

Isolation of the human genes encoding the Pyst1 and Pyst2 phosphatases: characterisation of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP and SAP kinases

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SUMMARY

We have isolated the human genes encoding the Pyst1 (MKP-3) and Pyst2 (MKP-X) MAP kinase phosphatases. Both genes consist of three exons interrupted by two introns and lack an intron which is conserved in all the other members of this gene family characterised to date. This reinforces the conclusion that Pyst1 and Pyst2 are members of a distinct and structurally homologous subfamily of dual-specificity (Thr/Tyr) MAP kinase phosphatases. We find that Pyst2 mRNA is constitutively expressed in a wide variety of human cell lines including those derived from ovarian, bladder and breast cancers. While there is no evidence for inducible expression of Pyst2 mRNA in human skin fibroblasts in response to cellular stress, Pyst2 mRNA levels are moderately increased in

response to serum stimulation. Pyst2 protein is predominantly cytosolic when expressed in COS-1 cells. In common with Pyst1, Pyst2 shows substrate selectivity for the classical p42 (ERK2) isoform of MAP kinase both in vitro and in vivo, displaying much reduced activity towards stress activated MAP kinase isoforms such as JNK-1 and p38/RK. Pyst2 binds p42 MAP kinase in vivo and both MAP kinase binding and substrate selectivity correlate with the ability of different recombinant MAP and SAP kinases to cause catalytic activation of the Pyst2 phosphatase in vitro.

Key words: MAP kinase phosphatase, Catalytic activation, Signal transduction

INTRODUCTION

The mitogen-activated protein (MAP) kinases are components of signal transduction pathways which are highly conserved from yeast to mammalian cells (Nishida and Gotoh, 1993; Marshall, 1994; Robinson and Cobb, 1997). These relay, amplify and integrate diverse signals, allowing the cell to coordinate a wide variety of cellular functions. These include proliferation, differentiation, development, inflammatory responses and apoptosis. Ten MAP kinase family members have been identified in mammalian cells thus far, and these are classified according to their differential activation by various agonists (Cohen, 1997). The most widely studied of the MAP kinases are the 'classical' 42 and 44 kDa isoforms (also known as ERK2/ERK1 or MAPK2/MAPK1) which respond vigorously to growth factors and phorbol esters and have been associated with cellular proliferation and differentiation (Cobb et al., 1994). A second group of kinases are preferentially activated by cellular stress including osmotic shock, oxidative stress, DNA-damaging agents and inhibitors of protein synthesis. These comprise the stress-activated protein (SAP) kinases and include the c-Jun kinase isoforms JNK-1, JNK-2 and JNK-3 (SAPK1c, SAPK1a and SAPK1b), p38/RK/CSBP

(SAPK2a), p38 β (SAPK2b), ERK6/p38 γ (SAPK3), SAPK4 and ERK5/BMK1 (SAPK5) (Cohen, 1997).

All MAP kinases are activated by phosphorylation of both threonine and tyrosine residues within the conserved signature sequence T-X-Y by a dual specificity MAP kinase kinase (MEK or MKK). In addition to mediating activation of MAP kinases, it has recently been demonstrated that phosphorylation of these key residues is responsible for promoting the dimerisation and nuclear translocation of ERK2/MAPK2 (Khokhlatchev et al., 1998). Other MAP and SAP kinases also form dimers indicating that this is a general mechanism of action of the MAP kinase family. This has important functional implications, as the magnitude and duration of MAP kinase signalling, coupled with nuclear translocation of the enzyme are critical determinants of biological effect (Marshall, 1995). This is best illustrated by studies of the differentiation of rat PC12 cells in culture. These cells proliferate in response to epidermal growth factor (EGF), while exposure to nerve growth factor (NGF) causes cell differentiation marked by the cessation of cell division and outgrowth of neurites. This differential response is entirely due to the ability of NGF to cause both sustained activation and nuclear translocation of MAP kinase. In contrast, EGF causes only a very transient

activation of MAP kinase and the kinase does not enter the cell nucleus (Traverse et al., 1992).

The magnitude and duration of MAP kinase signalling is likely to reflect a balance between the activities of upstream activators such as MEK/MKK and the activities of protein phosphatases. It is now clear that there are specific protein phosphatases which are responsible for regulating MAP kinase activities in yeasts, *Drosophila* and mammalian cells (Keyse, 1998). In particular, a family of dual-specificity (Thr/Tyr) MAP kinase phosphatases (MKPs) typified by the inducible, nuclear CL100 (MKP-1) enzyme appear to play a key role in the regulation of MAP kinase activity in mammalian cells (Keyse and Emslie, 1992; Alessi et al., 1993; Sun et al., 1993). At least nine CL100-like enzymes have now been isolated and characterised which show distinct patterns of transcriptional regulation, tissue/cell distribution and subcellular localisation (Keyse, 1998). These include the Pyst1 phosphatase (also called MKP-3) which is able to bind to p42 (ERK2) MAP kinase and shows remarkable substrate selectivity for the p42/p44 (ERK2/1) isoforms of MAP kinase both in vitro and in vivo (Groom et al., 1996; Muda et al., 1996a,b). Recent work has now shown that p42 (ERK2) substrate binding is accompanied by catalytic activation of Pyst1 (MKP-3) in vitro (Camps et al., 1998) and this may provide a mechanism for targeted inactivation of different MAP kinase isoforms.

In addition to Pyst1, the partial cDNA sequences of both human (Pyst2) and rat (MKP-X) enzymes closely related to Pyst1 (MKP-3) were reported (Groom et al., 1996; Muda et al., 1996a). Furthermore, a third enzyme designated MKP-4 which is more closely related to Pyst1 than to other MAP kinase phosphatases was isolated and found to exhibit substrate selectivity for the p42/p44 MAP kinases (Muda et al., 1997). This suggests that these enzymes might constitute a distinct and structurally homologous subfamily of dual-specificity (Thr/Tyr) MAP kinase phosphatases. Here we present evidence, based on the structure of both the human Pyst1 and Pyst2 genes, that this is indeed the case. Furthermore, we have shown that Pyst2 is a cytosolic protein which displays substrate selectivity for p42 MAP kinase both in vitro and in vivo. Finally we show that the ability of Pyst2 to bind to p42 MAP kinase (ERK2) in vivo correlates with the ability of recombinant MAP and SAP kinases to cause catalytic activation of Pyst2 in vitro.

MATERIALS AND METHODS

Isolation and characterisation of human genomic clones encoding Pyst1 and Pyst2

We screened approximately 2.4×10^5 plaques from a human genomic library constructed from human placental DNA cloned into EMBL3 (Clontech) using standard techniques (Sambrook et al., 1989). Plaques were initially probed using a 300 bp cDNA fragment derived from the 3' untranslated region of Pyst2. Filters were then rescreened using a 600 bp probe derived from the 3' untranslated region of Pyst1. Positive plaques were identified and purified. The inserts were then excised, subcloned and analysed using a combination of restriction analysis, PCR and DNA sequencing.

Plasmids

All manipulations were carried out using standard techniques (Sambrook et al., 1989) and plasmid structures were verified by DNA

sequencing (Sanger et al., 1977). The complete Pyst2 open reading frame was assembled from our partial cDNA (Groom et al., 1996) and the Pyst2 genomic clone by overlap extension PCR (Ho et al., 1989). The cDNA was then subcloned as an *NdeI-XhoI* fragment into the bacterial expression vector pET15b (Novagen) and as an *EcoRI-XhoI* fragment into a modified pSG5 mammalian expression vector (Stratagene) encoding a C-terminal myc-tag as previously described (Groom et al., 1996). Site directed mutagenesis of the putative start codons in our full length Pyst1 cDNA was performed by oligonucleotide directed mutagenesis using the Sculptor system (Amersham). The plasmids encoding myc-tagged p42 MAP kinase and p38/RK SAP kinase and HA-tagged JNK-1 have been described previously (Groom et al., 1996).

RNA extraction and northern blot analysis

Total RNA was prepared from cultured cells using TRIzol reagent (Gibco BRL) as recommended by the manufacturers and northern blot analysis of mRNAs was performed using standard techniques (Sambrook et al., 1989). ^{32}P -labelled cDNA probes were generated by random primed labelling (Feinberg and Vogelstein, 1984) using the following templates: the complete human Pyst2 cDNA; the complete human Pyst1 cDNA (Groom et al., 1996); the complete human CL100 cDNA (Keyse and Emslie, 1992) and a 1,400 bp *PstI* fragment of the rat cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (Piechaczyk et al., 1984).

Cell culture, transfection and indirect immunofluorescence

FEK4 primary human skin fibroblasts were cultured routinely and treated with radiation and chemicals as described previously (Keyse and Emslie, 1992). COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and transfected using a standard calcium phosphate method. Indirect immunofluorescence experiments in COS-1 cells transiently transfected with myc-tagged Pyst2, Pyst1 and CL100 proteins were performed using anti-myc monoclonal antibody 9E10 as described previously (Lewis et al., 1995). Cell staining was observed and photographed with an Olympus BX-60 microscope fitted with a $\times 100$ objective using excitation/emission filters for FITC and DAPI. At least 50 9E10-positive cells were examined for each cell type and staining patterns were entirely consistent with the representative cells photographed.

In cotransfection experiments, plasmids encoding kinase reporters were introduced at 2 μg per 6cm dish in combination with varying amounts (0.1–2.0 μg) of expression plasmid encoding Pyst2.

Antibodies, immunoblotting and immunoprecipitations

The anti-Pyst1 antibody was produced as described previously (Groom et al., 1996). Purified Pyst2 protein expressed in *Escherichia coli* was separated by SDS-PAGE and transferred to nitrocellulose before immunoblotting with the anti-Pyst1 antiserum using standard techniques (Harlow and Lane, 1988). For detection of the phosphorylated and non-phosphorylated forms of p42 MAP kinase in COS-1 cells, lysates were separated by SDS-PAGE (10%), transferred to nitrocellulose and immunoblotted with either an anti-MAP kinase monoclonal antibody (Zymed), or in the case of myc-tagged p42 MAP kinase, the anti-myc monoclonal antibody 9E10, exactly as described previously (Groom et al., 1996).

COS-1 cells transfected with either myc-tagged RK/p38, HA-tagged JNK-1 or myc-tagged p42 MAP kinase were lysed and the kinase was then immunoprecipitated before being assayed for kinase activities or analysed by SDS-PAGE as described previously (Groom et al., 1996).

Preparation of ^{32}P -labelled substrates and protein phosphatase assays

Phosphorylase a was ^{32}P -labelled exactly as described by Alessi et al.

(1993). MAP kinase was activated and ^{32}P -labelled using activated MAP kinase kinase as described by Alessi et al. (1995). Purified MalE-RK fusion protein was activated and phosphorylated as described previously (Groom et al., 1996).

Activated c-Jun kinase was specifically 'pulled-down' from lysates of UV-irradiated (80 J/m²) COS-1 cells using a GST-c-Jun (residues 1-191) fusion protein. Pyst2 and Pyst1 proteins were expressed in bacteria, purified and refolded exactly as described previously for CL100 (Keyse and Emslie, 1992). Assays of p42 MAP kinase, p38/RK, and c-Jun kinase phosphatase activities and phosphoamino acid analysis was carried out exactly as described previously (Keyse and Emslie, 1992; Alessi et al., 1993).

Catalytic activation of Pyst2 and Pyst1 was measured using *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis at 25°C. Phosphatase activities were measured in 96-well plates in 200 µl of 50 mM imidazole (pH 7.5) containing 10 mM DTT, 20 mM *p*-NPP and the indicated amounts of recombinant Pyst2 or Pyst1 and various purified recombinant MAP kinases. Reaction rates were monitored at an absorbance of 405 nm in a microplate reader (Bio-Rad) exactly as described (Camps et al., 1998).

Kinase assays

The activities of recombinant p42 MAP kinase or myc-tagged p42 MAP kinase and p38/RK immunoprecipitated from COS-1 cells using the 9E10 monoclonal antibody were assayed using myelin basic protein as substrate as described by Groom et al. (1996). Jun kinases 'pulled down' from COS-1 cell lysates using GST-c-Jun fusion protein or HA-tagged JNK-1 immunoprecipitated from COS-1 cells using the anti-12CA5 monoclonal antibody was assayed exactly as described by Hibi et al. (1993).

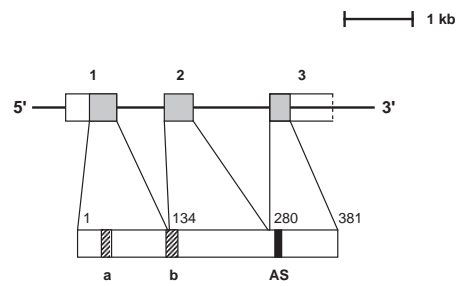
RESULTS

Isolation and characterisation of the human genes which encode Pyst1 and Pyst2

High stringency screening of the human genomic library with the full length Pyst1 cDNA and our partial Pyst2 cDNA yielded a number of positive signals. Upon further characterisation of these clones, the positive plaques were identified as overlapping or sister clones containing the Pyst1 and Pyst2 genes. These were then analysed in detail. The Pyst1 gene consists of three exons interrupted by two relatively short introns of about 700 bp (Fig. 1A). The size of the third exon of Pyst1 has not been determined since the polyadenylation signal for this gene has not yet been found. The Pyst2 gene spanned some 7 kb and, like the Pyst1 gene, contained three exons interrupted by two somewhat longer (about 2 kb) introns (Fig. 1B). The size of the first exon of the Pyst2 gene has not been determined as the transcriptional start site is unknown. This exon contains the DNA sequences encoding residues 1-46 of the Pyst2 protein which were absent in our original cDNA.

The organisation of the two genes is highly conserved, with exon 1 encoding the amino termini of the two proteins and ending within the second of two short regions of homology between all mammalian dual-specificity MAP kinase phosphatases and the *cdc25* proteins (Keyse and Ginsburg, 1993). The PTPase active site is found within exon 3 in both Pyst1 and Pyst2. Sequence comparison of the two genes reveals that all splice junctions conform to the donor and acceptor sequence consensus (Breathnach and Chambon, 1981) and that the intron/exon boundaries of the two genes are identical (Table 1). FISH analysis using our genomic clones

A. Pyst1



B. Pyst2

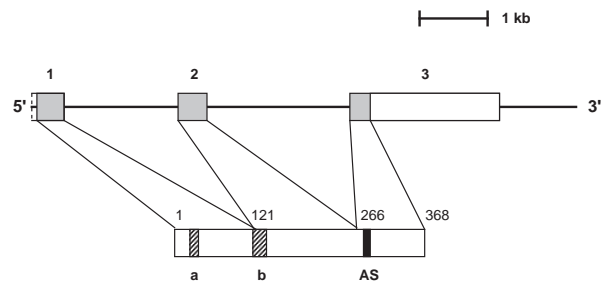


Fig. 1. Genomic structures of the human genes encoding Pyst1 (A) and Pyst2 (B). Exons are boxed and numbered and the shaded areas correspond to coding regions. Dashed lines indicate that the exon size has not yet been determined. The structure of the encoded proteins are also shown and amino acid residues are numbered. The positions of various domains within the proteins are indicated, including the regions of homology with the *cdc25* proteins (a and b) and the PTPase active site (AS).

maps the Pyst1 gene to human chromosome 12q21 and the Pyst2 gene to human chromosome 3p21 (data not shown).

To date, the structures of three human genes which encode dual specificity MAP kinase phosphatases have been analysed. These are CL100/MKP-1 (Emslie et al., 1994; Kwak et al., 1994) PAC-1 (Yi et al., 1995) and HB5/hVH-5 (Nesbit et al., 1997). In all of these genes both the positions and phase of the two introns which interrupt the second *cdc25*-like sequence and define the start of the exon containing the PTPase active site are conserved when compared with those found in Pyst1 and Pyst2. However, in contrast to Pyst1 and Pyst2, the intervening coding sequence (corresponding to exon 2) is interrupted by an additional intron. Furthermore, our preliminary analysis of the mouse gene encoding Pyst3 (MKP-4) has revealed an identical structure to the human Pyst1 and 2 genes (our unpublished data) indicating that the lack of this conserved intron is a hallmark of the Pyst subfamily of MAP kinase phosphatases.

Determination of the complete coding sequence of Pyst2 allows us to align the Pyst1, 2 and 3 amino acid sequences and shows that these proteins are highly homologous, particularly over the region containing the PTPase active site (Fig. 2). When compared with Pyst2 and Pyst3, Pyst1 appears to possess an amino-terminal extension of about 13 residues. However, western blotting of COS-1 cell lysates expressing a cDNA which encodes the Pyst1 protein fused with a single copy of the myc-epitope tag at the C terminus detects two distinct polypeptides and reveals that the smaller of these two

Table 1. Exon-intron boundary sequences of the human Pyst1 and Pyst2 genes

No.	Exon size (bp)	Boundary sequences*		Intron size (bp)
		5' boundary	3' boundary	
Pyst1				
1.	750	TAC CTG GAA G <u>gtacgccc...ttctggcag</u>	GT GGC TTC	~710
		T L E	G G F	
2.	437	TCT TTC ATA G <u>gtgagact...tggtcacag</u>	AT GAA GCC	~710
		S F I	D E A	
3.	>900			
Pyst2				
1.	>450	TAC CTC CAA G <u>gtgagggcg...tgacacacag</u>	GT GGT TTC	~2,100
		T L Q	G G F	
2.	435	AGC TTC ATT G <u>gtagttg...gtgtttcag</u>	AC GAA GCC	~2,100
		S F I	D E A	
3.	2,179			

*Exon sequences are shown in upper case letters and intron sequences in lower case letters. Splice donor and acceptor sequences are underlined and amino acid sequences are shown in upper case bold.

proteins is more efficiently translated (Fig. 3B). These correspond in size to translation products initiating at the first ATG and an internal ATG (Met 14) (Fig. 3A). Examination of the nucleotide sequences flanking these start codons reveals that the second ATG is in a more favourable context for initiation of translation (Kozak, 1991). Site directed mutagenesis of these putative start codons confirms that the major translation product of the Pyst1 cDNA corresponds to initiation at this internal ATG (Fig. 3B), indicating that this is most likely to represent the predominant form of Pyst1 expressed in vivo.

Pyst2 and Pyst1 are differentially expressed in human cells lines and Pyst2 mRNA is inducible by growth factors, but not stress, in human skin fibroblasts

We had previously detected the Pyst2 mRNA in a number of human tissues including muscle, brain heart and liver (Groom et al., 1996). However, using a probe corresponding to the 3' untranslated region of Pyst2, we were unable to detect the transcript in primary human skin fibroblasts. Using the complete Pyst2 cDNA we have now established that the Pyst2 mRNA is widely expressed in a variety of human cell

		** *	*** ** * ** *		
Pyst1	MIDTLRVPVFASEM	AISKTVAWLNEQLEL	GNERLLMDCRPQEYESSHIESAINVAI	58	
Pyst2		M P CKSAEWLQEELEARGGASLL	LLDCRPHELFESSTRDRAINLAI	45	
Pyst3/MKP-4		MEGLGRSCLWLRLRELSP	PRPRLLLDCRSRELYESARIGGALSVAL	46	
		**** * * *	* **		
Pyst1	PGIMLRRRLQKGNLPVRALFTRGEDRDRFTRRCGTDTVVLYDESSDW		NENTG	110	
Pyst2	PGLMLRRLRKGKGNLP	PIRSIIPNHADKERFATRCKAATVLLYDEATAEW	QPEPG	97	
Pyst3/MKP-4	PALLLRRRLRRGSLSVRALL		PGPPLQPPPPAPVLLYDQGGRRRRGEAAEAEEWE	101	
		* ** * ** * *	***		
Pyst1	G ESLGLLLKLLKDEG	CRAFYLEGGFSKFQAEFLS	HCEETNLDGSCS SSSPPLPVL	165	
Pyst2	APASVGLLLQKLRDDC	QAYYLQGGFNKFTQYSEHCE	TNVDSSSSP SSSPPTSVL	154	
Pyst3/MKP-4	A ESVLGTLQLKLRREEG	YLAYLQGGFSRFQAECPHLC	ETSLAGRAGSSMAPVPGVPVV	160	
		*** * ** * ** *	* ** * ** * ** *		
Pyst1	GLGGLRISSDSSDI	ESDLDRDPNSAT DSDG	SPLSNSQSPFVPEILPFLYLGCACKDS	222	
Pyst2	GLGGLRISSDC	SDGESDRELPP SSAT ESDG	SPVPSQPAFPVQILPFLYLGCACKDS	209	
Pyst3/MKP-4	GLGSLCLGSDC	SDAESEADRSMSGGLDSEGAT	PPVGLRASFPVQILPFLYLGSARDS	219	
		** * ** * ** * ** * ** *	* ** * ** * ** * ** *		
Pyst1	TNLDVLEEFGIKYLLNVTPNLP	PNLFENAGEFKYQIPISDHW	SQLNSQFFPEALSFIDEA	282	
Pyst2	TNLDVLEFVYGIKYLLNVTPNLP	NAFVGHGFEFTYQIPISDHW	SQLNSQFFPEALSFIDEA	269	
Pyst3/MKP-4	ANLES	LAKLGIRYLLNVTPNLP	NFFFKNGDFHYKQIPISDHW	SQLNSRFFPEALSFIDEA	279
		*****	*****		
Pyst1	RGKNCVGLVHCHLAGI	SRSVTVTVAYL	MQKLNLSMNDAYDIVKMKKSNISPNFNFMGQLLD	342	
Pyst2	RSKKCGVGLVHCHLAGI	SRSVTVTVAYL	MQKMNLSLNDAYDFVKRKKSNISPNFNFMGQLLD	329	
Pyst3/MKP-4	LSQNCVGLVHCHLAGI	SRSVTVTVAYL	MQKLHLSLNDAYDLVKKRKKSNISPNFNFMGQLLD	339	
		*** **	* ** *		
Pyst1	FERTLGL	SSPCDNRVPAQQLYFTTPSNQVNYQVDSLQT		381	
Pyst2	FERTLGL	SSPCDNHASSEQLYFSTPTNHNLPLNLEST		368	
Pyst3/MKP-4	FERSLRLEERHSQE	QSGGGQASAASNPPSFPTPTSDGAFA	LAPT	384	

Fig. 2. Sequence alignments of the Pyst1, Pyst2 and Pyst3 (MKP-4) proteins. Identical residues are marked with an asterisk and spaces represent gaps introduced to maximise matches. The highly conserved PTPase active site sequence of these enzymes is underlined and the two regions of homology between all of these proteins and the cdc25 phosphatase are boxed. The positions of two leucine-rich sequences are shaded.

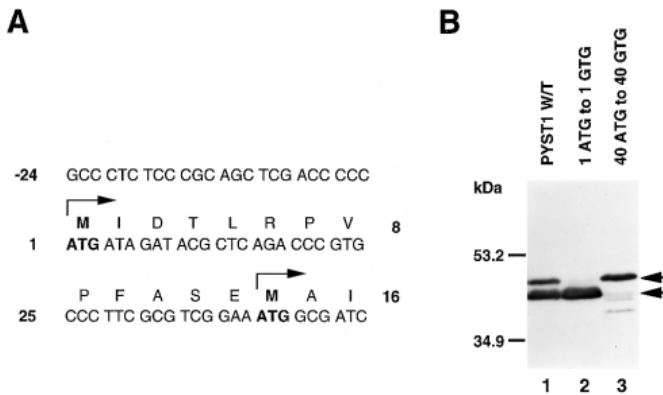


Fig. 3. The predominant form of Pyst1 expressed in COS-1 cells is initiated from an internal ATG. (A) The nucleotide sequence encoding the first 16 amino acids of the Pyst1 protein is shown with the corresponding amino acid sequence. The two putative start codons are shown in bold and indicated by arrows. (B) Western blot of myc-tagged wild-type (W/T) and mutant (1ATG to 1GTG and 40ATG to 40GTG) Pyst1 proteins expressed in COS-1 cells. Arrows indicate the positions of the encoded polypeptides.

lines including human skin fibroblasts (FEK4), HeLa cells, and ovarian (1847, Green et al., 1984), bladder (EJ138, O'Toole et al., 1983) and breast cancer (MCF-7, Soule et al., 1973)-derived cell lines (Fig. 4A). In certain of these, such as FEK4 fibroblasts and three of the cancer cell lines, both Pyst1 and Pyst2 mRNAs are coexpressed. This latter result indicates that loss of expression of these genes is not a characteristic of cells derived from at least some common

human tumours. However, Pyst1 mRNA was undetectable in HeLa cells which are derived from a human cervical carcinoma while CL100 mRNA, like Pyst2, was detected in all cell lines tested. We have also analysed the levels of Pyst2 transcript in FEK4 cells following exposure to growth factors and a variety of cellular stresses including heat shock, oxidative stress and UV-radiation. In contrast to Pyst1, Pyst2 mRNA is moderately inducible with rapid kinetics in FEK4 cells following serum stimulation (Fig. 4B). However, we can detect no significant increase in the levels of the Pyst2 transcript in these cells following exposure to a variety of stress conditions (Fig. 4C).

Pyst2 protein is localised in the cytosol of transfected COS-1 cells

Several of the CL100-like MAP kinase phosphatases are very tightly localised to the cell nucleus (Keyse, 1998). However, Pyst1 (MKP-3) is clearly localised predominantly within the cytosol of transfected COS-1 cells and sympathetic neurons (Groom et al., 1996; Muda et al., 1996a) and cytosolic localisation has also been reported for Pyst3/MKP-4 (Muda et al., 1997). Two leucine-rich sequences, reminiscent of the nuclear export sequences (NES) found in several proteins, were reported as being present in the amino terminus of rVH6, the rat homologue of Pyst1 (Mourey et al., 1996) and a comparison of the Pyst1, 2 and 3 sequences shows that these sequences are conserved in all three members of the Pyst subfamily of proteins (Fig. 2). Consistent with a functional role for these sequences we find that the Pyst2 protein shows predominantly cytosolic localisation in transfected COS-1 cells (Fig. 5) and we have obtained identical results in both HeLa cells and NIH 3T3 fibroblasts (data not shown).

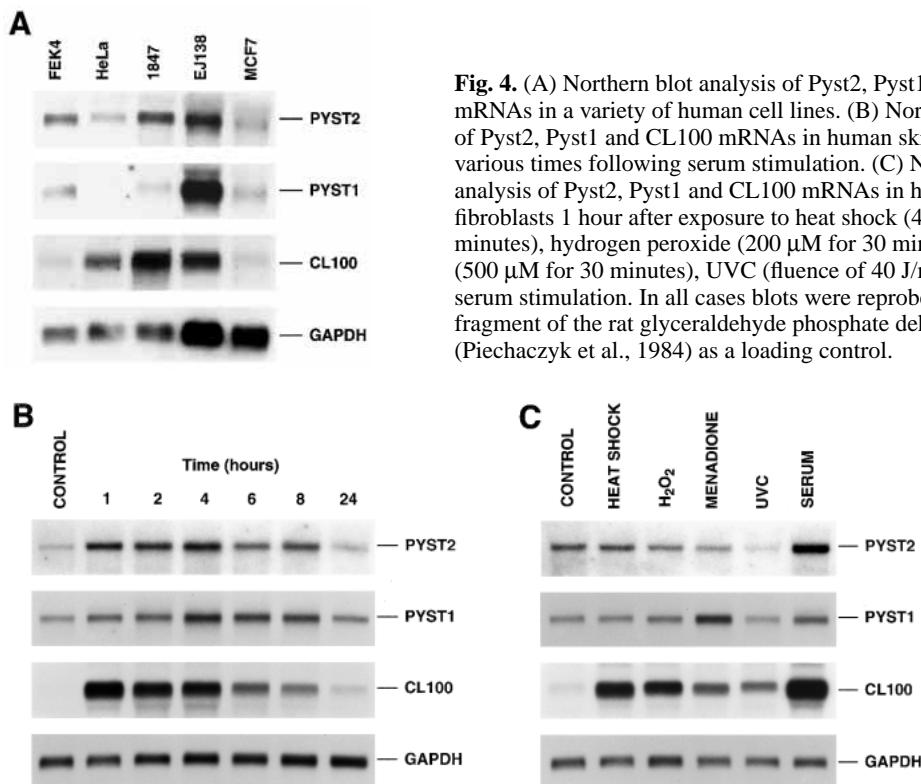


Fig. 4. (A) Northern blot analysis of Pyst2, Pyst1 and CL100 mRNAs in a variety of human cell lines. (B) Northern blot analysis of Pyst2, Pyst1 and CL100 mRNAs in human skin fibroblasts at various times following serum stimulation. (C) Northern blot analysis of Pyst2, Pyst1 and CL100 mRNAs in human skin fibroblasts 1 hour after exposure to heat shock (45°C for 15 minutes), hydrogen peroxide (200 μM for 30 minutes), menadione (500 μM for 30 minutes), UVC (fluence of 40 J/m²) or following serum stimulation. In all cases blots were reprobbed with the *PstI* fragment of the rat glyceraldehyde phosphate dehydrogenase gene (Piechaczyk et al., 1984) as a loading control.

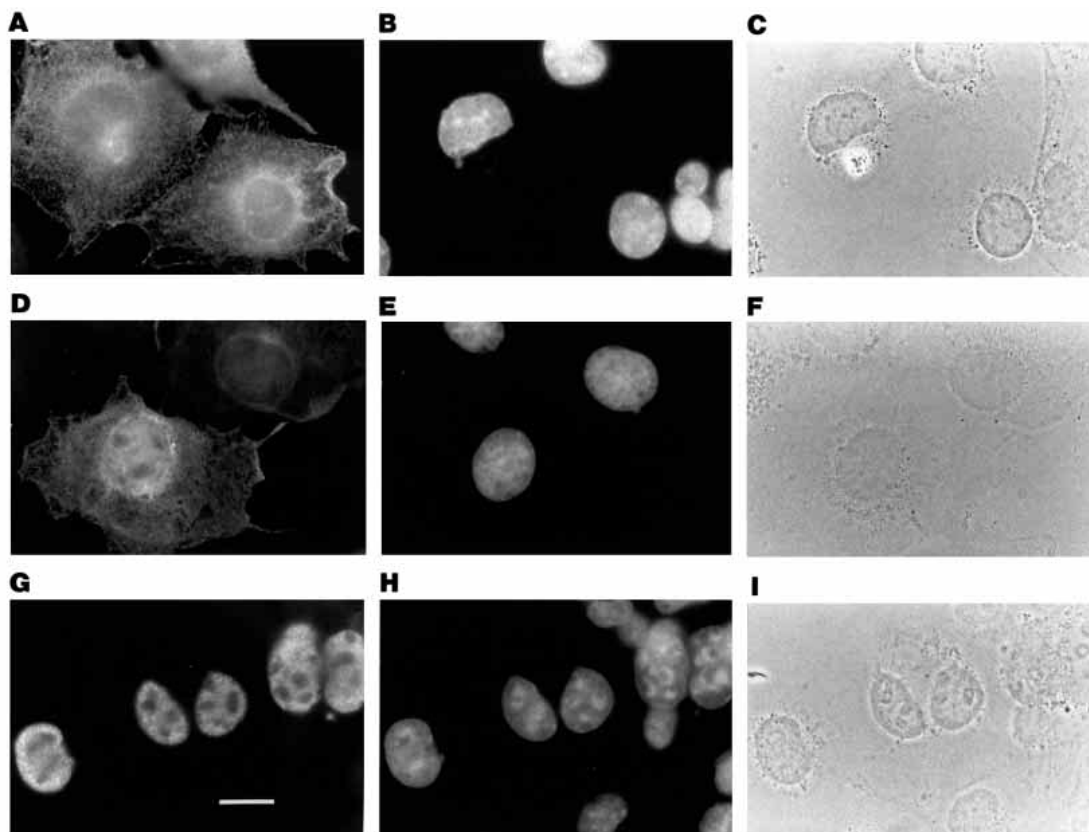


Fig. 5. The human Pyst2 protein is localised predominantly in the cytosol when expressed in COS-1 cells. COS-1 cells were transfected with the mammalian expression vector pSG5 containing either myc-tagged Pyst2 (A-C), Pyst1 (D-F) or CL100 (G-I), and analysed either by immunofluorescence using the anti-myc 9E10 monoclonal antibody (A,D,G), by DAPI fluorescence (B,E,H) or phase-contrast microscopy (C,F,I). Bar, 10 μ m.

Pyst2 preferentially dephosphorylates and inactivates p42 MAP kinase in vitro and in vivo

In order to study the activity and specificity of the Pyst2 enzyme we have expressed a His-tagged fusion protein in *E. coli* and obtained highly purified recombinant enzyme (Fig. 6A). Surprisingly, this protein is specifically recognised by a polyclonal antiserum raised against a peptide spanning the C-terminal 20 residues of the Pyst1 protein, despite only 50% amino acid identity between the two enzymes over this region (Fig. 6A). The Pyst2 protein is catalytically active, as it readily hydrolyses *p*-nitrophenyl phosphate (*p*-NPP) and this activity is abolished by sodium orthovanadate, a specific inhibitor of the protein tyrosine phosphatases (Fig. 6B).

We have examined the ability of recombinant Pyst2 to dephosphorylate a range of model substrates including MAP and SAP kinase isoforms in vitro. We find that Pyst2 efficiently dephosphorylates recombinant p42 MAP kinase (ERK2, MAPK2). Furthermore, its specific activity towards this substrate is approximately twofold greater than its activity towards recombinant RK/p38 (SAPK2a) (Table 2). This

contrasts with results previously obtained for Pyst1 in which the specific activity of this enzyme towards p38/RK was approximately 100-fold less than its activity against p42 MAP kinase (Groom et al., 1996). In the case of Pyst2, this largely reflects the higher activity of the enzyme towards p38/RK as the specific activity towards p42 MAP kinase is actually greater

Table 2. Phosphatase activity of purified Pyst2 protein

³² P-labelled substrate	Modified residue	Specific activity (mU/mg)*
GST-ERK2	Y,T	104
MalE-RK/p38	Y,T	47
Phosphorylase a	S	0

*One milliunit of activity is defined as the amount of phosphatase required to hydrolyse 1 nmol of phosphate from the substrate in 1 minute. Each assay was performed in triplicate and mean values are shown.

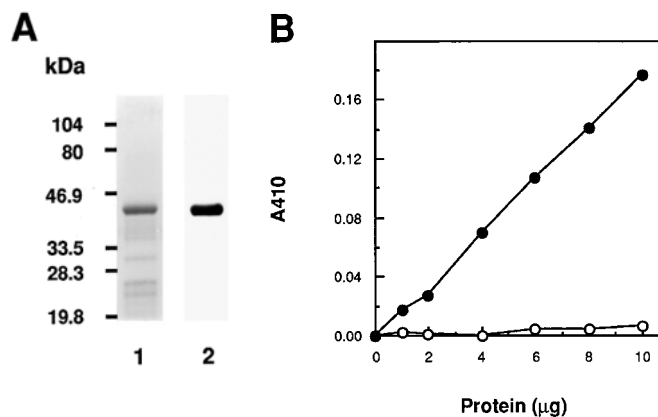


Fig. 6. (A) Expression of the Pyst2 protein in *E. coli*. SDS-PAGE of purified His-tagged Pyst1 fusion protein (lane 1) and immunoblot of this protein obtained using a polyclonal antiserum raised against a peptide spanning the C-terminal 20 residues of the Pyst1 protein (lane 2). Molecular mass markers are indicated on the left (kDa). (B) The Pyst2 protein possesses intrinsic phosphatase activity. The purified protein was assayed at the indicated concentrations for its ability to hydrolyse *p*-NPP either in the absence (●) or presence (○) of 1 mM sodium vanadate.

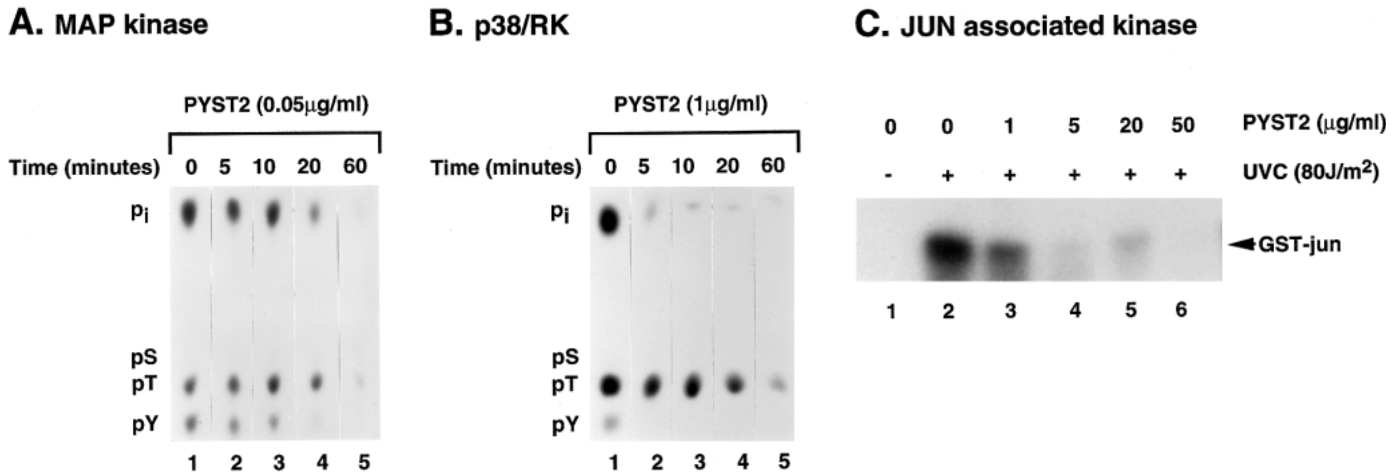


Fig 7. (A) Dephosphorylation of ³²P-labelled MAP kinase by Pyst2 in vitro. Activated MAP kinase was incubated with 0.05 µg/ml Pyst2 for the times indicated and assayed for phosphoamino acid content (lanes 1-5). (B) Dephosphorylation of ³²P-labelled p38/RK by Pyst2 in vitro. Activated MalE-p38/RK was incubated with 1 µg/ml Pyst2 for the times indicated and assayed for phosphoamino acid content (lanes 1-5). In A and B the positions of phosphotyrosine (pY), phosphothreonine (pT), phosphoserine (pS) and inorganic phosphate (Pi) are indicated. (C) Inactivation of Jun-associated kinases isolated from UVC-irradiated COS-1 cells by Pyst2. Autoradiogram showing SDS-PAGE analysis of ³²P-labelled GST-c-Jun fusion protein following incubation with Jun-associated kinases from non-irradiated (lane 1) or irradiated (lanes 2-6) cells in the presence of increasing concentrations of Pyst2 (lanes 3-6).

than that measured for Pyst1. In common with CL100 and Pyst1, Pyst2 shows no activity towards non MAP kinase substrates such as phosphorylase a (Table 2). Phosphoamino acid analysis of these ³²P-labelled substrates demonstrates that Pyst2 acts as a dual-specificity (Thr/Tyr) phosphatase towards p42 MAP kinase (Fig. 7A). In contrast, even at 20-fold higher levels, Pyst2 only dephosphorylates RK/p38 (SAPK2a) on the tyrosine residue of the T-G-Y signature sequence (Fig. 7B). In addition to p38/RK (SAPK2a), we have also determined the activity of recombinant Pyst2 towards the stress-activated Jun-kinase isoforms in vitro (Fig. 7C). We find that Pyst2 demonstrates measurable levels of activity towards these kinases in vitro.

We have extended these studies to look at the activity of Pyst2 towards MAP and SAP kinase isoforms under more physiological conditions. In these experiments we have co-

transfected COS-1 cells with epitope-tagged MAP kinase isoforms and increasing amounts of plasmid encoding the Pyst2 phosphatase. Following stimulation with the appropriate agonist to activate the transfected MAP kinase, cells were lysed, the kinases were immunoprecipitated using the appropriate monoclonal antibody and assayed for activity against either myelin basic protein (p42 MAP kinase and p38/RK) or a GST-c-Jun fusion protein (JNK-1/SAPK1c). Consistent with our in vitro results, Pyst2 was extremely effective in preventing the activation of p42 MAP kinase with activities reduced to background levels by as little as 0.5 µg of DNA encoding Pyst2 (Fig. 8A). In contrast, Pyst2 was much less potent towards both p38/RK and JNK-1 with measurable levels of kinase activity detected even in the presence of 1-2 µg of Pyst2 expression plasmid (Fig. 8B,C).

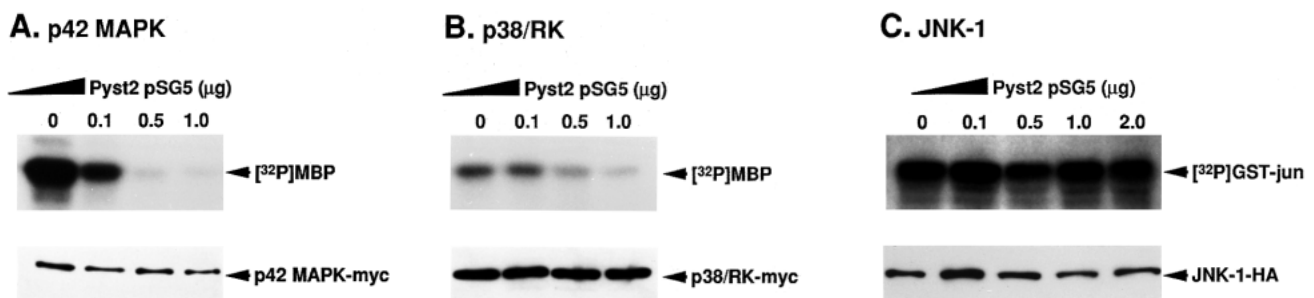


Fig. 8. Pyst2 inhibits the activity of p42 MAPK and p38/RK in vivo. COS-1 cells were transfected with either (A) p42 MAPK-myc, (B) p38/RK-myc or (C) JNK-1-HA (2.0 µg of plasmid) together with the indicated amount of pSG5 vector encoding Pyst2. Plasmid concentrations were maintained constant using pSG5 DNA. After 48 hours in culture cells were stimulated with serum (p42 MAPK), anisomycin (p38/RK) or UVC (JNK-1) before lysis and immunoprecipitation of MAP kinases using the appropriate monoclonal antibody. Immune complex kinase assays were then performed using MBP (p42 MAPK and p38/RK) or GST-c-Jun (JNK-1) as substrates in the presence of [γ -³²P]ATP before visualisation of phosphorylated substrates by SDS-PAGE and autoradiography (upper panels). Western blots showing expression of epitope-tagged MAP kinases are shown in the lower panels.

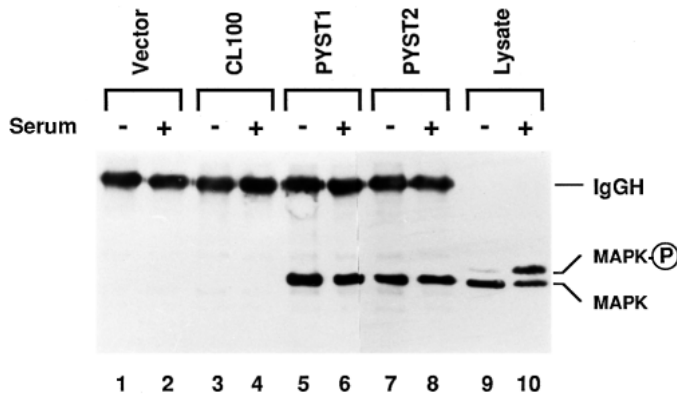


Fig. 9. Pyst2 is able to form a physical complex with endogenous p42 MAP kinase in COS-1 cells. Cells were transfected with either plasmid vector (lanes 1 and 2), expression vectors containing either myc-tagged wild-type CL100 (lanes 3 and 4), Pyst1 (lanes 5 and 6) or Pyst2 (lanes 7 and 8). Cells were either left unstimulated (minus sign) or serum stimulated for 10 minutes (plus sign) before immunoprecipitation using anti-myc 9E10 monoclonal antibody (lanes 1-10). MAP kinase was then detected in these immunoprecipitates using an anti-MAP kinase monoclonal antibody. Lysates from unstimulated (lane 9) and stimulated (lane 10) cells were also analysed directly for MAP kinase by immunoblotting with this antibody. The position of immunoglobulin heavy chain (IgGH) and the phosphorylated and nonphosphorylated forms of MAP kinase are indicated on the right.

Pyst2 is able to bind to p42 MAP kinase in vivo and demonstrates preferential catalytic activation by recombinant p42 MAP kinase in vitro

We have previously demonstrated that the Pyst1 MAP kinase phosphatase is able to bind to p42 MAP kinase in vivo. Furthermore this binding does not require activation of the MAP kinase (Groom et al., 1996). More recent in vitro studies have shown that MAP kinase binding is mediated by the amino terminus of the Pyst1 protein and results in catalytic activation of the enzyme as measured by *p*-NPP hydrolysis in vitro (Camps et al., 1998). Here we demonstrate that Pyst2, like Pyst1, is also able to bind to p42 MAP kinase in vivo (Fig. 9). Furthermore, incubation of Pyst2 with increasing concentrations of recombinant p42 MAP kinase stimulates the phosphatase activity of recombinant Pyst2 towards *p*-nitrophenyl phosphate in vitro (Fig 10A). Activation of Pyst2 by p42 MAP kinase was dose dependent and saturable. The kinetic parameters obtained for Pyst2 in the presence and absence of 10 μ g of p42 MAP kinase are given in Table 3. From these it is clear that both the affinity of Pyst2 for *p*-NPP and the catalytic rate of the enzyme are greatly increased on addition of p42 MAP kinase.

In order to relate the catalytic activation of Pyst2 by recombinant MAP kinases with its activity towards these enzymes in vitro and in vivo we have assayed a range of MAP and SAP kinase isoforms for the ability to catalytically activate both Pyst1 and Pyst2. In agreement with Camps et al. (1998), we find that Pyst1 is primarily activated by p42 MAP kinase with no significant activation by p38/RK (SAPK2a), p38 β (SAPK2b), ERK6/p38 γ (SAPK3) and SAPK4 and only a very small but significant (1.9-fold) activation by JNK-1 (SAPK1c). Pyst2, like Pyst1, is principally activated by p42 MAP kinase.

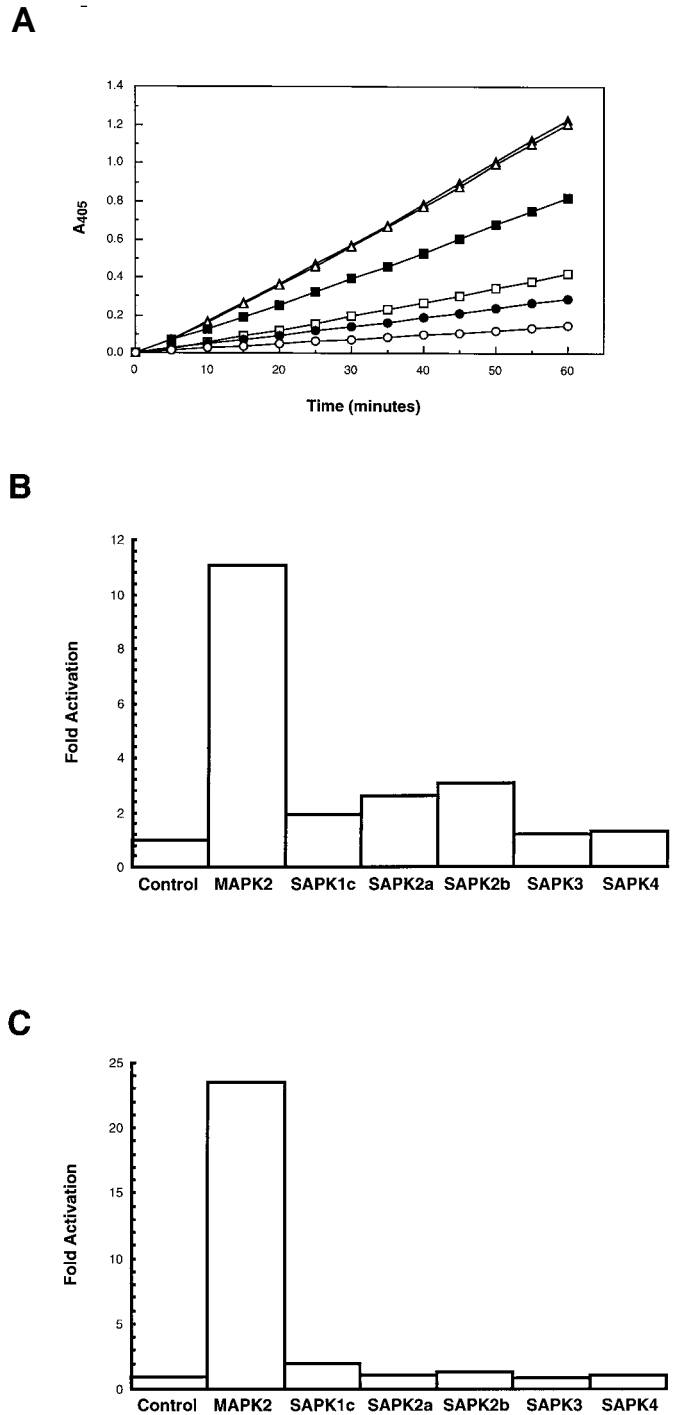


Fig. 10. (A) Activation of Pyst2 by p42 MAP kinase in vitro. Phosphatase activity of 5 μ g Pyst2 was assayed by time dependent hydrolysis of *p*-NPP at 25°C monitored at an absorbance of 405nm (A₄₀₅) in the absence (○) or presence of 1 μ g (●), 2 μ g (□), 5 μ g (■), 10 μ g (△) or 20 μ g (▲) purified recombinant p42 MAPK. (B) Activation of Pyst2 by recombinant MAP and SAP kinases expressed as an *n*-fold increase in specific activity of Pyst2 towards *p*-NPP assayed in the presence of 10 μ g of the indicated kinase. (C) Activation of Pyst1 by recombinant MAP and SAP kinases expressed as an *n*-fold increase in the specific activity of Pyst1 towards *p*-NPP assayed in the presence of 10 μ g of the indicated kinase. The nomenclature used for the recombinant MAP kinases in B and C is that proposed by Cohen (1997).

Table 3. Kinetic parameters for hydrolysis of *p*-NPP by Pyst2 in the absence and presence of p42 MAP kinase

Enzyme	K_m (mM)	V_{max} (pmoles of phosphate/minute)	K_{cat} (minutes ⁻¹)
Pyst2	6.82	8.74	0.35
Pyst2+p42 MAPK	0.76	55.96	2.27

However, we also detect significant (2- to 3-fold) catalytic activation of the enzyme following incubation with JNK-1 (SAPK1c), p38/RK (SAPK2a) and p38 β (SAPK2b).

DISCUSSION

The determination of the genomic structures of the human Pyst1 and Pyst2 genes has allowed a detailed comparison of five mammalian genes which encode dual specificity MAP kinase phosphatases. The absolute conservation of certain intron/exon boundaries amongst all of these suggests a common ancestral origin. In particular, the positions of the intron which interrupts the second of the *cdc25*-like sequences in the amino termini of all of these proteins and the intron which defines the start of the exon containing the PTPase active site sequence are likely to be conserved throughout this gene family.

The members of the Pyst subfamily are thus far unique in lacking an intron which interrupts the coding sequence corresponding to exon 2 in CL100 and PAC-1 (exon 3 in HB5/hVH-5). Furthermore, the sequences around this intron in CL100, PAC-1 and HB5/hVH-5 are more variable and it has been suggested that its position may reflect the evolutionary development of these genes (Nesbit et al., 1997). In this respect it is interesting that both Pyst1 and Pyst2, in which the intron is absent, and HB5/hVH-5, in which the position of this intron is shifted, exhibit different substrate specificities and subcellular localisation compared to the CL100 and PAC-1 proteins (Groom et al., 1996; Muda et al., 1996a,b). This may be particularly relevant, as recent work has shown that the amino-terminal domain of the Pyst1 (MKP-3) protein, which includes these sequences, is critical for substrate binding and catalytic activation (Camps et al., 1998).

Our analysis of the expression pattern of the Pyst2 transcript in mammalian cells indicates that the gene is expressed in a variety of human cell lines. The majority of these also express Pyst1 mRNA. However HeLa cells express only Pyst2 indicating that these two genes may be differentially regulated in certain cell types. We also find that, like Pyst1, the expression of the Pyst2 gene in human skin fibroblasts is not inducible by a wide variety of stress conditions. However, the Pyst2 transcript does accumulate rapidly in these cells following serum stimulation. This contrasts sharply with levels of Pyst1 mRNA, which rise only very modestly and with delayed kinetics (Groom et al., 1996). Such examples of differential expression and response to growth factors may indicate that the Pyst1 and Pyst2 genes may not be completely redundant in these cells.

The isolation of the Pyst2 gene has allowed us to complete our partial Pyst2 cDNA and undertake an analysis of the subcellular localisation and activity of the Pyst2 protein.

During the course of this work a cDNA sequence designated B59 was published which corresponds exactly to the sequence encoded by the Pyst2 gene (Shin et al., 1997). Ectopic expression of B59 was shown to inhibit morphological transformation by *H-ras* and *V-raf* oncogenes and it was concluded that these effects were mediated by p42/p44 MAP kinase inactivation. However, the substrate specificity, in vivo activity, and subcellular localisation of the phosphatase were not addressed. We find that the Pyst2 protein is predominantly cytoplasmic in transfected COS-1 cells. This pattern of localisation is distinct from the CL100 protein, which is tightly localised to the cell nucleus, and is confirmed as a general property of the Pyst subfamily of proteins. Studies of MAP kinase localisation reveal that up to 50% of the enzyme remains in the cytoplasm where it is primarily associated with the microtubule cytoskeleton (Reszka et al., 1995) serving to reinforce the conclusion that the function of the MAP kinase phosphatases is not restricted to the cell nucleus.

We have expressed and purified the Pyst2 protein and find that it exhibits both similarities and potentially important differences when compared with Pyst1. Firstly, Pyst2 preferentially dephosphorylates p42 MAP kinase when assayed in vitro. However, the degree of this selectivity is much reduced when compared to Pyst1. This is largely because Pyst2 exhibits a much higher specific activity towards p38/RK (SAPK2a) when compared to Pyst1. However, like Pyst1, Pyst2 is only able to dephosphorylate the p38/RK SAP kinase on the tyrosine residue of the T-G-Y activation sequence. This may be due to the inability of the catalytic domain of the Pyst1 and 2 proteins to efficiently recognise this SAP kinase as a substrate. Alternatively, it could reflect the inability of this MAP kinase isoform to cause full catalytic activation of these protein phosphatases (see below). Pyst2 also demonstrated measurable activity towards Jun-associated kinases (SAPK1 isoforms) in vitro. At similar concentrations Pyst1 protein is virtually inactive towards these kinases (Groom et al., 1996).

These results were broadly in agreement with our COS-1 cell cotransfection experiments in which Pyst2 demonstrated much higher activity in preventing activation of p42 MAP kinase when compared with its activity towards p38/RK (SAPK2a). Surprisingly, given our in vitro results, Pyst2 showed almost undetectable levels of activity towards the JNK-1 kinase expressed in these cells. However, the amounts of recombinant Pyst2 used in our in vitro assays are far greater than can be achieved by expression in vivo. In addition, the in vitro study was performed using a GST-c-Jun 'pull-down' assay from cell lysates following UV-radiation, and the Jun kinase activity measured could reflect the activities of multiple JNK (SAPK1) isoforms, certain of which may be more susceptible to inactivation by Pyst2.

We have previously demonstrated that wild-type Pyst1, unlike CL100, is able to bind to p42 MAP kinase in vivo (Groom et al., 1996). The ability of a dual specificity MAP kinase phosphatase to bind its substrate MAP kinase without mutation of the essential cysteine within the active site of the enzyme has also been demonstrated for the pmp1 phosphatase in the fission yeast *Schizosaccharomyces pombe* (Sugiura et al., 1998). These results indicate that such binding may be a more robust indicator of an in vivo substrate than binding observed using substrate trapping mutants. In agreement with this, recent work on Pyst1 (MKP-3) has now shown that specific binding

to p42 MAP kinase is mediated by the non-catalytic amino-terminal domain of the protein and results in catalytic activation of the phosphatase in vitro (Camps et al., 1998).

We have shown here that Pyst2 is also able to bind p42 MAP kinase in vivo. Furthermore, we have demonstrated that p42 MAP kinase is able to cause catalytic activation of the Pyst2 phosphatase in vitro. We have extended these studies by determining the ability of a range of MAP and SAP kinase isoforms to cause catalytic activation of both Pyst1 and 2 and our results indicate that, unlike Pyst1, Pyst2 can also be activated to a significant extent by certain SAP kinase isoforms. These include p38/RK (SAPK2a) and p38 β (SAPK2b). The ability of these SAP kinase isoforms to cause catalytic activation of Pyst2 may reflect the somewhat relaxed specificity of this enzyme towards these substrates when compared to Pyst1.

In conclusion, our data support the idea that the Pyst subfamily of proteins constitutes a distinct and structurally homologous subfamily of dual-specificity (Thr/Tyr) MAP kinase phosphatases. All of these proteins show a pattern of cytoplasmic localisation distinct from the nuclear phosphatases such as CL100, PAC-1, hVH2 and hVH3, indicating that they may act to regulate the activity of classical MAP kinase isoforms in the cytosol rather than in the nucleus. Although Pyst1, Pyst2 and Pyst3 show varying degrees of substrate selectivity, they are all most active towards the 'classical' p42 and p44 isoforms of MAP kinase and this is reflected by the ability of this MAP kinase isoform to bind to these phosphatases and cause catalytic activation in vitro.

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