April 2005

Evaluation of glucocorticoid sensitivity in 697 pre-B acute lymphoblastic leukemia cells after overexpression or silencing of MAP Kinase Phosphotase-1

Marc T. Abrams
Thomas Jefferson University

Noreen Robertson
Drexel University School of Medicine

Gerald Litwack
Thomas Jefferson University

Eric Wickstrom
Thomas Jefferson University, eric@tesla.jci.tju.edu

Evaluation of glucocorticoid sensitivity in 697 pre-B acute lymphoblastic leukemia cells after overexpression or silencing of MAP Kinase Phosphatase-1

Marc T. Abrams, Noreen Robertson[1], Gerald Litwack and Eric Wickstrom[*]

Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University Philadelphia, Pennsylvania 19107, U.S.A.

Running title: MKP-1 and glucocorticoid-induced apoptosis

Key words: Glucocorticoid, apoptosis, MKP-1, siRNA, ALL

[1]Present Address: A.J. Drexel Institute of Basic and Applied Protein Science, Drexel University School of Medicine, Philadelphia, PA 19102

Grant support: DOE/BER ER63055 to E. W.

[*]**Requests for reprints:** Dr. Eric Wickstrom, Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, BLSB #219, Philadelphia PA 19107. Phone: 215.955.4578, Fax: 215.955.4580. email: eric@tesla.jci.tju.edu

Abbreviations: GC, glucocorticoid; ALL, acute lymphoblastic leukemia; GR, glucocorticoid receptor; MKP-1; MAP kinase phosphatase-1; RNAi, RNA interference; siRNA, small-interfering RNA; FITC, fluorescein isothiocyanate; μF, microfarads; TA, triamcinolone acetonide; HU, hydroxyurea; RT-PCR, reverse transcriptase-polymerase chain reaction; 7-AAD, 7-aminoactinomycin D.

ABSTRACT

PURPOSE: To determine the effect of reducing MAP kinase phosphatase-1 (MKP-1) levels on cell death induced by glucocorticoid (GC) or hydroxyurea (HU) treatment in the human pre-B acute lymphoblastic leukemia cell line 697. METHODS: Stable MKP-1 overexpressing transformants of the 697 pre-B ALL cell line were created and tested for sensitivity to the GC triamcinolone acetonide (TA) and HU, and compared to a control 697 cell line containing normal MKP-1 expression levels. Small interfering RNAs (siRNAs) were designed to inhibit MKP-1 expression and evaluated for their effect on GC-mediated cell death. RESULTS: MKP-1 overexpression caused a phenotype of partial resistance to HU-induced apoptosis but not to GC-induced apoptosis. Electroporation of siRNAs effectively silenced MKP-1 expression, and increased sensitivity to TA by 9.6±1.9%. CONCLUSIONS: Because MKP-1 protects certain tumor cells from chemotherapy-induced apoptosis, its inhibition is being considered as a possible strategy for combination cancer therapy. However, this study suggests that while MKP-1 inhibition may improve the efficacy of DNA damaging agents, it may have only limited utility in combination with glucocorticoids.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, with approximately 5000 new cases reported annually (Cortes and Kantarjian 1995). The standard course of therapy includes both classical chemotherapeutics such as vincristine as well as glucocorticoids (GC) such as dexamethasone, prednisone, or triamcinolone (Cortes and Kantarjian 1995). Although this regimen leads to a cure rate of >70%, poor prognosis often correlates with resistance to GCs (Tissing et al. 2003). Precursor B cell ALL is the most prevalent form of this disease, ~10-20% of which contain a t(1;19)

chromosomal translocation (Raimondi et al. 1990). This translocation creates the E2A-Pbx chimeric transcription factor, which has oncogenic properties (Kamps et al. 1991) and causes aberrant expression of signaling proteins such as Wnt-16 (McWhirter et al. 1999) and N-Myc (Rutherford et al. 2001). The pre-B ALL cell line 697, a well-characterized GC-sensitive, t(1;19) positive cell line isolated from a 12 year old patient, is a useful model for studying signal transduction pathways in this disease (Findley et al. 1982).

GCs induce apoptosis in numerous hematopoietic tissues of both lymphoid and myeloid lineage in a process that requires the glucocorticoid receptor (GR), a GC-activated transcription factor of the nuclear receptor superfamily (Planey and Litwack 2000). GR activation by GC induces dissociation of GC-GR from a large cytoplasmic complex and translocation to the nucleus, where GC-GR affects mRNA levels of target genes by interacting directly with promoter DNA or by binding to other transcription factors (McEwan et al. 1997). Apoptosis occurs downstream of these transcriptional effects, and involves the biochemical hallmarks of the intrinsic cell death pathway: release of cytochrome c from the mitochondria, cleavage of caspase-9 and DNA fragmentation (Planey et al. 2002). In the cell line 697, GC rapidly induces or represses >100 genes, including a number of interesting candidates for mediators of this pathway. These target genes include *BCL2* family members, the *FOXO3A* transcription factor, a G-protein coupled receptor *GPCR18*, and MAP kinase phosphatase-1 (*MKP1*) (Planey et al. 2003).

MKP-1 protein is a dual-specificity phosphatase that exhibits anti-apoptotic effects by negatively regulating the stress-induced mitogen activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) and p38 (Slack et al. 2001). Hence, MKP-1 has emerged as an appealing GR target gene (Clark 2003). *MKP1*mRNA and MKP-1

protein are both strongly and rapidly repressed by GC-GR in 697 cells before they apoptose, which led us to the hypothesis that alleviating negative regulation of pro-apoptotic MAPKs contributes to this mechanism (Planey et al. 2003). On the contrary, MKP-1 protein is *induced* by GC in numerous non-hematopoietic tissues including mammary epithelial cells, which initiate a survival response after exposure to GC (Wu et al. 2004). Interestingly, it has been shown recently that silencing *MKP1* gene expression by RNA interference in MCF-7 breast cancer cells inhibits GC-mediated survival after paclitaxel treatment (Wu et al. 2004). Thus, it is possible that differential regulation of MKP-1 is a mechanism by which GC can cause opposite biological outcomes (survival versus apoptosis) in different tissues. Finally, given that MKP-1 is overexpressed in a variety of human cancers (Bang et al. 1998; Kurt et al. 1998), and that GCs are administered routinely to reduce the nausea and acute toxicity caused by chemotherapeutics, it is of considerable clinical interest to investigate the role of MKP-1 in GC-mediated effects.

In this study, we determined the effect of modulating MKP-1 expression levels on GC-induced apoptosis in the cell line 697. Based on the hypothesis stated above, MKP-1 overexpression would be expected to cause GC resistance, compared with cells containing only endogenous levels of MKP-1. However, strong overexpression of MKP-1 cDNA in 697 cells did not affect GC sensitivity or caspase-3 activation, even though it caused partial resistance to another chemotherapeutic agent, hydroxyurea. We also designed and validated small RNA duplexes (reviewed by Caplen and Mousses, 2003) that significantly reduced MKP-1 expression. Silencing of *MKP1* mRNA by RNA interference had a modest effect on GC sensitivity. These data establish that MKP-1 is not likely to be a critical mediator of GC signaling in pre-B ALL and underscore the importance of investigating the tissue-specific mechanisms of GC signal transduction.

MATERIALS AND METHODS

Compounds and reagents: Triamcinolone acetonide (TA) was purchased from Sigma (St. Louis, MO) and dissolved in 100% ethanol. The final concentration of ethanol in all cell treatments was kept constant at 0.1 %. Hydroxyurea (Sigma) was dissolved in phosphate buffered saline (PBS). All siRNAs were purchased from Dharmacon (Lafayette, CO). The GL2 firefly luciferase siRNA sequence (Elbashir et al. 2001) was used as the control for all siRNA experiments, and this sequence was also used for both the FITC and Cy3 labeled siRNAs. All tissue culture and electrophoresis reagents were purchased from Invitrogen (Carlsbad, CA) except where otherwise indicated.

Cell culture and cDNA overexpression: 697 pre-B ALL cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and penicillin/streptomycin mix. MKP-1 cDNAs were a generous gift from Dr. Steve Keyes and Dr. Chris Franklin. The BamH1/EcoR1 fragment from MKP-1/pSG5 (Groom et al. 1996) was subcloned into a pcDNA3.1 expression vector (Invitrogen). 60 μg of this plasmid, as well as the empty vector, were transfected by electroporation into 697 cells as described (Planey et al. 2003). 48 h later, 1 μg/mL G418 was added to the growth medium. After one week, resistant cells were seeded in 96-well plates at limiting dilutions. Clonal isolates were expanded for screening after approximately 4 weeks. 697 cells overexpressing dominant-negative caspase-9 were as described in (Planey et al. 2003).

Immunoblots: For all of the MKP-1 immunoblots, 40-50 μg of whole-cell lysate from each clone was electrophoresed on Tris-glycine 10% polyacrylamide gels. Samples were transferred to polyvinylidene fluoride membranes, blocked in PBS containing 5% powdered milk and 0.5% Tween-20, and immunoblotted with an anti-MKP-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS containing 0.5% Tween-20, or an anti-GAPDH antibody (Research Diagnostics, Flanders, NJ) diluted 1:20000. After washing, detection was performed using an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and a chemiluminescent substrate (SuperSignal, Pierce, Rockford, IL).

Real time RT-PCR: Total RNA was purified from 1×10^7 cells for each cell line using RNeasy columns (Qiagen, Valencia, CA). RT-PCR reactions were performed in duplicate using Taqman One Step reagents in an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 0.1 μg of template RNA was used per reaction. Primer/probe sequences and concentrations were the same as in Planey et al. (2003).

Flow cytometry: For cell cycle analysis, cells were treated in 6-well plates, washed with PBS, fixed in 70% ethanol, and stained with 50 μg/mL propidium iodide containing 1 mg/mL RNase A and 1 mg/mL glucose (all from Sigma) for 30 min. Samples were processed on a FACSCalibur instrument (BD Biosciences, San Jose, CA) and data were analyzed using FACSComp software. 10,000 cells were collected for each measurement. For viability analysis, samples were resuspended in 500 μL of 0.1 M HEPES-NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$ containing 0.5 μg/mL 7-aminoactinomycin-D (BD Pharmingen, San Diego, CA). 7-AAD content was measured using a Beckman Coulter

XL flow cytometer and data were analyzed using WinMDI software. 50,000 cells were counted for each measurement.

Caspase-3 activity: 1×10^5 cells per well were seeded in 96-well plates in triplicate for each condition. After drug treatment, cells were subjected to assay using ApoAlert (Clontech, Palo Alto, CA) reagents including the DEVD-AFC substrate, and detected fluorimetrically according to the manufacturer's protocol.

siRNA transfection: Exponentially growing 697 cells were washed twice with PBS and resuspended in serum-free RPMI-1640 media containing 25 mM HEPES-NaOH pH 7.4 (without phenol red or antibiotics) at 10^7 cells/mL. 200 μL aliquots of cells were added to 0.2 cm gap electroporation cuvets (Bio-Rad, Hercules, CA). siRNA (10 μL of a 20 μM stock solution for a final concentration of 1 μM) was added and incubated at room temperature for 20 min. Cells were then electroporated with a Bio-Rad Gene Pulser using voltage and capacitance settings of 220 V and 500 μF, respectively. Cells were incubated for an additional 20 min., and added to 3 mL of the above medium supplemented with 10% FBS (final siRNA concentration after dilution was 66 nM) and allowed to recover at 37ºC for the indicated times (excluding aggregated cell debris).

Cell Sorting: Cells were transfected as above except that FITC-siRNA was added to 0.25 μM in each cuvet. Three electroporations were pooled together for each condition to obtain enough cells to sort efficiently. After 18 h, cells were collected and resuspended in medium containing 5% FBS. Cells were sorted based on FITC content using a EPICS Elite cytometer (Beckman Coulter, Fullerton, CA) into 50% growth medium / 50% FBS

and allowed to recover for 30 min. Cells were then re-seeded and either harvested for immunoblotting or treated with compounds for viability assay after an additional 24 h.

RESULTS

We showed previously that GC-induced apoptosis is associated with rapid down-regulation of MKP-1 mRNA and protein in the pre-B ALL cell line 697, an event that is completely abrogated by the GR antagonist RU486 (Planey et al. 2003). This repression of MKP-1 correlated well with an increase in the activity of its primary substrate, c-Jun N-terminal linase (JNK) (Planey et al. 2003). To determine whether interfering with MKP-1 repression by GC-GR affects the sensitivity of 697 cells to GC, we created stable clones derived from the 697 line that strongly overexpress *MKP1* cDNA. After selecting plasmid-transfected clones derived from single cells for approximately 6 weeks, we screened 4 clones expressing *MKP1* cDNA by immunoblotting, in addition to a clone transfected with empty vector as a control (Fig. 1a). Two of those clones strongly overexpressed MKP-1 protein as compared to the empty vector control. We used the highest-expressing cell line (Fig. 1a, lane 5) for all subsequent experiments. To confirm overexpression at the mRNA level, we performed real-time RT-PCR (Fig. 1b). Duplicate RT-PCR data showed a reduction of 1.5 PCR cycles in the threshold cycle (C_t) value of MKP-1 overexpressing cells when compared to empty vector cells, which corresponds to a 3-fold increase in steady state mRNA concentration ($2^{1.5}$). A probe for 18S rRNA was included as a loading control. The large increase in steady-state protein levels, as compared to the moderate increase in steady-state mRNA levels, suggests that the exogenous MKP-1 protein is remarkably stable after synthesis.

To determine if the sensitivity of the 697 cells to GC was decreased after MKP-1 overexpression, we treated the MKP-1 and empty vector-expressing cells with the potent GC, triamcinolone acetonide (TA, 100 nM), for 48 h (Fig 2a), and then determined viability by Trypan blue exclusion. MKP-1 overexpression did not significantly change cell viability after TA exposure. In addition, lower concentrations of TA yielded similar results (data not shown). To evaluate MKP-1 in the context of an additional chemotherapeutic, we also treated both cell lines with the DNA replication inhibitor hydroxyurea (HU, 3 mM). MKP-1 overexpression caused partial resistance to HU, consistent with the role of the MKP-1 substrates JNK and p38 in the cellular DNA damage response. A derivative of the 697 line that stably expresses a dominant negative allele of caspase-9 (Planey et al. 2003), which was included as a positive control for drug resistance, retained viability after treatment with either drug. In addition to determining viability by microscopy, we also performed cell cycle analysis by propidium iodide staining followed by flow cytometry. Following a 48 h exposure to 100 nM TA, the majority of the cells contained <2n DNA content, indicating cell death. MKP-1 overexpression did not reduce the percentage of sub-G_0/G_1 cells after TA treatment (and surprisingly slightly increased this value). As expected for a compound that confers chemoresistance, MKP-1 overexpression decreased the accumulation of sub-G_0/G_1 cells after hydroxyurea treatment by 40%. Furthermore, we investigated the effect of exogenous MKP-1 expression on GC-induced activation of caspase-3, the primary effector caspase of the intrinsic apoptosis pathway (Fig. 2e). A 16 h exposure to 100 nM TA induced cleavage of the fluorescent caspase-3 substrate peptide, DEVD-AFC, to the same extent in both cell lines. Interestingly, a higher level of baseline caspase-3 activity was observed in the cells overexpressing MKP-1. Taken together, these data demonstrate

that increasing MKP-1 levels does not significantly modulate GC sensitivity in an ALL cell culture model.

In order to determine the effect of reducing the concentration of specific mRNAs on GC sensitivity, we optimized a method for electroporating small interfering RNAs (siRNAs) into 697 cells (Abrams et al. 2004). 48 h after electroporating a nonspecific Cy3-labeled siRNA, fluorescence was detected in nearly 100% of the cells (and was predominantly cytoplasmic as expected), although there was considerable heterogeneity in RNA uptake within the population (Fig 3a). We then designed 3 RNA duplexes targeting different regions of MKP-1 mRNA (Fig 3b). A BLAST search revealed no significant homology with other potential targets in the genome (data not shown). To determine if these siRNAs specifically reduce MKP-1 protein levels, 1 μM of each duplex was separately electroporated into the cells overexpressing MKP-1. A pool containing 0.33 μM of each in the same transfection mix was also tested in both the MKP-1 overexpressing cells and parental 697 cells. 48 h later, the cells were harvested and lysates were subjected to immunoblot analysis using an anti-MKP-1 antibody (Fig 3c). All three siRNAs, as well as the pool, reduced MKP-1 protein levels by more than 50% as compared to a control siRNA sequence. These lysates were also tested with an antibody against the housekeeping enzyme GAPDH as a loading control. To determine the effect of *MKP1* silencing on GC sensitivity, the *MKP1* siRNA pool was transfected into wild type 697 cells for 48 h, followed by 48 h exposure to 5 nM TA. This low concentration of TA, which is close to its K_d for binding to GR (Zhang and Danielsen 1995), was selected so that small changes in sensitivity could be detected. Cell viability was determined by flow cytometry after staining with the fluorescent DNA intercalator 7-aminoactinomycin-D, which can only penetrate dead or dying cells (Lecoeur et al. 2002) (Fig 3d). Viability data were also confirmed by Trypan blue exclusion (data not shown).

GC-induced cell death occurred in 9.6±1.9% more of the cells transfected with *MKP1* siRNA as compared to a control siRNA sequence that targets firefly luciferase. These data demonstrate that considerable silencing of MKP-1 expression only modestly increases GC sensitivity.

In order to achieve a greater level of *MKP1* silencing, we used flow cytometry to isolate a population of cells enriched for a high level of siRNA internalization after transfection. To accomplish this, we spiked the control or *MKP1* siRNAs with a FITC-labeled nonspecific siRNA at a ratio of 4:1. As a proof-of-principle experiment for this enriching method, the labeled siRNA was transfected into the 697 cells that overexpress MKP-1, and cell sorting was performed based on FITC content 18 h later (Fig 4a). Cells were allowed to recover for 24 h after sorting in normal growth medium at 37ºC, at which time they were harvested and immunoblotted. Both populations containing the *MKP1* siRNA had reduced MKP-1 protein levels compared to the control siRNA, but we observed enriched silencing in the high FITC fraction (Fig. 4b). After developing this protocol, we determined whether maximizing *MKP1* silencing by sorting increased GC sensitivity. Wild type 697 cells were sorted as above, and 5 nM TA was added after a 24 h recovery. After a 48 h drug exposure, viability was determined by 7-AAD staining as in Fig. 3d (Fig 4c). When comparing the control siRNA to the *MKP1* pool in each population (low FITC vs. high FITC), GC sensitivity increased only slightly in the cells enriched for siRNA internalization (Fig. 4d). Collectively, these data demonstrate that using genetic methods to cause modulations in MKP-1 protein levels have a relatively minor impact on GC-induced cell death in pre-B ALL cells.

DISCUSSION

MKP-1 overexpression confers resistance to ultraviolet UV radiation, cisplatin, paclitaxel, doxorubicin, and Fas ligand in well characterized *in vitro* models (Franklin et al. 1998; Sanchez-Perez et al. 2000; Srikanth et al. 1999; Wu et al. 2004). In this study, we also observed that MKP-1 overexpression renders 697 pre-B ALL cells resistant to hydroxyurea. Furthermore, siRNA, antisense or small molecule inhibitors of MKP-1 all exhibited drug sensitizing or antiproliferative effects in tumor cells (Guo et al. 1998; Liao et al. 2003; Wu et al. 2004). These data suggest that MKP-1 functions as a generalized resistance gene, affecting cell death induced by agents that initiate both intrinsic (caspase-9 mediated) apoptosis, such as DNA damage inducers, and extrinsic (caspase-8 mediated) apoptosis, such as Fas ligand. In this study, we investigated the role of MKP-1 in GC-induced apoptosis in pre-B ALL cells. We observed that MKP-1 overexpression did not confer GC resistance and that MKP-1 knockdown only slightly increased GC sensitivity (9.6±1.9%). These conclusions underscore the complexity and unique mechanism of glucocorticoid-induced apoptosis.

Inhibition of MKP-1 might contribute to therapy of solid tumors in the future. One recent study (Small et al. 2004) effectively demonstrated that antagonizing MKP-1 by either siRNA or anthracyclines increases the extent of apoptosis induced by proteasome inhibitors in breast carcinoma cells. In another, downregulation of MKP-1 by antisense DNA decreased growth of pancreatic tumors in a nude mouse model (Liao et al. 2003). Finally, in addition to its potential therapeutic value, MKP-1 expression may also have prognostic value, as it was shown to be a predictor of survival in non-small cell lung cancer. Based on our study, however, we predict that MKP-1 inhibition will have limited utility in sensitizing leukemias and lymphomas to GCs.

There are numerous examples of other GR target genes that, when overexpressed, confer GC resistance in leukemic cells. These include *BCL2* (Planey et al. 2003),

catalase (Tome et al. 2003), and dig120 (Wang et al. 2003b). In addition, we have recently reported that siRNA silencing of the pro-apoptotic *BCL2* relative *BIM*, a target for GC-mediated repression, partially inhibits GC-induced apoptosis (Abrams et al. 2004). Therefore, the mechanism of GC-induced apoptosis is likely to involve the combinatorial effect of activation or repression of numerous specific genes by the GR. MKP-1 is likely to be a key mediator of GC-induced survival in breast cancer cells (Wu et al. 2004), but not GC-induced apoptosis in leukemia as previously hypothesized (Planey et al. 2003).

One of the most important unresolved issues in GC biology is that GR activation causes apoptosis in some tissues and survival in others. These observations are directly related to the fact that the transcriptional program activated by GC is highly dependent on the cellular context. MKP-1 can either be induced or repressed by GC depending on the tissue (see Introduction). Another example of this phenomenon is that while Bcl-2 protein and its proapoptotic family member Bim are oppositely regulated by GC in 697 cells (Planey et al. 2003), CEM T-lymphocytes (Wang et al. 2003a) and S49A murine lymphoma cells (Wang et al. 2003a), neither appear to be affected in MCF-7 breast cancer cells (Wu et al. 2004) or U20S osteosarcoma cells (Rogatsky et al. 2003). Understanding the mechanism of tissue specificity of GR activation outcomes is critical to developing novel therapeutic strategies for GC resistant cancers.

ACKNOWLEDGEMENTS

The authors would like to thank Paul Hallberg for providing flow cytometry expertise, and Dr.'s Chris Franklin and Steve Keyes for providing *MKP1* cDNA constructs.

REFERENCES

Abrams MT, Robertson NM, Yoon K, Wickstrom E (2004) Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM with siRNA and shRNA. J Biol Chem

Bang YJ, Kwon JH, Kang SH, Kim JW, Yang YC (1998) Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinoma. Biochem Biophys Res Commun 250: 43-7

Caplen NJ, Mousses S (2003) Short interfering RNA (siRNA)-mediated RNA interference (RNAi) in human cells. Ann N Y Acad Sci 1002: 56-62

Clark AR (2003) MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? J Endocrinol 178: 5-12

Cortes JE, Kantarjian HM (1995) Acute lymphoblastic leukemia. A comprehensive review with emphasis on biology and therapy. Cancer 76: 2393-417

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-8

Findley HW, Jr., Cooper MD, Kim TH, Alvarado C, Ragab AH (1982) Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood 60: 1305-9

Franklin CC, Srikanth S, Kraft AS (1998) Conditional expression of mitogen-activated protein kinase phosphatase-1, MKP-1, is cytoprotective against UV-induced apoptosis. Proc Natl Acad Sci U S A 95: 3014-9

Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. Embo J 15: 3621-32

Guo YL, Kang B, Williamson JR (1998) Inhibition of the expression of mitogen-activated protein phosphatase-1 potentiates apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. J Biol Chem 273: 10362-6

Kamps MP, Look AT, Baltimore D (1991) The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes Dev 5: 358-68

Kurt RA, Urba WJ, Smith JW, Schoof DD (1998) Peripheral T lymphocytes from women with breast cancer exhibit abnormal protein expression of several signaling molecules. Int J Cancer 78: 16-20

Lecoeur H, de Oliveira-Pinto LM, Gougeon ML (2002) Multiparametric flow cytometric analysis of biochemical and functional events associated with apoptosis and oncosis using the 7-aminoactinomycin D assay. J Immunol Methods 265: 81-96

Liao Q, Guo J, Kleeff J, Zimmermann A, Buchler MW, Korc M, Friess H (2003) Down-regulation of the dual-specificity phosphatase MKP-1 suppresses tumorigenicity of pancreatic cancer cells. Gastroenterology 124: 1830-45

McEwan IJ, Wright AP, Gustafsson JA (1997) Mechanism of gene expression by the glucocorticoid receptor: role of protein-protein interactions. Bioessays 19: 153-60

McWhirter JR, Neuteboom ST, Wancewicz EV, Monia BP, Downing JR, Murre C (1999) Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A 96: 11464-9

Planey SL, Abrams MT, Robertson NM, Litwack G (2003) Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells. Cancer Res 63: 172-8

Planey SL, Derfoul A, Steplewski A, Robertson NM, Litwack G (2002) Inhibition of glucocorticoid-induced apoptosis in 697 pre-B lymphocytes by the mineralocorticoid receptor N-terminal domain. J Biol Chem 277: 42188-96

Planey SL, Litwack G (2000) Glucocorticoid-induced apoptosis in lymphocytes. Biochem Biophys Res Commun 279: 307-12

Raimondi SC, Behm FG, Roberson PK, Williams DL, Pui CH, Crist WM, Look AT, Rivera GK (1990) Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognostic implications of the t(1;19). J Clin Oncol 8: 1380-8

Rogatsky I, Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq CM, Darimont BD, Garabedian MJ, Yamamoto KR (2003) Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. Proc Natl Acad Sci U S A 100: 13845-50

Rutherford MN, Bayly GR, Matthews BP, Okuda T, Dinjens WM, Kondoh H, LeBrun DP (2001) The leukemogenic transcription factor E2a-Pbx1 induces expression of the putative N-myc and p53 target gene NDRG1 in Ba/F3 cells. Leukemia 15: 362-70

Sanchez-Perez I, Martinez-Gomariz M, Williams D, Keyse SM, Perona R (2000) CL100/MKP-1 modulates JNK activation and apoptosis in response to cisplatin. Oncogene 19: 5142-52

Slack DN, Seternes OM, Gabrielsen M, Keyse SM (2001) Distinct binding determinants for ERK2/p38alpha and JNK map kinases mediate catalytic activation and substrate selectivity of map kinase phosphatase-1. J Biol Chem 276: 16491-500

Small GW, Shi YY, Edmund NA, Somasundaram S, Moore DT, Orlowski RZ (2004) Evidence that Mitogen-activated Protein Kinase Phosphatase-1 Induction by Proteasome Inhibitors Plays an Anti-apoptotic Role. Mol Pharmacol

Srikanth S, Franklin CC, Duke RC, Kraft RS (1999) Human DU145 prostate cancer cells overexpressing mitogen-activated protein kinase phosphatase-1 are resistant to Fas ligand-induced mitochondrial perturbations and cellular apoptosis. Mol Cell Biochem 199: 169-78

Tissing WJ, Meijerink JP, den Boer ML, Pieters R (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 17: 17-25

Tome ME, Lutz NW, Briehl MM (2003) Overexpression of catalase or Bcl-2 delays or prevents alterations in phospholipid metabolism during glucocorticoid-induced apoptosis in WEHI7.2 cells. Biochim Biophys Acta 1642: 149-62

Wang Z, Malone MH, He H, McColl KS, Distelhorst CW (2003a) Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. J Biol Chem 278: 23861-7

Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F, Distelhorst CW (2003b) Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals. J Biol Chem 278: 27053-8

Wu W, Chaudhuri S, Brickley DR, Pang D, Karrison T, Conzen SD (2004) Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. Cancer Res 64: 1757-64

Zhang S, Danielsen M (1995) 8-Br-cAMP does not convert antagonists of the glucocorticoid receptor into agonists. Recent Prog Horm Res 50: 429-35

FIGURE LEGENDS

Figure 1. Overexpression of MKP-1 in 697 cells. *A*, Detection of MKP-1 protein in four stable, clonally-isolated cell lines derived from 697 after transfection of MKP-1/pcDNA3 plasmid and selection in G418. Vec, empty vector-transfected control cell line. *B*, Real-time RT-PCR detection of MKP-1 mRNA and 18S rRNA as a normalization control. Amplification plot shows accumulation of fluorescent PCR products as a function of PCR cycle. The data corresponding to the empty vector control and the MKP-1 overexpressing 697 cells (clone 4) are indicated. Each cell line was tested in duplicate.

Figure 2. Effect of MKP-1 overexpression on sensitivity to hydroxyurea and glucocorticoid. *A*, 697/pcDNA3 (white bars); 697/pcDNA3-MKP1 (black bars); and 697/pcDNA3-DNCasp9 (striped bars) were treated with 3 mM hydroxyurea (HU), 100 nM triamcinolone acetonide (TA), or vehicle (0.1% ethanol) for 48 h. Percent viability was determined by Trypan blue exclusion. Error bars on HU and TA data represent the SEM (n=3). *B*, Cell lines were treated as above with HU, TA, or vehicle as indicated, fixed, stained with propidium iodide and subjected to cell cycle analysis by flow cytometry. The stages of the cell cycle are indicated as follows: sub G0/G1 (black); G1 (white); S(striped); G2/M (dotted). Cell lines are abbreviated as Vec, 697/pCDNA3; MKP, 697/pcDNA3-MKP1; DN9, 697/pcDNA3-DNCasp9. *E*, Cell lines were treated as above in triplicate with TA (white bars) or vehicle (black bars) and assayed for caspase-3 activity by cleavage of fluorescent DEVD peptide. Error bars represent SEM (n=3).

Figure 3. Validation of MKP-1 siRNA and effect on GC sensitivity. *A*, 697 cells were transfected by electroporation with 1 μM Cy3-labeled siRNA and visualized 48 h later by fluorescence microscopy at 40x. *B*, Open reading frame of *MKP1* cDNA showing selected siRNA targets. *C*, 1 μM of control or *MKP1*-specific siRNAs, or a pool of 0.33 μM each of three *MKP1* siRNAs, were transfected into 697/pcDNA3-MKP1 cells (top) or parental 697 cells (bottom). After 48 h, whole-cell lysates were assayed by immunoblot with anti-MKP-1 and anti-GAPDH antibodies. Ctrl, control siRNA sequence; lanes labeled 1-3 correspond to the target sequences shown in *B*. *D*, 697 cells were transfected with the control siRNA (white bars) or the *MKP1* pool siRNA (black bars). After 48 h, cells were treated with vehicle or 5 nM TA for 48 h, then assayed for viability by 7-AAD as described in Materials and Methods. The error bars represent the SEM (n=3).

Figure 4. Cell sorting increases siRNA silencing potency. *A*, 697/pcDNA3-MKP1 cells were mock transfected (left) or transfected with the *MKP1* siRNA pool spiked with FITC-labeled siRNA (right) as described. Histograms show distribution of fluorescence in cell population, and "C" and "B" labels on the right histogram correspond to fractions collected for the low-FITC and high-FITC fractions (respectively) assayed in *B*. *B*, 24 h after sorting, whole cell lysates were subject to immunoblot with indicated antibodies. Ctrl, control siRNA sequence. C, 697 cells were transfected and sorted as above. 24 h after sorting, the low-FITC and high-FITC populations were treated with vehicle or 5 nM TA for 48 h, and assayed for viability by 7-AAD as described in Materials and Methods. Control siRNA, white bars; *MKP1* siRNA, black bars.

Figure 1

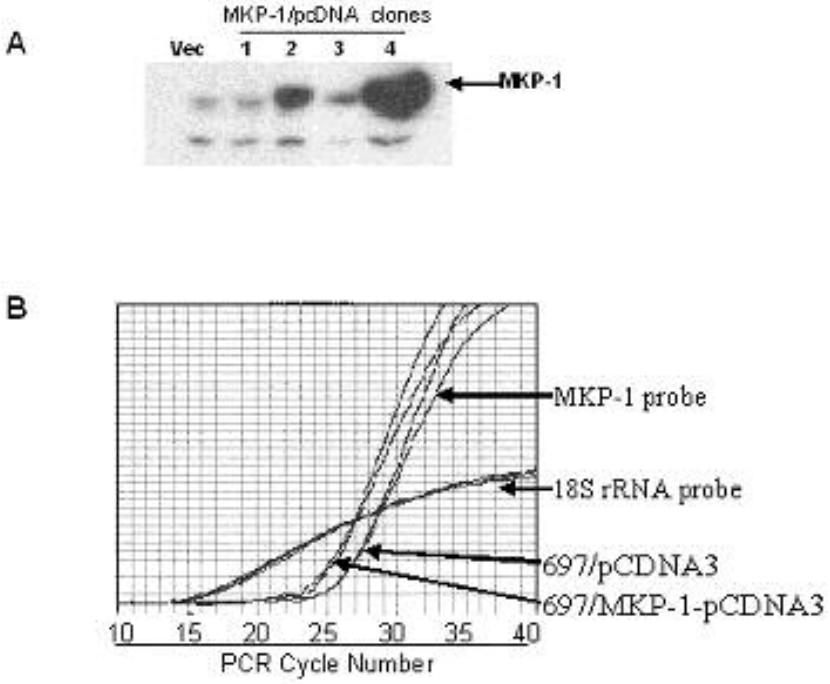
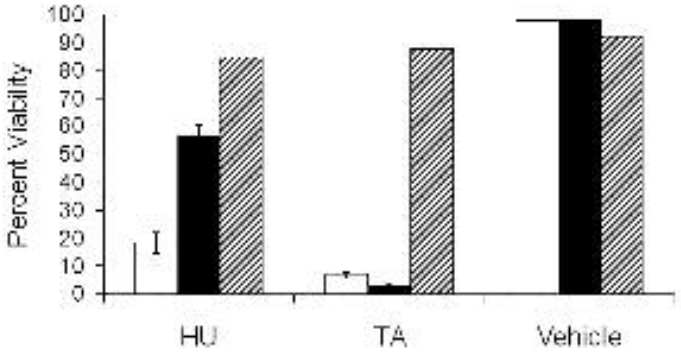
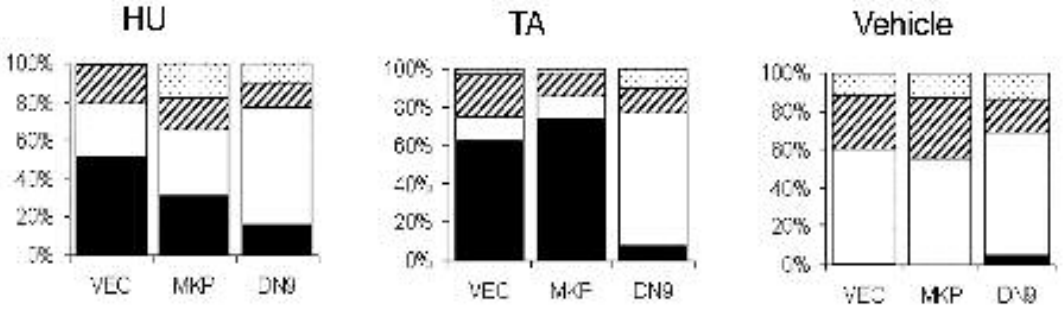

A
MKP-1/pcDNA clones
Vec
1
2
3
4
MKP-1
B
MKP-1 probe
18S rRNA probe
697/pCDNA3
697/MKP-1-pCDNA3
10
15
20
25
30
35
40
PCR Cycle Number

Figure 2

A


Percent Viability
100
90
80
70
60
50
40
30
20
10
0
HU
TA
Vehicle

B


HU
TA
Vehicle
100%
80%
60%
40%
20%
0%
VEC
MKP
DN9

C

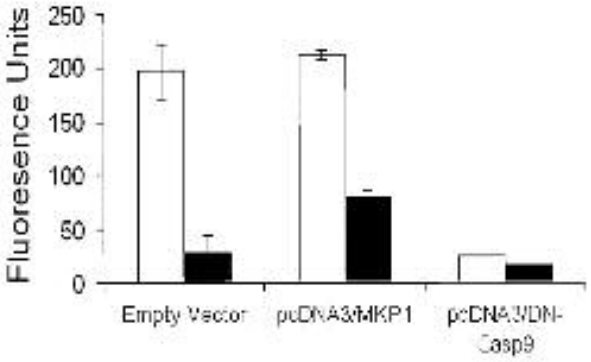
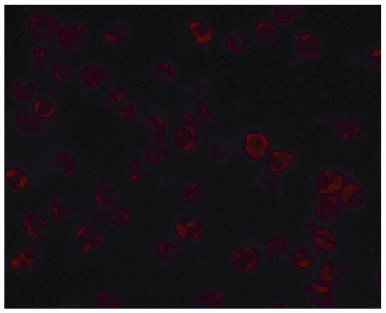

Fluoresence Units
250
200
150
100
50
0
Empty Vector
pcDNA3/MKP1
pcDNA3/DN-Casp9

Figure 3

A



B

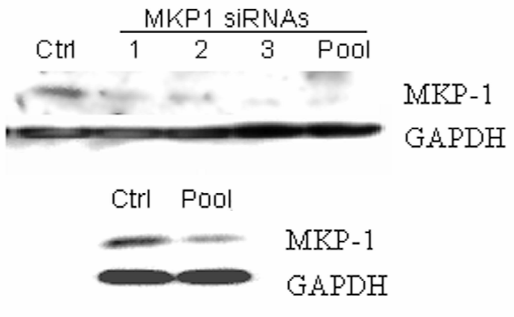
ATGGTCATGGAAGTGGGCACCCTGGACGCTGGAGGCCTGCGGGCGCTGCTGGGGGAGCGAGCGGCGCAAT
GCCTGCTGCTGGACTGCCGCTCCTTCTTCGCTTTCAACGCCGGCCACATCGCCGGCTCTGTCAACGTGCG
CTTCAGCACCATCGTGCGGCGCCGGGCCAAGGGCGCCATGGGCCTGGAGCACATCGTGCCCAACGCCGAG
CTCCGCGGCCGCCTGCTGGCCGGCGCCTACCACGCCGTGGTGTTGCTGGACGAGCGCAGCGCCGCCCTGG
ACGGCGCCAAGCGCGACGGCACCCTGGCCCTGGCGGCCGGCGCGCTCTGCCGCGAGGCGCGCGCCGCGCA
AGTCTTCTTCCTCAAAGGAGGATACGAAGCGTTTTCGGCTTCCTGCCCGGAGCTGTGCAGCAAACAGTCG
ACCCCCATGGGGCTCAGCCTTCCCCTGAGTACTAGCGTCCCTGACAGCGCGGAATCTGGGTGCAGTTCCT
GCAGTACCCCACTCTACGATCAGGGTGGCCCGGTGGAAATCCTGCCCTTTCTGTACCTGGGCAGTGCGTA
TCACGCTTCCCGCAAGGACATGCTGGATGCCTTGGGCA**TAACTGCCTTGATCAACGT**CTCAGCCAATTGT
(siRNA target 1)

CCCAACCATTTTCAGGGTCACTACCAGTACAAGAGCATCCCTGTGGAGGACAACCACAAGGCAGACATCA
GCTCCTGGTTCAACGAGGCCATTGACTTCATAGACTCCATCAAGAATGCTGGAGGAAGGGTGTTTGTCCA
CTGCCAGGCAGGCATTTCCCGGTCAGCCACCATC**TGCCTTGCTTACCTTATGA**GGACTAATCGAGTCAAG
(siRNA target 2)

CTGGACGAGGCCTTTGAGTTTGTGAAGCAGAGGCGAAGCATCATCTCTCCCAACTTCAGCTTCATGGGCC
AGCTGCTGCAGTTTGAGTCCCAGGTGCTGGCTCCGCACTGTTCGGCAGAGGCTGGGAGCCCCGCCATGGC
TGTGCTCGACCGAGGCACCT**CCACCACCACCGTGTTCAACTT**CCCCGTCTCCATCCCTGTCCACTCCACG
(siRNA target 3)

AACAGTGCGCTGAGCTACCTTCAGAGCCCCATTACGACCTCTCCCAGCTGC**TGA**

C



D

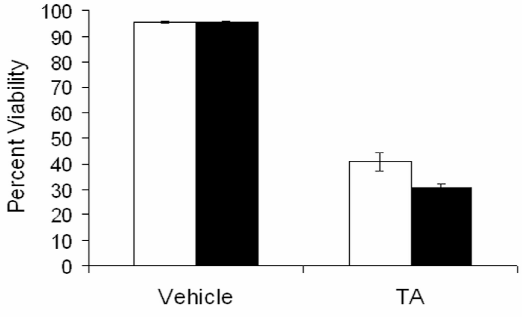
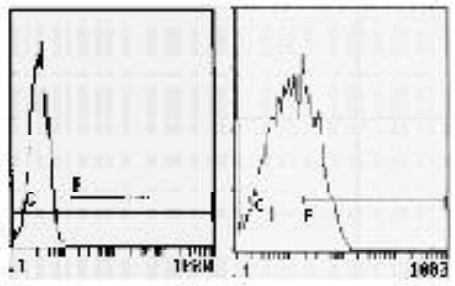
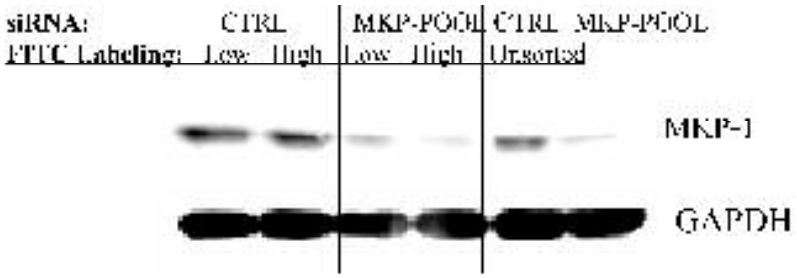


Figure 4

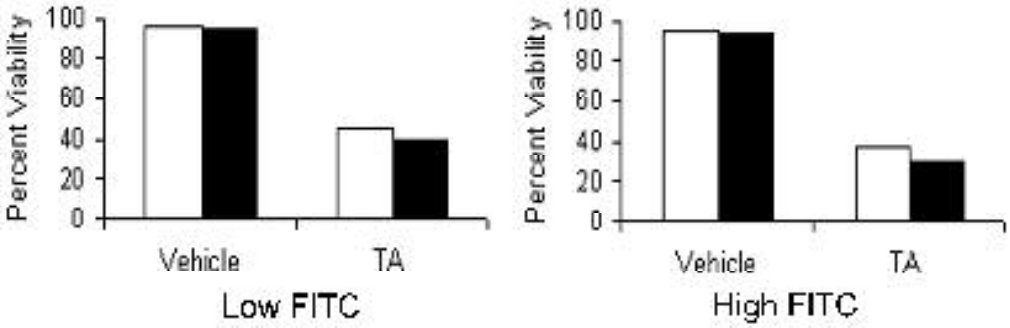
A



B


siRNA: CTRL MKP-POOL CTRL MKP-POOL
FITC Labeling: Low High Low High Unsorted
MKP-1
GAPDH

C


Percent Viability
100
80
60
40
20
0
Vehicle
TA
Low FITC
Percent Viability
100
80
60
40
20
0
Vehicle
TA
High FITC