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Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase

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The Pyst1 and Pyst2 mRNAs encode closely related proteins, which are novel members of a family of dual-specificity MAP kinase phosphatases typified by CL100/MKP-1. Pyst1 is expressed constitutively in human skin fibroblasts and, in contrast to other members of this family of enzymes, its mRNA is not inducible by either stress or mitogens. Furthermore, unlike the nuclear CL100 protein, Pyst1 is localized in the cytoplasm of transfected Cos-1 cells. Like CL100/MKP-1, Pyst1 dephosphorylates and inactivates MAP kinase *in vitro* and *in vivo*. In addition, Pyst1 is able to form a physical complex with endogenous MAP kinase in Cos-1 cells. However, unlike CL100, Pyst1 displays very low activity towards the stress-activated protein kinases (SAPKs) or RK/p38 *in vitro*, indicating that these kinases are not physiological substrates for Pyst1. This specificity is underlined by the inability of Pyst1 to block either the stress-mediated activation of the JNK-1 SAP kinase or RK/p38 *in vivo*, or to inhibit nuclear signalling events mediated by the SAP kinases in response to UV radiation. Our results provide the first evidence that the members of the MAP kinase family of enzymes are differentially regulated by dual-specificity phosphatases and also indicate that the MAP kinases may be regulated by different members of this family of enzymes depending on their subcellular location.

Keywords: MAP kinase phosphatase/SAP kinases/signal transduction

Introduction

The mitogen-activated protein kinases (MAPKs, also known as extracellular signal regulated kinases or ERKs) are key elements of highly conserved signalling pathways responsible for the inward passage of messages received at the cell surface by various growth factor and hormone receptors (reviewed in Nishida and Gotoh, 1993; Marshall, 1994; Seger and Krebs, 1995). The substrates of the MAP kinases include proteins such as cytosolic phospholipase A_2, the epidermal growth factor receptor and a number of transcription factors including $p62^{tcf}$/Elk-1 and c-Myc (reviewed in Davis, 1993). This latter finding, coupled with the observation that under certain conditions activated MAP kinase is translocated to the cell nucleus (Chen *et al.*, 1992; Traverse *et al.*, 1992; Lenormand *et al.*, 1993), has reinforced the idea that these kinases form an essential link in signal transduction events which are, in part, responsible for altered patterns of gene expression in response to extracellular stimuli.

The most extensively characterized of the mammalian MAP kinases are the 42 and 44 kDa isoforms (also known as ERK2 and ERK1, respectively) (reviewed in Cobb *et al.*, 1994). Unusually, these serine threonine kinases require phosphorylation on both tyrosine and threonine residues within the signature sequence T-E-Y for activity (Anderson *et al.*, 1990). This dual phosphorylation is catalysed *in vivo* by a dual-specificity (Thr/Tyr) kinase known as MAP kinase kinase or MAPKK (Kosako *et al.*, 1992; Nakielny *et al.*, 1992). MAPKK is itself activated by phosphorylation on either of two serine residues by a MAP kinase kinase kinase (MAPKKK) (Alessi *et al.*, 1994; Zheng and Guan, 1994). In mammalian cells, this latter group of enzymes includes members of the raf family of protein kinases (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Traverse and Cohen, 1994).

Recently, the family of mammalian MAP kinases has been extended by the discovery of a group of protein kinases which are activated by cellular stress. These comprise the stress-activated protein kinases (SAPKs) which include the c-Jun-associated kinase isoforms JNK-1 and JNK-2 (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). These kinases are 40–50% identical to the MAP kinases and contain the signature sequence T-P-Y. A distinct kinase known variously as reactivating kinase (RK), p38 or CSBP is activated in response to stress, bacterial endotoxin and proinflammatory cytokines (Freshney *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). This latter enzyme, which is responsible for the activation of mitogen-activated protein kinase protein kinase-2 (MAPKAP kinase-2) and subsequent phosphorylation of the small heat shock proteins (Rouse *et al.*, 1994), contains the signature sequence T-G-Y and is most closely related to the yeast MAP kinase HOG-1. As the name implies, the stress-activated protein kinases are characterized by their robust response to cellular stresses such as heat shock, sodium arsenite, UV radiation and osmotic shock, and are only poorly activated in response to growth factors. The differential responses of the MAP, SAP and RK/p38 kinases indicate that the signalling cascades containing these enzymes are distinct and exhibit very low levels of cross-talk. This idea is reinforced by the finding that MAP kinase kinase is unable to activate JNKs (Minden *et al.*, 1994) and conversely the upstream activators of either JNK-1 or RK/p38 are unable to activate the MAP kinases (Rouse *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995).

In contrast to the mechanisms of activation of the MAP kinases, the inactivation of these enzymes is less well understood. However, recent work has led to the isolation and characterization of a family of mammalian genes which encode dual-specificity (Thr/Tyr) MAP kinase phosphatases (reviewed in Keyse, 1995). To date, this group of enzymes comprises the CL100 phosphatase and its mouse homologue 3CH134/MKP-1 (Charles *et al.*, 1992; Keyse and Emslie, 1992), a T-cell-specific phosphatase Pac-1 (Rohan *et al.*, 1993), the B23/hVH-3 phosphatase (Ishibashi *et al.*, 1995; Kwak and Dixon, 1995) and a fifth enzyme designated hVH-2/MKP-2 (Guan and Butch, 1995; Misra-Press *et al.*, 1995). In addition to specificity for the MAP kinases, all of these enzymes share two common properties. First, the expression of their genes is highly inducible by cellular stress and/or mitogen stimulation. Secondly, all of these inducible enzymes are localized to the nucleus of the cell. This has led to the idea that these phosphatases constitute an inducible feedback mechanism leading to the regulated inactivation of MAP kinases and that the function of these enzymes is restricted to the cell nucleus.

Here we report the cDNA cloning of two closely related dual-specificity phosphatases, Pyst1 and Pyst2, which are novel members of the subfamily of CL100-like MAP kinase phosphatases. However, the gene encoding Pyst1 is expressed constitutively in human skin fibroblasts and, unlike the other enzymes in this family, it is neither an immediate early gene nor stress inducible. Furthermore, unlike the other MAP kinase phosphatases, Pyst1 is localized predominantly in the cytosol and not in the nucleus of the cell. Finally, unlike the CL100 enzyme, Pyst1 exhibits substrate selectivity for the MAP kinases. Pyst1 has only very low activity towards either the SAP kinases or RK/p38 *in vitro* and is unable to prevent the activation of either the stress-activated protein kinase JNK-1 or the RK/p38 kinase *in vivo*. Our results provide the first example of substrate selectivity among the dual-specificity MAP kinase phosphatases and also suggest that the MAP kinases may be differentially regulated by these enzymes depending on their subcellular location.

Results

Isolation of novel human dual-specificity phosphatases

We screened a human brain cDNA library with a 278 bp DNA fragment corresponding to an expressed sequence (EST) tag from infant brain (Adams *et al.*, 1993). This sequence was 62% identical to the human CL100 cDNA over the region encoding the highly conserved active site of the enzyme. Following analysis of positive clones and rescreening of human cDNA libraries, we obtained two novel sequences. The first of these, designated Pyst1, contains an open reading frame specifying a polypeptide of 381 residues (estimated molecular mass of ~42.3 kDa). The second cDNA, designated Pyst2, was identical in sequence to the probe used to screen the cDNA library and specifies the C-terminal 320 amino acids of a protein which is highly related to Pyst1 (73% identity, 86.5% similarity) (Figure 1A). Both proteins contain the highly conserved signature sequence of protein tyrosine phosphatases, HCXAGXXR (Fischer *et al.*, 1991). Database searching revealed that Pyst1 was most closely related over its entire length to the members of a subfamily of the vaccinia VH1-like enzymes which have previously been identified as dual-specificity (Thr/Tyr) MAP kinase phosphatases in mammalian cells (reviewed in Keyse, 1995). The Pyst1 and Pyst2 proteins are closer in sequence to each other (73% identity) than to the other members of this family

A

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                                                                  *
pyst1  MIDTLRPVPFASEMAISKTVAWLNEQLELGNERLLLMDCRPQELYESSHIESAINVAIPG  60
pyst2                                                           G

        ***** ***** *       *  **  **   ** ****    *    *   * *****
pyst1  IMLRRLQKGNLPVRALFTRGEDRDRFTRRCGTDTVVLYDESSSDWNENTGG ESLLGLLL 119
pyst2  LMLRRLRKGNLPIRSIIPNHADKERFATRCKAATVLLYDEATAEWQPEPGAPASVLGLLL

        ** * ** * ** *** *** * * ***** * * * *****  ************ *
pyst1  KKLKDEGCRAFYLEGGFSKFQAEFSLHCETNLDGSCS SSSPPLPVLGLGGLRISSDSSS 178
pyst2  QKLRDDGCQAYYLQGGFNKFQTEYSEHCETNVDSSSSPSSSPPTSVLGLGGLRISSDCS

            **  * *** *****   *** *** *** ***************   ******
pyst1  DIESDLDRD PNSATDSDGSPLSNSQPSFPVEILPFLYLGCAKDSTNLDVLEEFGIKYIL 237
pyst2    DGESDRELPSSATESDGSPVPSSQPAFPVQILPYLYLGCAKDSTNLDVLGKYGIKYIL

        ******** **  *** ***************************** * ***********
pyst1  NVTPNLPNLFENAGEFKYKQIPISDHWSQNLSQFFPEAISFIDEARGKNCGVLVHCLAGI 297
pyst2  NVTPNLPNAFEHGGEFTYKQIPISDHWSQNLSQFFPEAISFIDEARSKKCGVLVHCLAGI

        ************** *** ***** ** ******************************
pyst1  SRSVTVTVAYLMQKLNLSMNDAYDIVKMKKSNISPNFNFMGQLLDFERTLGLSSPCDNRV 357
pyst2  SRSVTVTVAYLMQKMNLSLNDAYDFVKRKKSNISPNFNFMGQLLDFERTLGLSSPCDNHA

          **** ** * *      * **
pyst1  PAQQLYFTTPSNQNVYQVDSLQST                                    381
pyst2  SSEQLYFSTPTNHNLFPLNTLEST
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of proteins, where the homology with Pyst1 ranges from 38% identity (58% similarity) for Pac-1 to 32% identity (52% similarity) for B23/hVH-3. The sequence relationship between Pyst1 and these other family members is illustrated in Figure 1B and C. Clearly, the highest level of sequence conservation occurs in the C-terminal region of these proteins, where the active site sequence containing the catalytically essential cysteine (residue 293 in Pyst1) is located. Interestingly, the N-terminal region of Pyst1 also shows regions of significant homology (designated A and B) with the cell cycle regulatory phosphatase cdc25. This sequence similarity has been observed previously in CL100 (Keyse and Ginsburg, 1993; Kwak *et al.*, 1994) and is characteristic of all the members of this subfamily of enzymes.

We have used the Pyst1 cDNA and a cDNA fragment derived from the 3′ untranslated sequence of the Pyst2 cDNA (to avoid cross-hybridization with Pyst1) as probes

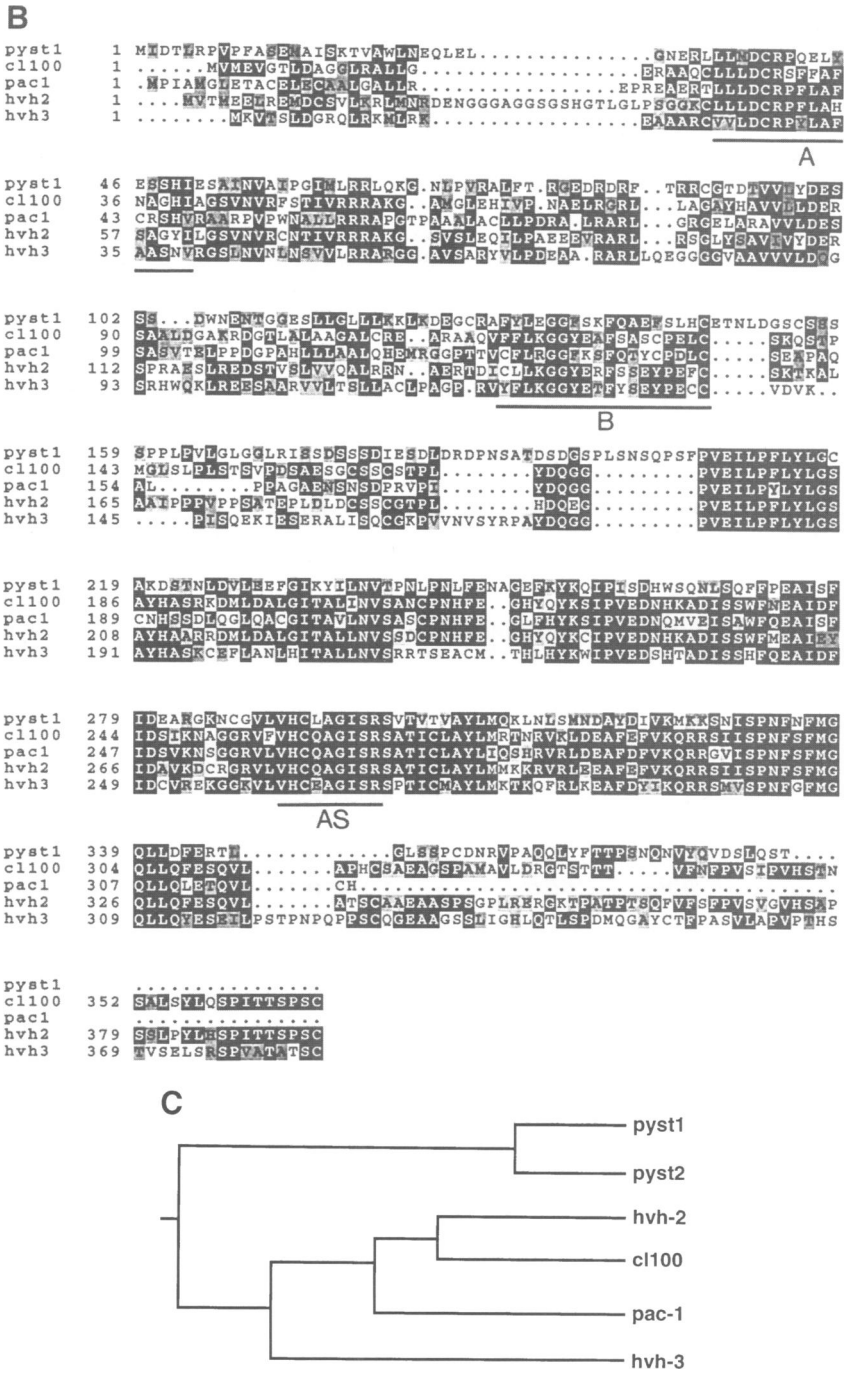

Fig. 1. (**A**) Amino acid sequences of the predicted Pyst1 and Pyst2 proteins. Identical residues are marked with an asterisk and spaces represent gaps introduced to maximize matches. (**B**) Sequence alignment of Pyst1 with the human MAP kinase phosphatases CL100 (Keyse and Emslie, 1992), Pac-1 (Rohan *et al.*, 1993), hVH-2 (Guan and Butch, 1995) and hVH-3 (Kwak and Dixon, 1995). Identical residues are shown white on black, while conserved residues are shaded. Gaps introduced to maximize matches are indicated by dots. The highly conserved active site (AS) sequences of these enzymes is underlined, as are two regions of homology (designated A and B) with the cdc25 phosphatase. (**C**) Phylogenetic tree representing these amino acid sequence comparisons generated using the method of Fitch and Margoliash (1967).

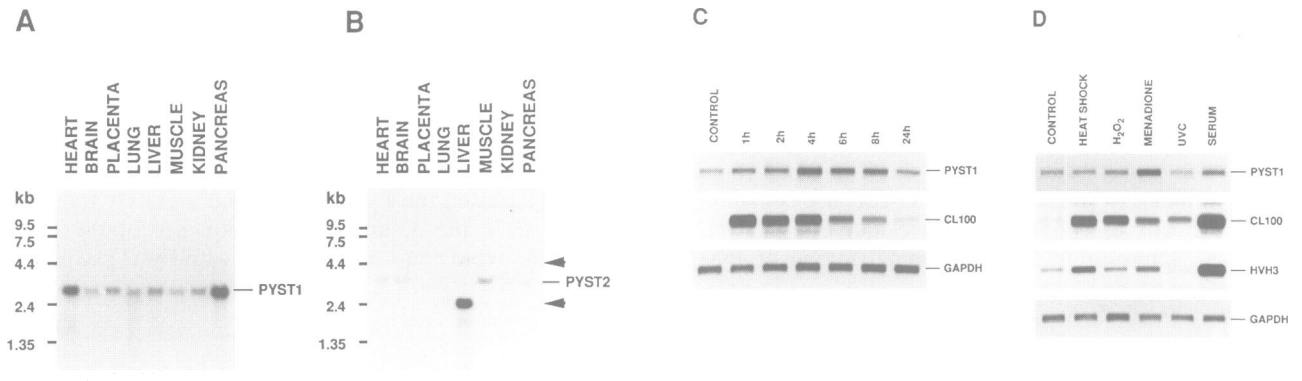

Fig. 2. Northern blot analyses of Pyst1 (**A**) and Pyst2 (**B**) mRNAs in a variety of human tissues. The positions of the major Pyst1 and Pyst2 transcripts are indicated on the right. In the case of Pyst2, two transcripts of differing size which are seen only in human liver are marked with arrows. (**C**) Northern blot analysis of Pyst1 and CL100 mRNAs in human skin fibroblasts at various times following serum stimulation. (**D**) Northern blot analysis of Pyst1, CL100 and hVH-3 mRNAs in human skin fibroblasts 1 h after exposure to heat shock (45°C for 15 min), hydrogen peroxide (200 μM for 30 min), menadione (500 μM for 30 min), UVC (fluence of 40 J/m^2, 254 nm) or following serum stimulation. In (C) and (D), blots were reprobed with the *Pst*I fragment of the rat glyceraldehyde phosphate dehydrogenase gene (Piechaczyk *et al.*, 1984) as a loading control.

to compare the distribution of these transcripts in human tissues by Northern blot analysis. Pyst1 mRNA (~3.0 kb in length) was expressed at moderate levels in a variety of human tissues, with the exception of peripheral blood leukocytes where it was undetectable. The highest levels of expression were seen in the heart and pancreas (Figure 2A). In contrast, a single Pyst2 transcript of ~3.5 kb was present at only very low levels in most tissues, with the exception of the liver which, instead of a single transcript, expressed a high level of a 2.5 kb mRNA and a much lower level of a larger transcript of ~5 kb (Figure 2B). The high degree of sequence similarity between Pyst1 and Pyst2, coupled with the observation that they are differentially expressed in human tissues, suggests that they may be tissue- or cell type-specific enzymes with highly related biochemical functions.

Pyst1 mRNA is expressed constitutively in human skin fibroblasts and is not inducible by stress or mitogens

The CL100 gene is inducible both by oxidative/heat stress and by mitogens (Charles *et al.*, 1992; Keyse and Emslie, 1992). Growth factor inducibility is a property shared by all of the members of this family of enzymes which have thus far been isolated. Pac-1 is mitogen inducible in human T cells (Rohan *et al.*, 1993), hVH-3/B23 is inducible by mitogens and heat shock in human skin fibroblasts (Ishibashi *et al.*, 1995), and hVH-2/MKP-2 is inducible by both EGF and NGF in rat PC12 cells (Misra-Press *et al.*, 1995). We have analysed mRNA from serum-stimulated human skin fibroblasts by Northern analysis using both Pyst1 and Pyst2 probes. First, we find that Pyst1, but not Pyst2, mRNA is constitutively expressed in these cells. Secondly, in contrast to CL100, Pyst1 mRNA does not accumulate to high levels immediately following serum stimulation (Figure 2C). In fact, levels of Pyst1 mRNA increase only moderately to 2- to 3-fold over the control level some 4–6 h after serum addition.

We have also analysed Pyst1 mRNA levels in human fibroblasts following various stress treatments and compared these with the levels of both the CL100 and hVH-3/B23 mRNAs. As previously reported, both the CL100 and B23/hVH-3 mRNAs are inducible by both heat shock and mitogens (Figure 2D). However, B23/hVH-3 responds less robustly to oxidative stress when compared with CL100 and, unlike CL100, is not inducible in response to UVC radiation. In contrast, Pyst1 mRNA levels remain completely unchanged following exposure to heat shock, hydrogen peroxide and UVC radiation, and show only a very slight increase in levels following exposure to menadione. Our results show that human skin fibroblasts express at least three genes which encode CL100-like dual-specificity phosphatases. However, unlike CL100 and B23/hVH-3, Pyst1 is not an immediate early gene in human skin fibroblasts nor does it show the pattern of stress inducibility characteristic of the CL100 and B23/hVH-3 MAP kinase phosphatases.

Pyst1 protein is localized in the cytosol of transfected Cos-1 cells

Previous studies have shown that the family of CL100-like MAP kinase phosphatases share the property of being very tightly localized to the cell nucleus (Rohan *et al.*, 1993; Guan and Butch, 1995; Kwak and Dixon, 1995; Lewis *et al.*, 1995). This has led to the suggestion that the function of these inducible enzymes is restricted to the nucleus. We have determined the subcellular location of Pyst1 by transiently transfecting an epitope-tagged Pyst1 cDNA into Cos-1 cells. Immunofluorescent staining using an antibody raised against the c-myc epitope revealed that, in contrast to the CL100 protein, Pyst1 is localized predominantly in the cytosol of transfected cells (Figure 3A). We have obtained identical results using either NIH 3T3 fibroblasts or HeLa cells (data not shown). In all cases, fluorescence was undetectable from either untransfected cells or cells transfected with the expression vector alone. In addition, in Cos-1 cells transfected with an expression vector containing untagged Pyst1, immunostaining with our polyclonal anti-Pyst1 antiserum gave an identical pattern of cytosolic staining, indicating that the presence of the 9E10 epitope tag does not alter the intracellular localization of Pyst1 (data not shown). We

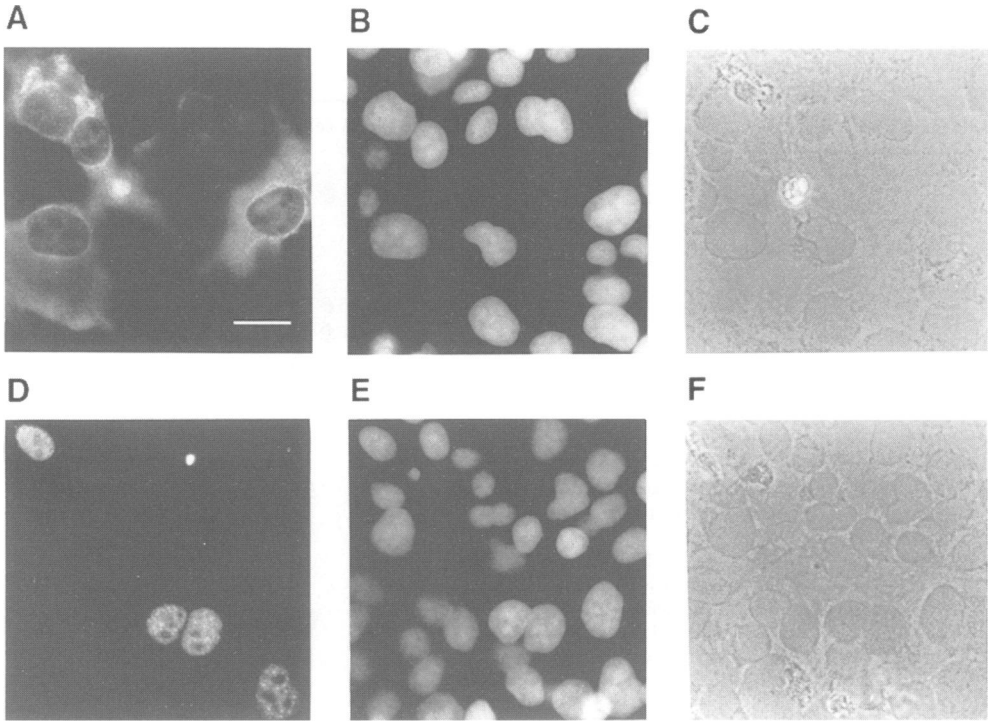

Fig. 3. The human Pyst1 protein is localized in the cytosol when expressed in Cos-1 cells. Cos-1 cells were transfected with the mammalian expression vector pSG5 containing either myc-tagged Pyst1 (**A–C**) or CL100 (**D–F**) and analysed either by immunofluorescence using the anti-myc 9E10 monoclonal antibody (A and D), by DAPI fluorescence (B and E) or phase-contrast microscopy (C and F). The bar in (A) is 10 μm.

have explored the possibility that the Pyst1 protein is cytosolic in unstimulated cells, but could possibly be translocated to the nucleus in response to mitogen stimulation. We can find no evidence of this, as the Pyst1 protein remained cytosolic in transfected cells even following serum stimulation (data not shown).

The Pyst1 phosphatase is highly specific for p42 MAP kinase but not the SAP kinases or RK/p38 in vitro

In order to study the activity and specificity of the Pyst1 phosphatase, we have expressed a His-tagged fusion protein in *Escherichia coli* and obtained recombinant enzyme in a highly purified form (Figure 4A, lane 1). This protein is full-length as it is recognized specifically by a polyclonal antiserum raised against a peptide spanning the C-terminal 20 residues of the Pyst1 protein (Figure 4A, lane 2). Furthermore, the purified Pyst1 protein is active as it readily hydrolyses *p*-nitrophenyl phosphate (pNPP) and this activity is abolished by sodium orthovanadate, a specific inhibitor of the protein tyrosine phosphatases (Figure 4B). In addition, like CL100 (and other protein tyrosine phosphatases), Pyst1 is insensitive to high concentrations (up to 1 μM) of either okadaic acid or microcystin, which are specific inhibitors of the type 1 and type 2 serine/threonine protein phosphatases (data not shown).

We have previously shown that the activity of the CL100 phosphatase is highly specific towards activated p42 MAP kinase *in vitro* (Alessi *et al.*, 1993). Therefore, we examined the ability of purified Pyst1 protein to dephosphorylate a range of model substrates containing either phosphotyrosine and/or phosphoserine/phosphothreonine (Table I). In common with CL100, Pyst1 was

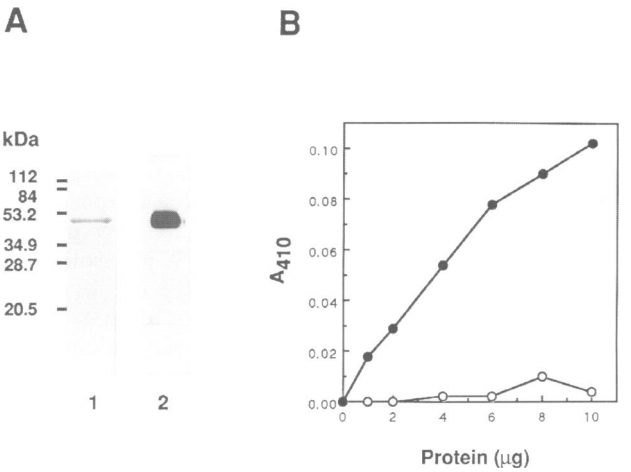

Fig. 4. (**A**) Expression of the Pyst1 protein in *E.coli*. SDS–PAGE of purified His-tagged Pyst1 fusion protein (lane 1) and immunoblot of this protein obtained using a polyclonal antiserum raised against a peptide spanning the C-terminal 20 residues of the Pyst1 protein (lane 2). Molecular mass markers are shown on the left ($\times 10^{-3}$). (**B**) The Pyst1 protein possesses intrinsic phosphatase activity. The purified protein was assayed at the indicated concentrations for its ability to hydrolyse pNPP either in the absence (●) or presence (○) of 1 mM sodium vanadate.

unable to dephosphorylate a range of protein and peptide substrates, including myelin basic protein, casein and phosphorylase. However, there was a major difference in the activities of Pyst1 and CL100 towards different members of the MAP kinase family of enzymes. While both Pyst1 and CL100 efficiently dephosphorylated p42 MAP kinase, Pyst1 was ~100-fold less active towards reactivating kinase (RK or p38). In order to rule out any influence of the fusion partners on the activity of these phosphatases

Table I. Phosphatase activities of purified Pyst1 and CL100 proteins

^{32}P-labelled substrate	Modified residue	Specific activity (mU/mg)	
		Pyst1	CL100
GST-MAP kinase	Y,T	57.9	499
MAP kinase	Y,T	45	389
MalE–RK/p38	Y,T	0.48	547
RK/p38	Y,T	0.48[a]	547[a]
Phosphorylase	S	0	0
Casein	S	0	0
Myosin light chain	S	0	0
AFLEDFFTSTEPQYQPGENL	Y	0	$<10^{-4}$
DRVYVHPF	Y	0	$<10^{-4}$
RCM lysozyme	Y	0	0
Myelin basic protein	Y	0	0

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

[a]Since it was not possible to obtain >50% cleavage of MalE–RK/p38, the dephosphorylation of the ^{32}P-labelled fusion protein was compared with the dephosphorylation of the free RK/p38 after incubation for 10 min at 30°C with different concentrations of either CL100 or Pyst1 by SDS–PAGE and phosphorimager analysis. Under these conditons, the initial rates of dephosphorylation of the two different forms were identical.

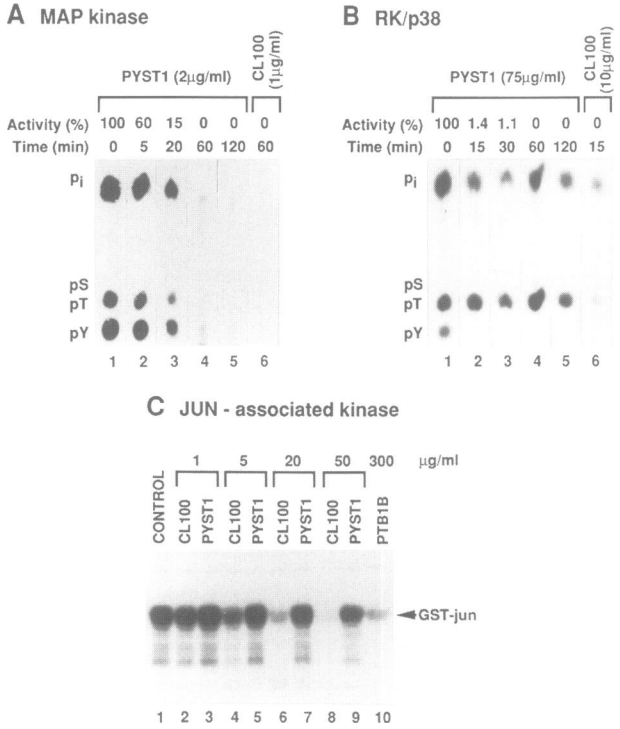

Fig. 5. (**A**) Inactivation and dephosphorylation of ^{32}P-labelled MAP kinase by Pyst1. Activated MAP kinase was incubated with 2 μg/ml Pyst1 for the times indicated and assayed for activity and phosphoamino acid content (lanes 1–5). CL100 was used as a control (lane 6). (**B**) Inactivation and dephosphorylation of ^{32}P-labelled reactivating kinase (RK/p38) by Pyst1. Activated MalE–RK was incubated with 75 μg/ml Pyst1 for the times indicated, and assayed for activity and phosphoamino acid content (lanes 1–5). CL100 was used as a control (lane 6). In (A) and (B), the positions of phosphotyrosine (pY), phosphothreonine (pT), phosphoserine (pS) and inorganic phosphate (p_i) are indicated. (**C**) Inactivation of Jun-associated kinases isolated from sodium arsenite-treated human fibroblasts by Pyst1. Autoradiogram showing SDS–PAGE analysis of ^{32}P-labelled GST–c-Jun fusion protein following incubation with activated Jun-associated kinase in the absence (lane 1) or presence of increasing concentrations of either Pyst1 (lanes 3, 5, 7 and 9) or CL100 (lanes 2, 4, 6 and 8). The tyrosine phosphatase PTP1B was included as a control.

towards MAP kinase or RK/p38, we have cleaved these using either thrombin (GST-p42 MAPK) or factor Xa (MalE–RK/p38) to obtain the free kinases. We again see that Pyst1 is ~90-fold less active towards RK/p38 compared with its activity towards MAP kinase (Table I).

Phosphoamino acid analysis of ^{32}P-labelled and activated p42 MAP kinase incubated for varying times with Pyst1 protein (2 μg/ml) revealed that, like CL100, Pyst1 was acting as a dual-specificity (Thr/Tyr) phosphatase (Figure 5A). In contrast to this activity towards p42 MAP kinase, Pyst1, even at high concentration (75 μg/ml), inactivated ^{32}P-labelled and activated RK/p38 solely by dephosphorylation of tyrosine residues (Figure 5B). This result was obtained using both the MalE–RK/p38 fusion protein and free RK/p38 as a substrate, and we conclude that Pyst1 only functions as a dual-specificity phosphatase towards MAP kinase *in vitro*.

In addition to RK/p38, we have also compared the activities of Pyst1 and CL100 towards the stress-activated family of JNKs *in vitro* (Figure 5C). Clearly CL100, but not Pyst1, is able to inactivate c-Jun-associated kinase activity. Thus, in addition to RK/p38, Pyst1 is unable to inactivate this second group of stress-activated protein kinases. Taken together, our *in vitro* data strongly suggest that the stress-activated protein kinases and RK/p38 may not be physiological substrates for the Pyst1 phosphatase.

Pyst1 blocks the activation of MAP kinase induced by serum and physically associates with MAP kinase in vivo

In order to study the effects of Pyst1 *in vivo*, we have co-transfected a plasmid encoding myc epitope-tagged p42 MAP kinase and prepared lysates from Cos-1 cells either with or without serum stimulation. These lysates were then analysed in two ways. First, the electrophoretic mobility of the p42 MAPK was determined by immunoblotting using anti-myc epitope antibody (Figure 6, upper panel). Secondly, the p42 MAP kinase was immunoprecipitated from the lysates and these immunoprecipitates were assayed directly for MBP kinase activity (Figure 6, lower panel). These results clearly show that expression of either Pyst1 or CL100 completely blocks both the serum-induced phosphorylation and activation of heterologously expressed MAP kinase.

It has been shown previously that a catalytically inactive mutant (Cys 258 to Ser) of 3CH134/MKP-1 (the mouse homologue of the CL100 phosphatase) is able to form a specific physical complex with the phosphorylated form of endogenous p42 MAP kinase in Cos-1 cells (Sun *et al.*, 1993). Similar results have also been reported for the T-cell-specific MAP kinase phosphatase Pac-1 (Ward and Kelly, 1994). We have transfected Cos-1 cells with myc-tagged wild-type and mutant forms of both the Pyst1 and CL100 enzymes. These phosphatases have then been immunoprecipitated from either serum-deprived or serum-stimulated Cos-1 cells and the immunoprecipitates analysed by immunoblotting using an anti-p42 MAP kinase monoclonal antibody (Figure 7). In agreement with Sun *et al.* (1993), we find that MAP kinase co-immuno-

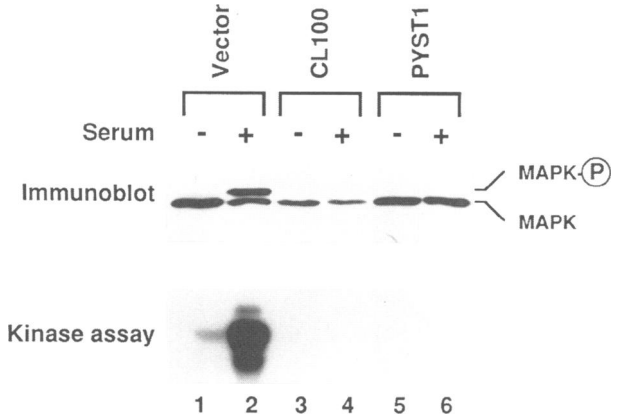

Fig. 6. Pyst1 prevents the activation of p42 MAP kinase by serum stimulation in Cos-1 cells. Cos-1 cells were co-transfected with the reporter plasmid pEXV3-p42^{MAPK}-myc together with plasmid vector (lanes 1 and 2) or expression vectors containing either CL100 (lanes 3 and 4) or Pyst1 (lanes 5 and 6). The phosphorylation state of the p42 MAP kinase was determined by immunoblotting with the 9E10 anti-myc monoclonal antibody (upper panel) in unstimulated cells (lanes 1, 3 and 5) or cells stimulated with serum for 10 min (lanes 2, 4 and 6). The positions of phosphorylated (active) and unphosphorylated (inactive) MAP kinase are indicated on the right. MAP kinase activity in 9E10 immunoprecipitates from these same cells was measured using myelin basic protein (MBP) as substrate (lower panel).

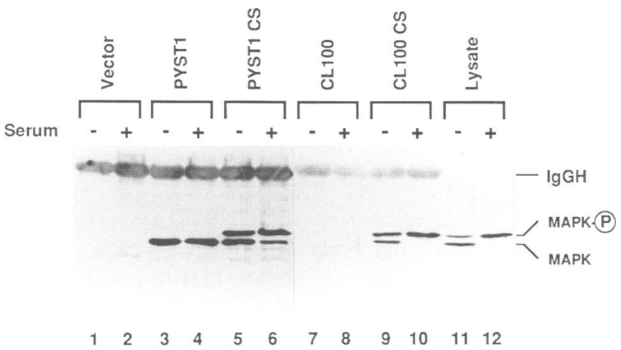

Fig. 7. Pyst1 is able to form a physical complex with endogenous p42 MAP kinase in Cos-1 cells. Cells were transfected with either plasmid vector (lanes 1 and 2), expression vectors containing either myc-tagged wild-type (PYST1, lanes 3 and 4) or mutant (PYST1 CS, lanes 5 and 6) Pyst1 or myc-tagged wild-type (CL100, lanes 7 and 8) or mutant (CL100 CS, lanes 9 and 10) CL100. Cells were either unstimulated (minus sign) or serum stimulated for 10 min (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 11) and stimulated (lane 12) cells were also analysed directly for MAP kinase by immunoblotting with this antibody. The position of immunoglobulin heavy chain (IgGH) and the phosphorylated and unphosphorylated forms of MAP kinase are indicated on the right.

precipitates with the mutant, but not the wild-type CL100 protein (Figure 7, lanes 7–10). However, in contrast to previous results, we find that the mutant CL100 protein will associate with both the unphosphorylated and phosphorylated forms of MAP kinase (Figure 7, lane 9). Furthermore, in contrast to the results obtained with CL100, Pyst1 does not require mutation of the essential cysteine within the active site of the enzyme to become complexed with MAP kinase (Figure 7, lanes 3 and 4). The MAP kinase complexed with wild-type Pyst1 is in the unphosphorylated form in both unstimulated and serum-stimulated cells (Figure 7, lanes 3 and 4). This

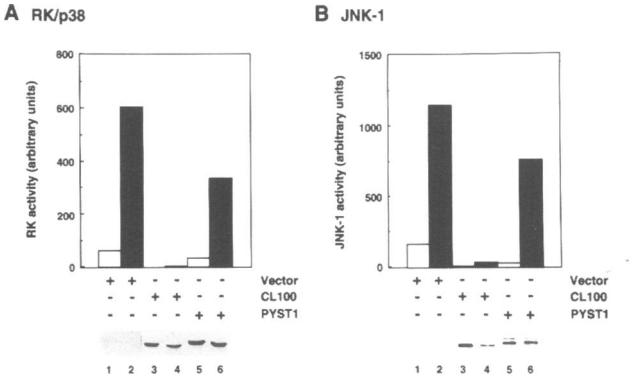

Fig. 8. The effects of expressing Pyst1 and CL100 on the activation of RK/p38 and JNK-1 kinases in Cos-1 cells. (**A**) Cells were co-transfected with 12CA5 epitope-tagged reactivating kinase and either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1. Following transfection, cells were either left untreated (open bars) or exposed to 0.5 mM sodium arsenite for 30 min (closed bars) before lysis and immunoprecipitation with anti-12CA5 monoclonal antibody. Immunoprecipitates were then assayed for RK activity by measuring the reactivation of MAPKAP kinase-2. (**B**) Cells were co-transfected with 12CA5 epitope-tagged JNK-1 and either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1. Following transfection, cells were either left untreated (open bars) or exposed to 80 J/m^2 UV-C radiation (closed bars) before lysis and immunoprecipitation with anti-12CA5 monoclonal antibody. Immunoprecipitates were then assayed for JNK-1 kinase activity in an immune complex assay using GST–c-Jun as substrate. Experiments were performed at least three times and the results of representative experiments are shown; kinase activities are the means of triplicate determinations. The lower panels in (A) and (B) show Western blots of the lysates from these experiments probed with 9E10 anti-myc monoclonal antibody to detect the CL100 (lanes 3 and 4) and Pyst1 proteins (lanes 5 and 6).

most probably reflects the phosphatase activity of Pyst1, rather than an inability to bind the activated form, as the mutant Pyst1 is found complexed to both phosphorylated and unphosphorylated MAP kinase (Figure 7, lanes 5 and 6).

Pyst1 is unable to block the activation of either RK/p38 or JNK-1 in vivo

Our *in vitro* results (Table I and Figure 5) strongly suggest that the Pyst1 phosphatase is able to discriminate between the MAP kinases and the distinct SAP and RK/p38 kinases. To address this question under more physiological conditions, we have co-transfected 12CA5 epitope-tagged reactivating kinase (RK/p38) with either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1 and prepared lysates from Cos-1 cells either unstimulated or treated with sodium arsenite, which has previously been shown to be a potent activator of RK/p38 (Rouse *et al.*, 1994). These lysates were then immunoprecipitated using the 12CA5 monoclonal antibody and assayed for RK activity. Figure 8A shows that sodium arsenite treatment leads to almost a 10-fold activation of heterologously expressed RK. However, expression of CL100 abolishes the basal RK activity and blocks the arsenite-induced activation of this kinase. In contrast, expression of Pyst1 causes only a modest decrease in the basal level of RK activity in unstimulated cells and is unable to block kinase activation by sodium arsenite.

In parallel experiments, 12CA5-tagged JNK-1 was co-transfected with either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1 and lysates were prepared from Cos-1 cells either unirradiated or exposed to UV light, which is a potent activator of JNK-1 (Derijard *et al.*, 1994). These lysates were then immunoprecipitated using the 12CA5 monoclonal antibody and assayed for JNK-1 activity. UV radiation activated heterologously expressed JNK-1 and this activation was blocked by expression of CL100, but not by Pyst1 (Figure 8B). The substrate selectivity demonstrated by Pyst1 in these experiments is not due to differences in the levels of expression of the two phosphatases, as Western blotting using an anti-myc epitope antibody shows that Pyst1 and CL100 are expressed at comparable levels in these cells (lower panels, Figure 8). One possible explanation for the results we have obtained is some differential effect of the Pyst1 and CL100 phosphatases on upstream elements of the signalling cascades. We have looked at the ability of both CL100 and Pyst1 to inactivate MAP kinase kinase and two isoforms of RK kinase (designated RKK1 and RKK2) which have been purified from rabbit skeletal muscle. We find that both CL100 and Pyst1 are unable to inactivate these enzymes (data not shown).

CL100 but not Pyst1 is able to block nuclear signalling mediated by the SAP kinases in response to UV radiation

The amino terminus of the ATF/CRE element binding protein ATF-2 contains a transcriptional activation domain that is regulated by phosphorylation (Livingstone *et al.*, 1995). This domain requires two threonine residues (Thr69 and Thr71) for activity, both of which are phosphorylated *in vivo* and are also efficiently phosphorylated by the SAP kinases *in vitro* (Livingstone *et al.*, 1995). Consistent with these findings, the activity of this domain is stimulated by treatments such as UV radiation which are known to activate either the stress-activated protein kinases such as JNK-1 or the RK/p38 kinase. In order to explore further the specificity of Pyst1 and CL100, expression vectors encoding the N-terminal activation domain of ATF-2 fused to Gal4 and a CAT reporter controlled by Gal4 binding sites were co-transfected into NIH 3T3 cells with either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1. These transfected cells were then grown in low serum, UV irradiated and incubated for 8 h prior to assay for CAT expression. Consistent with previous studies using these constructs (Livingstone *et al.*, 1995), Figure 9 shows that the activation domain of ATF-2 is induced by UV radiation. This activation is strongly inhibited by co-expression of the CL100 phosphatase, but is not prevented by co-expression of Pyst1. Identical results were obtained using an ATF-2–lexA DNA binding domain fusion in conjunction with a lexA-responsive CAT reporter. As was the case in the experiments shown in Figure 8, this difference in activity is not due to differences in the levels of expression of the two phosphatases as immunofluorescence analysis of the 9E10-tagged phosphatases shows that both CL100 and Pyst1 are expressed at comparable levels in these cells (data not shown).

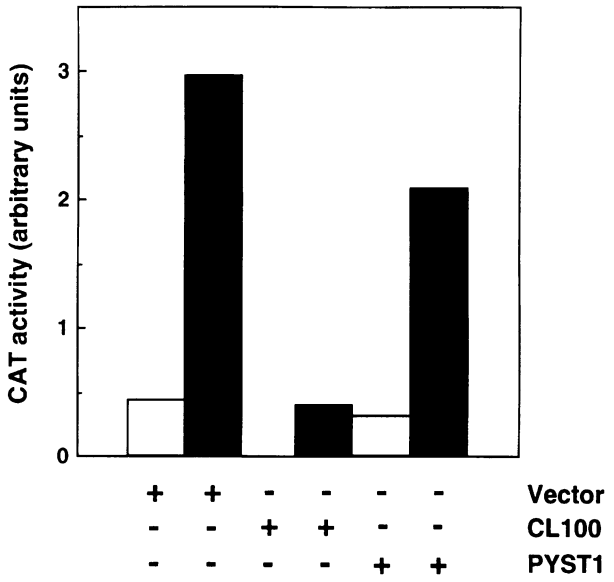

Fig. 9. The effects of Pyst1 and CL100 on the induction of the N-terminal activation domain of ATF-2 by UV radiation. Expression vectors encoding the N-terminal activation domain of ATF-2 fused to Gal4 and a CAT reporter controlled by Gal4 binding sites were co-transfected into NIH 3T3 cells with either plasmid vector, expression vector containing myc-tagged CL100 or expression vector containing myc-tagged Pyst1. Transfected cells were then grown in low serum (0.5%) and 40 h later were either untreated (open bars) or irradiated with 50 J/m^2 UVC (closed bars). Eight hours after UV treatment, the cells were harvested and CAT activity measured. Experiments were performed at least three times and the results of a representative experiment are shown.

Discussion

There is an increasing body of evidence which suggests that a subfamily of dual-specificity (Thr/Tyr) phosphatases, typified by CL100/MKP-1, play an important role in regulating the activity of the MAP kinases in mammalian cells (Keyse, 1995). The human CL100 phosphatase and its mouse homologue 3CH134/MKP-1 may be responsible for the inactivation of MAP kinase following growth factor stimulation or cellular stress in fibroblasts (Alessi *et al.*, 1993; Sun *et al.*, 1993; Zheng and Guan, 1993), while the Pac-1 protein may downregulate MAP kinase in T cells (Ward *et al.*, 1994). Furthermore, the isolation of CL100-like MAP kinase phosphatases from both *Chlamydomonas* (Haring *et al.*, 1995) and *Saccharomyces cerevisiae* (Doi *et al.*, 1994) indicates that this mechanism of MAP kinase inactivation is highly conserved.

Here we report the isolation of two human dual-specificity phosphatases, Pyst1 and Pyst2. The observation that these proteins are closely related to each other, but show differing patterns of mRNA expression in human tissues, strongly suggests that they may represent tissue- or cell-specific enzymes with closely related biological functions. The former conclusion is strongly supported by our finding that Pyst1, but not Pyst2, mRNA is expressed constitutively in human skin fibroblasts. Our conclusion that these proteins are novel members of the subfamily of CL100-like MAP kinase phosphatases is based on a number of structural and biochemical characteristics. First, these proteins are more similar in sequence to the existing members of this family of proteins than they are to other vaccinia VH-1-like dual-specificity phosphatases.

Furthermore, the Pyst1 sequence contains two amino acid sequence motifs within its amino-terminal domain which are homologous to regions of the cell cycle regulatory phosphatase cdc25 (Keyse and Ginsburg, 1993; Kwak *et al.*, 1994). The function of these sequences is as yet unknown, but their presence appears to be characteristic of this class of enzymes and distinguishes them structurally from other members of the family of VH1-like dual-specificity phosphatases. At the biochemical level, Pyst1, like CL100, is highly specific for the dephosphorylation and inactivation of MAP kinase *in vitro*. In addition, Pyst1 expressed in Cos-1 cells is able to block the serum-induced activation of MAP kinase and is able to form a physical complex with endogenous MAP kinase in these cells.

To date, all of the members of the MAP kinase phosphatase subfamily have exhibited two characteristic properties. First, the expression of their genes is strongly inducible by mitogens, and in some instances by cellular stress, and secondly, these proteins are all found to be localized in the cell nucleus. These properties have led to the hypothesis that these enzymes constitute an inducible feedback mechanism for the regulation of the MAP kinases and that the function of these enzymes is restricted to the cell nucleus. However, Pyst1 exhibits neither of these properties. Pyst1 mRNA levels in human skin fibroblasts increase only slightly some hours following growth factor stimulation and Pyst1 mRNA is not significantly induced in response to heat shock, oxidative or genotoxic stress. In addition, unlike CL100, the Pyst1 protein clearly localizes within the cytoplasm in transfected Cos-1, NIH 3T3 and HeLa cells. Although it could be argued that the Pyst1 gene may still be inducible, and that we have not been able to find the appropriate stimulus or that Pyst1 activity might be regulated at the post-transcriptional level, the above properties clearly set Pyst1 apart from the other dual-specificity MAP kinase phosphatases and suggest that this enzyme may play a role which is quite distinct from these inducible nuclear enzymes.

Much attention has recently been focused on the phosphorylation and subsequent activation of transcription factors by MAP kinases within the cell nucleus. However, MAP kinase is known to phosphorylate target proteins within the cytosol (Davis, 1993) and under certain conditions MAP kinase is transiently activated without being translocated to the nucleus (Traverse *et al.*, 1992). In addition, recent immunocytochemical and biochemical analyses have revealed that approximately one-third of the total MAP kinase in NIH 3T3 fibroblasts is associated with the microtubule cytoskeleton (Reszka *et al.*, 1995). Finally, it has been shown that in a variety of cell types MAP kinase can be inactivated in the absence of new protein synthesis, suggesting that the induction of CL100-like MAP kinase phosphatases is not required (Wu *et al.*, 1994; Alessi *et al.*, 1995; Lewis *et al.*, 1995). All of the above observations indicate that there may be constitutive cytosolic enzymes capable of inactivating MAP kinases. Recent evidence has suggested that the Ser/Thr protein phosphatase PP2A may inactivate MAP kinase in certain cells and there is biochemical evidence which suggests that as yet unidentified protein tyrosine phosphatases may also be involved (Sarcevic *et al.*, 1993; Alessi *et al.*, 1995). The constitutive expression and cytosolic localization of Pyst1 make this enzyme an attractive candidate for such a tyrosine phosphatase in human skin fibroblasts. With respect to the above, it is interesting that, unlike CL100, wild-type Pyst1 is able to form a stable complex with endogenous MAP kinase in Cos-1 cells. This raises the possibility that this phosphatase might be able to physically anchor MAP kinase (presumably in an inactive form) in the cytosol. It will be very interesting to look at the possible co-localization of Pyst1 and the different MAP kinase isoforms in cells under various conditions.

The cloning and characterization of Pyst1 and Pyst2 bring the total number of mammalian CL100-like enzymes so far discovered to six, and our data show that at least three of these, Pyst1, CL100 and B23/hVH-3, are expressed in human diploid fibroblasts. This would indicate that the regulation of MAP kinase by these enzymes may be complex. At one level, this may be a reflection of tissue specificity, as is the case with Pac-1, which is expressed only in haematopoietic cells (Rohan *et al.*, 1993) or of subcellular localization (as discussed above for Pyst1). However, a second level of complexity is suggested by the recent characterization of the SAPKs and RK/p38, and the finding that these kinases lie in distinct signalling pathways (Rouse *et al.*, 1994; Derijard *et al.*, 1995). This suggests that, in addition to distinct activators, these kinases may also be inactivated by distinct phosphatases, thus providing for the differential regulation of these pathways.

In order to study the substrate specificity of Pyst1, we have compared the activity of recombinant Pyst1 and CL100 proteins towards MAP kinase, the SAP kinases and RK/p38 kinase *in vitro*. We find that Pyst1 is able to discriminate between MAP kinase and these other enzymes in that this phosphatase is ~100-fold more active towards activated p42 MAP kinase than towards the RK/p38 kinase. In addition, Pyst1 is unable to inactivate stress-activated c-Jun-associated kinase activities isolated from sodium arsenite-treated human fibroblasts (Figure 5C). We conclude from the above that both the stress-activated protein kinases and RK/p38 are unlikely to be physiological substrates for Pyst1. This conclusion is underlined by the results of our experiments which show that Pyst1, unlike CL100, is unable to prevent the stress-mediated activation of either RK/p38 or JNK-1 in Cos-1 cells, and that Pyst1 is unable to block nuclear signalling mediated by the SAP kinases in UV-irradiated NIH 3T3 cells. While our data strongly suggest that the MAP kinases are physiological substrates for Pyst1, it should be noted that Pyst1 is somewhat less active towards MAP kinase than CL100 (see Table I). There are a number of possible explanations for this. First, the *in vivo* activity of Pyst1 could be increased by post-translational modification or by association with other proteins. Alternatively, it is possible that p42 MAP kinase is not the physiological substrate for Pyst1 in the cell. Recent work has identified novel members of the MAP kinase subfamily which also contain the T-E-Y signature sequence found in the p42 and p44 MAP kinase isoforms (Lee *et al.*, 1995; Zhou *et al.*, 1995). It will be of interest to determine whether Pyst1 shows activity towards these novel enzymes.

With respect to the substrate specificity of CL100, we note that a 30 min incubation with 50 μg/ml of CL100 phosphatase is required to inactivate 95% of c-Jun kinase

activity. We estimate from this result that CL100 is ~8- to 10-fold less active towards this class of enzymes when compared with its activity towards p42 MAP kinase or RK/p38. This finding is in agreement with a preliminary report which indicated that 10-fold greater concentrations of CL100/MKP-1 were required to inactivate JNK-1 compared with p42 MAP kinase (Sun *et al.*, 1994). It has recently been suggested that CL100 may play a physiological role in the regulation of the JNK family of c-Jun kinases. This conclusion was based on temporal correlations between the inactivation of c-Jun kinase activity and induction of CL100 mRNA in cells treated with DNA-damaging agents (Liu *et al.*, 1995). Based on our biochemical studies, we would suggest that a more likely candidate is RK/p38, which is both a good substrate for this phosphatase and is activated by many of the stress treatments which induce CL100 mRNA, including UV radiation (Rouse *et al.*, 1994; Raingeaud *et al.*, 1995).

In conclusion, we have isolated two novel human MAP kinase phosphatases, Pyst1 and Pyst2. The constitutive expression and non-inducibility of the Pyst1 mRNA, coupled with the cytosolic localization of the Pyst1 protein, suggest that this enzyme, unlike other members of this family of proteins, may be involved in MAP kinase inactivation within the cytosolic compartment of the cell. This provides the first indication that MAP kinases may be regulated by different MAP kinase phosphatases, depending on their subcellular localization. Finally, the demonstration that Pyst1 is able to discriminate between the MAP kinases and either SAP kinases or RK/p38 *in vitro* and *in vivo* provides evidence that different members of the MAP kinase family of enzymes may be regulated by different dual-specificity phosphatases. Furthermore, Pyst1 used in combination with CL100 will provide a powerful tool with which to dissect the biological roles of signalling through the MAP, SAP and RK/p38 kinase pathways, respectively, in determining the response of mammalian cells to various stimuli.

Materials and methods

cDNA cloning

A polymerase chain reaction (PCR) using a human brain cDNA library (Clontech) as template and the following oligonucleotides (GTCA-GAATTCTGGTGCACTGCCTGGCAGGC and CTCAGAATTCTCAC-GTGGACTCCAGCGTATTG) as primers, was used to obtain a 278 bp DNA fragment corresponding to an EST from infant brain (Adams *et al.*, 1993). This fragment of DNA, which was 62% homologous to the human CL100 cDNA, was then used as a probe to screen this brain cDNA library using standard techniques (Sambrook *et al.*, 1989). DNA from positive clones was sequenced in both strands using the dideoxy method (Sanger *et al.*, 1977), and partial cDNAs for Pyst1 and Pyst2 were identified. These partial cDNA clones were then used to screen a human skin fibroblast cDNA library (Keyse and Emslie, 1992), from which a clone containing the entire Pyst1 coding region was obtained. A second cDNA, which specified the C-terminal 320 amino acids of the Pyst2 protein, was obtained by subsequent screening of a human testis cDNA library (Clontech).

Plasmids

All manipulations were carried out by standard techniques and plasmid structures were verified by DNA sequencing. The Pyst1 open reading frame was subcloned as an *Nde*I–*Xho*I fragment into the bacterial expression vector pET15b (Novagen). The cDNAs encoding CL100 (Keyse and Emslie, 1992), Pyst1, JNK-1 (Derijard *et al.*, 1994) and *Xenopus* RK (XMpk2; Rouse *et al.*, 1994) were subcloned into a modified pSG5 mammalian expression vector (Stratagene), which contains an *Xho*I site followed by a single copy of either the myc epitope tag (EQKLISEEDL) or the 12CA5 epitope tag (YPYDVPDYA), followed by a stop codon and a *Bam*HI site. Substitution of Cys-293 for Ser in Pyst1 to produce the inactive mutant was performed by oligonucleotide-directed mutagenesis using the Sculptor system (Amersham). The plasmid pEXV3 ERK2-myc encoding myc-tagged wild-type p42 MAP kinase was provided by Dr C.Marshall (Institute of Cancer Research, London). The plasmids pAGC2, which encodes the amino terminal activation domain of ATF-2 fused to the Gal4 DNA binding domain, G5E4CAT, a Gal4-dependent CAT reporter plasmid, pAG (1-147), which encodes the DNA binding domain of Gal4 alone and pJATLac, which encodes β-galactosidase, were kindly provided by Dr N.Jones [Imperial Cancer Research Fund (ICRF), London].

Cell culture, transfection and indirect immunofluorescence

FEK4 primary human skin fibroblasts were cultured routinely and treated with radiation and chemicals exactly as described previously (Keyse and Emslie, 1992). Cos-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and transfected using a standard calcium phosphate method. Indirect immunofluorescence experiments in Cos-1 cells transiently transfected with myc-tagged CL100 and Pyst1 proteins were performed using anti-myc monoclonal antibody 9E10 exactly as described previously (Lewis *et al.*, 1995). Cell staining was observed and photographed with an Olympus BH-2 microscope fitted with a D-plan APO 40× objective using excitation/emission filters for fluorescein isothiocyanate (FITC) and DAPI. At least 50 9E10-positive cells were examined in each cell type and staining patterns were entirely consistent with the representative cells photographed.

In co-transfection experiments, plasmids encoding kinase reporters and either Pyst1 or CL100 were introduced at a ratio of 1:1. NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Co-transfection experiments, UV irradiations and CAT assays using the ATF-2 Gal4 fusion/Gal4 CAT reporter system in conjunction with either Pyst1 or CL100 were performed using DEAE–dextran exactly as described by Livingstone *et al.* (1995), except that plasmids were introduced in the following amounts (per 5 cm dish): pAGC2 or pAG (1-147), 4 μg; G5E4CAT, 2 μg; pSG5 vector and derivatives of it encoding CL100 or Pyst1, 8 μg; pJATLac, 2 μg.

RNA extraction and Northern blot analysis

Total RNA was prepared from FEK4 fibroblasts using TRIzol reagent (Gibco BRL) as recommended by the manufacturers. The human multiple-tissue Northern blot was obtained from Clontech and Northern blot analysis of mRNA from FEK4 fibroblasts 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 DNA templates: the complete human CL100 cDNA (Keyse and Emslie, 1992); the complete Pyst1 cDNA; a 300 bp cDNA fragment derived from the 3′ untranslated region of Pyst2; a cDNA fragment corresponding to nucleotides 1001–1921 within the 3′ untranslated region of B23/hVH-3 (Kwak and Dixon, 1995); a 1400 bp *Pst*I fragment of the rat cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (Piechaczyk *et al.*, 1984).

Antibodies, immunoblotting and immunoprecipitations

The anti-Pyst1 antibody was produced by immunizing rabbits with a synthetic peptide NH_2-CGG**LYFTTPSNQNVYQVPSLQST**-COOH (the bold letters correspond to residues 362–381 of the Pyst1 protein), coupled to keyhole limpet haemocyanin (Pierce). Purified Pyst1 protein expressed in *E.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 (15%), 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 by Nebreda and Hunt (1993). Cos-1 cells transfected with either 12CA5-tagged RK/p38, 12CA5-tagged JNK-1 or myc-tagged p42 MAP kinase were lysed in the following buffer: 1% Triton X-100, 0.27 M sucrose, 20 mM Tris–acetic acid (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 0.1% β-mercaptoethanol, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM Pefabloc). The kinase was then immunoprecipitated by the addition of 3.5 μg 12CA5 (BAbCO, USA) antibody (in the case of RK/p38 and

JNK-1) or 10.5 μg anti-myc monoclonal 9E10 (in the case of p42 MAP kinase) and protein A–Sepharose beads (12CA5) or protein G–Sepharose beads (9E10) and rocking for 2 h at 4°C. The immunoprecipitates were then washed once in lysis buffer and four times in 50 mM Tris–HCl (pH 7.5) before either being assayed for kinase activities or analysed by SDS–PAGE.

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

Phosphorylase, casein, myosin light chain, RCM lysozyme and myelin basic protein were ^{32}P-labelled exactly as described in Alessi *et al.* (1993). The peptide substrates (AFLEDFFTSTEPQYQPGENL and DRVYVHPF) were phosphorylated on tyrosine and ^{32}P-labelled using c-Fgr tyrosine kinase (Ruzzene *et al.*, 1993; kindly provided by Dr L.A.Pinna, Padova, Italy). MAP kinase was activated and ^{32}P-labelled using activated MAP kinase kinase as described in Alessi *et al.* (1995). Cleavage of activated GST-MAPK was performed using 10 μg/ml thrombin in a reaction containing 1.0 ml of ^{32}P-labelled GST-MAPK in 50 mM Tris–HCl (pH 7.5), 0.1 mM EGTA, 0.03% Brij-35, 0.5 mM $CaCl_2$ and 0.1% (by vol) β-mercaptoethanol which was incubated for 30 min at 30°C. Purified MalE–RK fusion protein (kindly provided by Dr A.R.Nebreda, ICRF Clare Hall Laboratories) was activated and phosphorylated equally at Thr-183 and Tyr-185 (to 1:1 mol phosphate/mol protein) using partially purified RK kinase (provided by Dr N.Morrice, University of Dundee) as follows. The incubation (total volume of 0.3 ml) was carried out for 20 min at 30°C in 50 mM Tris–HCl (pH 7.5), 0.1 mM EGTA, 0.03% (by vol) Brij-35, 0.1% (by vol) β-mercaptoethanol (buffer A) containing 2.3 μM MalE–RK, 30 μl RK kinase, 10 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (5 c.p.m./fmol), 1 μM okadaic acid and 1 μM sodium orthovanadate. Cleavage of activated MalE–RK was performed using 25 μg/ml factor Xa protease (New England Biolabs) in a reaction containing 1.0 ml of ^{32}P-labelled MalE–RK in 50 mM Tris–HCl (pH) 7.5, 0.1 mM EGTA, 0.03% Brij-35, 0.5 mM $CaCl_2$ and 0.1% (by vol) β-mercaptoethanol which was incubated for 60 min at 30°C, followed by 16 h at 4°C. Under these conditions, we were only able to achieve 50% cleavage of the MalE–RK fusion protein. The dephosphorylation of the fusion protein and the free kinase were therefore monitored in the same experiment, and analysis carried out after SDS–PAGE.

Activated c-Jun kinase was 'pulled down' specifically from lysates of sodium arsenite-treated human KB cells using a GST–c-Jun (residues 1–194) fusion protein (kindly provided by Dr R.Treisman, ICRF, London, and Dr R.Meier, University of Dundee). Pyst1 protein was expressed in bacteria, purified and refolded exactly as described previously for CL100 (Keyse and Emslie, 1992). Bacterially expressed PTP1B was provided by Dr D.Barford, University of Oxford. Assays of pNPP, MAP kinase, RK and c-Jun kinase phosphatase activities and phosphoamino acid analysis were carried out exactly as described previously (Keyse and Emslie, 1992; Alessi *et al.*, 1993).

Kinase assays

The activity of recombinant p42 MAP kinase or myc-tagged p42 MAP kinase immunoprecipitated from Cos-1 cells using the 9E10 monoclonal antibody was assayed using myelin basic protein as substrate as described in Alessi *et al.* (1993). The activity of recombinant RK/p38 or 12CA5-tagged RK/p38 immunoprecipitated from Cos-1 cells using the anti-12CA5 monoclonal antibody was assayed based on its ability to reactivate MAPKAP kinase-2 exactly as described in Rouse *et al.* (1994). Jun kinases 'pulled down' from KB cell lysates using GST–c-Jun fusion protein or 12CA5-tagged JNK-1 immunoprecipitated from Cos-1 cells using the anti-12CA5 monoclonal antibody were assayed exactly as described by Hibi *et al.* (1993). All kinase assays were performed in triplicate and mean values are shown.

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