Deletion of the Dual specific phosphatase-4 (DUSP-4) gene reveals an essential non-redundant role for MAP kinase phosphatase-2 (MKP-2) in proliferation and cell survival.

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ABSTRACT
Mitogen-activated protein kinase phosphatase-2 (MKP-2) is a type 1 nuclear dual specific phosphatase (DUSP) implicated in a number of cancers. We examined the role of MKP-2 in the regulation of MAP kinase phosphorylation, cell proliferation and survival responses in mouse embryonic fibroblasts (MEFs) derived from a novel MKP-2 (DUSP-4) deletion mouse. We show that serum and PDGF induced ERK-dependent MKP-2 expression in wild type MEFs but not in MKP-2-/- MEFs. PDGF stimulation of sustained ERK phosphorylation was moderately enhanced in MKP-2-/- MEFs whilst anisomycin-induced JNK was largely unaffected. However, marked effects upon cell growth parameters were observed. Cellular proliferation rates were significantly reduced in MKP-2-/- MEFs and associated with a significant increase in cell doubling time. Infection with adenoviral MKP-2 reversed the decrease in proliferation. Cell cycle analysis revealed a block in G2/M phase transition associated with cyclin B1 accumulation and enhanced cdc2 phosphorylation. Cardiac fibroblasts and macrophages from MKP-2-/- mice also showed similar deficits in proliferation rates compared to wild type controls. MEFs from MKP-2-/- mice also showed enhanced apoptosis when stimulated with anisomycin correlated with increased caspase-3 cleavage and H2AX phosphorylation. Increased apoptosis was reversed by adenoviral MKP-2 infection and correlated with selective inhibition of JNK signaling. Collectively, these data demonstrate for the first time a critical non-redundant role for MKP-2 in regulating cell cycle progression and apoptosis.

ABBREVIATIONS
MAP kinase, mitogen-activated protein kinase; MKP, MAP kinase phosphatase; DUSP, dual-specific phosphatase; MEFs, mouse embryonic fibroblasts; Adv, adenovirus.

INTRODUCTION
The amplitude and duration of MAP kinase signalling within a specific subcellular compartment is a key feature in the integration of extracellular stimuli and their effects on cellular function (1). Three main MAP kinase groups, the ERKs, JNK and p38 MAP kinases are involved in regulating functions such as proliferation, apoptosis
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and differentiation in response to growth factors, peptide hormones, stress and infection (2). Perturbations in MAP kinase signalling is a feature of several different types of diseases including several types of cancers (3), diabetes (4), atherosclerosis (5,6) and immune disorders.

The kinetics of MAP kinase activation are strictly controlled principally by the mitogen-activated protein kinases phosphatases (MKPs), a family of at least ten dual specific phosphatases (DUSPs) that function to terminate MAP kinase signalling within a defined subcellular location (7). They share a common C-terminal catalytic domain, and an N-terminal non-catalytic domain containing the MAP kinase interaction motif (KIM) (8). Each isoform has unique yet overlapping features including substrate specificity, subcellular distribution and factors regulating induction. For example MKP-1 is a nuclear DUSP of the type 1 class and selective for all three major MAP kinases in vitro, whilst MKP-3, a type II DUSP, is a cytosolic phosphatase selective solely for ERK over the other kinases (7). Due to effects upon MAP kinase signaling, perturbations in the MKPs have been implicated principally in cancer (9). However, more recently a role has been established in inflammation (10) and some cardiovascular disorders (11).

One poorly studied MKP is MKP-2 (12). This DUSP (DUSP-4) is a member of the type 1 family and has been shown to be induced in response to a number of stimuli including phorbol esters and growth hormones (12-14). Nuclear targeting is regulated by two distinct nuclear targeting sequences (15). Substrate specificity for ERK and JNK was originally demonstrated in vitro (16), however selective inhibition of JNK has been implicated in cellular studies (17,18). Whilst MKP-2 has been recently regarded as a surrogate for the more well described MKP-1, recent cellular studies demonstrate a role in protection against apoptosis (17) and in senescence (19). However, there is still a lack of information describing the function of DUSP4 in different cell types, in particular regarding substrate selectivity in vivo.

We have recently developed a DUSP-4 deletion mouse model and demonstrated a novel immunological phenotype in vivo (20). Using embryonic fibroblasts from DUSP-4 deletion mice we now examine the effect of deletion upon MAP kinase signalling and growth parameters. We find that despite very moderate increases in ERK and JNK activity, MKP-2 deletion has profound effects upon cellular proliferation. In particular, we identify a role for MKP-2 in G2/M phase transition and demonstrate that MKP-2 plays a role in cell survival in response to the apoptotic stimulus anisomycin. These data demonstrate that MKP-2 is a non-redundant DUSP and has overlapping but distinct functions relative to the prototypic DUSP, MKP-1.

EXPERIMENTAL PROCEDURES

DUSP-4 deletion mice were generated in collaboration with Geno-way, France and have been genetically characterised previously (20). Adenoviral MKP-2 was generated by Vector Bio Labs and used previously (21). Antibodies were purchased as follows: Rabbit polyclonal anti-JNK-1 (S-18, FL), anti-ERK-2 (K-23) and cyclin B1 antibodies were all purchased from Santa Cruz, (California, USA). Phospho-ERK, Anti-phospho-JNK1/2, p-cdc-2 and caspase-3 were purchased from Cell Signaling Technology, (UK). Anti-phospho-p38 was purchased from Biosource (Nivelles, Belgium). Anti-phospho-H2AX was purchased from Millipore (USA). HRP-conjugated anti-Rabbit antibody was purchased from Amersham (Little Chalfont, UK). HRP-conjugated anti-mouse, and conjugated anti-rabbit antibodies were purchased from Jackson Immunoresearch laboratories (Luton, Beds, UK). PE Annexin V Apoptosis detection Kit was purchased from BD Biosciences, (UK). All other materials used were of the highest commercial grade available and were obtained from Sigma Chemical Company (Poole, Dorset, UK) and Invitrogen (Paisley, UK).

Cell Culture

Mouse embryonic fibroblasts (MEFs) were isolated from MKP2 -/- or MKP-2 +/+ mice (backcross 5) at 13.5 day post coitum mice in compliance with British Home Office
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regulations. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% FCS, L-glutamine, Penicillin and Streptomycin, at 37°C, 5% CO². All experiments were performed within three passages. Cardiac fibroblasts (CFs) were prepared from the ventricles of adult MKP-2+/+ and MKP-2-/- mice, 8-10 weeks old, weighing between 20-30g as described previously (22). After 4-5 days, sub-confluent cultures were passaged by trypsinization and re-plated into 12 well plates. Passage 1 cells were used in all experiments. In all experiments, DMEM containing 20% FBS was washed out, and the cells were equilibrated in serum-free DMEM overnight before agonist stimulation. The purity of fibroblast cultures was established by immunofluorescence staining using anti-α-SMA antibody. Bone marrow mouse macrophages were cultured as outlined previously (20).

**G1 Synchronization**

MEFs were grown in 3.5cm dishes in DMEM, containing 10% FCS, L-glutamine, penicillin and streptomycin to 40% confluency. Thymidine was added to a final concentration of 2mM in the media. The cells were incubated at 37º C for 19h. After the incubation, cells were washed with PBS 3 times and fresh media was added without thymidine and incubated for 9h at 37º C. Then thymidine was added again to a final concentration of 2mM and incubated for another 16h. Cells were washed with PBS 3 times and fresh media was added. Cells were harvested at different time points and the cell-cycle profile was analysed by Western blotting.

**Cell Cycle Analysis**

Cell cycle profiles were analysed by staining intracellular DNA with propidium iodide, followed by flow cytometry using a BD FACs Diva software 2004 (Becton, Dickinson). MEFs were trypsinized and washed with PBS and prepared at 1x10⁶ in eppendorf tubes. Cells were fixed in ice-cold 70% ethanol while vortexing to ensure proper fixation of cells and prevent clumping) at 4°C overnight. Cells were washed with PBS and centrifuged at 2000g for 10min, then RNase A (50µg/ml) was added to ensure only DNA staining. Finally, cells were stained with propidium iodide (PI), at 50 µg/ml.

**Proliferation Assays**

Confluent MEFs or CFs were detached with trypsin-EDTA, seeded on coverslip into 24-well plates (5,000 cells/well) in 10% FCS-DMEM, and allowed to attach for 24h. Cells were starved in serum free media for 24h and then stimulated for either 24, 48, 72h with 10% FCS. Cultures were washed with PBS and stained with hematoxylin. The number of cells was determined by counting in 10 random fields per each coverslip.

**Infection of MEFs with MKP-2 Adenovirus**

MKP-2 adenovirus was generated as outlined previously (21) Cells were seeded on coverslips into 12 or 24 well plates and grown to approximately 50% confluency. The cell number was determined using a haemocytometer. MKP-2 adenovirus (100-300 pfu/cell) was added to the cells and incubated for 24h in normal growth medium, then serum starved for 24h before stimulation for 24, 48 or 72h with 10% FCS.

**Western Blotting**

Cells were lysed in SDS-PAGE sample buffer (63mM Tris-HCL, pH 6.8, 2mM Na₂P₂O₇, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue). The lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes which were incubated with phospho-specific antibodies followed by enhanced chemiluminescence detection (21).

**Flow cytometry assay of apoptosis**

Cells were infected for 24h then stimulated for a further 24h prior to analysis. Cells were trypsinized and then pelleted at 1000 rpm for 2 min. The pellet was then resuspended in 500µl of 1x annexin binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). Phycoerythrin-Annexin V and 7-AAD were added to the cells according to the manufacturer’s instructions and the samples read in a FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data was analysed using FACS Diva (Becton
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Dickinson, Oxford, UK) and RCS Express (De Novo Software, Canada) software. A total of 10,000 events were measured per sample. Gating was determined using PE-Annexin V FL-2 and 7–AAD FL-3 standards attached to beads (Becton Dickinson, Oxford, UK) and preliminary experiments conducted using paraformaldehyde and serum deprivation to define apoptotic and necrotic populations as outlined by the manufacturer’s instructions.

Data analysis

Each figure represents one of at least four separate experiments. Western blots were scanned using an Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software. For gels, densitometry measurement was performed using the Scion Image program. Data were expressed as mean ± s.e.m. Statistical analysis was performed by One-way ANOVA with Dunnett’s Post test (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

Mice deficient for DUSP4 have been genetically characterised elsewhere (20). However, in order to confirm MKP-2 deletion, quiescent mouse embryonic fibroblasts from wild type or DUSP-4 deletion mice were stimulated with FCS over a period of 24h (Fig. 1). In wild type MEFs, induction of MKP-2 was marked, reaching a peak between 2 and 4h before returning towards basal values by 24h (Figs. 1A & B). In contrast, there was no increase in MKP-2 in stimulated MEFs from the DUSP-4 KO mice (Fig. 1A & B). A number of other agents also increased MKP-2 expression including PDGF and PMA in wild type but not in DUSP-4 deletion mice1. In order to ensure MKP-2 protein expression was regulated in the normal manner in MKP-2+/+ fibroblasts we pre-incubated cells with different MAP kinase inhibitors prior to stimulation (Fig. 2). Specifically, pre-incubation with the MEK1/2 inhibitor, UO126, substantially and significantly reduced MKP-2 expression (Figs. 2A and B). However the JNK and p38 inhibitors, SP600125 (Figs. 2A & C) and SB203580 (Figs. 2A & D) did not decrease MKP-2 induction. These data suggests that by and large, an ERK-dependent mechanism of induction for MKP-2 exists in the fibroblast model.

We next sought to determine if the kinetics of ERK activation could therefore be modified in the absence of MKP-2 (Figure 3). PDGF stimulated a rapid and sustained increase in ERK activation which was maximal between 5 and 10 min and continued for up to 24h albeit at lower levels of phosphorylation. We found that over the early time period, ERK activation was not altered in MKP-2−/− MEFs (Figs. 3A & C). However, an extended time course revealed a small but significant potentiation between 2 and 24h (Figs. 3B & D).

Whilst there was a clear increase in ERK induction in the absence of MKP-2, changes in SAP kinase signalling were very much less marked. Anisomycin induced a strong and sustained phosphorylation of both JNK and p38 MAP kinase. MKP-2−/− deletion gave a very small but significant increase in the magnitude of JNK phosphorylation but had no effect upon the duration of the time course (Fig. 4A). Similarly, p38 MAP kinase phosphorylation was slightly but significantly increased at a single time point (Fig. 4B).

Nevertheless, despite observing only very small differences in kinase signaling, there were substantial changes in growth parameters following MKP-2 knockout. Figure 5 shows the effect of DUSP-4 deletion on the proliferative capacity of MEFs. In the absence of MKP-2 protein, serum stimulated proliferation assessed over 72h was significantly slowed compared to wild type (Figs. 5A & B). This was also manifest over a shorter time frame (Fig. 5C) and is reflected in an extended lag phase prior to exponential growth and a 2 fold increase in doubling time in MKP-2−/− MEFs (doubling time in days; MKP-2+/+ = 3.67 ± 1.25, MKP-2−/− = 8.00 ± 1.768). We then used Adv.MKP-2 in gain of function studies. Infection of either wild type or MKP-2−/− MEFs with adenoviral (Adv.) MKP-2 at 300 pfu (Fig. 5D) resulted in approximately 95% infection. Under these conditions the inhibition of proliferative responses in MKP-2−/− MEFs were effectively reversed and in fact, rates of proliferation increased...
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above growth rates for wild type MEFs (Figs. 5E &F).

To explain these differences in proliferation we examined a number of cellular growth parameters linked to cell survival (Fig. 6). Using propidium iodide staining we observed a decrease in the number of cells in G1 phase and an increase in the number of cells that accumulated at G2/M-Phase (Fig. 6A). In cells blocked in G1 using thymidine we found an increased number in sub G1 indicative of increased apoptotic cells (Fig. 6B). When we examined both cyclin B1 expression and the related cdc-2 phosphorylation, both these parameters were found to increase prematurely in MKP-2-/- MEFs in comparison to MKP-2 +/+ cells (Figs. 6C) indicative of retention in G2/M-phase. Furthermore, when cells were incubated with topotecan to cause G2/M phase block (23) then subsequently washed, p-cdc-2 levels remained higher over 24 and 48h again indicative of greater accumulation in G2/M-phase for MKP-2-/- MEFs (Fig. 6D).

To confirm that the effect of DUSP-4 gene deletion had a universal effect upon proliferation, we assessed proliferation in two other cell types including adult cardiac fibroblasts (CFs) and mouse bone marrow macrophages (Figure 7). For CFs the deficit in proliferation rates were similar to that observed for MEFs, cell number was over 50% less compared to wild type cells (Fig. 7A). The number of cells in G2/M phase were also increased in MKP-2-/- CFs but to a lesser extent than MEFs (Fig. 7B). A similar difference was observed for continuously growing macrophages, however when these cells where rendered quiescent prior to re-stimulation with FCS, as carried out for both MEFs and CFs, the cells failed to grow at all and subsequently died (Fig. 7C). For these cells only a marginal stage specific effect was observed (Fig 7D).

We also assessed apoptosis directly in MEFs from MKP-2 +/+ and MKP-2 -/- mice in response to anisomycin (Figure 8). Baseline apoptosis in quiescent MEFs was similar in both WT and KO. Anisomycin exposure increased apoptosis from 10 to 40% in MKP-2 +/+ MEFs, this was increased significantly in MKP-2 -/- MEFs to approximately 50% at 12h (Fig. 8A). This effect correlated with increased caspase-3 cleavage (Fig. 8B & D) and phosphorylation of γH2AX (Fig. 8C & E). UVC-induced apoptosis was also significantly increased in MKP-2 -/- MEFs.

In order to determine kinase involvement in the enhanced apoptotic effect due to MKP-2 deletion, cells were infected with Adv.MKP-2 (Fig. 9A). Following infection, anisomycin-induced JNK was substantially reduced in both MKP-2 +/+ and MKP-2 -/- MEFs. However no effect was observed in ERK or p38 MAP kinase phosphorylation (Fig. 9B). This correlated with an effective reversal of increased caspase-3 and γH2AX phosphorylation which was more pronounced in the MKP-2 -/- MEFs (Figs. 9C,D & E). Adv.MKP-2 also reversed the increased apoptosis in response to anisomycin in MKP-2 -/- MEFs but had little effect in wild type cultures (Fig. 9F).

DISCUSSION

The role of MKP-2 in cellular function has been hampered by the lack of a DUSP-4 deletion mouse model. Using such a model recently described (20) we make important new observations regarding the functional role of MKP-2 in cells. We identify a critical role for MKP-2 in the regulation of cellular proliferation and survival. We demonstrate a specific effect upon accrual of cells in G2/M phase and a role in attenuating stress induced apoptosis, which can be reversed by inhibiting JNK activity.

In wild type MEFs, MKP-2 induction was observed 1-2hr following serum stimulation, a time frame in keeping with previous studies in rodent systems (13). In addition, induction was specifically regulated by ERK signaling again consistent with previous publications implicating MKP-2 as a part of a negative feedback system for ERK signalling (24). Indeed, MKP-2 deletion revealed enhanced PDGF mediated ERK phosphorylation particularly over longer time frames, correlating with the time course of MKP-2 induction. This suggests that ERK regulation is a bona fide component of MKP-2 function. However, in
other cell types such as macrophages, we have observed no increase in ERK signalling following MKP-2 deletion (20) suggesting a cell type specific difference in the function of MKP-2. We also observed only minor increases in JNK signalling in response to anisomycin findings consistent with our own studies in macrophages (20) but at variance with other studies using siRNA run down (25). These results are not easily explained, as in wild type MEFs anisomycin did not cause any increase in MKP-2 expression, suggesting no potential for regulation of the corresponding kinase pathways. Deletion of MKP-2 also did not affect p38 MAP kinase activation, which was not unexpected as MKP-2 does not dephosphorylate this protein in vitro. This strongly suggests that the function of MKP-2 in terms of substrate specificity in vitro may be cell type specific and agonist dependent rather than being defined by dephosphorylation studies performed in vitro.

The small differences in MAP kinase signalling following DUSP4 deletion were nevertheless relevant to cellular proliferation. Deletion of MKP-2 resulted in a significant decrease in proliferation rate, an effect that was reversed by infection with Adv.MKP-2. This is similar to a recent study using MKP-1 KO fibroblasts (26) but not earlier studies which demonstrate no difference in cell growth parameters. Furthermore, MKP-2 deletion has been shown to be protective in pro B cells (27), suggesting a cell type dependent role in the regulation of cell survival. We pinpoint this defect in proliferation to an effect upon G2/M-phase transition, an effect not observed in MKP-1 deletion mice. A similar result has been obtained following MKP-2 knockdown in mice tumour cells (28) and this is associated with a complete loss in cyclin B expression and cdc2 phosphorylation. To our surprise however, we did not find any reduction in these parameters, in fact a small increase in cyclin B1 expression and cdc2 phosphorylation was observed which also apparent when cells were arrested in G2/M-phase with topotecan and then allowed to recover. This suggests a propensity for cells to accumulate within G2/M phase in MKP-2-/- MEFs. Usually, under conditions of cell cycle arrest, cyclin B is reduced due to p53 mediated transcriptional repression of the cyclin B1 promoter (29). However, it has been shown that following chemical treatment or ionising radiation, cyclin B1 can accumulate, particularly if p53 is non-functional (30) and this possibility is under investigation in our laboratory. Recently, MKP-2 has been identified as a target gene for p53 (31) and MKP-1 has also been shown to interact with p53, to regulate G1/S-phase progression (32). However, no study to date has demonstrated an effect of any DUSP within G2/M phase of the cell cycle. This further highlights the different functional role of MKP-2 versus the prototypic MKP-1 in cell cycle regulation.

Analysis of apoptosis demonstrated an essential role for MKP-2 in protecting cellular integrity. Both anisomycin and UVC stimulated apoptosis was significantly enhanced in MKP-2-/- cells and, for anisomycin stimulation, this was associated with increased caspase-3 cleavage and γH2AX phosphorylation. Caspase-3 is a well recognised apoptotic mediator protein whilst H2AX, a histone protein (33), mediates apoptotic DNA fragmentation and co-operates with the caspase-3/CAD pathway to mediate apoptosis (34). This aspect of MKP-2 function is essentially similar to that observed for MKP-1 where deletion resulted in increased apoptosis in response to hydrogen peroxide or anisomycin (26,31). In other studies MKP-2 is implicated in apoptosis suggesting potentially species or cell type specific differences (35).

A key issue however, was in the identification of the kinase mediating the effect of MKP-2 deletion. To do this we utilised adenoviral MKP-2 in gain of function studies. We have previously demonstrated the selective inhibitory effect of MKP-2 over expression on stress-induced JNK signaling either using stable or inducible MKP-2 expressing cells lines (17,36) or adenoviral infection in primary endothelial cell cultures (21). Our present study confirms this JNK sensitivity for anisomycin signaling (Fig. 8). Whilst caspase-3 activation is well recognised to be regulated by JNK, only recently have studies also identified H2AX as a target (34, 37, 38). Over expression of Ad-MKP-2 reversed enhanced anisomycin-mediated apoptosis in
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MKP-2<sup>−/−</sup> MEFs and also the increased caspase-3 degradation and γH2AX phosphorylation. This was also associated with a specific dephosphorylation of JNK. Thus, despite little apparent change in JNK signalling in MKP-2<sup>−/−</sup> MEFs per se, these findings link MKP-2 deletion to enhanced JNK-dependent signaling, DNA damage and resultant apoptosis. Preliminary studies using the JNK inhibitor SP600125 confirm this interpretation. Interestingly, a previous study demonstrated that pharmacological inhibition of p38 and not JNK was able to rescue MKP-1 deficient fibroblasts from enhanced cell death (26). Therefore, whilst the effects of MKP-2 and 1 deletion are similar in terms of apoptosis, the kinase mediating the effect of MKP deletion is clearly different.

Our findings also indirectly give an insight into the role of nuclear JNK in regulating apoptosis. Given that MKP-2 is a nuclear located enzyme and unlike MKP-1, which has been found in the mitochondria (39), there is little evidence for discrete pools within other compartments, this suggests that JNK mediated cell death is mediated in part by nuclear located JNK. Indeed JNK phosphorylation was found to be more sensitive to Adv.MKP-2 in the MKP-2<sup>−/−</sup> MEFs suggesting that JNK activation occurs within this compartment. Thus, it is surprising that in addition to increases in the phosphorylation of the nuclear substrate γH2AX, the cleavage of caspase-3, a cytosolic protein is also enhanced. Cleaved caspase-3 although identified in the nucleus (40) requires processing in the cytosol and whilst it is accepted that JNK has both nuclear and cytosolic substrates (41) our studies suggest that JNK could be translocated to the cytosolic compartments, such as the mitochondria, following activation within the nucleus. Alternatively long term modulation of JNK signalling could have profound effects on initiation of apoptosis presumably by changing expression of pro and anti-apoptotic genes which regulate initiator caspases such as c-FLIP (42,43), XIAP and A20 (44).

In conclusion, we demonstrate a critical non-redundant role of MKP-2 in cell survival. This might suggest that over-expression of MKP-2 may be a factor in the development of cancer. Indeed in other cellular studies we have consistently identified over-expression of MKP-2 as mediating resistance to apoptosis. (21). Furthermore, over-expression of DUSP-4 is observed in cancer cell lines (21) and high levels associated with oesophagogastric rib metastasis and pancreatic and liver tumours (28). However, in other cancers DUSP4 deletion is associated with tumour formation in lung (45) and breast, over expression in breast cancer cells substantially reduces tumour formation in mice (35). MKP-2 may also function to prevent ovarian metastatic spread (46). Thus it is possible that DUSP-4 plays opposing roles in different types of cancer depending on which kinase, ERK or JNK, is being preferentially regulated in that cell type.

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FOOT NOTES

1Unpublished observations.
2Unpublished observations.
3Unpublished observations.

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MKP-2 and cellular proliferation

FIGURE LEGENDS

Figure 1. Serum mediated induction of MKP-2 in wild type but not in MKP-2/− fibroblasts
Confluent MEFs were rendered quiescent for 24 h in serum free media. In A, cells were incubated with 10% FCS for the times indicated. Cell extracts were analysed by Western blotting using anti-MKP-2 and ERK1/2 antibodies as described in the Experimental procedures. Each blot is representative of five others. In B, gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. *p<0.01 and **P<0.001 vs wild type MEFs.

Figure 2. ERK mediates induction of MKP-2 in wildtype MEFs
Confluent MEFs were quiescent for 48 h in serum free medium, vehicle (-) or MAPK inhibitors (+) were added 1h prior to stimulation with 10% FCS for the times indicated. In A, cell extracts were analysed by Western blotting using anti-MKP-2 and p38 antibodies (loading control) as described in Experimental procedures. Each blot is representative of three others. In B-D, gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. **P<0.01 compared to vehicle control.

Figure 3. Enhanced PDGF-stimulated ERK phosphorylation in MKP-2/− MEFs
Confluent MEFs were rendered quiescent for 24h in serum free medium. Cells were incubated with 10ng/ml PDGF for up to 120 min (A) or up to 24h (B). Cell extracts were analysed by Western blotting using phospho-ERK, ERK1/2 and MKP-2 antibodies as described in Experimental procedures. Each blot is representative of three others. In C and D, gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. *P<0.05 compared to wild type MEFs.

Figure 4. Anisomycin-induced JNK and p38 MAP kinase phosphorylation in MKP-2/− MEFs
Confluent MEFs were quiescent for 24h in serum free medium, prior to stimulation with anisomycin (5µM) for the times indicated. In A, cell extracts were analysed by Western blotting using anti-MKP-2, p-JNK1/2, p-p38, JNK1/2 and p38 antibodies as described in Experimental procedures. In B and C, gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. *p<0.05 vs wild type MEFs.

Figure 5. MKP-2 is required for MEF proliferation
Sub-confluent quiescent MEFs from either WT or MKP-2/− were stimulated with 10% FCS for the times indicated and proliferation measured by cell counting at 24h intervals over 72hr (A and B), or 1-2 h intervals over 21 h (C) as outlined in Experimental procedures. In D, wild type and MKP-2/− MEFs were infected with increasing m.o.i of Adv.MKP-2 for 24h. In E and F, cells infected with 300pfu Adv.MKP-2 (or LacZ) were stimulated with 10% FCS and cell number counted over 72h. Each value represents the mean ± sem of at least 4 experiments. ***P<0.001 compared to wild type MEFs.

Figure 6. Delayed G2/M phase transition in MKP-2 deficient fibroblasts
Subconfluent quiescent fibroblasts were stimulated with FCS for 24 h and assessed by flow cytometry using propidium iodide staining (A) or blocked with thymidine (B). In C, quiescent cells were assessed for cyclin B1 and cdc2 phosphorylation following FCS stimulation as outlined in Experimental procedures. In F cells were incubated with topotecan (1µM) washed and then assayed for p-cdc-2 at 24 and 48h. Each experiment is representative of at least three others. In D and E gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. *p<0.05 vs wild type MEFs.

Figure 7. MKP-2 is also required for Cardiac fibroblast and Macrophage proliferation
MKP-2 and cellular proliferation

Sub-confluent quiescent CFs and macrophages from either WT or MKP-2−/− were stimulated with 10% FCS for the times indicated and proliferation measured by cell counting over 72h (A and C) as outlined in Experimental procedures. Sub-confluent quiescent CF and Macrophages were stimulated with FCS for 24h and assessed by flow cytometry using propidium iodide staining (B and D). Each value represents the mean ± sem of at least 3 experiments. **p<0.01 and ***P<0.001 vs wild type MEFs.

Figure 8. Increased rates of apoptosis in MKP-2 deficient MEFs
In A, cells were stimulated with anisomycin (5µM) for 8 and 12h and assessed for apoptosis by Annexin V and 7-AAD staining as outlined in Experimental procedures. Each value represents the mean ± sem of at least 4 experiments. In B and C cells were stimulated for 4h and 6h and lysates assessed for cleaved caspase 3 and γH2AX phosphorylation. Each gel is representative of four experiments. In D and E gels were quantified using densitometry and each value represents the mean ± sem of at least 4 experiments. *p<0.05 and ***P<0.001 vs wild type MEFs.

Figure 9. JNK inhibition reverses anisomycin-induced apoptosis in MKP-2−/− MEFs
MEFs were infected with Adv.MKP-2 (300 m.o.i) for 24h (A) then stimulated for anisomycin (5µM) for 30 min (B), 4h (C) and 12h (F). Samples were assessed for MKP-2 (A), pJNK, pERK and pp-38 MAP kinase (B), cleaved caspase 3 and γH2AX phosphorylation (C,D & E) and Annexin V and 7-AAD staining (F). Each gel is representative of three experiments. Gels were quantified using densitometry (D and E) and each value represents the mean ± sem of at least 4 experiments. *p<0.05, **p<0.01 and ***P<0.001 vs wild type MEFs.
Figure 1.

A

| +/+ | -/- |
|-----|-----|
| 0   | 0   | Time (hrs)
| 2   | 2   |
| 4   | 4   |
| 6   | 6   |
| 8   | 8   |
| 24  | 24  |

MKP-2 (43kDa)
ERK1 (44kDa)
ERK2 (42kDa)

B

Fold stim. Relative to control

0  1  2  3  4  5

MKP-2
MKP-2

Time (hrs)

0  2h  4h  6h  8h  24h

**  ***  ***  ***
Figure 2.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Deletion of the dual specific phosphatase-4 (DUSP-4) gene reveals an essential non-redundant role for MAP kinase phosphatase-2 (MKP-2) in proliferation and cell survival.

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