Elevated Expression of Mitogen-Activated Protein Kinase Phosphatase 3 in Breast Tumors: A Mechanism of Tamoxifen Resistance


Abstract
Antiestrogen resistance is a major clinical problem in the treatment of breast cancer. Altered growth factor signaling with estrogen receptor (ER)-α is associated with the development of resistance. Gene expression profiling was used to identify mitogen-activated protein kinase (MAPK) phosphatase 3 (MKP3) whose expression was correlated with response to the antiestrogen tamoxifen in both patients and in vitro–derived cell line models. Overexpression of MKP3 rendered ER-α-positive breast cancer cells resistant to the growth-inhibitory effects of tamoxifen and enhanced tamoxifen agonist activity in endometrial cells. MKP3 overexpression was associated with lower levels of activated extracellular signal-regulated kinase 1/2 phosphorylation in the presence of estrogen but that estrogen deprivation and tamoxifen treatment decreased MKP3 phosphorylation activity, leading to an up-regulation of pERK1/2 MAPK, phosphorylated Ser118-ER-α, and cyclin D1. The MAPK/ERK kinase inhibitor PD98059 blocked tamoxifen-resistant growth. Accumulation of reactive oxygen species was observed with tamoxifen treatment of MKP3-overexpressing cells, and antioxidant treatment increased MKP3 phosphatase activity, thereby blocking resistance. Furthermore, PD98059 increased the levels of phosphorylated c-Jun NH2-terminal kinase (JNK) in tamoxifen-treated MKP3-overexpressing cells, suggesting an interaction between MKP3 levels, activation of ERK1/2 MAPK, and JNK signaling in human breast cancer cells. MKP3 represents a novel mechanism of resistance, which may be a potential biomarker for the use of ERK1/2 and/or JNK inhibitors in combination with tamoxifen treatment.

Introduction
The steroid hormone estrogen can stimulate breast tumor growth, and agents that inhibit estrogen synthesis or antiestrogens, such as tamoxifen, which block its receptor, are the standard therapies offered to women with estrogen receptor (ER)-α–positive cancer. In many cases, however, these therapies eventually fail. One current hypothesis is that ER-α remains essential to the problem of resistance due to its molecular cross-talk with growth factors and/or downstream intracellular signaling molecules.

There is also a growing body of evidence implicating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) MAPKs in the growth factor phosphorylation cascade and its interaction with ER-α signaling in TR (1). Indeed, phosphorylation of ERK1/2 MAPK has been associated with a poorer quality of response to tamoxifen in breast cancer patients (2).

We know that ER-α can be phosphorylated by activated MAPK, resulting in ligand-independent transcriptional activity (3). An emerging area of research in MAPK signaling is the role of specific protein phosphatases in the control of MAPK activation and their role in specific biological responses. MAPK phosphatase 3 (MKP3; also called dual-specificity phosphatase 6 and Pyst1) is a member of a phosphatase family that inactivates MAPK function (reviewed in ref. 4). MKP3 is in a regulatory feedback loop with ERK1/2 MAPK (5, 6). How MKP3 affects ER-α and MAPK function in breast cancer cells and how a resultant feedback loop affects the generation of TR are the subject of the present study.

Materials and Methods

Reagents, hormones, and antibodies. 17β-Estradiol (E2) and 4-hydroxytamoxifen (4-OHT) were from Sigma (St. Louis, MO). ICI 182,780 was obtained from AstraZeneca (Macclesfield, United Kingdom). The MAPK/ERK kinase 1/2 (MEK1/2) inhibitor PD98059 and reduced glutathione (GSH) were from Calbiochem (La Jolla, CA). 2′,7′-Dichlorodihydrofluorescein (DCF) diacetate (H2DCF-DA) was obtained from Molecular Probes (Eugene, OR). p-Nitrophenyl phosphate (pNPP) and pNPP assay buffer were obtained from Upstate Biotechnology (Charlottesville, VA).

Construction of small interfering RNA plasmids. MKP3 small interfering RNA (siRNA) oligonucleotides containing the sequence GCTCAATCTGTCAAGAAC (corresponding to the MKP3 coding region from 1,283 to 1,301) and MKP1 siRNA oligonucleotides containing the sequence GGAGGATACGAGCGTTTTT (corresponding to the MKP1 coding region from 615 to 623) or GCATCATCTCTCCCAACTT (corresponding to the MKP1 coding region from 1,126 to 1,144) were inserted into RNAi-Ready pSiren-Retro-Q vector (Clontech, Palo Alto, CA). The control RNA interference oligonucleotide was purchased from Clontech.

Tumor specimens and expression microarray analysis. A cohort of frozen breast tumor specimens from nine patients who received adjuvant tamoxifen was selected from the tumor bank of Breast Center, Baylor College of Medicine (Houston, TX) for use in the RNA analyses. This study was approved by the Baylor College of Medicine Institutional Review Board in accordance with federal human research study guidelines. Within this cohort, metastatic tumors from five patients who developed their recurrent lesions within 1 to 11 months while undergoing tamoxifen treatment (tamoxifen resistant) and four primary tumors that were recurrent lesions within 1 to 10 months while undergoing tamoxifen treatment were collected at the time of initial diagnosis from patients who remained disease-free for 93 to 123 months with a median follow-up of 106 months (tamoxifen sensitive) were included.
Total cellular RNA was extracted from 100 mg pulverized tumor powder using Trizol reagent (Invitrogen, Carlsbad, CA) followed by QIAGEN RNeasy column purification (QIAGEN, Valencia, CA). cRNA was hybridized onto Affymetrix (Santa Clara, CA) HG-U95A GeneChips using recommended procedures for hybridization, washing, and staining with streptavidin-phycocerythrin. The GeneChips were scanned, and feature quantitation was done using MASS5.0 (Affymetrix). Data were normalized using the invariant set method and modeled to estimate expression using the perfect-match-only model-based expression index as implemented in dChip (7). Class comparisons were done using Biometrics Research Branch (BBB) array tools developed by Drs. Richard Simon and Amy Peng® and t tests were done with randomized variance modeling (8).

Quantitative reverse transcription-PCR. Real-time quantitative reverse transcription-PCR (RT-PCR) was done using the 7700 Sequence Detector (Applied Biosystems) following the recommended guidelines for MKP3 were developed using Primer Express software version 1.0 for Macintosh (Applied Biosystems) following the recommended guidelines based on sequences from Genbank. Primer sequences for human MKP3 were forward primer (GTGCGACCGACACAGTG), reverse primer (CCC-GTATTCCTGTTCCAG), and human MKP3 RNA probe sequence (TCTAC-GACGAGAGCAGCAGGC; assay length, 65 nucleotides). Primer sequences for human 18S rRNA were forward primer (CGCGCTAATTTGACTCAAC), reverse primer (ATCAATCTGTCAATCCTGTCC), and human 18S rRNA probe sequence (AAACCTCACCCGGCCCG; assay length, 68 nucleotides). Synthetic DNA oligonucleotides used as standards encompassed the entire 5’ to 3’ amplicon for the assay (Biosource, Camarillo, CA). Final normalized data are presented as the ratio of MKP3 to 18S rRNA.

Cells and stable transfection. The paired TR breast cancer cell lines, MCF-7/WT and MCF-7/TR or T47D/WT and T47D/TR, have been described previously (9). Ishikawa endometrial cancer cells were kindly provided by Dr. Carolyn Smith (Baylor College of Medicine, Houston, TX). MCF-7 cells were originally obtained from Dr. Benita Katzenellenbogen (University of Illinois-Urbana, Urbana, IL) but have been maintained in our laboratory for >15 years (10). These MCF-7 cells were maintained in MEM (Invitrogen) supplemented with 5% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO), 200 units/mL penicillin, and 200 μg/mL streptomycin. Cells were incubated at 37°C in 5% CO2. To generate MCF-7 and Ishikawa cells stably overexpressing MKP3, pcDNA3-MKP3-V5 (11) or empty vector (pcDNA3-His-V5, Invitrogen) was transfected as described (10), and positive clones were identified using immunoblot analysis with an anti-V5 antibody (1:5,000 dilution). Ishikawa cells were transfected with pcDNA3-MKP3-V5, empty vector (pcDNA3-His-V5), MKP3 siRNA vector, MKP1 siRNA vectors, or siRNA control vectors, and transfectants were selected with either 800 μg/mL G418 or 1 μg/μL puromycin.

Reactive oxygen species measurements. MCF3- and Tamoxifen Resistance

**Figure 1.** A, scatter plot of MKP3 RNA expression in nine tumors. Four tumors were tamoxifen sensitive (Tam Sens) and five were tamoxifen resistant (Tam Res). Individual RNA values in arbitrary units. Parametric P = 0.006; permutation P adjusted for multiple comparisons = 0.09. B, relative MKP3 mRNA levels were determined using quantitative RT-PCR in various cell lines. Relative levels were normalized to the levels of 18S rRNA in each RNA sample. C, relative MKP3 mRNA levels of MCF-7 cells treated with estrogen (E2; 10 nmol/L) or tamoxifen (Tam; 100 nmol/L) for 24 hours. Relative MKP3 RNA levels were normalized to the levels of 18S rRNA in each RNA sample.
**Figure 2.**

**A,** immunoblot (IB) analysis of two vector control transfected MCF-7 clones (Con 1 and Con 2) and two MKP3-transfected clones (MKP3-1 and MKP3-2). A V5 epitope tag was included in the expression vector and used for visualization of MKP3 levels. **B,** relative MKP3 levels in Con 1 and MKP3-1 transfectants were determined using quantitative RT-PCR. Levels were normalized to 18S rRNA in each sample. **C,** anchorage-independent colony formation assay with the above transfectants in the presence of 1 nmol/L estrogen, 100 nmol/L tamoxifen, 100 nmol/L tamoxifen plus 10 nmol/L ICI 182,780 (ICI+Tam), or 100 nmol/L tamoxifen plus 20 nmol/L PD98059 (PD+Tam). The mean colony number was assayed after growth under the respective treatment conditions for 14 days, and statistical significance was assessed using a two-tailed Student's t test. **C** and **D,** top, immunoblot analysis of vector control transfected Ishikawa cells (Con) and MKP3-transfected Ishikawa cell (MKP3). A V5 epitope tag was included in the expression vector and used for visualization of MKP3 levels. Anchorage-independent colony formation assay with the Ishikawa transfectants in the presence of ethanol (C), estrogen, or tamoxifen. The mean colony number was assayed after growth under the respective treatment conditions for 14 days, and statistical significance was assessed using a two-tailed Student's t test. **E,** knockdown of MKP3 in Ishikawa cells was done using a siRNA to MKP3. Top, relative MKP3 levels in control siRNA (Ctrl Si) and MKP3 siRNA (MKP3 Si) transfectants were determined using quantitative RT-PCR; bottom, colony formation assays were done in the presence of ethanol vehicle, 1 nmol/L E2, and 100 nmol/L tamoxifen. **F** and **G,** MCF-7 vector Con 1 and MCF-7-MKP3-2 cells were injected into athymic nude mice supplemented with E2 for 21 days and then randomly assigned to either continue E2 treatment (F) or have their E2 pellets removed and treated with tamoxifen (G). There were six animals each for the E2-treated vector control and MKP3-overexpressing cells, four animals for tamoxifen-treated vector control, and five animals for the tamoxifen-treated MKP3-overexpressing cells.
emission wavelength, 530 nm) was measured using a fluorimeter in a 96-well plate (300 μL/well) and normalized by subtracting fluorescence of DMSO-incubated samples. The experiment was done in triplicate.

**Immunoprecipitation and phosphatase assays.** MKP3-overexpressing and control transfected MCF-7 cells were treated as described above with ethanol, E2, or tamoxifen, with or without GSH, and lysed in buffer containing 20 mmol/L Tris-HCl (pH 7), 150 mmol/L NaCl, 1% Triton X-100, 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1% β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. A phosphatase reaction was done as described (5), and the nonenzymatic hydrolysis of the substrate was corrected by measuring the control vector-transfected immunoprecipitates. The amount of product p-nitrophenol was determined from the absorbance at 405 nm. The phosphatase assays were done in triplicate (n = 3 separate experiments).

**Xenograft studies.** MCF-7 vector control and MKP3-transfected cells were established as xenografts in ovariectomized 5- to 6-week-old BALB/c athymic nude mice (Harlan Sprague-Dawley, Madison, WI) supplemented with 0.25 mg, 21-day release E2 pellets (Innovative Research, Sarasota, FL) by inoculating the mice s.c. with 5 × 10^6 cells as previously described (12). Two vector control animals and one MKP3-injected animal failed to develop measurable tumors. These were eliminated from the analysis. When tumors reached ~250 mm^3 (~i.e., in 21 days), animals were randomly allocated to continue E2 (n = 6 per group) or estrogen withdrawal plus tamoxifen (n = 4-5 per group; 500 μg/animal given s.c. in peanut oil, 5 days weekly) for another 30 days. Tumor growth was assessed, and tumor volumes were measured as described previously (12). Tumor growth curves were fitted to an exponential growth model and tested for whether the growth rates were different for the cell line groups. This was implemented by log transforming the tumor sizes and using a mixed linear model with fixed main effects for subject within subject. Analyses were carried out using SAS version 9.1 (SAS Institute, Inc., Cary, NC).

**Anchorage-independent growth assays.** Cells were starved for 2 days in phenol red–free MEM supplemented with 5% FBS, 6 ng/mL insulin, 200 units/mL penicillin, and 200 μg/mL streptomycin. Soft agar assays were done in six-well plates. After 14 days, the colonies were fixed, and the colony number of colonies with >50 cells from quadruplicate assays was counted. Data are the mean colony number of four plates and representative of two independent experiments analyzed for statistical significance (P > 0.05) using a two-tailed Student's t test or one-way ANOVA test.

**Results**

**MKP3 overexpression and TR.** To identify genes whose expression was associated with the development of TR, we compared primary tumors from patients that did not relapse after tamoxifen treatment despite 7 to 10 years of follow-up with metastatic tumors from patients that progressed during adjuvant tamoxifen treatment using expression microarray analysis. Our rationale was that we might be more likely to identify genes whose differential expression was involved in the initiation of TR and whose expression might itself be affected by tamoxifen treatment with our selection criteria. Gene expression analysis of our cohort identified MKP3 as being more highly expressed in the tamoxifen-resistant tumor group compared with the tamoxifen-sensitive tumor group (P = 0.006; Fig. 1). The median level of MKP3 RNA was 2.5-fold higher in the tamoxifen-resistant group. Quantitative RT-PCR assay was also used to confirm differential MKP3 RNA expression levels in these tumors. The correlations between Affymetrix MKP3 RNA expression and quantitative RT-PCR levels were very high (r = 0.83; P = 0.0053; data not shown).

We next measured the endogenous levels of MKP3 RNA in two in vitro–derived models of TR using quantitative RT-PCR. Both models were derived by long-term culture in tamoxifen-containing medium (13). The levels of MKP3 RNA were 19- and 8-fold higher in MCF-7/TR and T47D/TR cells, respectively, compared with the parental WT control cell lines (Fig. 1B). In addition, we measured MKP3 RNA levels in the Ishikawa endometrial cancer cell line, where tamoxifen is known to act as an agonist (9). We found very high levels of MKP3 RNA in the Ishikawa line compared with that of breast cancer cell lines. Finally, we investigated whether estrogen or tamoxifen treatment of MCF-7 cells modulated MKP3 RNA levels (Fig. 1C); however, neither treatment altered MKP3 expression. These data collectively suggest that MKP3 levels may be correlated with the TR phenotype in patients and cell lines.

We next generated several models of TR, first stably transfecting ER-α-positive MCF-7 cells with either empty vector plasmid as a control or a plasmid encoding MKP3. Cells were drug selected for plasmid expression and cloned by limiting dilution, and several resulting clones were screened for expression of MKP3 using an anti-V5 antibody to the V5 tag introduced at the COOH terminal of MKP3 (11). An immunoblot analysis of two MCF-7 control clones (Fig. 2A, Con 1 and Con 2) and two MCF-7-MKP3-overexpressing clones (Fig. 2A, MKP3-1 and MKP3-2) showed similar ectopic expression of MKP3 in the stable transfectants. The relative MKP3 RNA levels in clone MKP3-1 were ~15-fold higher than RNA levels in the vector-transfected Con 1 cells (Fig. 2B), a difference that was similar to that found in the MCF-7/TR cells (Fig. 1B).

The growth characteristics of the MCF-7 stable MKP3 transfectants under different hormonal conditions were investigated using anchorage-independent soft agar assays (Fig. 2C). As expected, MCF-7 vector control cells exhibited low colony formation in the absence of E2 (Con 1 and Con 2, control treatment). Treatment with E2 increased the number of colonies, and tamoxifen treatment reduced the number of colonies of control cells. Ectopic expression of MKP3 reduced the number of colonies formed in soft agar under estrogen-deprived conditions (MKP3-1 and MKP3-2, control) compared with the control transfectants (P = 0.0037); however, estrogen-induced colony formation of the MCF-7-MKP3 clones was equivalent to those obtained with vector control cells. In contrast, the number of soft agar colonies formed in the presence of tamoxifen was increased ~10-fold in the two MKP3-overexpressing transfectants compared with vector control cells (P = 0.001). Both steroidal ER-α antagonist ICI 182,780 and MEK1/2 inhibitor PD98059 completely blocked tamoxifen-induced colony formation in vector control and MKP3-transfected cells. These results suggest that whereas the basal growth of MCF-7-MKP3 cells may be negatively affected concomitant with MKP3 overexpression, tamoxifen treatment either relieves this growth suppression or acts as an agonist to increase the colony-forming efficiency of MKP3-overexpressing breast cancer cells.

We also engineered TR Ishikawa cells to stably overexpress V5-MKP3 or used a siRNA to knockdown MKP3 in this cell line (Fig. 2D and E, respectively). In Ishikawa cells, increased levels of MKP3 did not affect control colony growth, but significantly enhanced growth was seen in the estrogen- and tamoxifen-treated MKP3 transfectants (P = 0.03 and 0.001, respectively; Fig. 2D, bottom). Conversely, when relative MKP3 RNA levels were decreased by ~45% using siRNA (P < 0.05; Fig. 2E, top), there was a decrease in tamoxifen colony growth in the tamoxifen-treated group (P = 0.02; Fig. 2E, bottom). MKP3 siRNA did not affect estrogen-stimulated growth, however, in Ishikawa cells.

To analyze the endocrine sensitivity of MKP3-overexpressing cells, we next examined their ability to form tumors in athymic nude mice. We established xenografts in ovariectomized mice supplemented with estrogen for 21 days. Mice were then randomized to
Figure 3. A, immunoblot analysis of two vector controls (Con 1 and Con 2) and two MKP3-overexpressing transfectants (MKP3-1 and MKP3-2) treated for 2 hours with ethanol vehicle (C), 100 nmol/L E2 (E), or 100 nmol/L tamoxifen (T). Immunoblots were stained with antibodies to V5 to show MKP3 levels or to pMAPK, total MAPK, pS118 ERα, total ERα antibodies, and anti-Rho GDI as a loading control. B, densitometric scan of the immunoblot in (A) showing levels of pMAPK normalized to Rho GDI levels. C, densitometric scan of the immunoblot in (A) showing levels of pS118 normalized to Rho GDI levels. D, an immunoblot analysis of MKP3 Con 1 and MKP3-2 transfectants treated with vehicle, E2, or tamoxifen for 2 hours in the presence (+) or absence (−) of 20 nmol/L PD98059. Immunoblots were stained with antibodies to V5, pMAPK and pS118 ERα, total MAPK and ERα. E, an immunoblot analysis of MKP3 Con 1 and MKP3-2 transfectants treated with vehicle, E2, or tamoxifen for 2 hours in the presence or absence of PD98059. Immunoblots were stained with antibodies to MKP1, pJNK, and total JNK. F, phosphatase assay using pNPP as a substrate using extracts prepared from MKP3 vector 1 and MKP3-2 cells treated for 2 hours with vehicle, E2, or tamoxifen. The nonenzymatic hydrolysis of the substrate was corrected (absorbance at 405 nm) by subtracting the control vector-transfected immunoprecipitates from MKP3 levels and expressed as MKP3 vector. Phosphatase assays were done in triplicate (n = 3 separate experiments). G, MKP3/MAPK binding assay was done with MKP3 Con 1 and MKP3-2 transfectants treated for 2 hours with ethanol vehicle, E2, or tamoxifen. Arrows, pre-V5 and post-V5 immunoprecipitated extracts (Pre-IP and Post-IP) were immunoblotted with antibodies to ERK2 and V5 to show levels of MAPK and MKP3, respectively. Immunoglobulin heavy chain (HC) and light chain (LC). The specificity of the V5 and ERK2 antibodies as in the previous figures.
continue estrogen treatment or subjected to estrogen withdrawal plus tamoxifen (Fig. 2F and G), and tumor growth was monitored over time. The main questions we addressed were whether MKP3-expressing tumors grew differently in the presence of estrogen and whether they responded similarly to tamoxifen treatment. This was examined by fitting the data in an exponential growth model and testing whether growth rates were different between the groups.

The analyses were done separately for estrogen-treated (Fig. 2F) and tamoxifen-treated animals (Fig. 2G). We found that although MKP3-expressing tumors were slightly larger in size at 21 days compared with control tumors, there was no difference in the estrogen-stimulated exponential growth rate of the MCF-7 vector control-transfected and MKP3-overexpressing cells (P = 0.52). In contrast, the growth rate of MKP3-expressing cells was significantly increased in tamoxifen-treated tumors (P = 0.047). These in vivo data thus recapitulate what we observed in the soft agar assay in MCF-7 and Ishikawa cells and with our discovery of higher levels of MKP3 RNA in tamoxifen-resistant breast tumors.

**Cross-talk between MKP3, ERK1/2 MAPKs, and ER-α signaling pathways.** Because there are conflicting reports about whether estrogen stimulation activates MAPK in MCF-7 breast cancer cells (14, 15), we next assessed the effects of estrogen and tamoxifen on the activation of MAPK in MCF-7 vector controls and MKP3 transfectants (Fig. 3A, Con 1, Con 2, MKP3-1, and MKP3-2). Cells were maintained under estrogen-depleted conditions for 2 days and treated for 2 hours with either estrogen or tamoxifen, and cellular extracts were prepared. Immunoblot analysis with anti-V5 was used to detect ectopic MKP3 expression in the two MKP3 transfectants (Fig. 3A, top). Immunoblotting with an antibody to Rho GDI was used as a loading control in these experiments. In vector transfectants, pMAPK was not induced with either short-term (2-30 minutes; data not shown) or 2-hour hormonal treatment (Fig. 3). In contrast, higher levels of pMAPK were seen in the control and tamoxifen-treated MKP3-overexpressing cells compared with that seen in the estrogen-treated cells (Fig. 3A and B). These results were rather paradoxical, in that we predicted to find lower levels of pMAPK in cells concomitant with MKP3 overexpression but instead observed hormonal influences on the ability of overexpressed MKP3 to modulate pMAPK. Levels of total MAPK did not seem to be affected by MKP3 overexpression. The highest activation of MAPK was observed in the tamoxifen-treated MKP3-overexpressing cells (Fig. 3B).

ER-α is a downstream target of pMAPK in breast cancer cells (3). We observed that levels of pS118 ER-α were highly induced with tamoxifen treatment in the MKP3 transfectants (Fig. 3A and C). The question is whether this dramatic induction with tamoxifen was coupled with changes in the levels of total ER-α. It has been shown that ER-α undergoes estrogen-dependent down-regulation via the proteosomal degradation pathway (16). Down-regulation of ER-α protein was observed in both control transfected and MKP3-overexpressing cells in the presence of estrogen. Similarly, it has been reported that tamoxifen stabilizes the receptor (17), and this stabilization was observed in both groups of transfectants. Thus, although ER-α hormonal regulation seemed to proceed normally, higher levels of pER-α were induced by tamoxifen in the MKP3-overexpressing cells, which was not associated with higher levels of total ER-α compared with control-treated cells.

We next tested the effect of the MEK1/2 inhibitor PD98059 on the ability of tamoxifen to induce phosphorylation of MAPK and ER-α in MKP3-overexpressing cells (Fig. 3D). We found that PK98065 effectively inhibited the increase in pMAPK and pS118 ER-α in control and tamoxifen-treated MKP3-2 cells. The MEK inhibitor also blocked activation of MAPK in vector control cells under all the treatment conditions. This result suggests that effects of tamoxifen in MKP3-overexpressing cells involve the MEK/ERK/MAPK signaling pathway.

As a control, we examined the levels of MKP1, which is also a major negative regulator of ERK1/2 but is more specific for JNK and p38, but did not see changes in the levels of MKP1 concomitant with MKP3 overexpression (Fig. 3E). We observed an increase in pJNK in MKP3 transfectants under all hormonal conditions. When vector control cells were treated with the MEK1/2 inhibitor, pJNK was elevated; similarly, we found higher levels of JNK activation in MKP3 transfectants in the presence of the inhibitor. These results were not expected and suggest that the MEK inhibitor might affect the decreased levels or activity of another MAPK, which could up-regulate pJNK. This possibility is currently under study but suggests that treatment of breast cancer cells with MEK inhibitors might concomitantly increase JNK signaling in cells, a consequence that might have therapeutic relevance in resistant disease necessitating combination therapy with signal transduction inhibitors.

We next questioned whether changes in either MKP3 phosphatase activity or binding between MKP3 and ERK2 might underlie the changes in pMAPK. Using the artificial substrate pNPP to measure endogenous phosphatase activity in MKP3 and vector control cells, we found an inverse relationship between measured MKP3 phosphatase activity and pMAPK levels in the MKP3 transfectants compared with the vector control. As shown in Fig. 3F, levels of MKP3 phosphatase activity were highest in the estrogen-treated cells, lowest in tamoxifen-treated cells, and intermediate in the control-treated cells. These findings in activity were inversely related to the levels of pMAPK in MKP3-overexpressing cells (Fig. 3A). These results suggest that tamoxifen might influence the ability of MKP3 to negatively regulate MAPK. We did not, however, observe changes in the ability of MKP3 to bind to MAPK as measured by immunoprecipitation and immunoblot analyses (Fig. 3G). When the levels of MKP3-bound ERK2 were compared between the different hormonal treatments, no differences were detected (Fig. 3G, C, E, T, IP: V5). Control levels of MKP3 and ERK2 were also examined in the preimmunoprecipitation and postimmunoprecipitation extracts to ensure that adequate pulldown of immunoprecipitated proteins were obtained; no differences were detected. Thus, hormonal modulation of MKP3 phosphatase activity, but not changes in the ability of MKP3 to interact with MAPK, may be a determinant of MAPK activation in breast cancer cells.

**ER-regulated gene transcription.** CCND1 is an estrogen-induced protein and a downstream marker of activated MAPK signaling in breast cancer cells (18). High levels of CCND1 were found in tamoxifen-treated MKP3-overexpressing cells using immunoblot analysis (Fig. 4A, top). Densitometric scanning of the CCND1 immunoblot is shown in Fig. 4B. Thus, the tamoxifen-stimulated soft agar growth and xenograft growth we observed concomitant with MKP3 overexpression was coupled with the induction of the MAPK downstream marker of proliferation, CCND1. These results suggest that the tamoxifen-resistant phenotype of the MKP3-overexpressing cells is associated with a biomarker of clinical resistance, elevated CCND1, downstream of MAPK activation. We also evaluated the RNA levels of...
CCND1 and c-fos, which is a known downstream marker of MAPK activation, in our TR patient samples and found significant correlations between CCND1 ($r = 0.83; P = 0.0053$), c-fos ($r = 0.74; P = 0.01$), and MKP3 (data not shown).

We have shown previously that prolonged treatment of MCF-7 cells grown as xenografted tumors in athymic nude mice results in oxidative stress (19), and it is well known that tamoxifen can induce oxidative stress and increases ROS (20). Furthermore, during oxidative stress, MKP3 phosphatase is inactivated in cortical neurons (21). To explore mechanisms associated with MKP3 TR, we therefore measured ROS using a fluorogenic probe in MKP3-overexpressing MCF-7 cells after hormonal treatments (Fig. 5A). Levels of ROS were significantly higher in both control (serum-deprived) and tamoxifen-treated cells but lowest in estrogen-treated cells ($P = 0.0029$, one-way ANOVA). Furthermore, treatment with the antioxidant GSH increased MKP3 phosphatase activity in control-treated ($P = 0.026$) and tamoxifen-treated ($P = 0.0012$) MKP3-overexpressing cells (Fig. 5B) but did not affect total levels of exogenous MKP3 (Fig. 5C). Treatment with GSH also effectively inhibited TR growth in soft agar but did not affect either control- or estrogen-induced growth (Fig. 5D). In agreement with the reversal of resistance after treatment with the antioxidant GSH, we observed inhibition of activated MAPK and reduction in pS118 ER-α levels with combined GSH and tamoxifen treatment of MKP3-2 transfectants (Fig. 3F). These combined results suggest that increases in ROS, which can be reversed with an antioxidant, may underlie the resistant phenotype in our model and, furthermore, that MKP3 activity and downstream signaling in TR cells are sensitive to changes in intracellular redox status in breast cancer cells.

MKP3 is a member of a large family of MAPK phosphatases with different specificities, so we next explored whether this identified TR mechanism extended to the prototypical dual-specificity phosphatase MKP1. This is of interest because MKP1 activity can be modulated by mitogens and oxidative stress (4). Using two siRNAs to MKP1, we were able to effectively knockdown MKP1 levels in Ishikawa cells (Fig. 6A). Reduction of MKP1 levels in the two MKP1 siRNA clones si615 and si126 resulted in increased TR growth of Ishikawa cells in soft agar compared with a control siRNA clone ($P = 0.0031$; Fig. 6B). Levels of pMAPK were elevated after both estrogen and tamoxifen treatment in MKP1 siRNA Ishikawa clones (Fig. 6C). Thus, enhanced tamoxifen activation of MAPK was associated with decreased MKP1 levels, but because MKP1 is at least as effective in phosphorylating JNK and p38 as MAPK, the precise role of MKP1 in TR remains to be elucidated. We conclude that activation of MAPK in the presence of tamoxifen is important for the resistant phenotype associated with MKPs.

Discussion

Tamoxifen has been the most frequently prescribed antiestrogen for the treatment of women with early-stage and metastatic ER-α-positive breast cancer. Although many patients will initially benefit from tamoxifen treatment, the emergence of resistance is a major clinical problem. It has been hypothesized that growth factor receptor pathways might affect ER-α activity and, hence, tamoxifen effectiveness. Increased dependence on MAPK signaling has been shown previously to be important for both TR and adaptive resistance to estrogen deprivation in MCF-7 cells (22, 23). We identified MKP3, a negative regulator of MAPK, as being expressed at higher levels in tamoxifen-resistant metastatic lesions and has been shown in preclinical studies that its overexpression can confer tamoxifen-resistant growth of cells in vitro or as xenografts in athymic nude mice.

We used microarray expression profiling to identify genes associated with tamoxifen resistance in breast cancer patients as a means of exploring new regulatory mechanisms operative during the selective pressure of tamoxifen treatment. Recently, a two-gene expression tamoxifen prediction model was developed using microarray analysis comparing primary breast tumors from patients treated with adjuvant tamoxifen who remained disease-free with those patients who developed distant recurrence (24). Although these results have been challenged recently (25), microarray technologies have shown great promise in identifying molecular features of hormone responsiveness (26).

Our study differed in both experimental design and goal compared with the microarray study of Ma et al. (24). Although we used a similarly defined tamoxifen-sensitive group of primary tumors, we chose to compare these with metastatic lesions from patients who recurred while on tamoxifen, with the goal to identify gene candidates that we could then examine further for a mechanistic role in resistance. Neither Ma et al. (24) nor a similarly designed microarray study reported by Jansen et al. (26) found MKP3 RNA levels to be differentially associated with the outcome of tamoxifen-treated primary tumors. We attribute this difference to be due to either experimental design or diverse array platforms used between the studies. Interestingly, we found that the levels of pMAPK protein were highest in tamoxifen-treated cells and lowest in the presence of E2 when MCF-7 cells were engineered to overexpress MKP3. We found that MEK inhibition reversed tamoxifen-induced soft agar growth of MKP3-overexpressing MCF-7 cells, further implicating MAPK signaling in resistance in our model. These results suggest that activated MAPK remains a common component of tamoxifen-resistant growth in preclinical models.

We showed that MKP3 enzymatic activity was particularly sensitive to regulation by hormones, and we propose that its hormonal modulation may be an alternative and novel mechanism by which ERK1/2 can be activated and regulated in breast cancer cells. Mechanistically, our data show that this hormonal regulation is associated with alterations in the redox state of the cell with high ROS coincident with TR growth and low MKP3 activity. The use of an antioxidant to reverse resistance confirms the role of oxidative stress in the process. Several MKPs are induced following exposure to stress and/or growth factor stimulation (4); however, MKP3 has not been shown previously to be regulated by these stimuli. Recently, it has been shown that the activity of MKP3, rather than its expression, was downregulated by oxidative signals (21) possibly through oxidizing critical cysteine residues.

There is also evidence for multiple, temporally discrete pathways, which differentially regulate MAPK depending on the external stimulus. For instance, fibroblast growth factor-1– and heregulin β1–induced TR in MCF-7 cells is also associated with prolonged MAPK activation that is incompletely susceptible to MEK inhibitors (27). Our results suggest that tamoxifen increases ERK1/2 activity via the loss of MKP3 phosphatase activity, an alternative “off-off” mechanism of resistance, which remains sensitive to MEK inhibition. Therefore, patients with tamoxifen-resistant disease and elevated MKP3 may be markedly sensitive to MEK inhibitors or antioxidants.
ER-α expression is lost in a minority of recurrent metastatic lesions after tamoxifen treatment (28). Thus, our model is different from hyperactivation of the Raf1/MEK1 signaling pathway, which leads to a down-regulation of ER-α and potentially reflects the more common resistance mechanisms associated with continued ER-α expression in patients. We have shown that protein kinase A signaling induces ER-α S305 phosphorylation, which is coupled to acetylation at K303 within the hinge domain and estrogen sensitivity (29). This site is important for tamoxifen resistance (30) and expression of CCND1 (31). The S118 ER-α site is phosphorylated in response to epidermal growth factor signaling possibly via pMAPK (32) and by activated ERK1/2 in breast cancer cells (3). S118 ER-α was phosphorylated in response to tamoxifen in MKP3-overexpressing cells, which was associated with higher pMAPK levels. However, high levels of pS118 ER-α are associated with a better disease outcome in breast cancer patients treated with tamoxifen in one clinical study (33). Therefore, the usefulness of pS118 as a clinical marker of resistance requires further study.

We hypothesize that MKP3 may be a novel target of tamoxifen action. It is possible that breast tumors will compensate for chronic

Figure 4. A, immunoblot analysis of two vector control (Con 1 and Con 2) and two MKP3-overexpressing transfectants (MKP3-1 and MKP3-2) treated for 2 hours with ethanol vehicle, 100 nmol/L E2, or 100 nmol/L tamoxifen. Immunoblots were stained with antibodies to CCND1 and anti-Rho GDI as a loading control. B, densitometric scan of the immunoblot in (A) showing levels of CCND1 normalized to Rho GDI levels.

Figure 5. A, ROS levels in MKP3-2 MCF-7 cells treated for 2 hours with ethanol vehicle, 100 nmol/L E2, or 100 nmol/L tamoxifen were measured using the fluorogenic DCF. B, MKP3 phosphatase activity was examined in MKP3-2 and vector Con 1 control cells in the presence of hormonal treatments and GSH. C, Immunoblot of exogenous MKP3 levels in cells from (B) using an antibody to the V5 tag. D, anchorage-independent colony formation assay with the above transfectants in the presence of vehicle, 1 nmol/L E2, or 100 nmol/L tamoxifen F100 A100 mol/L GSH. The mean colony number was assayed after growth under the respective treatment conditions for 14 days. E, an immunoblot analysis of MKP3 Con 1 and MKP3-2 transfectants treated with vehicle, E2, or tamoxifen for 2 hours in the presence or absence of GSH. Immunoblots were stained with antibodies to pMAPK, pS118 ER-α, total ERK1/2, total ER-α, and V5 to detect exogenous MKP3.
activation of MAPK by up-regulation of phosphatases, such as MKP3, which control these pathways. The emergence of tamoxifen resistance may therefore involve the disruption of this regulatory compensatory loop by inactivation of MKP3 phosphatase activity, explaining the seemingly paradoxical up-regulation of MKP3 levels but down-regulation of its activity in tamoxifen-treated MKP3-overexpressing cells.

We also detected activation of JNK with MEK1 inhibitor treatment of MCF-7 cells, with further enhanced JNK phosphorylation noted in our MKP3-overexpressing cells. Whether down-regulation of other MKPs may be associated with this effect and whether combined MAPK inhibitors with tamoxifen treatment are efficacious in our model are currently under study in our laboratory. It is tempting to speculate that MKP3-expressing tumors might be sensitive to estrogen deprivation (aromatase inhibitor treatment), given our finding that there was limited growth of MKP3-overexpressing breast cancer cells in the absence of estrogen. In summary, this study suggests that MKP3 may be an attractive new diagnostic and potentially therapeutic target in breast cancer.

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References

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