INHIBITION OF MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE 3 ACTIVITY BY INTERDOMAIN BINDING

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Mitogen-activated protein kinase phosphatase 3 is a cytoplasmic dual specificity phosphatase that functions to attenuate signalling via dephosphorylation and subsequent deactivation of its substrate and allosteric regulator, extracellular signal-regulated protein kinase 2. Expression of mitogen-activated protein kinase phosphatase 3 has been shown to be under the control of extracellular signal-regulated protein kinase 2, thus providing an elegant feedback mechanism for regulating the rate and duration of proliferative signals. Previously published studies suggest that mitogen-activated protein kinase phosphatase 3 might serve as a tumour suppressor; however, significantly elevated, rather than reduced, levels of this protein have been reported in early lesions. Since overexpression of this phosphatase is counter intuitive to a proposed tumour suppressor function, the observed cellular tolerance suggests a self-inactivation mechanism. Using surface plasmon resonance, we provide direct evidence of physical interaction between the N- and C-terminal domains. Kinetic analysis using dimethyl sulfoxide to activate the C-terminal fragment in the absence of extracellular signal-regulated protein kinase 2 showed that the isolated C-terminal domain had higher catalytic efficiency than the similarly activated full-length protein. Furthermore, when isolated N-terminal domain was added to activated C-terminal domain, a dose-dependant inhibition of catalytic activity was observed.

The similarity between the $K_I$ and $K_D$ values obtained indicate that interdomain binding stabilizes the inactive conformation of the catalytic site and implies that the N-terminal domain functions as an allosteric inhibitor of phosphatase activity. Finally, we provide evidence for oligomerization of mitogen-activated protein kinase phosphatase 3 in pancreatic cancer cells expressing elevated levels of this phosphatase.

Feedback regulation of cellular kinases and their cognate phosphatases is a fundamental component in many signal transduction cascades (1-3). In mitogen-activated protein (MAP)1 kinase signalling, an initiating stimulus from extracellular growth factors, stressors or damage, leads to a hierarchical signalling cascade that involves the serial activation of MAP-extracellular signal-regulated protein kinase/extracellular signal-regulated protein kinases (MEK/ERK).

1 DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP-extracellular signal-regulated protein kinase; MKP3, MAP kinase phosphatase 3; MKP3:WT, MKP3 wild type; MKP3:CT, C-terminal domain of MKP3; MKP3:NT, N-terminal domain of MKP3; PTPα, protein tyrosine phosphatase α (PTPα); pNPP, p-nitrophenol phosphatase; RAN, RAS-associated nuclear protein; SPR, surface plasmon resonance; TBS, Tris-buffered saline
followed by negative regulation of the latter by an antagonistic phosphatase (4-7). In the kinase/phosphatase pair that regulates the classical RAS-MAP kinase pathway, activation of extracellular signal-regulated kinase 1 or 2 (ERK1 or ERK2) leads to the concomitant upregulation and activation of MAP kinase phosphatase 3 (MKP3) which limits or abrogates the ERK signal (8-10).

Ultimately, the consequence of ERK dephosphorylation by MKP3 restrains cell cycling, an absolute requirement during the critical period of early embryonic development when growth factor stimulation leads to RAS-MAPK signalling, increased metabolic load and cellular expansion at key developmental loci. These loci, which include the neural plate and limb buds, then develop through synchronized periods of increased cell proliferation and apoptosis (11-13), a cycle which is believed to depend on the synchronization of ERK signalling and abrogation through ERK-dependant MKP3 protein synthesis (14-16). As a result, elevated levels of MKP3 generate the feedback signal needed for coordinated growth factor-induced cell division and, later, proper cell specification and differentiation (11-13;15;17;18).

Akin to its role in regulating the cell cycle via regulation of ERK signalling, MKP3 may also be involved in RAS/MEK/ERK-mediated oncogenesis. Indeed, MKP3 levels appear to correlate with the severity and outcome of pancreatic adenocarcinoma (19-21). The proposal for a role for MKP3 in oncogenesis arises from the observation that some pancreatic adenocarcinomas, as well as other solid tumours, show an allelic loss at the Mkp3 gene locus (22-24). Interestingly, pre-cancerous intraepithelial neoplasias have been observed to over-express MKP3, suggesting that MKP3 may serve a protective or tumour suppressive function through regulation of cell growth prior to transformation into adenocarcinoma (25). This concept appears further supported by histological observations that MKP3 protein levels appear to be elevated in both mildly and severely dysplastic lesions, but appeared to be reduced in advanced carcinomatous lesions that overexpress phosphorylated ERK2 (19;20;23).

The physiological importance of MKP3 regulation of the RAS-MAPK signalling cascade is reflected in its unusually tight substrate specificity for ERK1 and ERK2. This specificity, which prevents binding to, and thereby cross-reactivity with, the stress and oxidative damage-induced c-Jun N-terminal kinase (JNK) and p38 MAPKs , appears to be governed by MKP3’s ERK-specific N-terminal binding domain (26), and by the allosteric activation of MKP3’s C-terminal catalytic domain (27;28). This allosteric activation of MKP3 has been well documented in vitro using p-nitrophenol phosphate (pNPP), a small-molecule phospho-tyrosine analogue of its normal substrate, which allows for colorimetric determination of phosphatase activity. In the absence of ERK, MKP3 has little activity but in the presence of ERK, the ability of MKP3 to dephosphorylate pNPP increases nearly 100-fold. This increase in catalytic activity upon ERK binding has been attributed to a structural reorganization that triggers the closure of a flexible loop region near MKP3’s active site (26;27;29;30). In this model, loop closure repositions an aspartic acid (D262), placing it in proximity with the active site cysteine and arginine residues (C293 and R299), which enables the acid-catalyzed hydrolysis of phospho-amino acids. Unfortunately, while the structures of MKP3’s binding and catalytic domains have been determined (26;30-32), the structure of the full length protein, in either its activated or non-activated form, has not. However, a recent magnetization transfer NMR study using the MKP3 N-terminal binding domain and the PAC1 C-terminal catalytic domain (an MKP3 catalytic domain homologue) indicated that the MKP3 N-terminal domain interacts with an MKP3 catalytic domain homologue (31), suggesting binding between N- and C-terminal domains in the native, non-activated form of full-length MKP3. The effect of this putative interdomain binding on the function of MKP3 has not yet been studied.

This paper provides direct evidence of MKP3 self-association and determines the
effect of this phenomenon on MKP3 function. Specifically, we report the determination of the dissociation constants for the binding between full-length MKP3 and truncated analogues (corresponding to MKP3’s binding or catalytic domains) using surface plasmon resonance (SPR). An ERK-free activity assay was developed to enable the kinetic analysis of the activated C-terminal catalytic domain in the absence of ERK and in the presence and absence of the N-terminal ERK-binding domain. These data show that the addition of MKP3’s N-terminal domain to its catalytic C-terminal domain leads to a concentration-dependant decrease in enzyme activity. These experiments further demonstrate a relationship between MKP3 self-association and catalytic function, indicating that in the absence of ERK, MKP3’s N-terminal domain binds and inhibits the activity of its C-terminal domain. In principle, this interaction improves the fidelity of MKP3 and prevents non-specific dephosphorylation in the absence of ERK. Taken together, these data suggest an additional layer of functional control for MKP3. A model of MKP3 post-translational regulation, in which overexpression leads to the formation of inactive oligomers via interprotein interaction involving N- and C-terminal domains, is presented as an explanation for the maintenance of high levels of phosphorylated ERK in tumour cells (20).

EXPERIMENTAL PROCEDURES

MKP3 recombinant protein production and purification - Wild-type MKP3 protein (MKP3:WT) for the study was generated as previously described (33). Similar techniques were used to produce a truncated MKP3 N-terminal domain (corresponding to a.a. 1-154, MKP3:NT) using forward (5′-GGA ATT CCA TAT GAT AGA TAC GCT CAG AC-3′) and reverse primers (5′-TAG TTT ACA TAT GTC ACG TAG ATT GCA GAG AGT CC-3′). Underlined sections show EcoR IV cleavage sites that were used to clone the insert into a pET15b vector (Invitrogen, Burlington, ON). Cloning and sequence verification was carried out as previously described (33).

Verification of recombinant protein identity - Samples of the total cellular protein from recombinant E. coli cells expressing the MKP3:WT, MKP3:NT, or MKP3:CT proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Danvers, MA). To verify the identities of the immunodetected bands, MKP3-containing cell lysates were separated on a duplicate SDS-PAGE and stained with Sypro Ruby Red (Invitrogen). In-gel tryptic digests were performed on bands of interest, which were excised from the gel, diced to a uniform size (approximately 1 mm²) and then mixed with acetonitrile. The gel slurry was dried and resuspended in 1 ml, 100 mM ammonium bicarbonate containing 10 mM DTT to cleave disulphide bonds. The cysteine residues were then capped by the addition of iodoacetamide (50 mM final concentration). Gel pieces were washed and rehydrated in 1 ml, 50 mM ammonium bicarbonate and the proteins were digested by the addition of trypsin (20 μg). Peptide fragments were identified using an LTQ-FT mass spectrometer system ² (Thermo-Finnigan; Waltham, MA) coupled to an Agilent 1100 series HPLC using a Zorbax 300SB-C18 capillary HPLC column (Agilent technologies; Santa Clara, CA). PEAKS software (Bioinformatics Solutions; Waterloo, ON) was used to process the mass spectrometry data. The purity of MKP3 preparations after isolation and refolding was estimated by 12 % SDS-PAGE (34) using a Mini-Protean 3 cell (Bio-Rad, Hercules, CA) and staining by Coomassie blue. The MKP3 protein band density was assessed as a percentage of the total lane density using a Gel-doc XR with Quantity One 4.5.2 software (Bio-Rad, Hercules, CA).

CD spectroscopic verification of refolding - CD spectroscopy experiments were performed using a J-810 spectrometer (Jasco, Easton,
Protein solutions in TBS (pH 7.6) (Tris-buffered saline) were adjusted to concentrations between 0.5 and 1 mg/mL and then placed in a round cuvette with a 0.02 cm pathlength. Data were collected over 178-260 nm. A total of 5 scans were acquired and the average was used for final analysis. Due to interference at lower wavelengths, the CD spectra were cropped to include only the region encompassing 190-260 nm before analysis using Dichroweb (35;36) and CDSSTR (37).

Surface plasmon resonance - Surface plasmon resonance experiments were performed using a BIAcore 3000 (GE Healthcare, Piscataway, NJ). Experiments were carried out at 25°C, using HEPES buffered saline running buffer (HBS-EP, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.005% Tween-20, pH 7.4). MKP proteins were covalently bound to BIAcore CM5 (carboxymethyl-dextran-coated) sensor chips (GE Healthcare) using an EDC/NHS-based amine coupling reaction (0.4 M 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, EDC; 0.1 M N-hydroxysuccinimide, NHS) according to the manufacturer’s instructions. A moderate surface density of ligand (85-400 RU) was bound to sensor chips before addition of 1 M ethanolamine to deactivate the sensor surfaces. Following verification of a stable baseline, potential ligands were introduced at increasing concentrations (ERK2 and JNK1: 0, 0.595, 0.79, 1.19, 1.59, 2.38 μM or MKP3:CT: 0, 0.25, 0.5, 1, 5, 10, 25 μM). Binding curves were analyzed using BIAevaluation software (GE Healthcare) using a 1:1 Langmuir binding model.

Enzyme kinetic analysis using pNPP - Specific activities of MKP3:WT and MKP3:CT were assessed by nitrophenol phosphate assay in a 96-well plate. Assay buffer was prepared containing 20 mM pNPP in a 10 mM Tris, 50 mM NaCl solution at pH 8.0. A 75 μl aliquot of assay buffer was added to each well together with 14 μg MKP3 in 25 μl TBS. MKP3 was assayed in the presence of dimethyl sulfoxide (DMSO), which has been shown to induce an activity in wild-type MKP3 similar to the effect of ERK2 binding (27;33). Concentrations of DMSO ranging from 0 to 50% (v/v) were tested to determine an optimum concentration. Steady state kinetic analysis was performed using TBS buffer containing 0, 2, 4, 6, 8, 10, 25, or 50 mM pNPP and 14 μg MKP3 (2.1 μM MKP3:WT or 3.5 μM MKP3:CT) for inactive MKP3 assays or 1.4 μg MKP3 (0.21 μM MKP3:WT or 0.35 μM MKP3:CT) for active MKP3 assays. Experiments were performed to compare the activities of MKP3 under varying conditions by addition of 50 μl water, 50 μl DMSO, or 3.0 μg ERK2 in 50 μl TBS buffer (0.42 μM; Millipore). The production of nitrophenolate product was measured by absorbance at 405 nm, using an extinction coefficient of 18.2x10³ M⁻¹•cm⁻¹. Data analysis using non-linear regression in Graphpad Prizm 4.0 (Graphpad Software, San Diego, CA) was used to determine k_cat and K_M values.

Inhibition was determined using MKP3:NT added to MKP3:WT and MKP3:CT in 33% DMSO-containing 10 mM Tris, 50 mM NaCl, with 0.6 mM pNPP as a substrate. Each assay contained 1 μM MKP3:WT or MKP3:CT in a total assay volume of 150 μl. The amount of MKP3:NT was increased with concentrations ranging from 0 to 2.2 μM. IC₅₀, the concentration of inhibitor that led to a 50% decrease in activity, was determined by curve fitting to a sigmoidal-dose-response curve. K_i was determined by the equation K_i = IC₅₀ / [1+(substrate concentration)/(substrate K_D)] using Graphpad Prizm. Statistical analysis on MKP3 kinetic data was performed using an average of three independent experiments – each experiment comprised of three determinations. A Student’s t test was used to determine significance at a p-value < 0.05.

Detection and quantification of MKP3, ERK1/2 and phosphorylated ERK1/2 proteins in human pancreatic adenocarcinoma cell lines - Total protein isolates were prepared from HeLa (positive control), non-diseased human pancreas (obtained at surgery from an anonymous organ donor), immortalized human pancreatic duct cells (HPDE4, HPDE6), and human pancreatic adenocarcinoma cell lines.
(CRL1420, CRL1469, CRL1682, CRL1687, CRL1837, HTB79, HTB80, HTB134, and HTB147; American Type Culture Collection, Manassas, VA) were lysed in Laemmli reducing lysis buffer supplemented with orthovanadate and phosphatase and protease inhibitor cocktails (Roche Applied Sciences Inc, Laval, Québec). Aliquots were precipitated in 10% TCA and quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Twenty micrograms of protein were diluted in 1x SDS sample buffer + DTT (New England Biolabs, Ipswich, MA), loaded on 5% stacking/12% (or 15%) separating 29:1 acryl:bis SDS-PA mini-gels, fractionated by electrophoresis for 1.5 h at 100 V in 1x Running Buffer (BioRad, Hercules, CA) against molecular weight size markers (Invitrogen) and transferred to Immobilon-P membranes (Millipore) under standard conditions. Blots were probed with anti-human MKP3 mouse monoclonal antibody (1 μg/mL; catalogue # MAB3576; R&D Systems, Minneapolis, MN), rabbit polyclonal anti-human p44/42 antibody #9102 (1:5000; Cell Signalling Technology, Danvers, MA), rabbit monoclonal anti-human phosphorylated p44/42 Thr202/Tyr204 antibody 197G2 (1:1000; Cell Signalling Technology) and revealed with HRP-conjugated secondary antibodies (1:2000; Amersham, Piscataway, NJ) using 1:4 diluted SuperSignal reagent (Pierce, Rockford, PI). Chemiluminograms were subjected to semi-quantitative laser densitometry using a Molecular Dynamics Personal Laser Densitometer SI and ImageQuant v5.1 software.

**Detection of MKP3 oligomerization** – Human HPDE6 immortalized duct and pancreatic adenocarcinoma cell lines were lysed using a non-denaturating Radio-Immuno Precipitation Assay buffer to retain protein complexes. Cell lysates and purified recombinant MKP3:WT and MKP3:CT were diluted in a modified Laemmli loading buffer which contained reducing agent but not SDS. Samples (10 μg) were loaded, without prior heating, onto 5% stacking/7.5% separating polyacrylamide minigels. Electrophoresis, Western transfer and detection were performed as described above.

**Detection of MKP3 RNA** - Ten micrograms of total RNA isolated and purified using Trizol (Invitrogen, Mississauga, Ontario) were fractionated on a 1% agarose formaldehyde-MOPS gel and processed for Northern blot hybridization and autoradiography according to standard procedures. The blot was first probed with a 32P-radiolabelled 1.25 Kbp human MKP3 HindIII/XhoI cDNA fragment, stripped and re-probed with a human β-actin cDNA.

**Size exclusion chromatography** - MKP3 monomer and oligomers were separated on an AKTA-FPLC system (GE Healthcare; Piscataway, NJ) using a Superdex G200 10/300 gel filtration column (GE Healthcare). 1 ml of 1mg/ml purified recombinant MKP3 was injected at a flow rate of 0.5 ml/min.

**Transient transfection and inducible expression of epitope-tagged MKP3** – Log phase human pancreatic adenocarcinoma cell lines CRL1469 and CRL1682 (5x10^5 viable cells per 60 mm dia. dish) were co-transfected with 2.5 μg each form I pVgRXR and pInd/GS/MK3/V5 or pInd/V5-his/LacZ (control) plasmids (all constructs from Invitrogen) using Lipofectamine (Invitrogen). V5 epitope-tagged MKP3 or β-galactosidase expression was induced 16 h post-transfection with 5µM pronasterone A. Cell lysates were prepared 48 h later and processed for Western blot hybridization and laser densitometry as described above. Epitope-tagged MKP3 was detected with a mouse monoclonal anti-V5 antibody (Invitrogen). Transfected cells were photographed under phase contrast (Zeiss Axiovert 10) using Ilford FP-4 black and white film. Photographs were scanned and sharpened using Adobe Photoshop.
RESULTS

Production of MKP3 proteins – The integrity of the MKP3 proteins produced for use in this study was verified in several ways. Initial assessment was by Western blot and SDS-PAGE (Figures 1A and B). The apparent molecular weights of the protein bands detected in the BL21(DE3)/MKP3:WT, BL21(DE3)/MKP3:NT, and in the BL21(DE3)/MKP3:CT cell lysates were determined to be 45, 19, and 30 kDa, respectively. These values compared well to the theoretical molecular weights of MKP3:WT (44.5 kDa), MKP3:NT (19.6 kDa), and MKP3:CT (27.1 kDa). The identities of the proteins were subsequently verified by in-gel tryptic digestions followed by mass spectrometry, which confirmed the production of full-length MKP3:WT and the truncated MKP3:NT and MKP3:CT mutant proteins (data not shown).

Secondary structure analysis using CD spectroscopy – Once the identity of the recombinant MKP3 proteins had been verified by size and mass spectroscopic analysis, protein folding was assessed by circular dichroism (CD) spectroscopy. The secondary structural features were then compared to previously published data for the N-terminal ERK-binding domain (amino acids 1-154, PDB ID: 1HZM) and the C-terminal catalytic domain (amino acids 204-347, PDB ID: 1MKP) (30;31). CD of MKP3:NT and MKP3:CT showed well-structured domains with helical and sheet content generally consistent with published data (30;31) (Figure 2). These data show that the MKP3 proteins were successfully produced and refolded and thus suitable for use in subsequent experiments.

Surface plasmon resonance – The affinities between MKP3 and ERK were determined using surface plasmon resonance (SPR) as further verification that MKP3 protein produced was correctly folded and functional. Experiments were performed using sensor chip-immobilized MKP3:WT with the in vivo substrate, ERK2, to test for protein-protein interactions. JNK1 was used as a negative control. SPR data indicated that MKP3:WT and ERK2 associated with $k_a = 6.0 \times 10^6$ 1/Ms and $K_D = 2.63 \times 10^{-7}$ M (Table 1), which was comparable to the previously reported value obtained via enzyme assay ($K_D = 1.7 \times 10^{-7} +/-0.4 \times 10^{-7}$ M) (38). In contrast to MKP3-ERK2 binding, JNK1 showed no detectable association ($k_a$ and $K_D$ could not be determined using a 1:1 Langmuir binding curve), which was consistent with previous reports that MKP3 and JNK1 do not interact (10). These SPR data represent the first direct demonstration of MKP3:WT-ERK2 binding and confirm previous reports that assessed binding indirectly using enzymatic assays (26). Taken together with the CD data, these results provide further confirmation that MKP3:WT produced for this study is correctly folded and functional.

Previous studies have shown that the catalytic domain of PAC1 (a close structural homologue of MKP3’s C-terminal domain) could bind to the N-terminal domain of MKP3 (31). Thus, this series of experiments was designed to examine directly the putative binding between MKP3’s N- and C-terminal domains. Samples of MKP3 proteins (MKP3:WT, MKP3:NT, MKP3:CT) were immobilized on the sensor chip and MKP3:CT was added as an analyte. These data showed that while the MKP3 C-terminal domain possesses little ability to self associate, it can bind both the full-length MKP3:WT and the N-terminal MKP3:NT proteins (Figure 3, Table 2). These results show that the MKP3’s C-terminal domain is directly binding the N-terminal domain in the full-length MKP3 protein. Furthermore, the data indicating low micromolar values for $K_D$ suggests that, under normal physiological conditions the N- and C-terminal domains exist in the bound conformation.

DMSO-induced MKP3 phosphatase activity – To measure MKP3:CT activity in the absence of ERK, a phosphatase assay was developed in which MKP3:CT was assayed in the presence of DMSO, a compound which has been shown previously to induce activity in wild-type MKP3 similar to that seen with ERK2 binding (27;33). $pNPP$ was used as a colorimetric
substrate. MKP3:WT and MKP3:CT both showed a dose-dependent enhancement of activity with increasing concentrations of DMSO (Figure 4). The MKP3:CT activity peaked at approximately 40% DMSO then declined; probably because higher concentrations of DMSO resulted in protein denaturation. These data are consistent with previous studies of full-length MKP3 that showed maximal activation at 33% DMSO (27;33). Thus, a DMSO concentration of 33% was chosen for this assay as it results in significantly increased activity with no protein denaturation.

After confirming that MKP3:CT could be activated using DMSO, steady state kinetic analyses were performed to compare the effects of ERK and DMSO on MKP3 activity. Results showed the expected low activity in the non-activated MKP3:WT. The $k_{cat}$ determined for MKP3:WT in aqueous buffer was $9.86 +/− 0.41 \times 10^{-3}$ s$^{-1}$ (Table 2), a value which was lower than the $k_{cat}$ of $12.0 +/− 0.41 \times 10^{-3}$ s$^{-1}$ displayed by the non-activated MKP3:CT. $K_M$ values for MKP3:WT and MKP3:CT in the absence of DMSO or ERK2 were similar at $7.87 +/− 0.99$ mM and $7.60 +/− 0.82$ mM, respectively. These data indicated that the removal of MKP3’s non-catalytic N-terminal domain significantly improved the basal function of MKP3 in the absence of ERK.

The $k_{cat}$ for MKP3:WT increased three-fold with the addition of ERK2 and four-fold with the addition of DMSO (Table 2). In contrast, MKP3:CT showed no increase in $k_{cat}$ with the addition of ERK2 but a marked increase with the addition of DMSO. The $K_M$ values of MKP3:WT and MKP3:CT also showed differing responses with the addition of ERK2: $K_M$ of MKP3:WT decreased 5-fold with the addition of ERK2 but no further with DMSO, in comparison with MKP3:CT, which decreased slightly with the addition of ERK2, and decreased further with DMSO. These data are consistent with previous reports on the MKP3 C-terminal catalytic domain that showed a similar two-fold decrease in $K_M$ when the MKP3 catalytic domain was tested in the presence of ERK2 (30). These data also demonstrate, for the first time, that DMSO is able to induce activity in MKP3:CT, similar to ERK or DMSO-based activation of the full-length MKP3.

**Inhibition of MKP3:CT by MKP3:NT** - After confirming our ability to assay the activity of activated MKP3:CT in an ERK-free system (using DMSO), the activity of MKP3:CT was determined in the presence of increasing concentrations of MKP3:NT. These data showed that the addition of MKP3:NT led to a dose-dependent decrease in the specific activity of the MKP3:CT (Figure 5). A similar addition of increasing amounts of MKP3:NT to DMSO-activated MKP3:WT led to a similar dose-dependent decrease in activity. When these data were subsequently analysed using a non-linear regression fit to a sigmoidal-dose response, the half-maximal inhibition of MKP3:CT by MKP3:NT (i.e., IC$_{50}$), was estimated to be approximately $\sim 0.97$ μM. This IC$_{50}$ value was then used to calculate $K_I$, which was determined to be $0.68 +/− 0.37$ μM. The value determined for the $K_I$ of MKP3:NT compares well with the SPR determined $K_D$ for MKP3:NT and MKP3:CT binding. This indicates that, while DMSO is capable of stabilizing the active structure of the MKP3:CT protein, MKP3:NT acts as an inhibitor of the C-terminal catalytic domain through interdomain binding.

**Evaluation of MKP3, ERK1/2 and phosphorylated ERK1/2 expression in pancreatic adenocarcinoma cell lines** – The ability of MKP3:NT domains to bind and inhibit MKP3:CT activity suggests that at high levels of MKP3 expression, the auto-inhibitory effect of the MKP3 N-terminal domain results in a loss of enzymatic function. To explore the effects of MKP3 overexpression, a panel of pancreatic cells was tested for MKP3 levels using Western blot. MKP3 protein levels were evaluated in HeLa cells, normal human pancreatic tissue, immortalized (but not tumourigenic) pancreatic duct cell lines, and a series of pancreatic adenocarcinoma cell lines. This panel of cells included 3 adenocarcinoma cell lines used in a previously published study (20). Initial blots were probed with the same...
antibody (Santa Cruz Biotechnology, sc-8599) as that used in the previous studies (20) and showed negligible expression of MKP3 (data not shown) as previously reported. In contrast, duplicate blots probed with a more recent, more robust and selective monoclonal antibody to MKP3 (R&D Systems, MAB3576) revealed that while MKP3 was detected at low levels in normal human pancreatic tissue, all other lines exhibited strong expression of MKP3 (Figure 6A). We are in the process of resolving this apparent discrepancy by defining the amino acid residues involved in this interdomain binding. Preliminary evidence suggests that the epitope recognized by the sc8599 antibody may be masked as a result of interaction between N- and C-termini (Mark and Hefford, in preparation).

Cellular expression of MKP3 was confirmed using Northern Blot analysis to detect \textit{Mkp3} RNA transcripts in normal human pancreatic tissue, immortalized pancreatic duct cell lines and adenocarcinoma cell lines (Figure 6B). Human pancreas showed a lower level of \textit{Mkp3} RNA but in seven of the nine adenocarcinoma lines, RNA levels were significantly elevated. These results correlate well with the protein levels detected in Figure 6A; however, in cell lines CRL1420 and 1469, \textit{Mkp3} RNA levels were decreased, relative to other pancreatic adenocarcinoma cell lines in the panel. Despite the lower RNA levels, the cells displayed high levels of MKP3 protein. These results suggest that MKP3 protein displays a long half-life within these cell lines.

In addition to MKP3, total and phosphorylated ERK1/2 were detected on parallel blots and juxtaposed with the MKP3 data (Figure 6A). Densitometric analysis of the ERK data showed that despite upregulated MKP3 in all pancreatic adenocarcinomas, the cells concurrently expressed high levels of total and phosphorylated ERK1/2. A consistent relationship between MKP3 levels and those of phosphorylated ERK1 and/or ERK2 could not be discerned.

**Immunodetection of MKP3 oligomerization** – The presence of MKP3 in cells, that possessed high levels of phosphorylated ERK (20), implied a loss of function. From Arkinstall and coworkers’ findings (26) and Hafen’s model of cognate kinases and phosphatase (5), one would expect the presence of MKP3 to lead to a downregulation of ERK expression/activity rather than to the high levels of ERK expression observed. To investigate this apparent discrepancy, MKP3 interdomain binding was examined by additional Western blots using samples of non-denatured pancreatic adenocarcinoma cell lysates and samples of purified recombinant MKP3:WT and MKP3:CT. The results in Figures 7A (7.5% cross-link PAGE separation) and 7B (immunoblot) show that significant amounts of cellular MKP3 were readily detectable in the adenocarcinoma cells. Also supporting previous surface plasmon resonance data, is the finding that recombinant MKP3:CT was able to bind and form a dimer, detected as a band at ~60 kDa (Figure 7B). Recombinant MKP3:WT (Figure 7A and 7B) was also able to form dimers (90 kDa), but was also found in higher molecular weight trimers (135 kDa). Presumably this occurs through interdomain binding, analogous to “3D domain swapping” (39;40) of the N- and C-termini from different MKP3:WT molecules. When recombinant MKP3 was compared to CRL1687 pancreatic adenocarcinoma cell lysate, an identical set of immuno-reactive bands (corresponding to monomer, dimer, and trimer) was identified. This result demonstrates that MKP3 can exist in progressively oligomerized states when overexpressed in tumour cells.

The MKP3 immunodetection was repeated on an expanded panel of pancreatic adenocarcinoma cell lines (Figure 7C). Densitometric analysis revealed that MKP3 existed predominantly in an oligomerized state and this oligomerized state was detected in excess of the monomeric species (Table 3). **Enzyme activity of oligomerized MKP3** – We next wished to determine if MKP3 oligomers were catalytically active. To this end, size exclusion chromatography was used purify the oligomerized MKP3 for enzymatic activity assays. MKP3 monomeric and oligomeric forms were separated using gel filtration. The fractions that contained oligomerized MKP3
Expression of epitope-tagged MKP3 in pancreatic adenocarcinoma cells – The effect of elevated MKP3 production on pancreatic adenocarcinoma cells was tested by transient transfection and inducible expression of epitope-tagged MKP3. To confirm that the commercial Mkp3 vector construct (which contains a C-terminal V5 epitope) produced fully functional enzyme, the Mkp3/v5 cDNA cassette was excised and subcloned into a pET15b plasmid suitable for production in E. coli. The MKP3/V5 protein was then purified and tested for phosphatase activity using pNPP. The results indicated that the V5 epitope did not compromise ERK-binding or ERK-induced pNPP catalysis (Figure 8A). Epitope-tagged MKP3 was next upregulated in the presence of pronasterone A following transient transfection into CRL1469 and CRL1682 cell lines. Despite dramatic induction, total and phosphorylated ERK1/2 levels showed essentially no change when compared to the non-induced controls (Figure 8B). In order to determine the phenotypic effect of MKP3 transgene induction, we surveyed cell morphology and general culture health by phase contrast microscopy (Figure 8C) and noted that despite significant upregulation of MKP3, pancreatic adenocarcinoma cell viability was unaffected. Transfection of epitope-tagged β-galactosidase (control) also had no effect on ERK and phosphorylated ERK expression and cell viability (data not shown).

DISCUSSION

In the currently accepted model, the regulation of MKP3 function is dependant upon the presence ERK1 or ERK2. When either ERK isoform is present, MKP3 undergoes a structural reorganization at its catalytic domain that leads to enzyme activation, ERK phosphorylation and signal abrogation. The data presented here complement this model in an important way by suggesting an additional level of control of MKP3 activity: self-inhibition of MKP3 even in the absence of ERK (Figure 9). Evidence suggesting interdomain binding in MKP3 has been reported previously by Farooq et al. (31), using the PAC1 C-terminal domain, which indicated that the N-terminal MKP3 binding domain was able to bind C-terminal PAC1 catalytic domain with an approximate K_D of ~100 μM. The current study directly demonstrates that the C-terminal of MKP3 can bind to the N-terminal and shows that this binding is actually stronger than suggested by the PAC1 study (the dissociation constant for the MKP3:NT-MKP3:CT interaction was found to be ~70-fold lower than for the PAC1 interaction). This K_D is still substantially higher, indicating weaker affinity, than the K_D for ERK2-MKP3:WT interaction. These data imply that while the MKP3 N-terminal domain can bind the catalytic domain, when ERK is present, the ERK-MKP3 interaction is favoured. Additionally, pNPP hydrolysis experiments were performed using an ERK-free assay that tested the activity of the MKP3 C-terminal domain in the presence and absence of the MKP3 N-terminal domain. These experiments demonstrated the ability of the N-terminal domain to inhibit pNPP hydrolysis by the activated C-terminal catalytic domain. The determination of similar values for the MKP3:NT/MKP3:CT dissociation and inhibition constants suggests that MKP3:NT directly inhibits MKP3:CT function. This operational model is consistent with the detection of oligomerized endogenous MKP3 and the lack of effect observed for induced epitope-tagged MKP3 expression in pancreatic adenocarcinoma cell lines.

The N-terminal domain has previously been shown to inhibit wild type MKP3 activity (26): Arkinstall and coworkers reported a decrease in the activity of ERK-activated MKP3 that was titrated with N-terminal MKP3 domain protein. This decrease in ERK-activated MKP3 activity was attributed to ERK binding to the isolated N-terminal domain, which resulted in a decrease in the
effective concentration of ERK available for MKP3 activation. In this study, we have exploited the ability of DMSO to uncouple the activity of MKP3 from ERK. DMSO has previously been used to activate MKP3:WT in the absence of ERK: at DMSO concentrations less than 33%, MKP3 is activated, but can be further activated by the addition of ERK; at DMSO concentrations greater than 33%, MKP3 is maximally activated (27). Here we report the extension of this assay to the activation of MKP3:CT activity in the absence of either MKP3:NT or ERK. This modified assay allowed for the testing of the effect of the MKP3 binding domain (MKP3:NT) on the DMSO-activated C-terminal domain, an experiment that shows, for the first time, that the N-terminal domain plays a role in modulating MKP3 activity at its C-terminal catalytic domain. This role for the N-terminal domain in the modulation of C-terminal activity is further supported by the observation that MKP3:CT exhibits a small, but significant increase in $k_{cat}$ in comparison with the full-length MKP3:WT in the inactive and DMSO-activated states. These results indicate that the removal of the N-terminal non-catalytic domain is responsible for the higher rate of pNPP hydrolysis observed for basal and activated MKP3.

Consistent with these data is a model of inhibition of MKP3:CT by MKP3:NT. This inhibition can be attributed to stabilization of the inactive enzyme structure or to blockage of the MKP3:CT active site. In the presence of ERK2, competitive binding for the MKP3:NT between MKP3:CT and the higher affinity ERK2 ligand, results in the release of MKP3:NT from the MKP3 catalytic domain and enables the subsequent structural reorganizations that are necessary for high specific activity hydrolysis (Figure 9A).

This proposed model is also consistent with the observation that MKP3 is found in abundance in many pancreatic carcinoma cell lines that exhibit constitutive ERK phosphorylation (Figure 6). Under pathophysiological conditions, this model suggests that high levels of potentially tumour suppressive MKP3 phosphatase are constrained through interprotein binding and oligomerization (Figure 7), in order to maintain levels of monomeric MKP3 suitable for allowing cells to cycle.

To be catalytically active, MKP3 must be present in monomer form. Thus, the activity of any given C-terminal domain depends on two factors: that it is itself not bound to an N-terminal domain (whether inter- or intramolecular binding), and that it is allosterically activated through the binding of ERK to its own N-terminal domain. As a result of oligomerization, these two conditions are unlikely to be satisfied simultaneously (Figure 9C).

We have shown, through SPR, that the N-terminal domain has a higher affinity for ERK than for the C-terminal domain. ERK would normally displace the C-terminal from the N-terminal, but at high MKP3 concentrations the N- to C-termini binding results in oligomerization. Although the preferential binding of ERK to any particular N-terminal binding domain releases a C-terminal catalytic domain, this catalytic domain remains inactive as its own N-terminal domain is still not bound to ERK. This model of interprotein binding and oligomerization would suggest that while ERK binds to MKP3, the release of the MKP3 catalytic domain is unlikely to occur at a domain properly positioned to dephosphorylate ERK’s active site residues (Figure 9C).

The self-inactivation mechanism proposed by our study is similar to one recently described for src-homology 2 domain-containing tyrosine phosphatase, which possesses an autoinhibitory domain that blocks the phosphatase catalytic site (41). It is also similar to a domain-domain interaction that was recently described for protein tyrosine phosphatase $\alpha$ (PTP$\alpha$) where interaction between internal domains favour the stabilization of a dimeric state until ligand binding disrupts this interaction and leads to activation (42). Our study now suggests that a similar scheme might be applicable to other members of the dual specificity and multi-domain phosphatases (43). We propose that dimerization/oligomerization of MKP3 could
provide an effective means for restricting the attenuation of RAS-MAPK signalling to within physiologically acceptable limits. This added level of regulation complements the feedback loop elicited by downstream ERK2 activation and nuclear translocation (26). This proposal is consistent with significantly elevated MKP3 protein levels seen in the tumour cell lines used in this study and in the dysplastic pancreatic adenocarcinoma specimens reported previously (20). Thus, elevated MKP3 does not appear to exercise intrinsic tumour suppressor activity. Self-inactivation by oligomerization becomes all the more relevant in this context since loss-of-function mutations in MKP3 have not yet been found in cultured cancer cells or clinical specimens (22).

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FOOTNOTES

1 DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP-extracellular signal-regulated protein kinase; MKP3, MAP kinase phosphatase 3; MKP3:WT, MKP3 wild type; MKP3:CT, C-terminal domain of MKP3; MKP3:NT, N-terminal domain of MKP3; PTPα, protein tyrosine phosphatase α (PTPα); pNPP, p-nitrophenol phosphatase; RAN, RAS-associated nuclear protein; SPR, surface plasmon resonance; TBS, Tris-buffered saline

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FIGURE LEGENDS

Fig 1. Detection of expressed recombinant proteins. MKP3 protein expression was detected by Western analysis and SDS-PAGE. A. Western analysis: MKP3 variants were separated on 16% SDS-PAGE and then transferred to Immobilon-P membrane for immunodetection using an anti-(His)6 antibody. Lane M: (His)6-tagged molecular weight markers, Lane 1: MKP3:CT (31 kDa), Lane 2: MKP3:NT (18 kDa), Lane 3: MKP3:WT (45 kDa). B. SDS-PAGE separation. Following protein purification, the MKP3 variants were separated by 12% SDS-PAGE. Lane M: Molecular weight markers, Lane 1: MKP3:WT, Lane 2: MKP3:NT, Lane 3: MKP3:CT, Lane M: Molecular weight markers.

Fig 2. Secondary structure analysis by circular dichroism spectroscopy. Folding of the recombinant MKP3 proteins was assessed using circular dichroism spectroscopy to determine the secondary structure composition. Data shows the residue-weighted, CD spectra traces from MKP3:NT, MKP3:CT, and MKP3:WT. The spectra were deconvoluted using Dichroweb as described in the Methods to obtain the average secondary structure content from each recombinant protein.

Fig 3. Biacore analysis of association kinetics. Binding between the N- and C-terminal domains of MKP3 was measured using surface plasmon resonance. MKP3:WT, MKP3:NT or MKP3:CT were immobilized on BIacore SPR sensorchips with MKP3:CT used in the mobile phase. A: MKP3:WT, B: MKP3:NT, C: MKP3:CT. Different curves for each figure reflect increasing MKP3:CT concentrations (0 to 25 μM). These data indicate that increasing concentrations of MKP3:CT lead to an increase in the SPR plateau-phase response, which confirms protein binding. These data were analysed by curve fitting to a 1:1 Langmuir association to determine k a and K D values (Table 1).

Fig 4. DMSO concentration dependence of MKP3 catalytic domain. As a first step in the development of an ERK-free MKP3:CT activity assay, the ability of MKP3:CT and MKP3:WT to hydrolyze pNPP was tested in the presence of increasing DMSO concentration. DMSO, which has been shown to increase the activity of the full-length MKP3, was observed to increase the phosphatase activity of both MKP3:CT and MKP3:WT, and show that DMSO can be used as a chemical activator to the binding-deficient MKP3:CT mutant in the absence of ERK.
**Fig 5. Inhibition of enzyme activity by N-terminal MKP3.** The effect of MKP3:NT binding on activated MKP3:CT was tested using the DMSO-based phosphatase assay, with increasing concentrations of MKP3:NT. Non-active MKP3:CT (o, MKP3:CT/H2O) was used as a baseline for MKP3:CT activity. DMSO-activated MKP3:CT (Δ, MKP3:CT/DMSO) was tested in the absence of MKP3:NT and set to 100% activity. MKP3:NT concentration was then increased up to a 2.2:1 concentration relative to MKP3:CT (plotted on the logarithmic scale). The increased MKP3:NT led to a dose-dependant decrease in MKP3:CT activity. The same experiment was performed on DMSO-activated MKP3:WT (○, MKP3:WT/DMSO), which showed that increasing MKP3:NT was capable of further inhibiting the full-length MKP3 activity.

**Fig 6. Analysis of MKP3 and ERK1/2 expression in human pancreatic adenocarcinoma cell lines.** MKP3 and ERK1/2 proteins were detected using Western blot hybridization and laser densitometry as described in Methods to confirm their presence in pancreatic adenocarcinomas. A. Western blot was performed on 20 μg total cellular protein from each of the indicated cell lines. RAN (RAS-associated nuclear protein) was used as the protein loading control. Lane C: HeLa cells (positive control); Lane 1: Normal pancreas (homogenized human pancreatic tissue used to detect the baseline MKP3 level); Lane 2: HPDE4 (immortalized, non-tumourigenic ductal pancreatic cells); Lane 3: HPDE6 (immortalized, non-tumourigenic ductal pancreatic cells); Lane 4-11 utilized pancreatic adenocarcinoma cell lines. Lane 4: CRL1420 (MIAPaCa-2); Lane 5: CRL1469; Lane 6: CRL1682 (BxPC-3); Lane 7: CRL1687; Lane 8: CRL1837 (Su86.86); Lane 9: HTB79; Lane 10: HTB80; Lane 11: HTB134; Lane 12: HTB147. Figure shows immunodetection for each specified protein. Densitometric data, adjusted to the RAN loading control, are shown relative to HPDE6, which was set to 1. B. Mkp3 Northern blot analysis. Total cellular RNA was fractionated and processed as described in Methods. Mkp3 detection was confirmed using 1.25 kbp human Mkp3 HindIII/XhoI cDNA fragment, while human β-actin cDNA was used for loading controls. Lane 1: normal human pancreas; Lane 2: CRL1420 (MIAPaCa-2); Lane 3: CRL 1469; Lane 4: 1682; Lane 5: 1687 (BxPC-3); Lane 6: 1837 (Su86.86); Lane 7: HTB79; Lane 8: HTB80; Lane 9: HTB134; Lane 10: HTB147. The Northern blot correlated well with the Western blot, and indicated that Mkp3 RNA was actively transcribed in 7 of the 9 pancreatic adenocarcinomas tested.

**Fig 7. Western analysis of MKP3 under non-denaturing condition.** Western analysis was performed to test for MKP3 oligomerization in pancreatic adenocarcinoma cell lines and purified recombinant MKP3. Proteins were prepared in non-denaturing RIPA buffer to retain any bound complexes. Cell lysate (10 μg) or purified, recombinant MKP3 (0.4 μg) were separated by 7.5% SDS-PAGE prior to Western detection. A. Sypro stained 7.5% SDS-PAGE separation of MKP3:WT protein standards. Lane 1: Molecular weight markers, Lane 2: purified monomeric MKP3:WT, Lane 3: purified MKP3:WT allowed to oligomerize. B. Western-detected MKP3 proteins. Lane 1: CRL1687, Lane 2: purified MKP3:WT, Lane 3: purified MKP3:CT. C. Western-detected MKP3 proteins in pancreatic adenocarcinoma cell lysates. Lane 1: HPDE6; Lane 2: CRL1420; Lane 3: CRL1469; Lane 4: CRL1682; Lane 5: CRL1687; Lane 6: CRL1837; Lane 7: HTB80; Lane 8: HTB134, Lane 9: HTB147.

**Fig 8. In vitro kinetic and post-transfection biological activity of recombinant MKP3/V5 enzyme.** A. V5 epitope-tagged MKP3 was purified from E. coli and subjected to steady-state kinetic analysis under DMSO (○) or ERK2 (Δ) inducing conditions or under non-inducing condition (o) as described in Methods. Both DMSO and ERK2 were capable of increasing the catalytic efficiency of MKP3, resulting in an increased $v_{max}$ and decreased $K_M$ relative to the uninduced control. B. Effect of transient MKP3/V5 upregulation on ERK1/2 and phosphorylated ERK1/2 levels in human pancreatic adenocarcinoma cell lines.CRL1469 and
CRL1682 cultures were co-transfected with 2.5 μg each form I pVgRXR and pInd/GS/MK3/V5 as described in Methods. V5 epitope-tagged MKP3 expression was induced 16 h post-transfection with 5μM pronasterone A. Cell lysates were prepared 48 h later and processed for Western blot hybridization and laser densitometry as described in Methods. Epitope-tagged MKP3 was detected with a mouse monoclonal anti-V5 antibody. Dramatic upregulation of catalytically active epitope-tagged MKP3 had no significant effect on ERK1/2 or phosphorylated ERK1/2 levels. C. Cell morphology and general culture health of MKP3/V5 transfected and pronasterone A induced CRL1469 and CRL1682 adenocarcinoma cells. Transfected cells were photographed under phase contrast 48 h following pronasterone A induction. Cell morphology and viability were unaffected despite upregulation of epitope-tagged MKP3.

**Fig 9.** Models of ERK-induced activation and function in MKP3. The results of the binding and autoinhibition studies support a model of MKP3 deactivation due to oligomerization of MKP3. A. Under normal conditions, the presence of ERK (purple) leads to competitive binding and a two-step MKP3 activation: displacement of the C-terminal domain (yellow oval) from the N-terminal domain (green oval) followed by allosteric activation of the C-terminal domain (yellow square) by the N-terminally bound ERK. The allosteric activation results in dephosphorylation of phospho-tyrosine and phospho-threonine residues on ERK (red circles). B. If MKP3 is overexpressed, it undergoes interdomain binding, leading to the formation of high molecular weight oligomers. C. ERK binding to the N-terminal of the oligomerized MKP3 can release the C-terminal catalytic domain, but this catalytic domain may be unable to become allosterically activated or to bind and consequently dephosphorylate ERK. Thus, the presence of oligomers adversely affects MKP3’s inability to dephosphorylate ERK.
Table 1. Summary of surface plasmon resonance data

Surface plasmon resonance experiments were performed on MKP3 (WT, NT and CT) and MAPK (ERK2 and JNK) proteins that were covalently bound to SPR sensor chips. Binding data was obtained with increasing concentrations of either MKP3:WT or MKP3:CT. Binding curves were fitted to a 1:1 Langmuir binding model $[A] + [B] \leftrightarrow [AB]$ using the BIAevaluation 4.0 program to obtain association and dissociation constants.

| Immobilized protein | MKP3:WT $k_a$ (M$^{-1}$s$^{-1}$) | $K_D$ (μM) | MKP3:CT $k_a$ (M$^{-1}$s$^{-1}$) | $K_D$ (μM) |
|---------------------|---------------------------------|------------|---------------------------------|------------|
| ERK2                | 6.0 x 10$^6$                    | 0.263      |                                 |            |
| JNK1                | No fit                          | No fit     |                                 |            |
| MKP3:WT             | 456 +/- 11                      | 0.3 +/- 0.2|                                 |            |
| MKP3:NT             | 574 +/- 23                      | 1.4 +/- 0.4|                                 |            |
| MKP3:CT             | 23 +/- 4                        | 48 +/- 18  |                                 |            |
Table 2. Recombinant MKP3 steady state analysis

MKP3 proteins were assayed using pNPP as a colorimetric substrate in the presence or absence of ERK2 and DMSO, which were used to activate MKP3’s phosphatase function. MKP3 proteins were tested using 0-50 mM substrate (for non-active MKP3 tests) or 0-10 mM substrate (for ERK- or DMSO-activated tests). To detect MKP3 phosphatase activity, the hydrolysis of substrate was measured at 405 nm, following a 60 minute incubation at 25°C. To determine $k_{\text{cat}}$ and $K_M$, the data was analysed in Graphpad Prizm 4.0.

| Enzyme/Activator | $k_{\text{cat}}$ (s$^{-1}$) x10$^{-3}$ | $K_M$ (μM) x10$^{-3}$ | $k_{\text{cat}}/K_M$ (M$^{-1}$s$^{-1}$) |
|------------------|---------------------------------|-----------------|----------------------------------|
| MKP3:WT         | 9.9 +/- 0.4                     | 7.9 +/- 1       | 1.3 +/- 0.2                      |
| MKP3:WT/ERK2    | 30 +/- 6                        | 1.6 +/- 0.1     | 18 +/- 2                         |
| MKP3:WT/DMSO    | 40 +/- 4                        | 1.3 +/- 0.04    | 30 +/- 4                         |
| MKP3:CT         | 12 +/- 0.5                      | 7.6 +/- 0.8     | 1.6 +/- 0.2                      |
| MKP3:CT/ERK2    | 14 +/- 0.9                      | 4.5 +/- 0.7     | 3.1 +/- 0.7                      |
| MKP3:CT/DMSO    | 58 +/- 0.4                      | 1.4 +/- 0.07    | 40 +/- 2                         |
Table 3. Relative amounts of monomer, dimer, trimer and oligomer in pancreatic adenocarcinoma cells

Western blot analysis was performed on pancreatic adenocarcinoma cell lysates as described in Methods. The total MKP3 and MKP3 band pixel counts were determined by densitometry, then the relative count for each band was used to obtain a percentage of MKP3 existing as monomer, dimer, trimer, and oligomer.

| Cell line | Total MKP3 density | % Monomer | % Dimer | % Trimer | % Oligomer |
|-----------|-------------------|------------|--------|----------|------------|
| HPDE6     | 3,502             | 8          | 10     | 35       | 47         |
| CRL1420   | 2,064             | 6          | 3      | 15       | 80         |
| CRL1469   | 4,421             | 8          | 8      | 30       | 54         |
| CRL1682   | 3,143             | 8          | 3      | 17       | 72         |
| CRL1687   | 4,364             | 9          | 6      | 28       | 57         |
| CRL1837   | 1,790             | 12         | 4      | 35       | 49         |
| HTB80     | 1,674             | 7          | 2      | 16       | 75         |
| HTB134    | 3,817             | 11         | 17     | 29       | 43         |
| HTB147    | 4,314             | 9          | 6      | 21       | 64         |
Figure 1

A

75 kDa
50 kDa
25 kDa
15 kDa

M       1              2          3

B

50 kDa
40 kDa
30 kDa
25 kDa
20 kDa

M          1                 2             3

M
Figure 2

[Graph showing measurements of per molar residue ellipticity (°M⁻¹cm⁻¹) across different wavelengths (nm). The graph compares MKP3:WT, MKP3:CT, and MKP3:NT samples.]
Figure 3

A

B

C

[MKP3:CT] 25 μM
0 μM

[MKP3:CT] 25 μM
0 μM

[MKP3:CT] 25 μM
0 μM
Figure 5
Figure 6

A

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| C | - | - | 2.0 | 1.0 | 3.6 | 1.5 | 3.2 | 2.3 | 1.7 | 2.6 | 1.3 | 1.5 | 0.9 |
|   |   |   | MKP3 |

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| C | - | - | 0.3 | 1.0 | 0.5 | 0.5 | 1.5 | 1.0 | 3.7 | 2.6 | 0.5 | 2.5 | 1.3 |
|   |   |   | Ran |

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| C | - | - | 0.6 | 1.0 | 0.8 | 0.5 | 0.6 | 0.7 | 1.1 | 1.5 | 0.9 | 0.8 | 0.7 |
|   |   |   | ERK |

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|----|
|   |   |   | Ran |

B

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|----|
|   |   |   | MKP3 |

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|----|
|   |   |   | β-actin |
Figure 7.

A

B

MKP3:CT dimer
MKP3:CT

C

oligomer
trimer
dimer
monomer
Figure 8.

A

\[ v \text{ (nmol/min)} \]

\[ [S] \text{ (mmol/L)} \]

B

CRL1469  CRL1682
- + - +

MKP3/V5

1.0 0.7 1.0 1.1

ERK

Ran

pERK

1.0 1.2 1.0 0.7

Ran

- CRL1469 +

- CRL1682 +

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Figure 9
Inhibition of mitogen-activated protein kinase phosphatase 3 activity by interdomain binding
John K. Mark, Rémy A. Aubin, Sophie Smith and Mary Alice Hefford
J. Biol. Chem. published online August 11, 2008

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