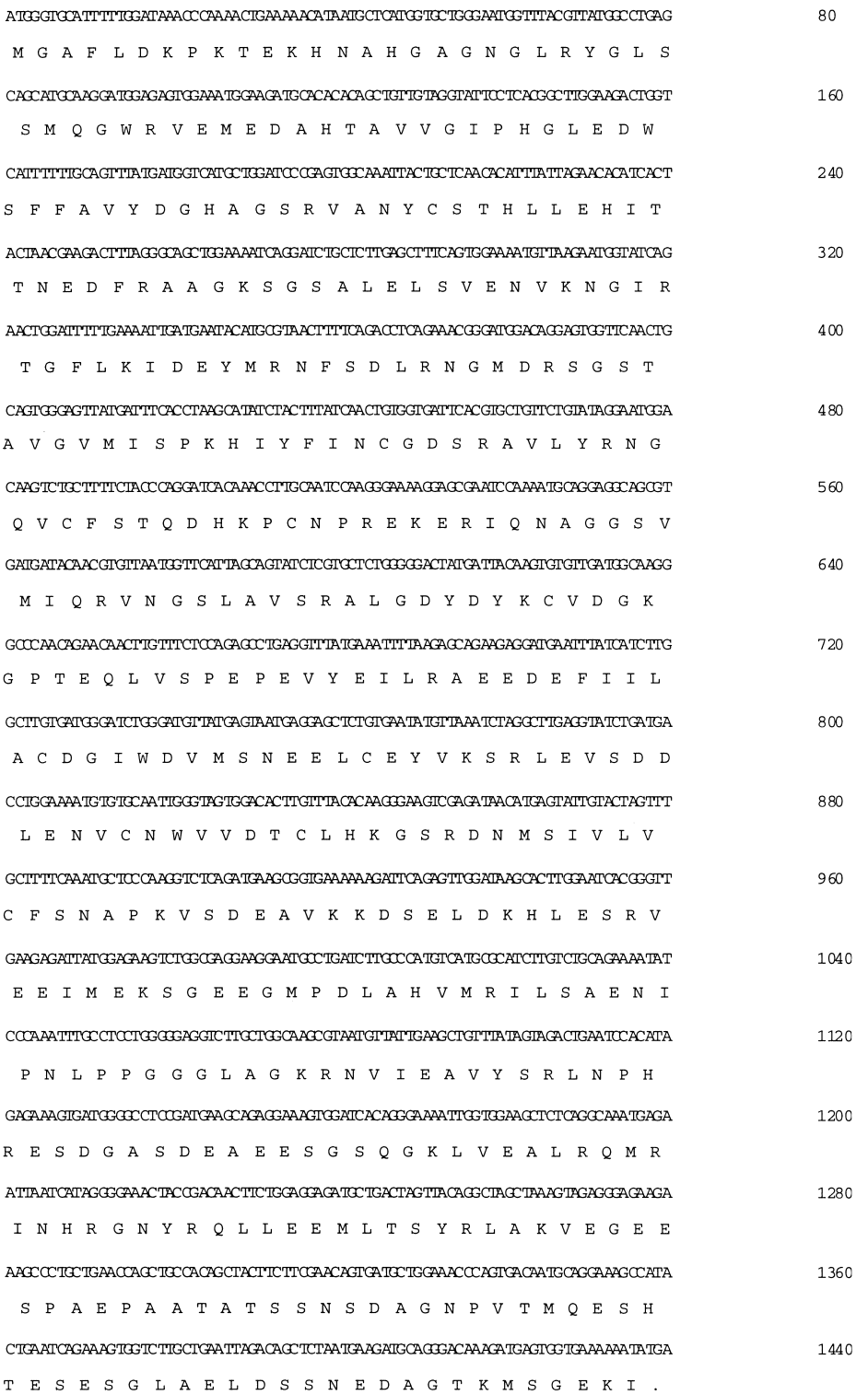

Fig. 1. DNA and protein sequence of PP2CβX.

fied almost to homogeneity on a nickel histidine binding column (Fig. 3). We found that the purified recombinant protein was biochemically indistinguishable from recombinant human PP2Cα when assayed against *p*-nitrophenyl phosphate (NPP) as described previously [4]. The K_m with NPP as substrate was 3.5 mM in the presence of 10 mM Mn^{2+} and no inhibition was seen with okadaic acid. No activity was seen in the absence of Mn^{2+} (results not shown). We recently published a paper in which the region of PP2Cα important for catalytic activity was defined by deleting the C-terminus of the protein [4], and found that the C-terminal part of the protein (9 kDa) was not required for catalytic activity. It is possible that the

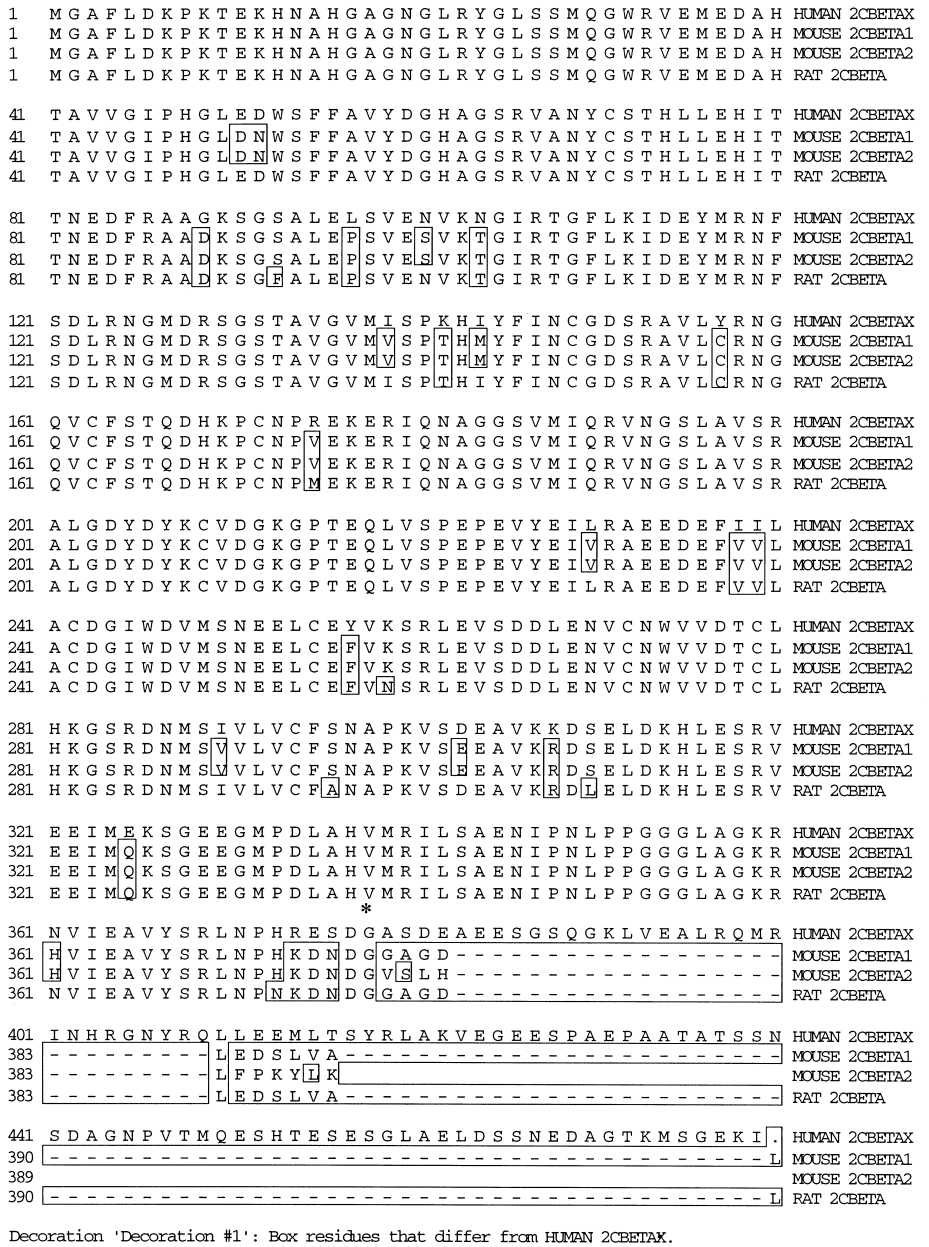

Fig. 2. Alignment of human PP2CβX, rat PP2Cβ and mouse PP2Cβ1 and PP2Cβ2.

differential C-termini of the PP2Cs play a role in substrate targeting, although other workers have failed to identify proteins interacting with purified PP2Cβ from rat liver and muscle [6]. Since the method used involved protein purification it is possible that interacting proteins were dissociated. The availability of cDNAs for the PP2C isoforms will make it possible to search for interactions in a more physiological environment e.g. in a two-hybrid system.

3.2. Northern analysis

PP2CβX and PP2Cα had a similar expression profile being most predominant in skeletal muscle, heart and liver but also evident in other tissues (Fig. 4). The widespread expression of PP2C suggests that it may play a role in regulating phosphorylation/dephosphorylation in a number of tissues. Identification of intracellular substrates for PP2C has been hampered by the lack of specific inhibitors, leaving the role of this enzyme in vivo unclear. However AMPK (AMP-activated protein kinase) has been identified as an intracellular target in hepatocytes using okadaic acid, a phosphatase inhibitor to which PP2C is insensitive [7]. It is interesting to note that the tissue distribution of the PP2C isoforms is similar to that of AMPK being highly expressed in skeletal muscle [8]. However, despite the high level of protein expression, the activity of AMPK in this tissue is low and this might be due to the high level of PP2C in this tissue. The identification of proteins which interact with the PP2C isoforms and in particular with their unique C-termini would help to find substrates of this enzyme and begin to unravel its intracellular function.

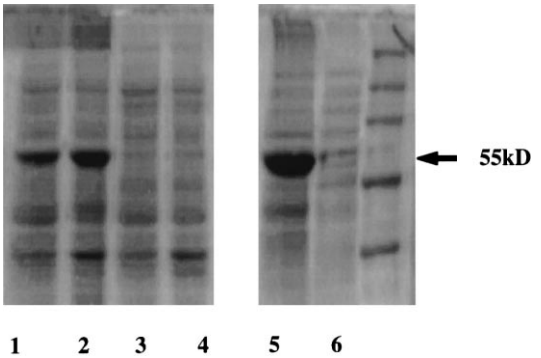

Fig. 3. SDS page gel analysis of recombinant PP2Cβ. Lane 1, soluble PP2C; lane 2, insoluble PP2C; lane 3, soluble control; lane 4, insoluble control; lane 5: nickel column purified PP2C; lane 6, nickel column purified control.

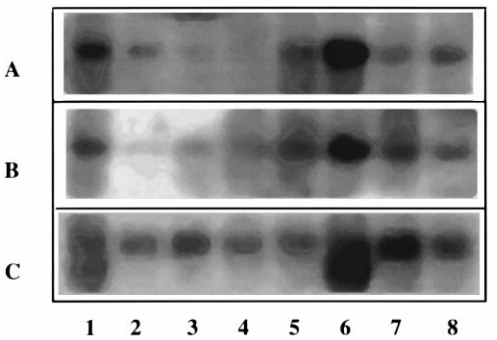

Fig. 4. Northern blots of human PP2CβX (A), PP2Cα (B) and β-actin (C), nucleic acid probes against the following human tissue poly(A)+ RNAs: Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas.

References

[1] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[2] Cohen, P., Klumpp, S. and Schelling, D.L. (1989) FEBS Lett. 250, 596–600.
[3] Wenk, J., Trompeter, H.I., Pettrich, K.G., Cohen, P.T.W., Campbell, D.G. and Mieskes, G. (1992) FEBS Lett. 297, 135–138.
[4] Marley, A.E., Sullivan, J.E., Carling, D., Abbott, W.M., Smith, G.J., Taylor, I.W.F., Carey, F. and Beri, R.K. (1996) Biochem. J. 320, 801–806.
[5] Travis, M. and Welsh, M.J. (1997) FEBS Lett. 412, 415–419.
[6] McGowan, C.H. and Cohen, P. (1987) Eur. J. Biochem. 166, 713–722.
[7] Moore, F., Weekes, J. and Hardie, D.G. (1991) Eur. J. Biochem. 199, 691–697.
[8] Verhoven, A.J., Woods, A., Brennan, C.H., Hawley, S.A., Hardie, D.G., Scott, J., Beri, R.K. and Carling, D. (1995) Eur. J. Biochem. 228, (2) 236–243.