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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

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SUMMARY

We have cloned the *Xenopus laevis* homologue (*XCL100)* of the human CL100 (Thr/Tyr) MAP kinase phosphatase. Expression of the *XCL100* mRNA and protein is inducible by serum stimulation and oxidative/heat stress in a *X. laevis* kidney cell line. In contrast, *XCL100* is constitutively expressed in growing *Xenopus* oocytes. Recombinant XCL100 protein is able to dephosphorylate both tyrosine and threonine residues of activated p42 MAP kinase in vitro and both the *Xenopus* and human CL100 proteins were localised predominantly in the nucleus in transfected COS-1 cells. As nuclear translocation of activated MAP kinase is necessary for some of its essential functions in proliferation and cell differentiation our results indicate a role for CL100 in the regulation of these nuclear signalling events. In *Xenopus* kidney cells both heat shock and serum stimulation lead to transient activation of MAP kinase. However, in contrast to results previously reported from studies on mammalian fibroblasts the inactivation of MAP kinase in these epitheloid cells is rapid and is not dependent on synthesis of new protein. These results indicate that the induction of CL100 (or CL100-like enzymes) may not be required for MAP kinase inactivation in all cell types. Finally, during early embryogenesis, levels of *XCL100* mRNA are greatly increased at the mid-blastula transition, suggesting that this enzyme may be involved in the regulation of MAP kinase activity during early development.

Key words: *Xenopus*, MAP kinase phosphatase, nuclear localisation, oocyte, development localisation

INTRODUCTION

The *CL100* cDNA was originally isolated from human skin fibroblasts as corresponding to an mRNA which is inducible by either oxidative stress or heat shock (Keyse and Emslie, 1992). The mouse homologue of *CL100* (*3CH134* or *erp*) was independently isolated as a growth factor inducible gene in mouse fibroblasts (Charles et al., 1992; Noguchi et al., 1993). Recombinant CL100 and 3CH134 proteins are able to dephosphorylate and inactivate recombinant MAP kinase in vitro (Alessi et al., 1993; Charles et al., 1993; Zheng and Guan, 1993). This activity is highly specific for MAP kinase, the enzyme showing no activity towards a wide range of other protein and peptide substrates phosphorylated on either tyrosine or serine/threonine (Alessi et al., 1993). In addition, CL100 is able to block the ras dependent activation of MAP kinase in extracts of *Xenopus* oocytes (Alessi et al., 1993) and causes selective dephosphorylation of MAP kinase when expressed in COS-1 cells (Sun et al., 1993).

Recent work has now identified three further genes which show sequence homology to *CL100*. The human *PAC-1* gene encodes a mitogen inducible MAP kinase phosphatase, which, unlike *CL100*, is only expressed in haematopoetic tissues (Rohan et al., 1993; Ward et al., 1994). PAC-1 is 55% identical to CL100 over the amino terminus and the catalytic domain but lacks the carboxy-terminal domain that is present in the CL100 enzyme. More recently, an additional human gene (*B23*) has been isolated from mammary epithelial cells by low stringency hybridisation (Ishibashi et al., 1994). This gene is 45% identical to *CL100* and, like *CL100*, is inducible by both heat shock and mitogens. Finally, the *Saccharomyces cerevisiae* gene *MSG5*, which is more distantly related to *CL100* (22% identity), has been identified as encoding a dual specificity phosphatase which is responsible for dephosphorylating and inactivating the yeast MAP kinase homologue FUS3 (Doi et al., 1994).

The mitogen-activated protein (MAP) kinases are key elements of the signalling pathways responsible for the inward passage of messages from various activated growth factor and hormone receptors at the cell surface (reviewed by Nishida and Gotoh, 1993; Blenis, 1993; Marshall, 1994). The sustained activation of MAP kinase appears to be required for its translocation from the cytosol to the nucleus of mammalian cells, where its targets include transcription factors such as c-myc, ATF-2 and ELK-1 (Davis, 1993). The phosphorylation of this class of proteins is thought to control the transcriptional

activity of key genes involved in either cell proliferation or differentiation.

The most extensively characterised of the mammalian MAP kinases are the 42 kDa and 44 kDa isoforms (also referred to as ERK2 and ERK1, respectively) (Cobb et al., 1994). These serine/threonine kinases are unusual in requiring phosphorylation on both threonine and tyrosine 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 termed MAP kinase kinase (MAPKK) (Nakielny et al., 1992; Kosako 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, which lie downstream of activated ras proteins in the mitogenic signalling cascade (Roberts, 1992).

In many cells the activation of MAP kinases is a transient event, even in the continuing presence of the activating stimulus. Examples of this latter phenomenon are seen in rat phaeochromocytoma (PC12) cells exposed to epidermal growth factor (EGF) and in Chinese hamster lung fibroblasts stimulated with phorbol esters (Traverse et al., 1992; Kahan et al., 1992). All of these observations suggest that the inactivation of MAP kinases, like their activation, might be tightly regulated, and that the activity of the MAP kinases might be controlled by the balance of MAP kinase kinase and specific MAP kinase phosphatase activities such as CL100.

MAP kinase is thought to play a number of pivotal roles during early development in *Xenopus laevis*. These include the regulation of key steps in progression through meiosis (Gotoh et al., 1991; Kosako et al., 1994) such as the control of entry into meiosis I, inhibition of DNA synthesis following meiosis I, and induction of metaphase arrest during meiosis II (Furuno et al., 1994; Haccard et al., 1993). This cell cycle arrest is relieved by fertilisation, and inactivation of MAP kinase normally occurs 30 minutes after fertilisation, suggesting that the inactivation of MAP kinase is subject to cell cycle dependent regulation (Ferrell et al., 1991).

As the first step in analysing the regulation of MAP kinase inactivation in *Xenopus* we have undertaken the cloning of the *Xenopus* homologue of the human CL100 phosphatase. We have determined the subcellular localisation of the CL100 protein and studied its role in the inactivation of MAP kinase in a *Xenopus* cell line following exposure to mitogens or cellular stress. In addition, we show that in contrast to the pattern of inducible expression of the *CL100* gene in *Xenopus* and human somatic cells, the *XCL100* mRNA and protein are constitutively present in growing *Xenopus* oocytes. Finally, we show that the expression of *CL100* is dramatically up-regulated during embryogenesis, suggesting an important role for this protein in the developmental programme.

MATERIALS AND METHODS

Cell culture

The *Xenopus laevis* kidney cell line, XIK-2 (McStay et al., 1991), was cultured routinely in half-strength (diluted 1:1 with water) Liebowitz medium (Gibco-BRL) supplemented with penicillin, streptomycin, glutamine and 10% foetal calf serum (FCS) at ambient temperature (18-22°C). For serum starvation, subconfluent cultures were incubated in medium containing 0.5% FCS for 36 hours prior to stimulation by addition of medium containing 10% FCS. Drug treatments were carried out as described previously (Keyse and Emslie, 1992) and cells were heat shocked by immersion of cultures in a waterbath at 37°C for 15 minutes. Where indicated, protein synthesis was blocked by addition of puromycin (Sigma) to a final concentration of 10 μg/ml. COS-1 cells were cultured routinely in Dulbecco's modified Eagle's medium (Gibco-BRL) suplemented with penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% FCS.

Synchronous fertilisation of *Xenopus* eggs and staging of embryos during development

Freshly laid *X. laevis* eggs were obtained by squeezing gonadotrophin treated frogs into 1× MMR buffer (5 mM Hepes, pH 7.8, 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA). Immediately prior to fertilisation, excess MMR was poured off and eggs were mixed with sperm, obtained by gentle sqeezing of freshly isolated bisected testes. After a 5 minute incubation, water (5 ml) was added and the eggs allowed to stand for a further 5 minutes. After 25 minutes, eggs were dejellied in 2% cysteine (pH 7.8) for 5 minutes, after which they were transferred to 0.2× MMR. Development was monitored using a Wild M8 stereomicroscope and embryos were staged according to the method of Nieuwkoop and Faber (1967). At the indicated times and stages, 25 embryos were removed and snap frozen in liquid nitrogen.

cDNA cloning and DNA sequence analysis

Approximately 10^5 recombinants from a λgt11 cDNA library derived from the *X. laevis* kidney cell line XIK-2 (McStay et al., 1991) were screened with a ^{32}P-labelled human *CL100* cDNA probe using standard techniques (Sambrook et al., 1989). Three clones which gave positive signals with this probe were plaque purified and the cDNA inserts isolated using the purified phage DNAs as template in a polymerase chain reaction with *Taq* polymerase (Promega) and the following oligonucleotide primers: 5′-GGATCCCATGGTCAATATGGAAACCTG-3′ and 5′-TTGACACCAGACCAACTGGTAATG-3′, which are complementary to sequences flanking the *Eco*RI site of the λgt11 vector. Following digestion with *Eco*RI, all three clones yielded two DNA fragments, indicating the presence of an internal *Eco*RI site. These DNA fragments were then subcloned into the vector pTZ (Pharmacia) for DNA sequence analysis. The entire DNA sequence from both strands of the cDNAs was determined by the dideoxy method (Sanger et al., 1977) using the enzyme Sequenase (USB) and the orientation of the fragments with respect to the internal *Eco*RI sites was confirmed by direct analysis of the original PCR products. All three recombinants were found to contain identical DNA sequences, which differed only in the length of the 5′ *Eco*RI fragment.

RNA extraction and northern blot analysis

Total RNA was prepared from cultured XIK-2 cells using the guanidine thiocyanate method of Chomczynski and Sacchi (1987) and from *Xenopus* oocytes, *Xenopus* eggs and staged *Xenopus* embryos using Trizol reagent (Gibco-BRL). Northern blot analysis 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 *XCL100* cDNA; a *Xenopus* c-*mos* cDNA fragment (nucleotides 118-1350) (Sagata et al., 1988) isolated by using *X. laevis* genomic DNA as template in a polymerase chain reaction with *Taq* polymerase (Promega); and the following oligonucleotide primers: 5′-GGCAGCCATATGCCTTCCCCAATCCCCGTGGAG-3′ and 5′-CCGCTCGAGCCTCACTAGTGCACGACTGAGCTG-3′; and a 1,400 bp *Pst*I fragment of the rat cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (Piechaczyk et al., 1984).

Expression of XCL100 in a rabbit reticulocyte lysate

The *XCL100* reading frame was first modified using purified *XCL100* phage DNA as template in a polymerase chain reaction using *Taq* polymerase (Promega) and the following oligonucleotide primers: 5′-GGATCCCATGGTCAATATGGAAACCTG-3′ and 5′-CATCGACTAGTTCAAGATCTGCTTGGTGAT-3′. This introduces an *Nco*I site at the initiating ATG codon and alters the sequence immediately following codon 368 to a *Bgl*II site followed directly by a TGA stop codon and an *Spe*I site. The element containing the internal ribosome entry site (IRES) was isolated from the pCITE vector (Novagen) as an *Eco*RI-*Nco*I fragment and subcloned into pBluescript (Stratagene) to create pIRESBlue. The *Nco*I-*Spe*I fragment containing the modified *XCL100* reading frame was then inserted into this vector to create pXCL100Blue. Finally this vector was cut with *Bgl*II and a DNA fragment encoding tandem copies of the myc epitope (EQKLISEEDL) flanked by *Bam*HI and *Bgl*II linkers was inserted to produce pXCL100mycBlue. To produce the XCL100 protein 1 μg of this plasmid was added, together with T7 RNA polymerase (100 units), to a 50 μl coupled transcription/translation lysate (TNT, Promega) and incubated at 30°C for 90 minutes.

MAP kinase phosphatase assays and phosphoamino acid analysis

A 1 μl sample of reticulocyte lysate which had been incubated either with or without the plasmid pXCL100mycBlue was added to 1 μl of ^{32}P-labelled activated p42 MAP kinase (provided by D. R. Alessi, University of Dundee) and Microcystin-LR (provided by C. Mackintosh, University of Dundee) was added to a final concentration of 2 μM. Microcystin was added to inhibit Ser/Thr protein phosphatases present in the reticulocyte lysates and does not affect the activity of the recombinant human CL100 enzyme. Where sodium orthovanadate was required, this was added to a final concentration of 2 mM. Reactions were then adjusted to a final volume of 15 μl with 50 mM Tris-HCl, pH 7.5, and incubated at 30°C for 60 minutes. Following incubation, 5 μl was removed and analysed using SDS-PAGE. A trace amount of ^{32}P-labelled MAP kinase kinase is present in our preparation of activated MAP kinase and is visible after separation in these gels. The remainder of the proteins in the reaction were then precipitated with 25% trichloroacetic acid and, following centrifugation, the amount of ^{32}P released into the supernatant was determined by scintillation counting and expressed as a proportion of the total amount of labelled p42 MAP kinase in the reaction. For phosphoamino acid analysis the precipitated proteins were subjected to acid hydrolysis in 6 M HCl at 110°C for 60 minutes (Kamps and Sefton, 1989) and the products were analysed by thin layer electrophoresis at pH 3.5.

Transient transfection and indirect immunofluorescence

The IRES element and *XCL100* reading frame including the tandem myc epitope tag were excised from pXCL100Blue by partial digestion using *Nco*I and *Eco*RI, and subcloned into the vector pSG5 (Stratagene). The human *CL100* reading frame was first modified by using the *CL100* cDNA as template in a polymerase chain reaction using *Taq* polymerase (Promega) and the following oligonucleotide primers: 5′-CATCAAGAATGCTGGAGGAAGGGTGTTTGTCCACTGCCAGGCAGGC-3′ and 5′ GTGGTGCTCGAGTCTGCAGCTGGGAGAGGTCGTAATGGGGCTCTGAAG-3′. This PCR product spans the unique *Bsm*I site at nucleotide 983 of the *CL100* cDNA and replaces the stop codon in *CL100* with an in-frame *Xho*I site. Following digestion with *Bsm*I and ligation to the 5′ *Eco*RI-*Bsm*I fragment (nucleotides 1-983) of CL100, the modified cDNA was then cut with *Xho*I and subcloned into the pSG5 vector (Stratagene), which contains an *Xho*I site followed by a single copy of the myc epitope EQKLISEEDL followed by a stop codon and a *Bam*HI site. COS-1 cells were transfected with these expression plasmids (5 μg) using the standard calcium phosphate method. Indirect immunofluorescence was carried out 48 hours after transfection. Cells were washed three times in phosphate buffered saline (PBS), fixed for 10 minutes in methanol/acetone (3:7, v/v) at −20°C and then washed twice with PBS. After permeabilisation in 0.2% Triton X-100 for 5 minutes, the cells were washed three times in PBS containing 0.02% BSA and incubated with affinity-purified anti-myc epitope monoclonal antibody 9E10 (1:100 dilution of 0.7 mg/ml stock) for 1 hour. Cells were then washed in PBS containing 0.02% BSA and incubated with rabbit anti-mouse FITC-conjugated antibody (1:100 diluted) (Sigma) for 1 hour in the dark. Finally, the cells were washed 5 times with PBS containing 0.02% BSA, with the addition of 4,6-diamidino-2-phenylindole (DAPI) at 0.5 μg/ml in the final wash, before mounting. 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 FITC and DAPI.

Production of antibodies, immunoblotting and immunoprecipitations

The anti-XCL100 antibody was produced by immunising rabbits with a synthetic peptide NH_2-LCANNVPGSADSNCTPC-COOH (corresponding to residues 150-166 of the XCL100 protein), coupled to keyhole limpet haemocyanin (Pierce). For detection of myc epitope-tagged XCL100 protein expressed in reticulocyte lysates, 5 μl of lysate was separated by SDS-PAGE (9% gel) and transferred to nitrocellulose membranes before immunoblotting with affinity-purified anti-myc epitope monoclonal antibody 9E10 using standard techniques (Harlow and Lane, 1988). For detection of the phosphorylated and non-phosphorylated isoforms of MAP kinase in XIK-2 cells, lysates were separated by SDS-PAGE (15%), transferred to nitrocellulose membranes and immunoblotted with an anti-MAP kinase monoclonal antibody (Zymed) exactly as described (Nebreda and Hunt, 1993).

For XlK-2 cell labelling and immunoprecipitation [^{35}S]methionine (1,000 Ci/mmol, 10 mCi/ml, Amersham) was added to the culture medium at a final concentration of 0.5 mCi/ml and cells were labelled for 2 hours. Cells were then rinsed 3 times with 0.5× PBS and lysed in RIPA (minus SDS) buffer (10 mM Tris-HCl, pH 7.5, 1% NP40, 150 mM NaCl, 1 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate) plus protease inhibitors (1 μg/ml leupeptin, 1 mM Pefabloc, 2 μg/ml aprotinin and 1 μg/ml pepstatin A; all from Boehringer Mannheim). Lysates were then microcentrifuged for 2 minutes and precleared by addition of 25 μl Protein A-Sepharose beads (Pharmacia), which had been equilibrated with lysis buffer, and 5 μl of pre-immune serum for 4 hours at 4°C, with rocking. The beads were then removed by centrifugation and the supernatant incubated with 25 μl of anti-XCL100 antiserum for 30 minutes on ice before addition of 25 μl Protein A-Sepharose beads (equilibrated with cold XlK-2 cell lysate) and a further incubation for 16 hours at 4°C with rocking. The beads were then washed 5 times with RIPA plus SDS (as above but with 0.1%SDS and 1% sodium deoxycholate) and twice with 50 mM Tris-HCl, pH 7.5. The samples were then resuspended in 2× Laemmli sample buffer and analysed by SDS-PAGE. Fixed gels were treated with Intensify (Amersham) before drying and ^{35}S-labelled proteins were visualised by exposure to X-ray film (Fuji-RX) at −70°C.

Immunoprecipitations from coupled transcription/translation reactions were carried out exactly as above, except that lysates were diluted into 500 μl of RIPA (minus SDS) buffer and the preclearing step was ommitted. For labelling of *Xenopus* oocytes 80 oocytes per sample were incubated in MMR containing 1 mCi/ml [^{35}S]methionine (1,000 Ci/mmol, 10 mCi/ml; Amersham) in the presence or absence of progesterone (8 μg/ml) for 11 hours. Oocytes were then homogenised in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1% Triton X-100 with protease inhibitors (as before), centrifuged at 10,000 ***g*** for 5 minutes and the protein-containing layer removed. The samples were then split in two and XCL100 protein was immunoprecipitated as before using anti-XCL100 antiserum which had been preincubated for 30 minutes on ice either with or without 25 μg of the XCL100 peptide

a

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   1                                GAAAAGTCTGGCTTTATTATTTTTTTATTGAAACATTAA
  40 ATCAGCGACAGTATTTATTTTATTATTTTTTAACAGTCGGTATAAGCTTTTATTGGAGCTACAGAGG
 107 CGCCGGAGCGTAAAAGAAGCGGGAGAGTGGAGCCTATTGTCAAGGATTGTTCGGCTCATCCCCAGCA
     Met Val Asn Met Glu Thr Cys Ala Met Asp Cys Cys Val Leu Lys Ala Leu   17
 174 ATG GTC AAT ATG GAA ACC TGC GCT ATG GAT TGC TGC GTG TTG AAA GCG CTG
     Leu Ala Glu Arg Ala His Lys Cys Leu Ile Leu Asp Cys Arg Ser Phe Phe   34
 225 TTG GCA GAG AGA GCT CAC AAA TGC CTC ATA TTG GAC TGC AGG TCG TTC TTC
     Ser Phe Ser Ser Cys Ser Ile Val Gly Ser Ser Asn Val Arg Leu Ser Thr   51
 276 TCC TTC AGC TCG TGC AGC ATC GTG GGA TCC AGC AAT GTT CGC CTG AGC ACT
     Ile Val Lys Arg Arg Ala Lys Gly Ser Met Gly Leu Glu His Ile Val Pro   68
 327 ATC GTC AAG AGG AGG GCG AAA GGC AGC ATG GGC TTG GAG CAC ATC GTC CCC
     Asn Glu Glu Gln Arg Cys Arg Leu Val Ala Gly Met Tyr Glu Ala Val Val   85
 378 AAT GAG GAG CAG AGG TGC CGG CTG GTG GCT GGG ATG TAC GAG GCG GTG GTG
     Leu Leu Asp Glu Arg Thr Ser Glu Leu Asp Met Leu Arg Lys Asp Ser Thr  102
 429 CTG CTG GAT GAG AGG ACA TCA GAG CTG GAC ATG CTC AGG AAA GAC AGC ACC
     Met Met Leu Ala Val Asn Ala Leu Cys Arg Asp Ser Arg Gly Ser Ser Ile  119
 480 ATG ATG CTT GCA GTC AAT GCC CTG TGT AGG GAC TCC AGG GGC AGC AGC ATC
     Tyr Phe Leu Lys Gly Gly Tyr Glu Thr Phe Ser Ala Gln Cys Pro Glu Phe  136
 531 TAC TTC CTA AAA GGT GGC TAT GAG ACA TTT TCT GCA CAG TGC CCA GAA TTC
     Cys Thr Lys Asn Ser Pro Pro Val Gly Leu Ser Leu Pro Leu Cys Ala Asn  153
 582 TGC ACC AAA AAC TCT CCT CCA GTG GGT CTG AGT TTG CCT CTT TGT GCC AAC
     Asn Val Pro Gly Ser Ala Asp Ser Asn Cys Thr Pro Cys Gly Thr Pro Leu  170
 633 AAC GTG CCA GGC AGT GCT GAC TCC AAT TGT ACT CCC TGT GGA ACT CCA CTC
     Tyr Asp Gln Gly Gly Pro Val Glu Ile Leu Pro Phe Leu Tyr Leu Gly Ser  187
 684 TAT GAC CAG GGG GGA CCA GTG GAA ATT CTG CCC TTC CTG TAT TTG GGC AGT
     Ala Tyr His Ala Ser Arg Lys Asp Met Leu Asp Thr Leu Gly Ile Thr Ala  204
 735 GCA TAC CAC GCA TCA AGG AAA GAC ATG CTG GAT ACC CTT GGC ATT ACA GCC
     Leu Ile Asn Val Ser Ala Asn Cys Pro Asn His Phe Glu Gly His Phe Gln  221
 786 CTC ATT AAT GTG TCC GCA AAC TGT CCT AAT CAC TTT GAG GGC CAC TTC CAG
     Tyr Lys Ser Ile Pro Val Glu Asp Ser His Lys Ala Asp Ile Ser Ser Trp  238
 837 TAC AAA AGC ATT CCC GTG GAA GAC AGT CAC AAG GCA GAC ATC AGT TCA TGG
     Phe Asn Glu Ala Ile Asp Phe Ile Asp Ser Val Lys Asn Ser Gly Gly Arg  255
 888 TTC AAT GAA GCT ATC GAT TTC ATA GAC TCT GTA AAA AAT TCT GGT GGA AGG
     Val Phe Val His Cys Gln Ala Gly Ile Ser Arg Ser Ala Thr Ile Cys Leu  272
 939 GTG TTT GTC CAC TGC CAA GCT GGT ATA TCC CGA TCT GCT ACC ATC TGC CTT
     Ala Tyr Leu Met Arg Thr Asn Arg Val Lys Leu Asp Glu Ala Phe Glu Phe  289
 990 GCC TAT CTT ATG AGA ACT AAC CGA GTG AAG CTA GAT GAA GCG TTT GAG TTT
     Val Lys Gln Arg Arg Ser Ile Ile Ser Pro Asn Phe Ser Phe Met Gly Gln  306
1041 GTG AAG CAA AGG AGG AGT ATA ATC TCC CCA AAC TTC AGT TTT ATG GGA CAG
     Leu Leu Gln Phe Glu Ser Gln Val Leu Ala Pro Ser Cys Ser Ala Glu Ala  323
1092 CTT CTT CAG TTT GAG TCT CAA GTG CTA GCA CCA TCA TGT TCA GCA GAA GCA
     Gly Ser Pro Thr Ile Ser Val Leu Asp Arg Gly Thr Ser Thr Thr Thr Val  340
1143 GGT AGC CCT ACA ATA TCT GTT CTA GAT CGA GGG ACA TCC ACC ACC ACC GTC
     Phe Asn Phe Pro Val Ser Ile Pro Val His Ser Gly Ala Asn Ser Leu Ser  357
1194 TTC AAT TTT CCT GTT TCT ATA CCT GTC CAC TCA GGA GCA AAC TCT CTC AGT
     Tyr Leu Gln Asn Pro Ile Thr Thr Ser Pro Ser Cys Stop                 369
1245 TAC CTT CAA AAT CCC ATC ACT ACA TCA CCA AGC TGC TGA GGTACTGAGCAAACA
1299 GACACAGACTGTAGCAAATGGACAATTTGTGTTTTTATCAAGTGCAATAATTATTTTTATATCCAAC
1366 ACTTTTATTTTACCTTCTGTTGAGTTAAAGGAGCAACATGTACTTTGAGCCAGTTGAACTGGTAAAA
1433 CTACAAAAAAAACCAAACTGACATGACTTGTTGGACAATATTTTAGATGCTTATCCTAACCACTAAG
1500 GAAGTCAAGAAAAACAGGTCACAATTGCCTTTTGTGTTTTTGTCCCGTTTTTATTTGTTATCACCCT
1567 TTTTGGGGGGTGATTTAGCAGTATTTTCATTCCTGAACTCCTTTTTTATTGTTGATTGATGAAGAGA
1634 GGCTGCTGCCTAAACCTGTAATATAATATTTATTCTGCAAGCTGTGTTCTGCCTACAGAAGCCTAAG
1701 ATTTCAGCTTTTCAAAGAAGCAAAGAAAGAGATACCTCAGTATTTTCTGGTACTGTAATTCCTGTGT
1768 GTAATTCAAGACCAGCTTCAGTTTCCAAAGCACTGATGTCAAAGCACTAGGCCCTAGCCTTCTTCAG
1835 AGTTTGCGGACTATTGTGTGTGTGTGTGTATATGTTTGTGTGTATATGTATATACATATATACAC
1902 ATATATATACTTGTATGCTATGATTTTATTTATGATGCAAAATAATATATATCTTCTACTGTGAGGT
1969 GAGAAGACGTTCTTAATACAGAACTATGACTTTTTATACTGATAGAATAA
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Fig. 1. (a) Nucleotide and deduced amino acid sequences corresponding to the *XCL100* cDNA. Nucleotides are numbered on the left, beginning with the first nucleotide of the cDNA. Amino acids are numbered on the right, beginning at the first predicted Met. Several repeats of a message destabilisation signal within the 3′ untranslated sequence are underlined. The position of the conserved active site sequence of protein tyrosine phosphatases at amino acids 259-266 is indicated by a black background. (b) Sequence alignment of the predicted *XCL100* open reading frame with those of the human *CL100* cDNA and its mouse homologue 3CH134. Amino acids which are identical in all three sequences are marked with an asterisk, and residues which are conserved are shaded. The active site sequences are boxed and two sequences (designated A and B) which represent regions of homology between the human CL100 sequence and the cdc25 proteins are underlined. Spaces represent gaps introduced to maximise matches. Full stops indicate the end of an amino acid sequence.

b

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          ** **    *   * *** * *  ** ******* *    * ** *** **** ***** ********* * * ** ** * *
XCL100    MVNMETCAMDCCVLKALLAERAHKCLILDCRSFFSFSSCSIVGSSNVRLSTIVKRRAKGSMGLEHIVPNEEQRCRLVAGMYEA 83
CL100     MV MEVGTLDAGGLRALLGERAAQCLLLDCRSFFAFNAGHIAGSVNVRFSTIVRRRAKGAMGLEHIVPNAELRGRLLAGAYHA 82
3CH134    MV MEVGILDAGGLRALLREGAAQCLLLDCRSFFAFNAGHIAGSVNVRFSTIVRRRAKGAMGLEHIVPNAELRGRLLAGAYHA 82
                                                  A
          *******   **    * *  **  ****  *     ** **** *** *** * * * * ******    ** ** * *  *
XCL100    VVLLDERTSELDMLRKDSTMMLAVNALCRDSRGSSIYFLKGGYETFSAQCPEFCTKNSPPVGLSLPLCANNVPGSADSNCTPC 166
CL100     VVLLDERSAALDGAKRDGTLALAAGALCREARAAQVFFLKGGYEAFSASCPELCSKQSTPMGLSLPLST SVPDSAESGCSSC 164
3CH134    VVLLDERSASLDGAKRDGTLALAAGALCREARSTQVFFLQGGYEAFSASCPELCSKQSTPTGLSLPLST SVPDSAESGCSSC 164
                                                        B
           **** ******** ***************** ******************* ********* ******************
XCL100    GTPLYDQGGPVEILPFLYLGSAYHASRKDMLDTLGITALINVSANCPNHFEGHFQYKSIPVEDSHKADISSWFNEAIDFIDSV 249
CL100     STPLYGQGGPVEILPFLYLGSAYHASRKDMLDALGITALINVSANCPNHFEGHYQYKSIPVEDNHKADISSWFNEAIDFIDSI 247
3CH134    STPLYDQGGPVEILSFLYLGSAYHASRKDMLDALGITALINVSANCPNHFEGHYQYKSIPVEDNHKADISSWFNEAIDFIDSI 247
                                                .
          *  *************************************** ******************* ********   ***
XCL100    KNSGGRVFVHCQAGISRSATICLAYLMRTNRVKLDEAFEFVKQRRSITSPNFSFMGQLLQFESQVLAPSCSAEAGSPTISVLD 332
CL100     KNAGGRVFVHCQAGISRSATICLAYLMRTNRVKLDEAFEFVKQRRSIISPNFSFMGQLLQFESQVLAPHCSAEAGSPAMAVLD 330
3CH134    KDAGGRVFVHCQAGISRSATICLAYLMRTNRVKLDEAFEFVKQRRSIISPNFSFMGQLLQFESQVLAPHCSAEAGSPAMAVLD 330


          *****************     * **  ********
XCL100    RGTSTTTVFNFPVSIPVHSGANSLSYLQNPITTSPSC• 369
CL100     RGTSTTTVFNFPVSIPVHSTNSALSYLQSPITTSPSC• 367
3CH134    RGTSTTTVFNFPVSIPVHPTNSALNYLKSPITTSPSC• 367
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antigen. The labelled proteins were then analysed by SDS-PAGE as above. The ^{35}S-labelled XCL100 protein used as a size marker was generated by transcription/translation of the vector pXCL100Blue, which contains the *XCL100* reading frame minus the myc epitope tag.

Preparation of XIK-2 cell lysates and 'in gel' MAP kinase assays

Cells were washed twice with ice-cold 0.5× PBS. They were then lysed in 20 mM Tris-HCl, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1% Triton X-100, 0.1% 2-mercaptoethanol and protease inhibitors (as before). Lysates were microcentrifuged briefly and the supernatant was analysed for kinases with activity towards myelin basic protein using an in gel assay exactly as described (Kameshita and Fujisawa, 1989). For quantitation of kinase activities dried gels were analysed using a phosphor-imager (Bio-Rad GS-250 molecular imager). In experiments where cell lysates were treated with human CL100 protein prior to the in gel assay, cells were lysed in RIPA (minus SDS) buffer (containing protease inhibitors and 2 μM Microcystin-LR) and 20 μl of lysate was incubated with 20 μl of either recombinant CL100 protein (80 μg/ml) or buffer for 5 minutes at 30°C.

RESULTS

Isolation of the *XCL100* cDNA and sequence analysis

Genomic Southern blotting of DNA from a variety of species using the human *CL100* cDNA as a probe revealed that homologous sequences were present in *Xenopus laevis* (Emslie et al., 1994). To isolate the *Xenopus CL100* cDNA we screened a cDNA library derived from the *X. laevis* kidney cell line XIK-2 (McStay et al., 1991) using the human *CL100* cDNA at high stringency. This screen yielded three positive clones. Subcloning and sequencing of these cDNA inserts revealed that they all corresponded to the same mRNA. The sequence of the longest cDNA (designated *XCL100)* is shown in Fig. 1a. The 2018 bp cDNA contains a single continuous open reading frame starting at nucleotide 174 and extending to nucleotide 1280. This specifies a protein of 369 amino acids (calculated M_r = 40,256). Comparison of the *XCL100* cDNA sequence with the human *CL100* cDNA revealed that the nucleotide sequences

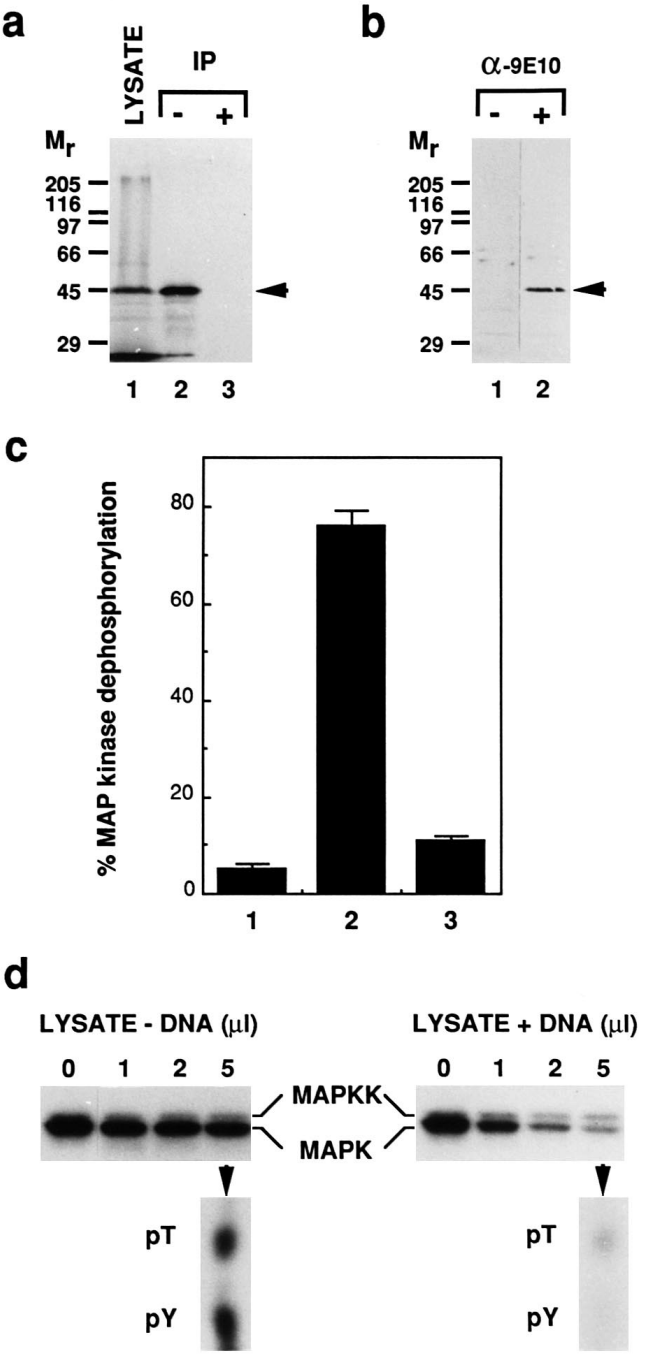

Fig. 2. (a) Expression of the XCL100 protein in rabbit reticulocyte lysates. SDS-PAGE analysis of ^{35}S-labelled proteins from coupled transcription/translation reactions programmed with the pXCL100mycBlue plasmid. Lane 1, total lysate. Lane 2, XCL100 protein immunoprecipitated from this lysate with the anti-XCL100 antibody. Lane 3, immunoprecipitation carried out in the presence of competing XCL100 peptide. Molecular mass markers are shown on the left ($\times10^{-3}$) and the position of the XCL100 protein is marked with an arrow on the right. (b) Immunodetection of the XCL100 protein using the anti-myc 9E10 monoclonal antibody. Western blot of transcription/translation reactions in reticulocyte lysates with either no DNA (– lane 1) or with the pXCL100mycBlue plasmid (+ lane 2) using the anti-myc epitope 9E10 monoclonal antibody. Relative molecular mass markers are shown on the left ($\times10^{-3}$) and the position of the XCL100 protein is marked with an arrow on the right. (c) Dephosphorylation of p42 MAP kinase by recombinant XCL100 protein. Release of ^{32}P from labelled activated MAP kinase expressed as a percentage of total label after incubation with 1 μl of reticulocyte lysate for 1 hour at 30°C in the presence of 2 μM microcystin-LR. Lane 1, unprogrammed lysate. Lane 2, lysate programmed with pXCL100mycBlue. Lane 3, lysate programmed with pXCL100mycBlue and incubated with MAP kinase in the presence of 2 mM sodium orthovanadate. (d) SDS-PAGE and phosphoamino acid analysis of ^{32}P-labelled MAP kinase following incubation with recombinant XCL100 protein. ^{32}P-labelled MAP kinase (MAPK) was analysed by SDS-PAGE after incubation with increasing amounts (0-5 μl) of reticulocyte lysate as above, either unprogrammed (on the left) or progammed with the plasmid pXCL100mycBlue (on the right). The position of ^{32}P-labelled MAP kinase kinase (MAPKK), which is also visible in these reactions, is marked. The MAP kinase incubated with 5 μl of lysate was then subjected to phosphoamino acid analysis (bottom panels) The positions of phosphothreonine (pT) and phosphotyrosine (pY) are marked.

were 71% identical. The region of similarity extends from the initiating ATG and includes some 500 bp of 3′ untranslated sequence (data not shown). The 3′ untranslated sequence of the *X. laevis* cDNA contains several copies of the mRNA destabilisation sequence element AUUUA (Shaw and Kamen, 1986).

At the amino acid sequence level the XCL100 protein is 76% identical to the human CL100 protein. The sequence relationships between the *Xenopus*, human and mouse CL100 proteins are shown in Fig. 1b. Clearly the proteins are most highly conserved over the catalytic domain, which contains the signature active site sequence (Ile/Val-His-Cys-X-Ala-Gly-X-X-Arg) (Fischer et al., 1991) of the protein tyrosine phosphatases. Within the amino-terminal domain of the human CL100 protein there are two short regions of sequence similarity with the cdc25 phosphatases (Keyse and Ginsburg, 1993; Kwak et al., 1994). Interestingly, these are also present in the PAC-1 and B23 proteins. Examination of the corresponding region of the XCL100 protein (Fig. 1b) revealed that these sequences (designated A and B) are conserved.

The XCL100 protein is a dual specificity MAP kinase phosphatase in vitro

In order to characterise the biochemical activity of XCL100 we have expressed residues 1-368 of XCL100 fused at the C terminus with tandem copies of a myc epitope tag (calculated M_r = 43,375) in coupled transcription/translation lysates. Fig. 2a shows that these lysates make a single ^{35}S-labelled protein of about 44 kDa, which can be immunoprecipitated with a polyclonal antiserum raised against residues 150-166 of the XCL100 protein. This protein can also be detected by immunoblotting using the anti-myc epitope antibody 9E10 (Fig. 2b). The XCL100 protein in these lysates brought about the near complete dephosphorylation of both tyrosine and threonine residues in activated recombinant ^{32}P-labelled p42 MAP kinase (Fig. 2c,d). Furthermore, this dephosphorylation is blocked by addition of the specific protein tyrosine phosphatase inhibitor, sodium orthovanadate.

Expression of *XCL100* is inducible by both growth factors and oxidative/heat stress in the *X. laevis* kidney cell line XIK-2

We exposed XIK-2 cells to various stress treatments and to serum stimulation, and assayed by northern blotting for *XCL100* mRNA expression. The *XCL100* mRNA was inducible by heat shock, hydrogen peroxide, the superoxide generating drug, menadione, and also in response to growth factor stimulation (Fig. 3a). Following serum stimulation an increased level of the *XCL100* mRNA was detected within 30 minutes of serum addition, with maximum levels of transcript accumulation seen after 1-2 hours (Fig. 3b). We have used our polyclonal anti-peptide antiserum to immunoprecipitate the XCL100 protein from lysates of ^{35}S-labelled XIK-2 cells. Consistent with our mRNA analysis, XCL100 protein was undetectable in control cells. However, following both serum stimulation and heat shock the synthesis of XCL100 protein was induced (see Fig. 6b, and data not shown).

Both the *Xenopus* XCL100 and human CL100 proteins are localised in the nucleus when expressed in COS-1 cells

The subcellular location of both the *Xenopus* and human CL100 proteins was investigated by transient expression of myc epitope-tagged fusion proteins in COS-1 cells followed by immunofluorescent staining. COS-1 cells transfected with either an *XCL100* or a human *CL100* expression vector demonstrated positive staining on approximately 5-10% of cells. No staining was observed in any cells following transfection of the expression vector alone. In both cases the CL100 protein was located predominantly in the nucleus with a punctate staining pattern (Fig. 4a,c). This staining pattern was clearly distinct from that obtained using the DNA specific dye, DAPI, indicating that CL100 is not associated generally with chromatin, but rather is localised to discrete sites within the nucleus. Some cytoplasmic staining above background was seen in a small proportion of cells expressing the human CL100 protein but was more apparent in cells expressing the XCL100 protein (see Fig. 4a).

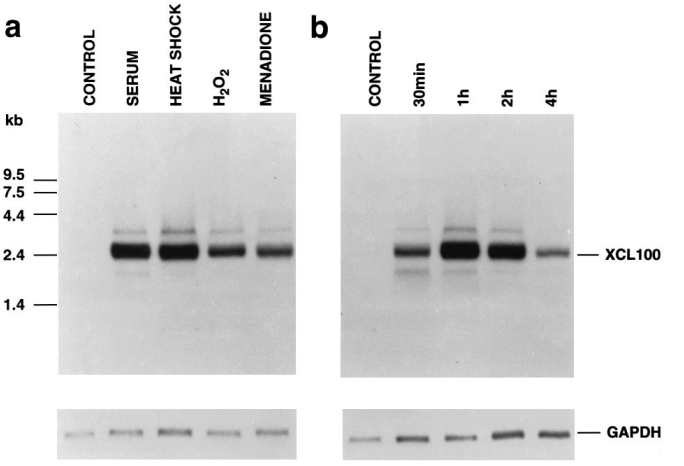

Fig. 3. (a) Accumulation of *XCL100* mRNA in XIK-2 cells after serum stimulation, heat shock and oxidative stress. Northern blot analysis of total RNA isolated 1 hour after stimulation of XIK-2 cells with 10% serum, heat shock (15 minutes at 37°C), exposure to H_2O_2 (200 μM for 30 minutes) or treatment with menadione (250 μM for 30 minutes). (b) Time course of *XCL100* mRNA accumulation in XIK-2 cells following serum stimulation. Northern blot analysis of total RNA isolated at the indicated times following addition of 10% serum to serum starved (0.5% serum for 36 hours) XlK-2 cells. RNA size markers are shown on the left. Both blots were reprobed with the *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase gene as a loading control.

Synthesis of the XCL100 protein is not required for MAP kinase inactivation in *X. laevis* XIK-2 cells following both serum stimulation and heat shock

We have determined the kinetics of MAP kinase activation in the XIK-2 cell line following both serum stimulation and heat shock by using an electrophoretic mobility retardation assay to monitor the phosphorylation state of MAP kinase in conjunction with direct measurements of MAP kinase activity using an in gel myelin basic protein (MBP) kinase assay. Both serum stimulation and heat shock (15 minutes at 37°C) lead to maximal activation of MAP kinase within 5-15 minutes (Fig. 5a), as revealed by the complete shift of the MAP kinase detected by immunoblotting into the upper (phosphorylated) band. This shift is accompanied by a 5- to 8-fold increase in the activity of an MBP kinase activity with an apparent

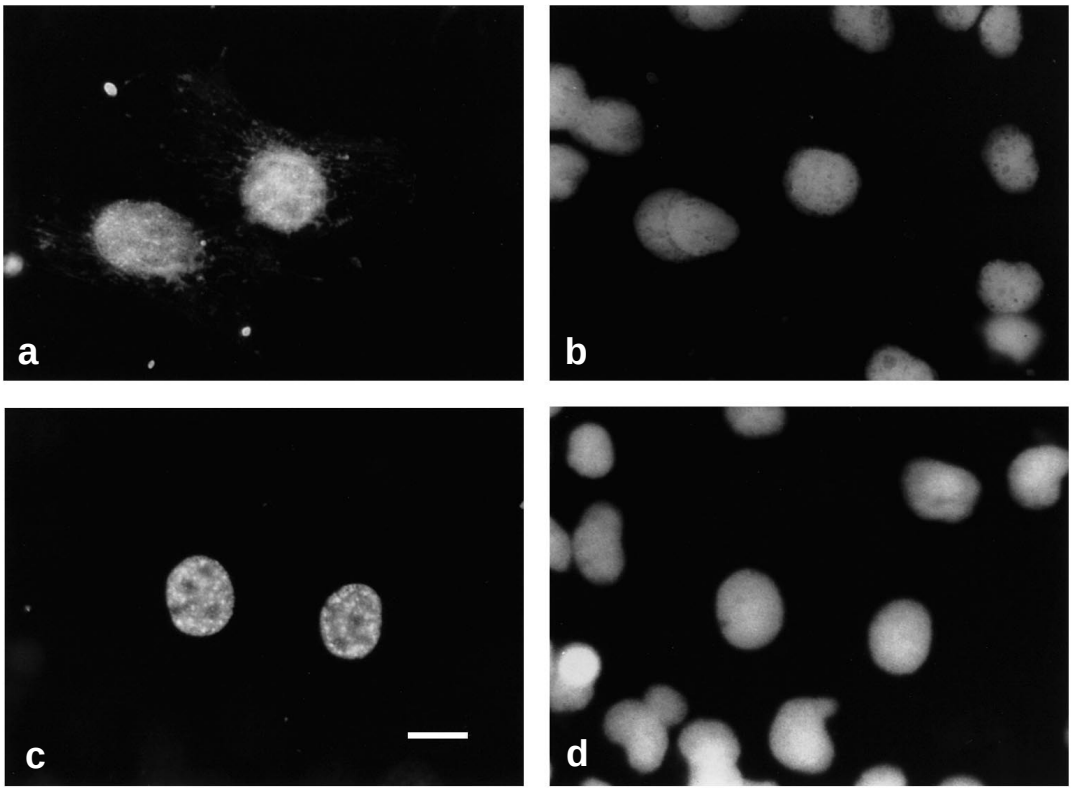

Fig. 4. Both *Xenopus* and human CL100 proteins are localised in the nucleus when expressed in COS-1 cells. COS-1 cells were transfected with the mammalian expression vector pSG5 containing either myc-tagged *XCL100* (a and b) or myc-tagged human *CL100* (c and d) and analysed by indirect immunofluorescence using the anti-myc 9E10 monoclonal antibody (a and c) or by DAPI fluorescence (b and d). Bar in c, 10 μm.

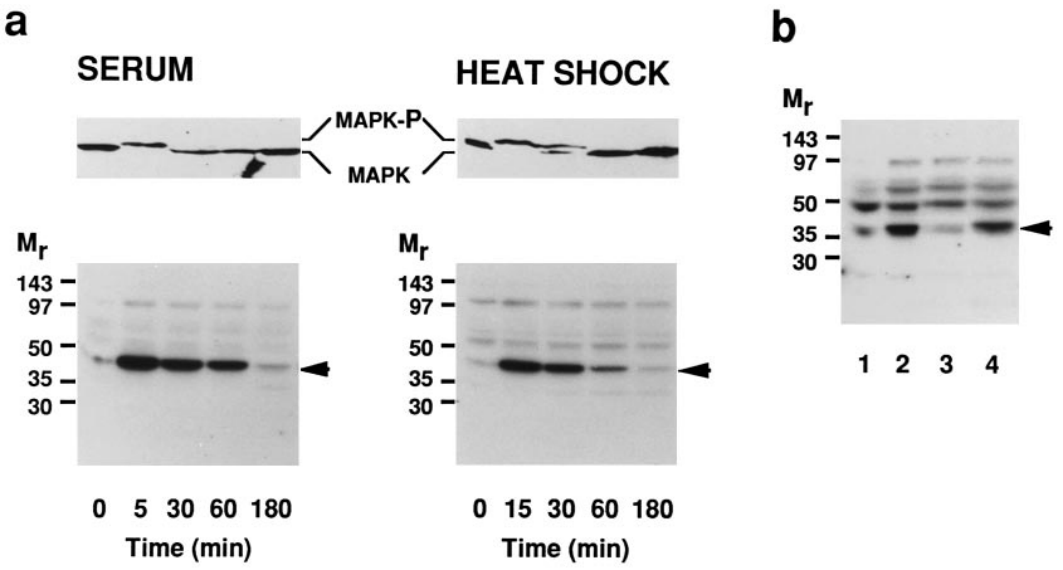

Fig. 5. (a) Transient activation of MAP kinase in XlK-2 cells following serum stimulation or heat shock. The upper panels show western blots of total cell lysates at the indicated times following serum stimulation (left) or heat shock (right) probed with an anti-MAP kinase monoclonal antibody to detect the phosphoshift of MAP kinase (MAPK) to its activated isoform (MAPK-P). The lower panels show the same lysates analysed using an in gel MBP kinase assay. The arrows on the right show the migration of the inducible MBP kinase activity attributed to MAP kinase. Relative molecular mass markers are shown on the left ($\times 10^{-3}$). (b) The inducible MBP kinase activity is inactivated by recombinant human CL100 MAP kinase phosphatase. In gel MBP kinase assays of total cell lysates from untreated cells (lane 1); heat-shocked cells (lane 2); heat-shocked lysate incubated with recombinant human CL100 protein (lane 3); and heat-shocked lysate incubated with buffer alone (lane 4). The arrow on the right shows the migration of the inducible MBP kinase activity attributed to MAP kinase. Molecular mass markers are shown on the left ($\times 10^{-3}$).

molecular mass of 42-44 kDa. This activity is attributed to MAP kinase on the basis of the following criteria. Firstly, it can be immunoprecipitated using our anti-MAP kinase monoclonal antibody (result not shown). Secondly, this inducible kinase is inactivated by incubation with recombinant human CL100 MAP kinase phosphatase (Fig. 5b). These data are

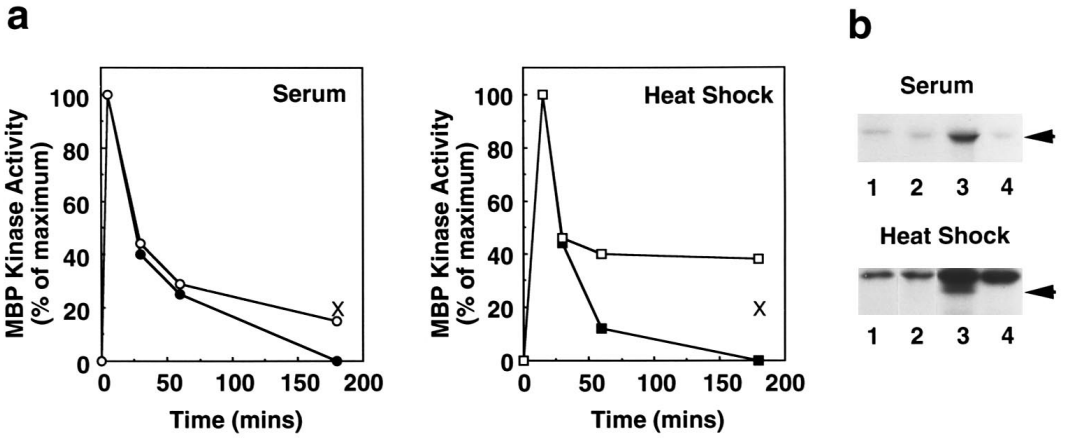

Fig. 6. Inhibition of protein synthesis does not prevent MAP kinase inactivation in XIK-2 cells following serum stimulation or heat shock. (a) MBP kinase activity analysed using the in gel kinase assay and quantitated by phosphor-imaging expressed as a percentage of maximum induction at various times after serum stimulation (left) or heat shock (right). Serum alone (●); serum stimulation in the presence of 10 μg/ml puromycin (○); heat shock alone (■); heat shock in the presence of 10 μg/ml puromycin (□). In both panels the cross represents the level of MBP kinase activation following treatment with 10 μg/ml puromycin alone. (b) Puromycin blocks the synthesis of the XCL100 protein in XIK-2 cells following serum stimulation or heat shock. SDS-PAGE analysis of immunoprecipitations of XCL100 protein from ^{35}S-labelled XIK-2 cell lysates 2 hours after serum stimulation (upper panel) or heat shock (lower panel). For serum stimulation, [^{35}S]methionine was added to the cells at the same time as the serum. For heat shock, cells were prelabelled for 3 hours with [^{35}S]methionine. Preimmune controls (lane 1); uninduced cell lysates (lane 2); lysates immunoprecipitated 2 hours after serum stimulation or heat shock (lane 3); lysates immunoprecipitated 2 hours after serum stimulation or heat shock in the presence of 10 μg/ml puromycin (lane 4). The migration of the XCL100 protein is marked with an arrow on the right.

quantitated in Fig. 6a, and our results show that significant inactivation of MAP kinase following serum stimulation or heat shock occurs within 30 minutes in the XIK-2 cell line. Furthermore, the treatment of these cells with the protein synthesis inhibitor puromycin does not modify the kinetics of MAP kinase inactivation over this period, despite the fact that the synthesis of XCL100 protein in response to either serum stimulation or heat shock is completely blocked (Fig. 6b). However, there is some evidence, at least in heat-shocked cells, that inhibition of protein synthesis does affect the inactivation of MAP kinase which occurs between 1 and 3 hours (Fig. 6a, right panel). Similar results were obtained when accumulation of XCL100 protein was blocked with cycloheximide or when the transcription of the XCL100 gene was inhibited by actinomycin D (data not shown).

XCL100 is expressed as maternal mRNA and *XCL100* mRNA is regulated during embryogenesis

We have examined the expression of *XCL100* mRNA in oocytes and early embryos of *X. laevis* and compared this with the expression of the c-*mos*^{xe} mRNA, which encodes a MAP kinase kinase kinase (Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). Northern analysis of a fixed amount (30 μg) of total RNA extracted from stage I (smallest, 50-150 μm in diameter) to stage VI (fully grown, 1.2 mm in diameter) oocytes shows that *XCL100* mRNA can be detected throughout oocyte growth (left panel, Fig. 7a). In addition, *XCL100* mRNA, like c-*mos*^{xe}, appears to be expressed at a higher level in the earlier stages of oocyte growth (stages I-IV) relative to the later stages (stages V and VI). However, loading of RNA on a per oocyte basis reveals that, as is the case for c-*mos*^{xe} mRNA (Sagata et al., 1988), *XCL100* is in fact expressed at a constant level during oocyte growth as well as during oocyte maturation both in vitro and in vivo (right panel, Fig. 7a). Further analysis of total RNA from different developmental stages (Fig. 7b) reveals that levels of the *XCL100* mRNA decline somewhat during cleavage (stages 3-6). However, in contrast to the c-*mos*^{xe} mRNA, which is no longer detected shortly after gastrulation, *XCL100* mRNA levels rise dramatically during the mid-blastula transition, coincident with the onset of zygotic transcription. This higher level of expression is then maintained through gastrulation and all subsequent developmental stages.

XCL100 protein is present in both immature oocytes and progesterone-induced mature eggs

Both growing oocytes and oocytes induced to undergo meiotic maturation with progesterone (which is a physiological inducer of amphibian oocyte maturation; Masui and Clarke, 1979) were metabolically labelled and subjected to immunoprecipitation analysis using the anti-XCL100 antiserum (Fig. 8). A ^{35}S-labelled polypeptide which co-migrates with recombinant XCL100 protein was specifically immunoprecipitated (i.e. blocked in the presence of competing peptide antigen). The XCL100 protein is present both in the immature and progesterone (in vitro) matured *Xenopus* oocytes. In addition to XCL100 three other polypeptides (approximate molecular masses of 80 kDa, 150 kDa and 200 kDa) are present specifically in these immunoprecipitates.

DISCUSSION

XCL100 is highly homologous with *CL100* at both the nucleotide (71% identity) and amino acid (76% identity) levels. In addition, the pattern of inducible expression of the *CL100* gene in response to serum growth factors and cellular stress is conserved in a *Xenopus* kidney cell line. We have

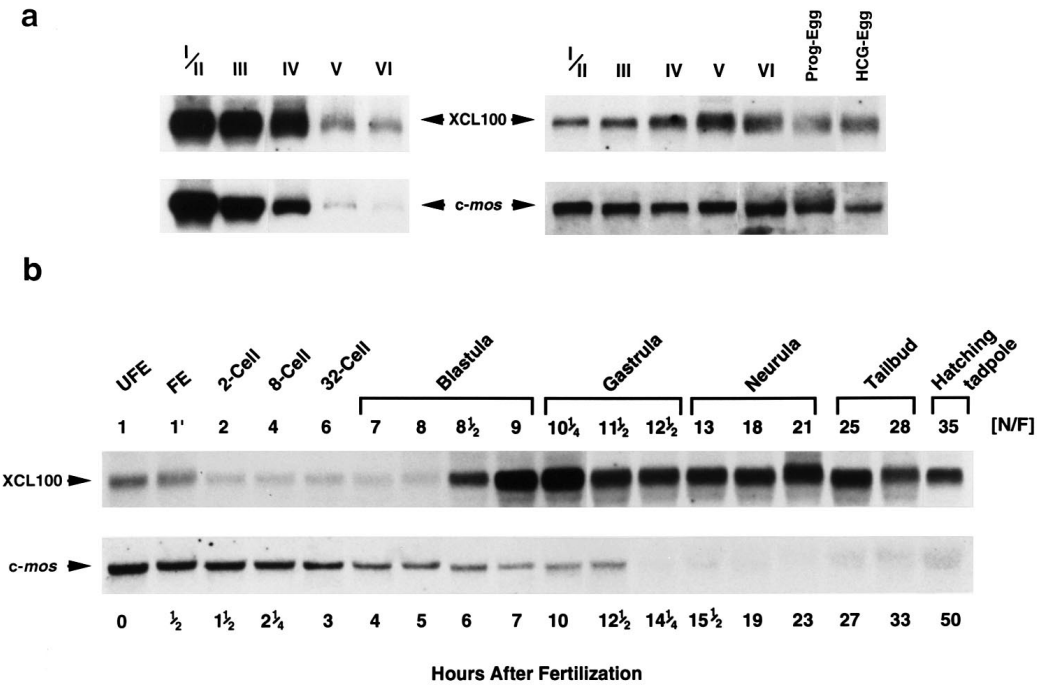

Fig. 7. *XCL100* mRNA expression during oogenesis and embryogenesis. (a) Northern blot analysis: of total RNA (30 μg) isolated from oocytes at various stages (left); or of total RNA equivalent to six oocytes (or eggs) (right). Prog-Egg, progesterone-induced mature eggs (in vitro); HCG-Egg, human chorionic gonadotrophin-induced mature eggs (in vivo). Blots were reprobed with the c-*mos*^{xe} cDNA (lower panels). (b) Northern blot analysis of total RNA (30 μg, equivalent to six eggs or embryos) isolated from eggs or embryos at various stages. UFE, unfertilised eggs; FE, fertilised eggs; N/F, Nieuwkoop and Faber's stage. Blots were reprobed with the c-*mos*^{xe} cDNA (lower panels)

expressed recombinant XCL100 protein in a coupled transcription/ translation system and shown that it is able to dephosphorylate activated p42 MAP kinase on both threonine and tyrosine residues. We conclude, on the basis of its sequence similarity, common pattern of mitogen and stress inducibility, and the biochemical properties of the encoded protein, that *XCL100* is the *X. laevis* homologue of the human *CL100* MAP kinase phosphatase.

The CL100 protein is the founding member of a subfamily of dual specificity protein phosphatases which are related in sequence to the vaccinia VH-1 enzyme. Certain members of this subfamily of proteins are distinguished by their substrate specificity for the MAP kinase group of enzymes (Keyse, 1995). Activated MAP kinases are known to be translocated to the nucleus under certain conditions (Chen et al., 1992; Traverse et al., 1992; Seth et al., 1992). The subsequent phosphorylation of nuclear targets, which include several well characterised transcription factors, is thought to mediate changes in gene expression resulting in cell proliferation or differentiation. We have found that both the *Xenopus* and the human CL100 proteins are translocated to the nucleus when transiently expressed in COS-1 cells. This result strongly suggests that the inactivation of MAP kinases by the CL100 family of enzymes will occur in the cell nucleus and, by implication, that these dual specificity phosphatases act to control the programme of gene expression mediated by MAP kinases when in the nucleus. The haematopoetic cell specific MAP kinase phosphatase PAC-1 is also localised in the nucleus and its overexpression is able to block MAP kinase dependent transcriptional activation (Rohan et al., 1993; Ward et al., 1994). Interestingly, the most recently identified member of the CL100 subfamily, the B23 phosphatase, contains a consensus bipartite nuclear localisation sequence motif, although nothing is yet known about the subcellular localisation of this protein. This motif is only partially conserved in the *Xenopus* and human CL100 proteins. Future work will be directed towards

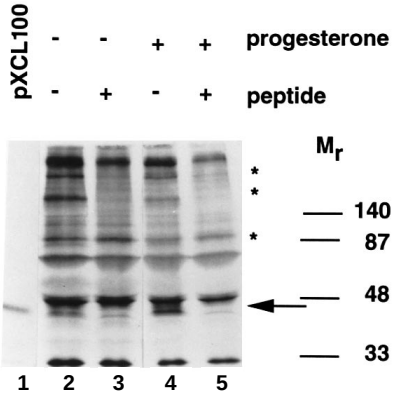

Fig. 8. Immunoprecipitation of XCL100 protein from ^{35}S-labelled immature and mature oocytes. SDS-PAGE analysis of immunoprecipitations using the anti-XCL100 antibody from metabolically labelled stage 6 oocytes in the absence (lanes 2-3) or presence (lanes 4-5) of progesterone; and in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of an excess of the competing XCL100 peptide antigen. A 1 μl sample of a ^{35}S-labelled transcription/translation reaction expressing recombinant XCL100 protein (without the myc epitope tag, pXCL100) has been run in lane 1. The position of the XCL100 protein is marked with an arrow on the right, and relative molecular mass markers are also shown on the right ($\times 10^{-3}$). The positions of three labelled proteins that appear to co-immunoprecipitate with the XCL100 protein are indicated by asterisks.

the dissection of the sequences necessary for the translocation of the CL100 proteins to the nucleus. The significance of the punctate nuclear staining pattern observed with both *Xenopus* and human CL100 proteins is unclear. Similar patterns are observed with a range of nuclear proteins whose functions necessitate attachment to the nuclear matrix (Hoffman, 1993; Lamond and Carmo-Fonseca, 1993). However, following translocation to the nucleus MAP kinase exhibits a diffuse

staining pattern (Lenormand et al., 1993). Further studies, and in particular the determination of the subnuclear location of endogenous as opposed to ectopically expressed CL100 protein, will be required before any firm conclusions can be drawn as to the degree to which CL100 and its putative substrate are co-localised within the nucleus.

Recent work has shown that the kinetics of synthesis of the mouse *CL100* homologue (*3CH134*) correlate with the inactivation of MAP kinase in serum stimulated NIH3T3 cells (Sun et al., 1993). Furthermore, treating these cells with cycloheximide, which blocks the synthesis of the 3CH134 protein, leads to sustained activation of MAP kinase (Sun et al., 1993). These results have been taken as evidence that CL100 is solely responsible for the inactivation of MAP kinase in vivo (Sun et al., 1993). In the present study we have examined the kinetics of activation and inactivation of MAP kinase in the *X. laevis* kidney cell line XIK-2, following both serum stimulation and heat shock. Firstly, we find that in these epitheloid cells MAP kinase activation is rapid and complete, occurring within 5-15 minutes of either serum stimulation or heat shock. Secondly, in both cases the subsequent inactivation of MAP kinase occurs relatively rapidly, with significant loss of activity within the first 30 minutes. Thus MAP kinase is significantly inactivated in the XIK-2 cells well before the maximum levels of *XCL100* mRNA (and protein) have accumulated. Finally, we have shown that this rapid inactivation of MAP kinase is not prevented by protein synthesis inhibitors under conditions where synthesis of the XCL100 protein is blocked.

These results argue that, despite the induction of XCL100, this enzyme (or any other inducible phosphatase) plays no role in the inactivation of MAP kinase, at least at times up to 60 minutes, in XlK-2 cells, and suggests that the mechanism of MAP kinase inactivation may reflect the activities of other enzymes which are able to dephosphorylate and inactivate MAP kinase. However, we cannot exclude the possibility that CL100 is involved in the inactivation of MAP kinase at longer times. For instance, after heat shock there is some indication that protein synthesis inhibition prevents the loss of the remaining 20% of MAP kinase activity, which occurs between 1 and 3 hours (see right-hand panel in Fig. 6a).

A pattern of rapid inactivation of MAP kinase which is independent of new protein synthesis has recently been noted in chromaffin (PC12) cells exposed to epidermal growth factor (EGF) (Alessi et al., 1995; Wu et al., 1994). We suggest two possible interpretations of our results. Firstly, a requirement for new protein synthesis in order to bring about the inactivation of MAP kinase may be restricted to fibroblast cell lines. This model would be compatible with the idea that CL100 inactivates MAP kinase in the nucleus as the sustained activation (60-120 minutes) of MAP kinase seen in fibroblasts is accompanied by nuclear translocation of the enzyme (Lenormand et al., 1993). However, in some cell types, which would include the epitheloid kidney cell line used in the present study, MAP kinase may not be translocated to the nucleus and, as is apparently the case in PC12 cells treated with EGF (Alessi et al., 1995), the rapid inactivation of MAP kinase may take place largely in the cytosolic compartment of the cell. The fact that the XCL100 enzyme is still induced in the latter case may reflect a requirement to dephosphorylate low levels of activated MAP kinase which translocate to the nucleus of these cells at later times. Secondly, it is possible that the cellular targets of the CL100 phosphatase are not the classical p42 and p44 isoforms of MAP kinase but other members of the MAP kinase family. In addition to its activity towards p42 and p44 MAP kinases in vitro, CL100 is able to dephosphorylate other MAP kinase isoforms, including the yeast MAP kinase homologue FUS3 (S. M. Keyse and A. Gartner, unpublished data) and a stress activated mammalian homologue of the yeast HOG-1 MAP kinase (Rouse et al., 1994). Given the fact that the expression of the *CL100* gene is also induced by cellular stress, it is possible that stress activated MAP kinases might be physiological targets for the CL100 enzyme. Further experimental work will be required to determine the relationship between the induction of CL100 and the inactivation of other MAP kinase family members.

We have investigated the expression of *XCL100* in oocytes and during embryonic development. In contrast to the *Xenopus* and human cell lines discussed above, growing *Xenopus* oocytes constitutively express moderate levels of *XCL100* mRNA. In addition, the protein product of the *XCL100* gene is detectable in oocytes both before and during the events of meiosis. Previous characterisation of the human and mouse homologues of *CL100* indicated that it is an immediate early gene, expressed in response to growth factors early in the G_1 phase of the cell cycle. The presence of XCL100 protein in immature oocytes, which are arrested in G_2, suggests that CL100 may also function at other points in the cell cycle. Entry into meiosis I, the suppression of DNA synthesis following meiosis I, and induction of metaphase arrest during meiosis II are controlled by MAP kinase (Furuno et al., 1994; Haccard et al., 1993). This cell cycle arrest is relieved by fertilisation, and inactivation of MAP kinase normally occurs 30 minutes after fertilisation (Ferrell et al., 1991). Indeed, ectopic overexpression of a C-terminally truncated form of CL100 in metaphase arrested extracts of *Xenopus* eggs results in premature inactivation of MAP kinase and exit from metaphase arrest (Minshull et al., 1994). Taken together these results suggest a role for CL100 in the regulation of meiotic events. In contrast, Sarcevic et al. (1993) have recently reported the purification of MAP kinase phosphatase activities from the cytosolic fraction of *Xenopus* eggs. These activities correspond to the catalytic subunit of the Ser/Thr protein phosphatase PP2A and a previously uncharacterised 47 kDa protein tyrosine phosphatase. Further experiments involving the manipulation of XCL100 protein and the 47 kDa enzyme in intact oocytes and egg extracts will be required to probe the roles of these proteins in the control of MAP kinase activity during early development.

During the early cleavage stages of development levels of the maternal *XCL100* mRNA were barely detectable but are markedly upregulated after the 12th cleavage, which corresponds to the mid-blastula transition (MBT). The MBT marks the point at which zygotic transcription is initiated and is characterised by a change in the nature of the cell cycle, with introduction of G_1 and G_2 phases, and asynchronous cell divisions (Gerhart, 1980). By analogy with *Drosophila* and *Caenorhabditis elegans* development models, in which MAP kinase activation/inactivation is implicated in the determination of correct differentiation of retinal and vulval cells, respectively (Biggs et al., 1994; Lackner et al., 1994), we predict that up regulation of *XCL100* mRNA will coincide with a necessity to regulate MAP kinase activity in specific signalling events that determine cell fates in the developing *Xenopus* gastrula. Further work on the localisation of *XCL100* transcripts in

whole embryos during blastulation and gastrulation will be necessary to test this hypothesis.

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