Characterization of a murine gene encoding a developmentally regulated cytoplasmic dual-specificity mitogen-activated protein kinase phosphatase

Robin J. DICKINSON*, David J. WILLIAMS*, David N. SLACK*, Jill WILLIAMSON†, Ole-Morten SETERNES* and Stephen M. KEYSE*[1]

*Cancer Research UK, Molecular Pharmacology Unit, Biomedical Research Centre, Ninewells Hospital, Dundee DD1 9SY, Scotland, U.K., and †Cancer Research UK, Cytogenetics Laboratory, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Mitogen-activated protein kinases (MAPKs) play a vital role in cellular growth control, but far less is known about these signalling pathways in the context of embryonic development. Duration and magnitude of MAPK activation are crucial factors in cell fate decisions, and reflect a balance between the activities of upstream activators and specific MAPK phosphatases (MKPs). Here, we report the isolation and characterization of the murine *Pyst3* gene, which encodes a cytosolic dual-specificity MKP. This enzyme selectively interacts with, and is catalytically activated by, the 'classical' extracellular signal-regulated kinases (ERKs) 1 and 2 and, to a lesser extent, the stress-activated MAPK p38α. These properties define the ability of this enzyme to dephosphorylate and inactivate ERK1/2 and p38α, but not JNK (c-Jun N-terminal kinase) *in vivo*. When expressed in mammalian cells, the Pyst3 protein is predominantly cytoplasmic. Furthermore, leptomycin B causes a complete redistribution of the protein to the nucleus, implicating a CRM (chromosomal region maintenance)1/exportin 1-dependent nuclear export signal in determining the subcellular localization of this enzyme. Finally, whole-mount *in situ* hybridization studies in mouse embryos reveal that the *Pyst3* gene is expressed specifically in the placenta, developing liver and in migratory muscle cells. Our results suggest that this enzyme may have a critical role in regulating the activity of MAPK signalling during early development and organogenesis.

Key words: catalytic activation, dephosphorylation, signal transduction, subcellular localization.

INTRODUCTION

Dual-specificity (threonine/tyrosine) protein phosphatases play a key role in the regulation of mitogen-activated protein kinase (MAPK) activity in eukaryotes ranging from yeast to humans [1]. These MAPK phosphatases (MKPs) act in direct opposition to MAPK kinases ['MKKs' or MAP/ERK kinases (MEKs, where 'ERK' represents extracellular signal-regulated kinase)], with the balance of these two activities determining the level of activation of the MAPK, and hence the biological outcome of signalling. Studies of MKPs in *Saccharomyces cerevisiae* and *Drosophila* have shown that the expression of the genes encoding these enzymes is itself regulated by signalling via MAPK cascades, suggesting that they function as components of negative feedback loops [2,3].

Thus far, ten distinct dual-specificity MKPs have been identified and characterized in mammalian cells. These can be divided into two main groups on the basis of expression properties and subcellular localization [1]. The first, which includes MKP-1/CL100/erp, MKP-2/hVH2/Typ1, hVH3/B23 and Pac-1, are encoded by growth factor- and/or stress-inducible genes, and these proteins are localized within the nucleus when expressed in mammalian cells. The second, which includes Pyst1/MKP-3/rVH6, Pyst2/MKP-X/B59, MKP-4, hVH5/M3/6, MKP-5 and MKP-7, are not encoded by immediate early genes and are localized predominantly within the cytoplasm. Recent excitement surrounding the MKPs has come from the finding that they exhibit exquisite substrate selectivity both *in vitro* and *in vivo*. This was demonstrated first for the cytosolic enzyme Pyst1/MKP-3, which efficiently dephosphorylates the 'classical' growth factor-activated MAPKs, ERK1 and ERK2, while exhibiting no activity towards stress-activated MAP kinases, such as p38α and c-Jun N-terminal kinase (JNK)1 [4,5].

The selective dephosphorylation of ERK2 by Pyst1/MKP-3 is accompanied by the formation of a physical complex between the two proteins [4]. Furthermore, ERK2 binding, which is mediated by the N-terminal non-catalytic domain of Pyst1/MKP-3 [6], is accompanied by catalytic activation of the phosphatase, as revealed by a greatly increased ability to hydrolyse *p*-nitrophenyl phosphate (*p*-NPP) *in vitro* [7]. A combination of structural and biochemical studies suggest that catalytic activation results from the displacement of the general acid (Asp^{262} in Pyst1) to a more favourable position for catalysis [8,9]. On the basis of these results, specific recognition and binding to MAPKs, coupled with catalytic activation, has been proposed to mediate the substrate selectivity of all dual-specificity MKPs, and recent work lends support to this idea. The closely related enzymes Pyst2/MKP-X and MKP-4 also bind directly to ERK2, and undergo catalytic activation [7,10], and the inducible nuclear enzyme MKP-1/CL100 binds selectively to ERK2, JNK and

Abbreviations used: ATF2, activating transcription factor-2; CRM, chromosomal region maintenance; DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FISH, fluorescence *in situ* hybridization; GFP, green fluorescent protein; HA, haemagglutinin; HGF, hepatocyte growth factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP/ERK kinase; MKP, MAPK phosphatase; NES, nuclear export signal; *p*-NPP, *p*-nitrophenyl phosphate.

[1] To whom correspondence should be addressed (e-mail S.Keyse@cancer.org.uk).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ430230.

p38α and is catalytically activated by these MAPK isoforms [11,12]. For all of these MKPs, binding and catalytic activation exactly mirrors the substrate selectivity of the phosphatase towards MAPKs *in vivo*.

Despite our expanding knowledge of the molecular basis of substrate recognition and catalysis by the mammalian MKPs, information about their physiological roles in mammalian cells and tissues is lacking. Studies in budding yeast and *Drosophila* suggest that these enzymes have key roles in the regulation of MAPK signalling in response to extracellular stimuli and during early embryonic development respectively [2,3]. More recent studies in the nematode *Caenorhabditis elegans* have shown that a dual-specificity MKP encoded by lip-1 (lateral signal induced phosphatase-1) plays a pivotal role in the regulation of MAPK signalling in cell fate specification during vulval development [13]. Thus far, the only genetic studies of a mammalian dual-specificity MKP have involved the generation of mice that lack a functional copy of the inducible nuclear phosphatase MKP-1 [14]. The broad distribution of *MKP-1* gene expression during mouse embryogenesis and development of tissues coupled with the observation that expression was localized to post-mitotic cell populations suggested an important role for MKP-1 in the process of cellular differentiation [15]. However, MKP-1-deficient mice are phenotypically normal and fertile, a result interpreted as indicating that other phosphatases must compensate for the loss of MKP-1 activity during development and in the adult animal [14].

As part of ongoing studies to understand the physiological roles of the Pyst subfamily of cytosolic MKPs, we report the isolation and characterization of the murine Pyst3 gene and its protein product. Pyst3 encodes a dual-specificity MKP that is highly related to, but not identical with, the human MKP-4 phosphatase. We demonstrate that this protein interacts with, and is catalytically activated by, both mitogen- and stress-activated MAPK isoforms, and is able to dephosphorylate and inactivate both ERK1/2 and p38α MAPKs *in vivo*. Like Pyst1, the Pyst3 protein is cytosolic, and we show that this localization is maintained by the activity of a chromosomal region maintenance (CRM)1-dependent nuclear export pathway. Finally, using whole-mount *in situ* hybridization we have determined the expression of Pyst3 during early mouse development. This reveals a very restricted distribution in which Pyst3 mRNA is found in the placenta, developing liver and migrating muscle cells. Our results suggest that Pyst3 may play a critical role in the regulation of MAPK signalling during early development and organogenesis.

EXPERIMENTAL

Isolation and characterization of murine genomic clones encoding Pyst3

A 129Sv/lDashII mouse genomic library was screened using the full-length human Pyst2 cDNA as a probe, and positive plaques were identified and purified. The inserts were then excised, subcloned and analysed using a combination of restriction analysis, PCR and DNA sequencing. This screen yielded clones containing the murine homologue of Pyst2 and a 9 kb *Eco*RI fragment (2.1pTZ) containing a related gene, which was distinct from both *Pyst1* and *Pyst2*. This gene was designated *Pyst3*.

Fluorescence *in situ* hybridization (FISH)

Metaphase spreads were prepared from normal mouse spleen as described by Monier et al. [16]. Pyst3 DNA (1 μg of 2.1pTZ) was labelled with biotin-14-ATP using a Bionick kit (Invitrogen, Carlsbad, CA, U.S.A.) and purified using a Sephadex G50 column, before ethanol precipitation with the addition of 50 μg of sheared salmon-sperm DNA and 50 μg of *Escherichia coli* tRNA. Hybridization and post-hybridization washes were performed exactly as described by Ragoussis et al. [17]. For detection of biotin-labelled probes, slides were incubated in avidin–FITC at 1:500 (Vector Laboratories, Burlingame, CA, U.S.A.) and then counterstained with 0.06 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma, Poole, Dorset, U.K.) in Citifluor (Citifluor Ltd, London, U.K.). Images were captured using a Zeiss Axioscop microscope equipped with a digital camera (Carl Zeiss Ltd, Stuttgart, Germany). Separate images of probe signal, banding pattern and counterstain were pseudo-coloured and merged using an Apple Powermac 8100 and Smartcapture software (Vysis Inc., Downers Grove, IL, U.S.A.).

Plasmids

All manipulations were performed using standard techniques [18] and plasmid structures were verified by DNA sequencing [19]. The complete Pyst3 open reading frame was assembled from the mouse genomic clone 2.1pTZ by overlap extension PCR [20]. The cDNA was then subcloned as an *Nde*I–*Xho*I fragment into the bacterial expression vector pET15b (Novagen, Madison, WI, U.S.A.), as an *Eco*RI–*Xho*I fragment into a modified pSG5 mammalian expression vector (Stratagene, La Jolla, CA, U.S.A.) encoding a C-terminal Myc-epitope tag [4] and as a *Bgl*II–*Sal*I fragment into the green fluorescent protein (GFP)-containing mammalian expression vector pEGFP-N1 (Clontech, BD Biosciences, Palo Alto, CA, U.S.A.). Finally, Pyst3 was subcloned as an *Nde*I–*Xho*I fragment into the GAL4 DNA-binding and activation domain fusion yeast expression vectors pGBKT7 and pGADT7 (Clontech Matchmaker 3). GAL4 DNA-binding and activation domain fusion yeast expression vectors containing the various mitogen- and stress-activated kinase isoforms have been described previously [12]. Mammalian expression plasmids encoding haemagglutinin (HA)-tagged ERK1, p38α and JNK-1 have been described previously [4,21].

Northern blot analysis

^{32}P-Labelled probe was generated by random-primed labelling [22] using a 701 bp fragment derived from the 5′ end (−212 to 489 bp) of the Pyst3 genomic DNA as template. A mouse Multiple Choice™ tissue Northern blot (OriGene Technologies Inc., Rockville, MD, U.S.A.) was hybridized and washed, as recommended by the manufacturer.

Cell culture, transfection and fluorescence microscopy

COS1 and HeLa cells were maintained in Dulbecco's modified Eagle's medium ('DMEM') supplemented with 10% (v/v) foetal bovine serum and transfected using a standard calcium phosphate method, as described previously [4]. Localization of the Pyst3 protein in HeLa cells was determined by transfection of 2 μg of pEGFP-N1-Pyst3, which encodes a C-terminal fusion between Pyst3 and GFP, or empty vector as a control. Cells were then cultured for 48 h. To treat cells with leptomycin B (kindly given by Dr Minoru Yoshida, University of Tokyo, Japan), the drug was added at a final concentration of 10 μg/ml for the final 3 h of culture. Cells were then washed with PBS and fixed for 5 min in chilled 4% (w/v) paraformaldehyde. Following extensive washing with PBS, nuclei were counterstained with DAPI. Cell staining was observed and photographed using a Zeiss Axiovert S100 microscope fitted with a digital camera (Hamamatsu

Photonics, Bridgewater, NJ, U.S.A.) by fluorescence microscopy using excitation/emission filters for FITC and DAPI. Images were processed using Openlab (Improvision, Warwick, U.K.) and Photoshop (Adobe, San José, CA, U.S.A.). At least 50 GFP-positive cells were examined in each experiment, and staining patterns were entirely consistent with the representative cells photographed.

Antibodies, immunoblotting and immunoprecipitations

The anti-Pyst3 antibody was produced by immunizing sheep with the synthetic peptides NH_2-Ser-Asp-Phe-Ser-Asp-Ala-Glu-Ser-Glu-Ala-Asp-Arg-Asp-Thr-Leu-Ser-Cys-Gly-Leu-Asp-Ser-Glu-Asn-Thr-Thr-Ser-COOH (corresponding to residues 233–258 of the Pyst3 protein) and NH_2-Ser-Thr-Val-Ser-Asp-Pro-Pro-Ser-Phe-Phe-Thr-Thr-Pro-Thr-Ser-Asp-Gly-Val-Phe-Glu-Leu-Asp-Pro-Thr-COOH (corresponding to residues 425–448 of the Pyst3 protein) coupled with keyhole-limpet haemocyanin (Pierce, Rockford, IL, U.S.A.). Purified Pyst3 protein expressed in *E. coli* or 5 μg of total protein from freshly prepared mouse tissue lysates was separated by SDS/PAGE and transferred to PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA, U.S.A.) before immunoblotting with the anti-Pyst3 antiserum using standard techniques [23]. The phosphorylation status of ERK2 was analysed by Western blotting using rabbit polyclonal antibodies specific for either pThr^{183}-ERK2 (Promega Corp., Madison, WI, U.S.A.) or pTyr^{185}-ERK2 (Calbiochem, La Jolla, CA, U.S.A.). As a loading control, blots were also probed with an anti-ERK2 monoclonal antibody (Zymed Laboratories, San Francisco, CA, U.S.A.). HA-tagged ERK1, p38α and JNK1 were detected in transfected COS1 cell lysates by Western blotting using the anti-HA monoclonal antibody 12CA5, and Myc-tagged Pyst3 was detected using the anti-Myc monoclonal antibody 9E10. HA-tagged MAPKs were immunoprecipitated from transfected COS1 cell lysates as described previously [4].

Expression and purification of recombinant Pyst3

The pET15b expression plasmid encoding His-tagged Pyst3 was transformed into the host bacterial strain BLR-DE3 (Novagen, Madison, WI, U.S.A.) using standard techniques. Following expression, the protein was purified using nickel-agarose resin (Ni^{2+}-nitriloacetate; Qiagen Inc., Valencia, CA, U.S.A.) under denaturing conditions and refolded by sequential dialysis exactly as described previously [24].

Protein phosphatase assays

Phosphatase activity and catalytic activation of recombinant Pyst3 was determined using *p*-NPP hydrolysis at 25 °C. This was measured in 96-well plates in 200 μl of 50 mM imidazole, pH 7.5, containing 10 mM dithiothreitol, 20 mM *p*-NPP and the indicated amount of Pyst3 protein supplemented with either 1 mM sodium orthovanadate or the indicated amount of various purified recombinant MAPKs. Reaction rates were monitored by absorbance at 405 nm, in a microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.) as described previously [10], and initial rates of *p*-NPP hydrolysis were determined using Microplate Manager III-Vs 1.57 (Bio-Rad Laboratories).

To measure dephosphorylation of ERK2 by Pyst3 *in vitro*, 500 ng aliquots of recombinant activated ERK2 (kindly provided by Dr Neil McDonald, Cancer Research UK, Lincoln's Inn Fields, London, U.K.) were incubated with increasing amounts of recombinant Pyst3 protein for 30 min at 37 °C. Following incubation, aliquots of each reaction equivalent to 5 ng of recombinant ERK2 were analysed using SDS/PAGE, transferred to nitrocellulose, and the phosphorylation status of the ERK2 was determined by Western blotting, as described above.

Kinase assays

The activity of Pyst3 towards HA-tagged ERK1, p38α and JNK1 MAPKs was determined by co-transfection of COS1 cells with 2 μg of expression vector encoding HA-tagged MAPK isoforms in combination with increasing amounts of pSG5-Pyst3-Myc. Following expression, cells were exposed to an appropriate activating stimulus (serum for ERK1; anisomycin for JNK1 and p38α), followed by immunoprecipitation and assaying of kinase activities, exactly as described previously [10].

Yeast two-hybrid assays

Plasmids encoding GAL4 DNA-binding domain and activation domain fusion proteins were transformed into the yeast strains PJ69-4A and PJ69-4α respectively, according to the manufacturer's instructions (Clontech). Semi-quantitative analysis of two-hybrid interactions was performed using a β-galactosidase assay, according to the manufacturer's instructions (Clontech). To ensure all protein–kinase interactions were detected, binding capability was assessed using each kinase isoform expressed as GAL4 DNA-binding domain and activation domain fusions, where possible.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to a protocol modified from that of Wilkinson [25]. Embryos were incubated overnight with 1 μg of digoxigenin-labelled cRNA probe, generated by run-off transcription with T7 polymerase from a pBluescript construct containing a 635 bp *Xba*I–*Pst*I restriction fragment derived from the 3′UTR of Pyst3. Photographs were taken on a Leitz dissecting microscope equipped with an Olympus 35 mm camera. Images were scanned and processed using Photoshop (Adobe).

RESULTS

Isolation and characterization of the murine *Pyst3* gene

High-stringency screening of the mouse 129Sv/lDashII genomic library with the full-length human Pyst2 cDNA yielded a number of positive signals. Upon further characterization of these clones, several were found to contain a murine gene related to, but distinct from, both *Pyst1* and *Pyst2*, and this was designated *Pyst3*. The *Pyst3* gene spans approx. 4.5 kb, and comprises three coding exons interrupted by two introns (Figure 1A). In terms of gene structure, both the positions and the phase of the two introns are conserved when compared with the human *Pyst1* and *Pyst2* genes [10].

Determination of the complete coding sequence of Pyst3 reveals that it specifies a protein of 448 amino acids, and sequence comparison indicates that it is most similar (85% identity; 87% similarity) to the human enzyme MKP-4 (Figure 1B). However, when compared with MKP-4, Pyst3 contains an insertion of 63 amino acids within the N-terminal non-catalytic domain. This insertion comprises mainly repeats of the sequence Ala-Glu-Ala-Lys, and lies between two conserved regions of sequence similarity found in all dual-specificity MKPs and the cdc25 phosphatase [26].

PCR analysis of DNAs from a panel of radiation hybrids has mapped the human *MKP-4* gene to chromosome Xq28 [27]. FISH analysis using our Pyst3 genomic clone maps the mouse

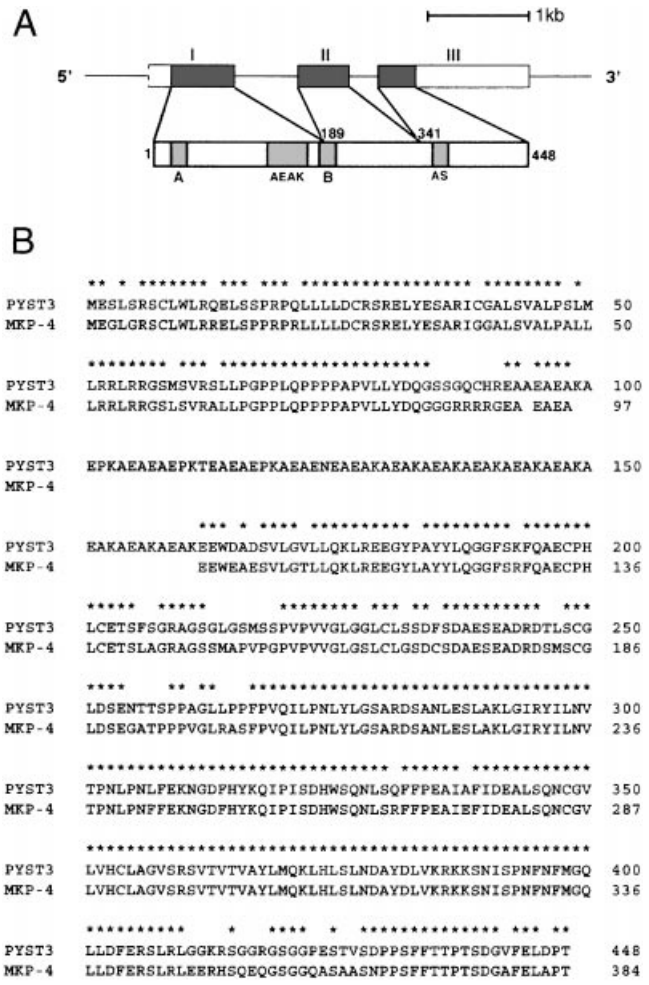

Figure 1 Genomic structure, amino acid sequence comparison of the encoded protein and chromosomal localization of the mouse Pyst3 gene

(**A**) Genomic structure of the mouse *Pyst3* gene. Exons are boxed and numbered, and the dark grey boxes correspond to coding sequences. Dashed lines indicate that the exon size has not yet been determined. The structure of the encoded Pyst3 protein is also shown, and amino acid residues are numbered. The positions of various domains within the protein are indicated by light grey boxes. These include the regions of homology with the cdc25 phosphatase (labelled 'A' and 'B'), the stretch of AEAK repeats (AEAK) and the position of the protein tyrosine phosphatase active site (AS). (**B**) Amino acid sequence alignment of the Pyst3 and human MKP-4 proteins. Identical residues are marked with an asterisk and spaces represent gaps introduced to maximize matches. (**C**) Processed image of a partial metaphase spread showing the specific chromosomal hybridization of the biotinylated Pyst3 probe to the mouse X chromosome in region B (arrowed). The signal was detected using avidin–FITC and counterstained with DAPI. Separate images of probe signal and DAPI banding pattern were pseudo-coloured and merged.

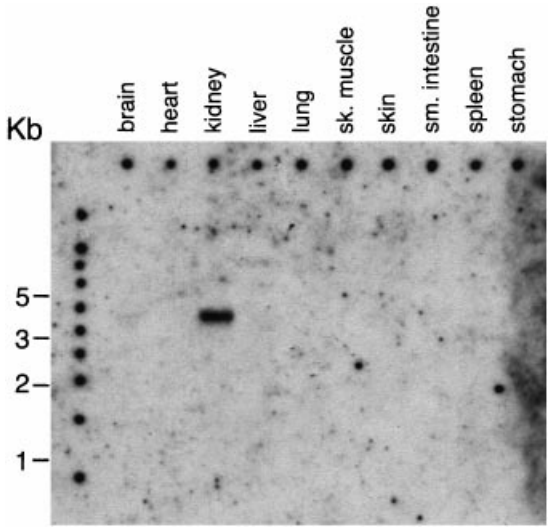

Figure 2 Northern blot analysis of Pyst3 mRNA in a variety of adult mouse tissues

RNA size markers are indicated on the left. sk. muscle, skeletal muscle; sm. intestine, small intestine.

gene to the X chromosome in region B (Figure 1C), which is syntenic with Xq28 [28]. Furthermore, we have performed a Northern blot analysis on mRNA derived from a range of adult mouse tissues. Using a probe from the 5′ end of the Pyst3 cDNA, we were able to detect a single mRNA species of approx. 3.8 kb, which, of the ten adult tissues tested, is expressed only in the kidney (Figure 2). Interestingly, an extensive tissue Northern blot analysis of the human MKP-4 transcript also revealed that expression was restricted to the adult kidney [27].

Pyst3 interacts with both mitogen- and stress-activated MAPKs *in vivo*

The specific dephosphorylation of ERK2 by Pyst1 and Pyst2 is accompanied by the formation of a tight physical complex between phosphatase and MAPK [4,10]. The N-terminal non-catalytic domain of Pyst1 mediates ERK2 binding, since removal of this domain abrogates substrate selectivity *in vivo* [6]. Furthermore, this binding is direct, as demonstrated by GST 'pull-down' experiments using purified proteins [7]. Pyst3 contains an insertion of 63 amino acids within this region, and it was important to determine whether Pyst3 was capable of interaction with MAPKs and also the full extent of its binding specificity. To answer this question, a yeast two-hybrid assay was used to determine the specificity of interactions between Pyst3 and a comprehensive panel of nine distinct mitogen- and stress-activated MAPK isoforms *in vivo*. In this assay, a DNA-binding-domain-fused 'bait' protein interacts with an activation-domain-

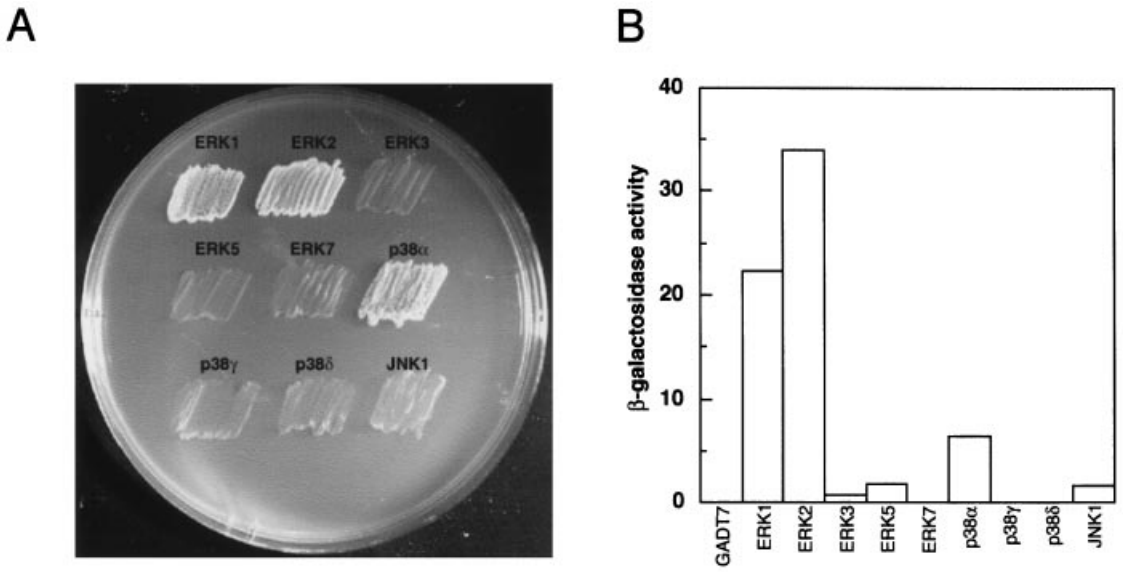

Figure 3 Analysis of two-hybrid interactions between Pyst3 and a panel of mitogen- and stress-activated MAPK isoforms

(**A**) pGBKT7.Pyst3 was transformed into PJ69-4A and mated with PJ69-4α expressing the GAL4 activation domain (AD) fusions pGADT7.ERK1, pGADT7.ERK2, pGADT7.ERK3, pGADT7.ERK5, pGADT7.ERK7, pGADT7.JNK1, pGADT7.p38γ and pGADT7.p38δ. p38α was expressed in PJ69-4A as a GAL4 DNA-binding domain (BD) fusion (pGBKT7.p38α), and mated with pGADT7.Pyst3 transformed into PJ69-4α. Yeast diploids expressing both BD and AD fusions were selected on synthetic dropout (SD) media deficient in leucine and tryptophan. —Leu/—Trp positives were re-streaked on to SD—Leu/—Trp/—His/—Ade. Protein–protein interactions were assessed by growth on this selective medium. (**B**) Semi-quantitative analysis of the two-hybrid interactions on the basis of the level of induction of the β-galactosidase reporter gene. Assays were performed in triplicate, and the results of a representative experiment are shown.

fused partner protein to reconstitute a sequence-specific transcriptional activator. The interacting pair then binds a specific sequence motif, thus activating transcription of at least two separate reporter genes. In our case, protein–protein interactions were detected by the activation of GAL4-dependent ADE2/HIS3/LacZ reporters. Activation of HIS3 and ADE2 was assessed by selection on synthetic dropout medium deficient in histidine and adenine (Figure 3A), and the strength of the interactions was quantified in a β-galactosidase assay (Figure 3B). Western blot analysis using GAL4-specific antibodies (Clontech) verified expression of each of the MAPK-fusion proteins used in the screen (results not shown). Pyst3 specifically interacts with ERK1, ERK2 and p38α with a rank order of binding strength ERK2 > ERK1 $\gg$ p38α. Very weak interactions, which were not revealed by growth on selective media, were also detected with JNK1, ERK3 and ERK5, whereas no significant interaction was seen with ERK7, p38δ or p38γ (Figure 3B). We consistently found that ERK1 has a lower affinity for Pyst3 when compared with ERK2, and this was also the case when either Pyst1 or MKP-1/CL100 was used as the bait in these assays [12].

Pyst3 dephosphorylates ERK2 *in vitro*

Pyst3 was expressed as a His-tagged fusion protein and purified from bacteria (Figure 4A, lane 1). A polyclonal antiserum raised against two C-terminal Pyst3 peptides detects this recombinant protein (Figure 4A, lane 2), and, in agreement with our tissue expression data (Figure 2; also see Figure 8), also recognizes endogenous Pyst3 protein in lysates of mouse placenta and adult kidney, but not of adult liver (Figure 4A, lanes 3–5). The Pyst3 protein is catalytically active, since it hydrolyses *p*-NPP, and this activity is abolished by sodium orthovanadate, a selective inhibitor of protein tyrosine phosphatases (Figure 4B). To determine whether Pyst3 has activity towards MAPK, recombinant activated ERK2 was incubated with increasing amounts of purified enzyme, and the phosphorylation state of the ERK2 was monitored using antibodies which specifically recognize either the phosphothreonine or phosphotyrosine residues within the Thr-Xaa-Tyr motif. Consistent with the ability of Pyst3 to interact with ERK2 in our two-hybrid assays, our results clearly show that Pyst3 dephosphorylates both residues with approximately equal efficiency (Figure 4C), and thus acts as a dual-specificity MKP *in vitro*.

Catalytic activation of Pyst3 by MAPKs

Significant progress in understanding MKP function was made with the demonstration that the catalytic activity of MKP-3 (Pyst1) could be specifically increased on binding to its substrate ERK2 *in vitro* [7]. Catalytic activation by MAPK isoforms has subsequently been demonstrated for Pyst2/MKP-X [10], CL100/MKP-1 [11,12] and MKP-2 [29]. To determine whether Pyst3 is activated in this way, *p*-NPP hydrolysis was monitored in the absence or presence of increasing amounts of recombinant ERK2, p38α and JNK1 (Figure 5A). The activity of Pyst3 is stimulated in a dose-dependent manner by these three MAPKs, with ERK2 being clearly the most potent. In order to determine the specificity of Pyst3 catalytic activation, we extended our study to a panel of six distinct recombinant mitogen- and stress-activated MAPK isoforms (Figure 5B). In addition to ERK2, p38α and JNK1, Pyst3 activity was also increased on incubation with ERK5. Although these stimulations are not in the order of the 20–30-fold effects seen on incubation of Pyst1/MKP-3 with ERK2, they would appear to be significant, since incubation with the closely related p38 MAPK isoforms γ and δ did not cause a significant increase in the catalytic activity of Pyst3. Interestingly, although p38α, JNK1 and ERK5 all increase the activity of Pyst3, of these only p38α appears to be able to interact strongly

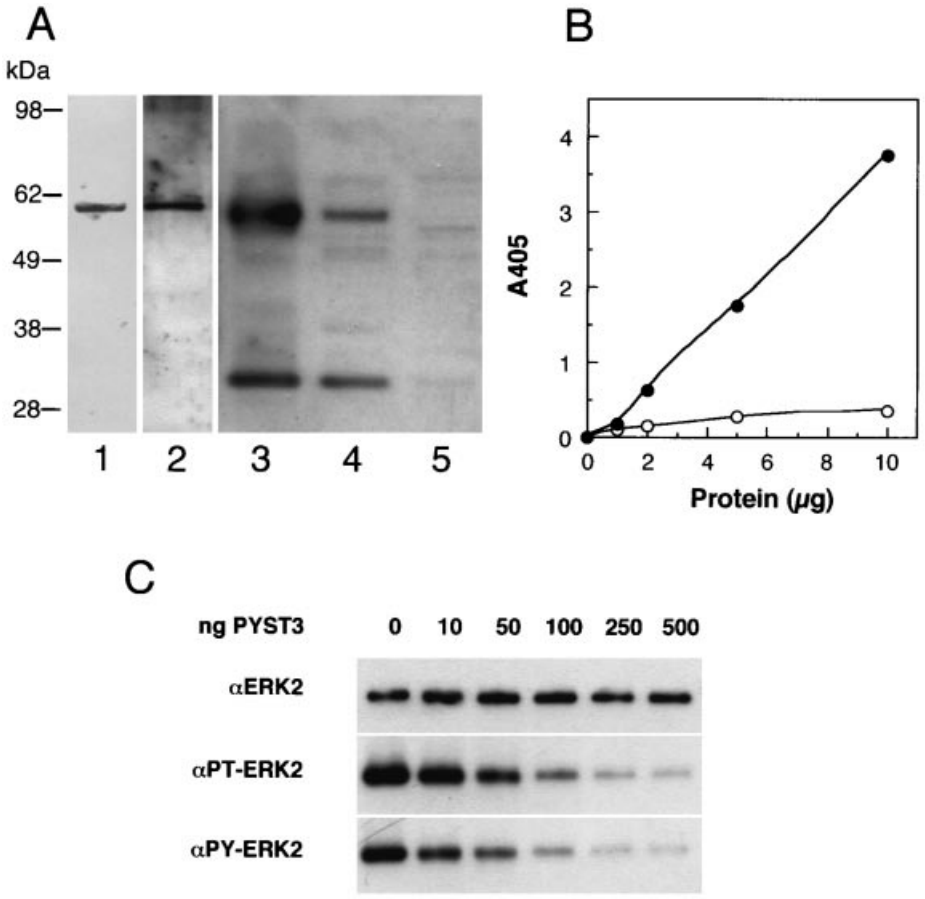

Figure 4 Expression and characterization of recombinant Pyst3 protein *in vitro*

(**A**) Expression of Pyst3 protein in *E. coli*. Coomassie Blue-stained SDS/PAGE gel of purified His-tagged Pyst3 fusion protein (lane 1), and an immunoblot of this protein (lane 2). Also shown are immunoblots of tissue lysates obtained from mouse placenta (lane 3), adult kidney (lane 4) and adult liver (lane 5). All blots were obtained using a polyclonal antiserum raised against peptides derived from the C-terminus of Pyst3. Molecular-mass markers are indicated on the left (kDa). (**B**) The Pyst3 protein possesses intrinsic phosphatase activity. The indicated amount of purified protein was assayed for its ability to hydrolyse *p*-NPP either in the absence (●) or presence (○) of 1 mM sodium vanadate. (**C**) Dephosphorylation of recombinant activated ERK2 by Pyst3 *in vitro*. Immunoblot of activated ERK2 protein following incubation with the indicated amount of recombinant Pyst3. The blot was probed with an anti-ERK2 antibody as a loading control (upper panel), and then with antibodies that specifically recognize either the phosphorylated threonine (PT; middle panel) or tyrosine (PY; lower panel) residues within the conserved T-E-Y motif of ERK2.

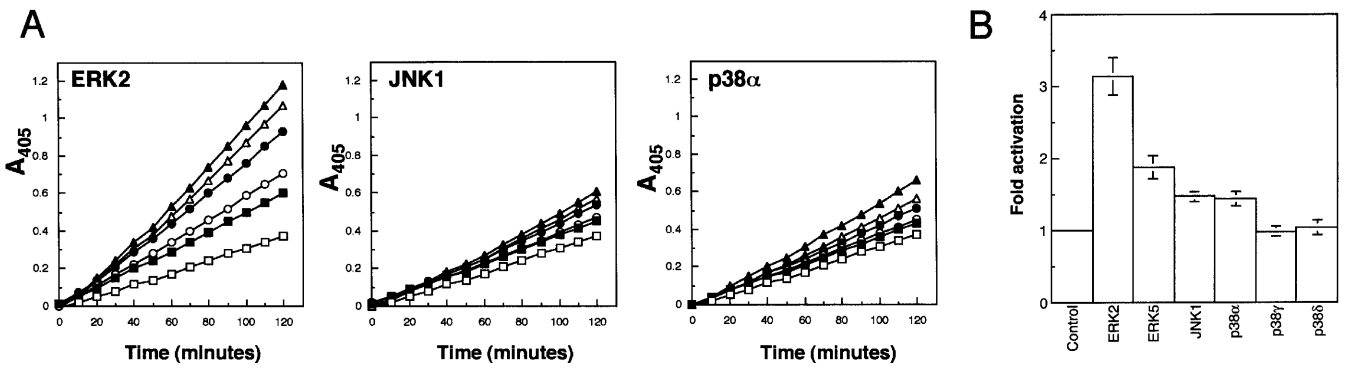

Figure 5 Catalytic activation of Pyst3 by MAPKs *in vitro*

(**A**) Dose-dependent catalytic activation of Pyst3 on incubation with MAPKs *in vitro*. Phosphatase activity of 5 μg of Pyst3 was assayed by time-dependent hydrolysis of *p*-NPP at 25 °C monitored at an absorbance of 405 nm (A_{405}) in the absence (□) or presence of 1 μg (■), 2 μg (○), 5 μg (●), 10 μg (△) or 20 μg (▲) of the indicated purified recombinant MAPK. Assays were performed in triplicate and the results of a representative experiment are shown. (**B**) Activation of Pyst3 by recombinant mitogen- and stress-activated MAPK expressed as an *n*-fold increase in specific activity towards *p*-NPP assayed in the presence of 10 μg of the indicated kinase. The amalgamated results of six independent experiments are shown.

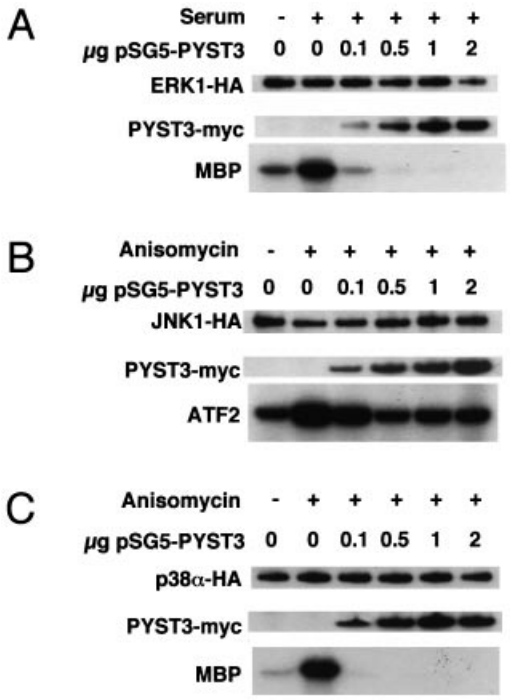

Figure 6 Pyst3 inhibits the activities of ERK1 and p38α MAPKs *in vivo*

COS1 cells were transfected with (**A**) HA-tagged ERK1, (**B**) HA-tagged JNK1 or (**C**) HA-tagged p38α expression constructs (2 μg of plasmid), together with the indicated amount of plasmid encoding Myc-tagged Pyst3. Cells were then stimulated either with serum (HA-tagged ERK1) or anisomycin (HA-tagged JNK1 and p38α) before lysis, immunoprecipitation and kinase assay using either myelin basic protein (MBP) (for ERK1 and p38α) or activating transcription factor-2 (ATF2) (for JNK1) as substrates (bottom panels of **A**–**C**). Immunoblots were performed using either anti-HA (top panels of **A**–**C**) or anti-Myc (middle panels of **A**–**C**) to verify expression of the MAPKs and Pyst3 respectively.

with Pyst3 *in vivo* (Figure 3A). However, a similar mismatch between binding and catalytic activation has been noted for the inducible nuclear phosphatase MKP-2 [29].

Inactivation of MAPK isoforms by Pyst3 in mammalian cells

Our yeast two-hybrid data indicate that Pyst3 is likely to interact with ERK1/2 and p38α in mammalian cells, and both kinases are also able to catalytically activate Pyst3 *in vitro*. However, other MAPKs, including JNK, also stimulate the activity of Pyst3 *in vitro* and therefore could be substrates for Pyst3. In order to determine the substrate selectivity of Pyst3 *in vivo*, co-transfection experiments were performed in which increasing amounts of Myc-tagged Pyst3 were expressed in COS1 cells, together with HA-tagged ERK1, JNK1 or p38α. Following activation of the respective kinases using an appropriate stimulus, cells were lysed and immunocomplex kinase assays were performed to determine the effects of Pyst3 expression on activity. Pyst3 clearly acts potently on ERK1 and p38α even at the very lowest levels of expression (Figures 6A and 6C). In contrast, Pyst3 elicits markedly lower inhibition of JNK, and is unable to abolish the activity of this kinase even at the highest levels of Pyst3 expression (Figure 6B). Thus it would appear that, at least with respect to p38α and JNK1, binding in the yeast two-hybrid assay is a better predictor of substrate selectivity for Pyst3 than is catalytic activation of the phosphatase *in vitro*.

Pyst3 is localized in the cytoplasm of mammalian cells by a leptomycin B-sensitive mechanism

The Pyst1 (MKP-3) and Pyst2 (MKP-X) proteins are localized predominantly in the cytoplasm when expressed in mammalian cells [4,10,30]. In contrast, inducible MKPs, such as MKP-1, MKP-2 and Pac-1, are found in the cell nucleus [31–33]. However, nothing is yet known about the mechanisms determining the

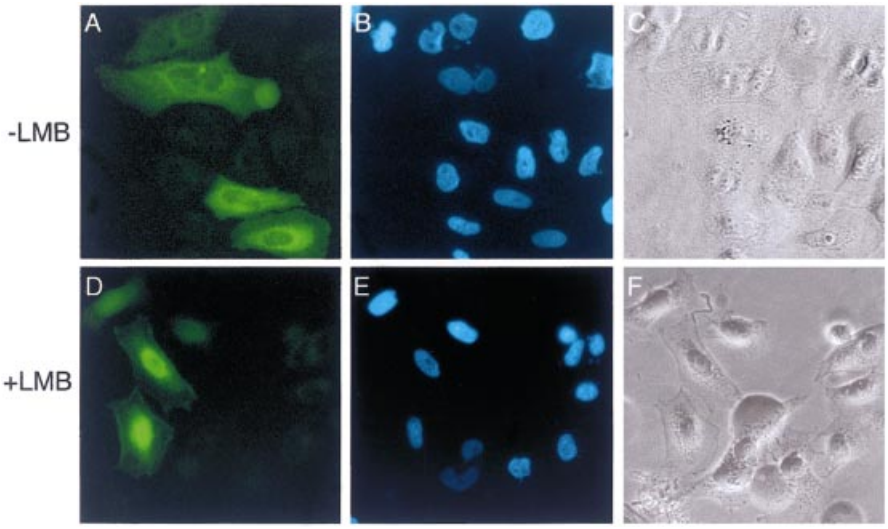

Figure 7 Pyst3 is localized in the cytoplasm of mammalian cells by a leptomycin B-sensitive mechanism

Localization of the Pyst3 protein in HeLa cells was determined by transfection of 2 μg of pEGFP-N1-Pyst3, which encodes a C-terminal fusion between Pyst3 and GFP. Cells were then cultured for 48 h before fusion protein expression was observed by fluorescence microscopy using excitation/emission filters for FITC (GFP; **A** and **D**) and DAPI (**B** and **E**) or phase contrast microscopy (**C** and **F**). To treat cells with leptomycin B (**D**–**F**), the drug was added at a final concentration of 10 μg/ml for the final 3 h of culture. At least 50 GFP-positive cells were examined for each transfection, and staining patterns were entirely consistent with the representative cells photographed here.

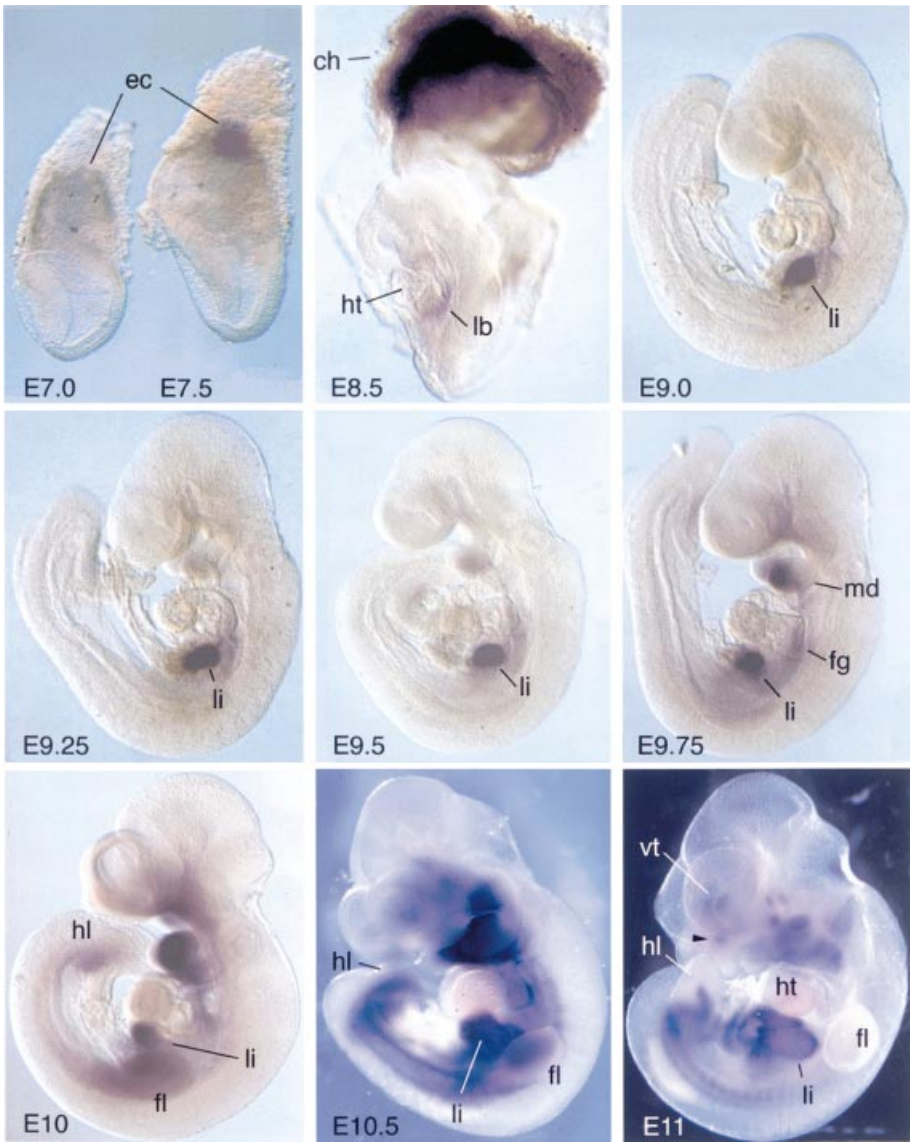

Figure 8 Pyst3 expression during murine embryogenesis

Expression of Pyst3 in developing embryos at the indicated stages from E7–E11 was determined by whole-mount *in situ* hybridization. Embryos before stage E9 were either left completely within the decidua (E7–E7.5), or removed from the yolk-sac to separate embryonic and placental expression patterns (E8.5). Thereafter, embryos were dissected completely clear of the extra-embryonic tissues (E9–E11). The E11 arrowhead indicates the telencephalic mid-line expression domain. In each case, the embryo has been photographed from the left side of the main axis. Abbreviations: ch, chorion; ec, ectoplacental cone; fg, foregut; fl, forelimb; hl, hind-limb; ht, heart; lb, liver bud; li, liver diverticulum; md, mandible; vt, ventral telencephalon.

differential localization of these enzymes, or its physiological significance. An expression vector encoding Pyst3 fused to GFP was transfected into HeLa cells and the expressed protein was found to be predominantly cytoplasmic (Figure 7A). Identical results were obtained when this fusion protein was expressed in COS1 cells, and treatment of cells with serum or anisomycin, classical agonists for mitogen and stress-activated MAPKs respectively, had no effect on Pyst3 localization (results not shown).

Two leucine-rich sequences reminiscent of nuclear export sequences (NESs) found in proteins such as MEK1, PKIα and Rev were reported as being present in the non-catalytic N-terminus of rVH6/Pyst1 [34], and sequence comparison shows that these are also conserved in Pyst2 and MKP-4 [10]. NESs are responsible for mediating interactions with the nuclear export protein CRM1, but to date it has not been demonstrated that the cytoplasmic localization of Pyst family proteins is dependent on this export pathway. The fungal toxin leptomycin B blocks nuclear export of proteins containing a leucine-rich NES by preventing the binding of the NES to the export receptor CRM1 [35–38]. Treatment of cells expressing Pyst3-GFP with leptomycin B results in almost complete re-localization of Pyst3 to the nucleus, indicating that cytoplasmic localization of Pyst3 is CRM1-dependent (Figure 7B, lower panels).

Expression of Pyst3 in developing mouse embryos

Although our understanding of the MKPs has increased greatly in recent years, very little is known of their physiological roles in mammalian cells and tissues, as studies to date have mostly

concentrated on biochemical and structural aspects of their function. Expression studies have, in the main, relied on tissue Northern blots and expression profiles in cultured cell lines. Differential regulation of various MKPs has been shown in the rat brain following excitotoxic cell death in seizures [39,40], whereas limited embryonic expression patterns using radiolabelled riboprobes on sectioned material have been obtained for the murine MKP-1/CL100 orthologue 3CH134/erp [15].

Using whole-mount *in situ* hybridization, we have analysed the expression pattern of Pyst3 in intact mouse embryos from stage E7 through to stage E11, a period spanning establishment of the body plan and development of most major organ systems. Three main zones of expression were identified by these methods. In the earliest phases from stages E7 to E8 (Figure 8; stages as indicated), Pyst3 is expressed with increasing levels in the ectoplacental cone and chorion components of the developing placenta. This expression continues through E9 and E10, although by the later stages the hybridization signal declines slightly (results not shown). The second domain of expression commences at E8.5, in a group of cells of the ventral foregut endoderm induced by signals from the cardiac mesoderm to acquire a hepatocytic fate [41]. Expression of Pyst3 increases in these cells through E9, coincidently with their migration into the septum transversum region to become the liver diverticulum. These cells continue to proliferate throughout the stages examined, during which time expression of Pyst3 is maintained.

Finally, expression of Pyst3 commences in diverse groups of future muscle cells from E9. This includes transient expression in both dorsal and ventral muscle groups of fore- and hind-limbs at stages E9–E10, although the forelimb expression is lost entirely by E11, and is limited to a group of ventral muscle cells in the hind-limbs at this stage. At E10, Pyst3 is expressed in small groups of cells beneath and lateral to the developing liver, which give rise to musculature of the diaphragm. Expression of Pyst3 is evident from E9 in the face, expanding from a group of cells in the mandible at E10 to occupy locations around the oral cavity comprising musculature derived from the hypoglossal cord by E11. At E11, expression is also detected in the ventral telencephalon, and additionally in a punctate central domain at the base of the mid-line separating the telencephalic hemispheres.

DISCUSSION

The murine *Pyst3* gene encodes a dual-specificity MKP that is most highly related to the human enzyme MKP-4, and this, coupled with the mapping of *Pyst3* to a region of the X-chromosome syntenic with the location of the human gene, strongly suggests that Pyst3 and MKP-4 are orthologous. The major difference between Pyst3 and MKP-4 is the presence in the former of an insertion of 63 amino acids within the N-terminal non-catalytic domain of the protein essential for substrate binding and recognition. Surprisingly, this insertion does not impair the ability of Pyst3 to interact with MAPKs *in vivo*, nor does it affect the catalytic activation of Pyst3 on incubation with MAPKs *in vitro*. The solution structure of the N-terminal ERK2-binding domain of the related enzyme Pyst1/MKP-3 was reported recently [42]. This reveals that the insertion in Pyst3 is located within one of two β–α–β motifs, which are in the most conserved parts of the protein. However, within this motif the insertion lies in a highly variable region forming a surface loop, and it must be presumed that this can be accommodated without affecting the overall fold and function of the ERK2-binding domain.

We have used the yeast two-hybrid assay to probe interactions between Pyst3 and a panel of nine distinct MAPKs *in vivo*, and find that the strongest interaction partners for this enzyme are ERK1 and ERK2, with a much weaker interaction observed with p38α. Recombinant Pyst3 also undergoes catalytic activation and, in agreement with our binding data, ERK2 is the most potent activator of the enzyme. However, unlike Pyst1/MKP-3 and Pyst2/MKP-X, where activities are increased between 12 and 30-fold on incubation with ERK2 [4,10], maximal stimulation of Pyst3 is only 3.2-fold. Stress-activated MAPKs, such as p38α and JNK1, also increase Pyst3 activity, but only with a factor of between 1.5 and 1.8-fold. The significance of these small effects is questionable in relation to the substrate selectivity of Pyst3, because our *in vivo* assays show that Pyst3 inactivates ERK1 and p38α, but not JNK1. Thus our interaction data are a more reliable indicator of the ability of Pyst3 to inactivate stress-activated MAPK isoforms than the ability of these enzymes to stimulate the *in vitro* activity of Pyst3 towards *p*-NPP. Similar discrepancies have been observed between MAPK binding and catalytic activation for the dual-specificity phosphatases MKP-5 and MKP-2 [29,43]. Interestingly, the human MKP-4 enzyme was reported to be capable of dephosphorylating JNK [27]. However, as stated above, we can find no evidence for binding or significant inactivation of JNK by Pyst3. We conclude that Pyst3 is most likely to target ERK1 and ERK2 *in vivo*, but a role for this enzyme in the regulation of signalling via the p38/MAPK pathway cannot be excluded.

It has become clear that the outcome of MAPK signalling is determined not only by the magnitude and duration of activation, but also by the subcellular localization of the MAPK itself [44,45]. Thus far, scant attention has been paid to the mechanisms determining the subcellular localization of critical regulators such as the MKPs, and how this relates to the function of these enzymes. All members of the Pyst subfamily studied thus far are predominantly cytoplasmic when expressed in mammalian cells. Although the presence of canonical leucine-rich NESs has been noted [10,34], nothing is yet known about the mechanisms which govern localization of these proteins. We have demonstrated that cytoplasmic localization of Pyst3 is maintained by a CRM1-dependent nuclear export pathway. Furthermore, the size of our Pyst3–GFP fusion protein and the observation that it becomes exclusively nuclear in the presence of leptomycin B indicates that this export pathway must normally overcome an opposing active process of nuclear import to maintain the localization of Pyst3. Experiments are currently underway to determine whether the Pyst3 protein is capable of 'shuttling' between these two subcellular compartments, and to define the sequence motif(s) involved in these critical export and import pathways.

The generation of knockout mice is a powerful tool to probe gene function. As part of this process, it is essential to perform a detailed survey of the expression pattern of the gene during embryogenesis in order to allow any eventual phenotype to be interpreted on the basis of when and where the gene of interest is expressed. To this end, we instigated an *in situ* hybridization analysis of murine *Pyst3* expression during a range of embryonic stages covering important organogenic and morphogenic events from E7 through to E11. Pyst3 is expressed in a strikingly specific range of locations both within the developing embryo and in its extra-embryonic tissues. Initially, Pyst3 is found in the trophoblast from the very earliest stages examined. This structure contributes to the labyrinthine layer of the placenta by E10, which constitutes the principal exchange surface between maternal and embryonic circulatory systems from this stage onwards [46].

Loss of function of a number of gene products functioning either as activators or components of MAPK-signalling cascades results in ablation or substantial impairment of labyrinth formation, indicating that MAPK signalling has a crucial role in the

regulation of cell growth and differentiation in these cell layers. These include hepatocyte-growth-factor (HGF), the HGF receptor c-Met, the son-of-sevenless 1 ('Sos-1') gene product, MEK1, the MAPK p38α and its upstream activator MEK kinase-3 ('MEKK3') [46–48]. We have demonstrated that Pyst3 is a potential regulator of both ERK1/2 and p38α MAPKs, and this, coupled with the expression pattern of Pyst3, suggests an important role for this enzyme in placental development.

The second major expression zone for Pyst3 is in the developing liver, again suggesting a role for Pyst3 in regulation of organogenesis. Initiation of the liver occurs at E8, when signals emanating from the cardiac mesoderm induce ventral cells of the underlying foregut endoderm to proceed along a path of hepatocyte cell fate. This bud of cells then proliferates and migrates into the surrounding septum transversum mesenchyme, and the entire domain becomes delineated or encapsulated as the liver bud grows rapidly into an organ [49]. Proliferation of hepatocytes following initial specification relies on reciprocal signalling between mesenchymal and endodermal cells expressing HGF and its receptor c-Met respectively [48]. Fibroblast growth factor ('FGF') signals from the cardiac mesoderm have been demonstrated to be both necessary and sufficient to induce a hepatic fate within the endoderm [41]. *Pyst3* is expressed from the very earliest stages that these signals are known to be effective, and it is possible that *Pyst3* is induced directly as a very early marker of this event. The p38 MAPK pathway has also been implicated as a negative regulator of hepatocyte proliferation [50] and its activity is maintained at negligible levels throughout the early phases of liver development, during which time the cells undergo considerable proliferation. It is possible, given our findings both *in vivo* and *in vitro*, that Pyst3 may be the implied negative regulator of p38 activity in the liver, and thus a promoter of hepatocyte proliferation during embryonic development.

The third major zone of Pyst3 expression in the stages examined comprises lineages of migratory skeletal-muscle cells originating in the lateral dermamyotomal lips of the somites. These provide musculature for the tongue, diaphragm and limbs. All of these cells depend on signalling via the c-Met receptor for correct migration [47,51]. Conversion of muscle cells from committed precursors into differentiated myotubes requires the activity of both ERK1/2 and p38 MAPKs [34,52]. This strongly suggests a role for Pyst3 in inhibition of differentiation of migratory muscle cells via blockade of ERK1/2 and/or p38 signals *in vivo*.

In conclusion, we have isolated and characterized a mouse gene encoding a functional dual-specificity MKP that is most closely related to the human enzyme MKP-4. We have demonstrated that this enzyme interacts with and inactivates both mitogen- and stress-activated MAPK isoforms *in vivo*, and shown that the cytoplasmic localization of the enzyme is maintained by a CRM1-dependent nuclear export pathway. Finally, our determination of the pattern of *Pyst3* expression in the developing mouse embryo suggests a role for this enzyme in the regulation of MAPK signalling during placental, hepatic and muscular development. In particular, the tight correlation between Pyst3 expression and domains of c-Met expression strongly suggests that Pyst3 may lie downstream of, and regulate signals transduced by, the c-Met receptor itself.

We thank Iain Goldsmith (Oligonucleotide Synthesis Laboratory, Cancer Research UK, Clare Hall Laboratories, Herts., U.K.) for oligonucleotide synthesis, Andy Cassidy (Ninewells Hospital, Dundee, Scotland, U.K.) for DNA sequencing, Chris Armstrong (MRC Protein Phosphorylation Unit, University of Dundee, Scotland, U.K.) for providing recombinant MAPKs, Neil McDonald for providing recombinant activated p42 MAPK, and Dr Minoru Yoshida for providing leptomycin B. We also thank Professor Cheryll Tickle, Dr Andrea Munsterberg and Dr Peter Ashby (Wellcome Trust Biocentre, University of Dundee, Scotland) for advice and assistance to R.J.D. in performing whole-mount *in situ* hybridization. This work was supported by a Ph.D. studentship from the Biomedical Research Centre, University of Dundee (to R.J.D.) and by the Imperial Cancer Research Fund (now Cancer Research UK).

REFERENCES

1 Keyse, S. M. (2000) Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr. Opin. Cell Biol. **12**, 186–192
2 Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K. and Matsumoto, K. (1994) MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. EMBO J. **13**, 61–70
3 Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M. and Martinez Arias, A. (1998) *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. Genes Dev. **12**, 557–570
4 Groom, L. A., Sneddon, A. A., Alessi, D. R., Dowd, S. and Keyse, S. M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J. **15**, 3621–3632
5 Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A. and Arkinstall, S. (1996) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for the inactivation of distinct mitogen-activated protein kinases. J. Biol. Chem. **271**, 27205–27208
6 Muda, M., Theodosiou, A., Gillieron, C., Smith, A., Chabert, C., Camps, M., Boschert, U., Rodrigues, N., Davies, K., Ashworth, A. and Arkinstall, S. (1998) The mitogen-activated protein kinase phosphatase-3 N-terminal non-catalytic region is responsible for tight substrate binding and enzymatic specificity. J. Biol. Chem. **273**, 9323–9329
7 Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. Science (Washington, D.C.) **280**, 1262–1265
8 Stewart, A. E., Dowd, S., Keyse, S. M. and McDonald, N. Q. (1999) Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. Nat. Struct. Biol. **6**, 174–181
9 Rigas, J. D., Hoff, R. H., Rice, A. E., Hengge, A. C. and Denu, J. M. (2001) Transition state analysis and requirement of Asp-262 general acid/base catalyst for full activation of dual-specificity phosphatase MKP3 by extracellular regulated kinase. Biochemistry **40**, 4398–4406
10 Dowd, S., Sneddon, A. A. and Keyse, S. M. (1998) 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. J. Cell Sci. **111**, 3389–3399
11 Hutter, D., Chen, P., Barnes, J. and Liu, Y. (2000) Catalytic activation of mitogen-activated protein (MAP) kinase phosphatase-1 by binding to p38 MAP kinase: critical role of the p38 C-terminal domain in its negative regulation. Biochem. J. **352**, 155–163
12 Slack, D. N., Seternes, O.-M., Gabrielsen, M. and Keyse, S. M. (2001) Distinct binding determinants for ERK2/p38α and JNK MAP kinases mediate catalytic activation and substrate selectivity of MAP kinase phosphatase-1. J. Biol. Chem. **276**, 16491–16500
13 Berset, T., Frohli-Hoier, E., Battu, G., Canevascini, S. and Hajnal, A. (2001) Notch inhibition of RAS signaling through MAP kinase phosphatase LIP-1 during *C. elegans* vulval development. Science (Washington, D.C.) **291**, 1055–1058
14 Dorfman, K., Carrasco, D., Gruda, M., Ryan, C., Lira, S. A. and Bravo, R. (1996) Disruption of the erp/mkp-1 gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts. Oncogene **13**, 925–931
15 Carrasco, D. and Bravo, R. (1993) Expression of the non-transmembrane tyrosine phosphatase gene *erp* during mouse organogenesis. Cell Growth Differ. **4**, 849–859
16 Monier, K., Usson, Y., Mongelard, F., Szepetowski, P., Robert-Nicoud, M. and Vourc'h, C. (1996) Metaphase and interphase mapping by FISH: improvement of chromosome banding and signal resolution in interphase nuclei by means of iterative deconvolution. Cytogenet. Cell. Genet. **72**, 200–204
17 Ragoussis, J., Senger, G., Trowsdale, J. and Campbell, I. G. (1992) Genomic organization of the human folate receptor genes on chromosome 11q13. Genomics **14**, 423–430
18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
19 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
20 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**, 51–59
21 Brunet, A. and Pouyssegur, J. (1996) Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science (Washington, D.C.) **272**, 1652–1655

22 Feinberg, A. P. and Vogelstein, B. (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. **137**, 266–267

23 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

24 Keyse, S. M. and Emslie, E. A. (1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature (London) **359**, 644–647

25 Wilkinson, D. G. (1992) *In situ* Hybridisation: A Practical Approach, IRL Press at Oxford University Press, Oxford

26 Keyse, S. M. and Ginsburg, M. (1993) Amino acid sequence similarity between CL100, a dual-specificity MAP kinase phosphatase and cdc25. Trends Biochem. Sci. **18**, 377–378

27 Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C., Camps, M., Martinou, I., Ashworth, A. and Arkinstall, S. (1997) Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. J. Biol. Chem. **272**, 5141–5151

28 Searle, A. G., Peters, J., Lyon, M. F., Hall, J. G., Evans, E. P., Edwards, J. H. and Buckle, V. J. (1989) Chromosome maps of man and mouse. IV. Ann. Hum. Genet. **53**, 89–140

29 Chen, P., Hutter, D., Yang, X., Gorospe, M., Davis, R. J. and Liu, Y. (2001) Discordance between the binding affinity of mitogen-activated protein kinase subfamily members for MAP kinase phosphatase-2 and their ability to activate the phosphatase catalytically. J. Biol. Chem. **276**, 29440–29449

30 Muda, M., Boschert, U., Dickinson, R., Martinou, J.-C., Martinou, I., Camps, M., Schlegel, W. and Arkinstall, S. (1996) MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J. Biol. Chem. **271**, 4319–4326

31 Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U. and Kelly, K. (1993) PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. Science (Washington, D.C.) **259**, 1763–1766

32 Guan, K. L. and Butch, E. (1995) Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. J. Biol. Chem. **270**, 7197–7203

33 Lewis, T., Groom, L. A., Sneddon, A. A., Smythe, C. and Keyse, S. M. (1995) XCL100, an inducible nuclear MAP kinase phosphatase from *Xenopus laevis*: its role in MAP kinase inactivation in differentiated cells and its expression during early development. J. Cell Sci. **108**, 2885–2896

34 Mourey, R. J., Vega, Q. C., Campbell, J. S., Wenderoth, M. P., Hauschka, S. D., Krebs, E. G. and Dixon, J. E. (1996) A novel cytoplasmic dual specificity protein tyrosine phosphatase implicated in muscle and neuronal differentiation. J. Biol. Chem. **271**, 3795–3802

35 Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. Cell (Cambridge, Mass.) **90**, 1051–1060

36 Wolff, B., Sanglier, J. J. and Wang, Y. (1997) Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem. Biol. **4**, 139–147

37 Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature (London) **390**, 308–311

38 Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. and Yoshida, M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell Res. **242**, 540–547

39 Boschert, U., Muda, M., Camps, M., Dickinson, R. and Arkinstall, S. (1997) Induction of the dual specificity phosphatase PAC1 in rat brain following seizure activity. Neuroreport **8**, 3077–3080

40 Boschert, U., Dickinson, R., Muda, M., Camps, M. and Arkinstall, S. (1998) Regulated expression of dual specificity protein phosphatases in rat brain. Neuroreport **9**, 4081–4086

41 Jung, J., Zheng, M., Goldfarb, M. and Zaret, K. S. (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science (Washington, D.C.) **284**, 1998–2003

42 Farooq, A., Chaturvedi, G., Mujtaba, S., Plotnikova, O., Zeng, L., Dhalluin, C., Ashton, R. and Zhou, M.-M. (2001) Solution structure of ERK2 binding domain of MAPK phosphatase MKP-3: structural insights into MKP-3 activation by ERK2. Mol. Cell **7**, 387–399

43 Tanoue, T., Moriguchi, T. and Nishida, E. (1999) Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. J. Biol. Chem. **274**, 19949–19956

44 Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E. and Cobb, M. H. (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell (Cambridge, Mass.) **93**, 605–615

45 Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S. M. and Pouyssegur, J. (1999) Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. **18**, 664–674

46 Rossant, J. and Cross, J. (2001) Placental development: lessons from mouse mutants. Nat. Rev. Genet. **2**, 538–548

47 Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature (London) **376**, 768–771

48 Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E. and Birchmeier, C. (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature (London) **373**, 699–702

49 Zaret, K. (2000) Liver specification and early morphogenesis. Mech. Dev. **92**, 83–88

50 Awad, M. M., Enslen, H., Boylan, J. M., Davis, R. J. and Gruppuso, P. A. (2000) Growth regulation via p38 mitogen-activated protein kinase in developing liver. J. Biol. Chem. **275**, 38716–38721

51 Birchmeier, C. and Brohmann, H. (2000) Genes that control the development of migrating muscle precursor cells. Curr. Opin. Cell Biol. **12**, 725–730

52 Puri, L. P. and Sartorelli, V. (2000) Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-translational modifications. J. Cell Physiol. **185**, 155–173

Received 7 December 2001/7 February 2002; accepted 7 March 2002