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Partial Depletion of Intracellular ATP Mediates the Stress-Survival Function of the PCPH Oncoprotein[1]

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ABSTRACT

Promotion of cellular resistance to stressful stimuli, including ionizing radiation and chemotherapeutic drugs, contributes to the transforming activity of the *PCPH* oncogene. The mechanism of this action, however, has remained unknown. Consistent with its intrinsic ATP diphosphohydrolase activity, expression of the PCPH oncoprotein in cultured cells has now been shown to result in partial depletion of intracellular ATP and consequent inhibition of the c-JUN NH_2-terminal kinase-mediated stress signaling pathway. Supplementation of cells expressing the PCPH oncoprotein with exogenous ATP restored both stress-response signaling and sensitivity to cisplatin-induced apoptosis. In contrast, overexpression of the wild-type PCPH protein had a minimal effect on stress-induced signaling and on the cellular ATP content and did not protect cells from apoptosis. These results suggest that the PCPH oncoprotein confers resistance to stressors by reducing the cellular ATP concentration to levels below those required for optimal stress-induced signaling and apoptosis. Treatment with adenosine or nucleoside analogues may thus enhance the response to radiation or chemotherapy of tumors that express the *PCPH* oncogene.

INTRODUCTION

The *PCPH* oncogene was isolated from primary Syrian hamster embryo fibroblasts that had been treated with 3-methylcholanthrene. The *PCPH* proto-oncogene is conserved from yeast to humans and is expressed in many tissues and developmental stages. The expression of *PCPH* is frequently altered in human tumor cells and solid tumors (1). Furthermore, the characteristic pattern of PCPH polypeptides in human breast tumor cells is reproduced in a rat model of mammary carcinogenesis (2), suggesting a possible role for *PCPH* in human cancer development. We showed previously that, whereas ectopic expression of the PCPH oncoprotein (mt-PCPH[5]) increased cell survival after exposure to various stressful stimuli, including serum deprivation, hyperthermia, ionizing radiation, and chemotherapeutic drugs, expression of the wild-type protein provided only marginal protection against such stressors (3). The generalized nature of the survival-promoting effect of mt-PCPH suggested that this protein might alter signaling mediated by a central stress-response pathway.

We have now investigated the mechanism by which mt-PCPH protects cells against stress. We show that mt-PCPH negatively regulates the stress-response pathway mediated by JNK (or SAPK) and reduces the intracellular ATP concentration. Signaling by the JNK pathway was restored, and the resistance to cisplatin-induced apoptosis was reversed in cells expressing mt-PCPH by supplementation with exogenous ATP. Together with our recent observation that PCPH possesses ATP diphosphohydrolase activity (4), these results suggest that this protein promotes cell survival by depleting cells of ATP and thereby depriving protein kinases activated by stress of their phosphate donor.

MATERIALS AND METHODS

Reagents and General Methods. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics, including G418 (200 μg/ml) for stably transfected cell lines. Expression vectors included pcDNA3-*PCPH* and pcDNA3-*mt-PCPH*, containing full-length cDNAs for wild-type PCPH and mt-PCPH, respectively, under the transcriptional control of the cytomegalovirus early promoter; vectors containing the H-*ras*^{Val12} and v-*raf* oncogenes, under the control of the Rous sarcoma virus promoter; a vector encoding the constitutively active MEKK1 mutant ΔMEKK1 (5); and pAP1-Luc and pSRE-Luc luciferase reporter plasmids (Stratagene, La Jolla, CA). Luciferase activity was determined with an assay kit (Promega Corp., Madison, WI) and a Lumat LB9501 luminometer (Berthold; EG&G Wallac, Gaithersburg, MD). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Transfection Assays. For trans-activation experiments, cells were transiently transfected with 3 μg of pcDNA3-*mt-PCPH*, 1 μg of H-*ras*^{Val12}, v-*raf*, or ΔMEKK1 plasmids, and 0.7 μg of pAP1-Luc or pSRE-Luc with the use of Superfect (Qiagen, Valencia, CA). For treatment with EGF, transfected cells were incubated for 4 h in low-serum (0.1%) medium and then stimulated with EGF (100 ng/ml; Calbiochem, San Diego, CA) for up to 30 min. In cotransfection experiments, the total amount of DNA was kept constant by the addition of empty pcDNA3 vector. Each experiment was performed with triplicate cultures, which usually exhibited <10–12% variation; data are presented as mean ± SE of values from at least three experiments.

EMSA. EMSA analysis was performed with 5 μg of nuclear extract in a reaction mixture containing ~30,000 cpm of ^{32}P-labeled AP-1 consensus oligonucleotide (5′-CGCTTGATGAGTCAGCCGGGA-3′; Promega), 0.1 μg of poly(deoxyinosinic-deoxycytidylic acid), 40 mM HEPES (pH 7.0), 140 mM NaCl, 4 mM DTT, 0.01% NP40, BSA (100 μg/ml; Roche, Indianapolis, IN), and 4% Ficoll (Amersham Pharmacia Biotech, Piscataway, NJ). Protein-DNA complexes were resolved by electrophoresis on nondenaturing 6% polyacrylamide gels in Tris-borate-EDTA buffer and were visualized by autoradiography.

Immunoblot Analysis. Cells were lysed by sonication in a solution containing 20 mM Tris-HCl (pH 7.5), 20 mM *p*-nitrophenyl phosphate, 1 mM EGTA, 50 mM NaF, 50 μM sodium orthovanadate, and 5 mM benzamidine. Lysates (50 μg of protein) were fractionated by SDS-PAGE on 4–15% gradient gels (Bio-Rad, Hercules, CA) and then subjected to immunoblot analysis as described (1, 2). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL; Amersham Pharmacia Biotech). Primary antibodies included those to phospho-SEK1 (MKK4), phospho-JNK, phospho-ERK1 or -ERK2, phospho-p38, JNK, and p38 (New England Biolabs, Beverly, MA) as well as those to glyceraldehyde-3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD), enhanced GFP (Clontech, Palo Alto, CA), c-FOS (kindly provided by M. A. Avila, University of Navarra, Pamplona, Spain), and PCPH (367-10W; a polyclonal rabbit antiserum prepared with recombinant hamster wild-type PCPH; Ref. 1).

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[5] The abbreviations used are: mt-PCPH, PCPH oncoprotein; JNK, c-JUN NH_2-terminal kinase; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase kinase kinase; SRE, serum response element; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Determination of Cellular ATP Content. Cells were washed twice, and the total cellular ATP concentration was measured with a bioluminescence somatic cell assay system (Sigma), as recommended by the manufacturer.

Apoptosis Assays. Transfected cells were incubated overnight in medium containing 0.5% serum and then treated with cisplatin (10 μg/ml) for 24 h. Cells were fixed in 80% ethanol, stained with propidium iodide (50 μg/ml), and analyzed by flow cytometry. The percentage of apoptotic cells was determined by integrating the area of the peak corresponding to cells with hypodiploid DNA in histograms of DNA content. Cells were also stained with 4′,6-diamidino-2-phenylindole (0.5 μg/ml) and visualized by epifluorescence microscopy to detect nuclear changes typical of apoptosis.

RESULTS AND DISCUSSION

The PCPH Oncoprotein Inhibits Stress-Response Signaling. To evaluate whether mt-PCPH affects signaling by stress-response pathways, we subjected NIH 3T3 cells to transient transfection with vectors encoding mt-PCPH and a constitutively active mutant of MEKK1 (ΔMEKK1), the upstream kinase shared by the JNK and p38 stress signaling pathways (6). Transfection with the ΔMEKK1 vector alone resulted in activation of the downstream kinases SEK1 (MKK4) and JNK, whereas transfection with the mt-PCPH vector alone had little effect on these kinases (Fig. 1*A*). However, cotransfection with the ΔMEKK1 vector and pcDNA3-*mt-PCPH* resulted in marked inhibition by mt-PCPH of the activation of SEK1 and JNK induced by ΔMEKK1. The PCPH oncoprotein induced only slight inhibition of ΔMEKK1-induced p38 phosphorylation.

Activation of ERK1 or ERK2 by MEKK1 occurs as a result of cross-talk between mitogenic and stress signaling pathways (7). Moreover, we showed previously that mitogenic stimulation with an activated H-*ras* oncogene and expression of mt-PCPH cooperated in the induction of ERK1/2 activation (8). We therefore investigated whether mt-PCPH affected the activation of ERK1/2 by ΔMEKK1. Whereas transfection with pcDNA3-*mt-PCPH* alone had no effect on ERK1/2 phosphorylation, transfection with the ΔMEKK1 vector alone induced a 4.4-fold increase in this parameter. Coexpression of mt-PCPH, however, prevented ERK1/2 activation by ΔMEKK1 (Fig. 1*A*). The PCPH oncoprotein thus markedly inhibited the activation of JNK and ERK but had little effect on p38 activation, demonstrating pathway specificity in the action of this protein. The simultaneous inhibition of JNK and ERK by mt-PCPH may be of importance, given that the activation of both kinases is required for the induction of apoptosis by various stressors (9). These various effects of mt-PCPH were also observed in transfected monkey COS-7 and human 293T cells (data not shown), suggesting that the ability of this protein to inhibit the activation of JNK and ERK signaling by MEKK1 is not restricted to mouse NIH 3T3 cells.

We also examined the effect of mt-PCPH on stress signaling mediated by the activation of endogenous MEKK1. MEKK1 participates in the activation of ERK by EGF in COS-7 cells (10). Consistent with this previous observation, EGF induced marked activation of ERK1/2 (5.7-fold) and SEK1 (2.4-fold) in COS-7 cells transiently transfected with pcDNA3. However, EGF had virtually no effect on the activation ERK1/2 (1.3-fold) and SEK1 (0.9-fold) in cells expressing mt-PCPH. Expression of mt-PCPH thus inhibited signaling by endogenous MEKK1.

The PCPH Oncoprotein Inhibits AP-1- and SRE-mediated Trans-activation and c-FOS Induction. We examined trans-activation mediated by AP-1 and by the SRE as well as the induction of c-FOS expression as end points to determine the effects of mt-PCPH on gene expression in response to stress signaling (11). NIH 3T3 cells were transiently cotransfected with pcDNA3-*mt-PCPH*, the ΔMEKK1 vector, and reporter plasmids in which luciferase expression is regulated by AP-1 or by the SRE. Expression of mt-PCPH inhibited the increase in AP-1-mediated trans-activation induced by ΔMEKK1 (Fig. 1*B*). Trans-activation of the SRE-luciferase construct by ΔMEKK1 was also inhibited by mt-PCPH, although to a lesser extent than was AP-1-mediated trans-activation (29 *versus* 52% inhibition). Qualitatively similar results were obtained with transfected COS-7 and 293T cells (data not shown). MEKK1 induces c-*Fos* expression through the SRE (12). Expression of mt-PCPH inhibited by ~60% the increase in the abundance of c-FOS induced by ΔMEKK1 in transfected NIH 3T3 cells (Fig. 1*A*, *bottom*).

TPA activates both AP-1 (13) and transcription factors that bind to the SRE (12). Moreover, through its interaction with specific isoforms of protein kinase C, TPA activates several kinases in stress signaling pathways (12). To examine the possible effect of mt-PCPH on TPA-induced trans-activation mediated by AP-1 or the SRE, we subjected NIH 3T3 cells to transient transfection with pAP1-Luc or pSRE-Luc together with pcDNA3 or pcDNA3-*mt-PCPH*. Cells were then treated with TPA (100 nM) for 24 h before determination of luciferase activity. Expression of mt-PCPH inhibited TPA-induced trans-activation mediated by AP-1 (by 58%) or by the SRE (by 56%; Table 1). Similar results were obtained with COS-7 cells expressing mt-PCPH (65% inhibition for AP-1, 44% inhibition for the SRE).

The possible effect of mt-PCPH on the DNA-binding activity of AP-1 was examined by EMSA with nuclear extracts prepared from NIH 3T3 cells after cotransfection, both with vectors encoding the oncoproteins RAS^{Val12} or v-RAF as activators of AP-1 and with pcDNA3-*mt-PCPH* or pcDNA3. Nuclear extracts prepared from control cells exhibited a relatively high basal extent of protein binding to the AP-1 consensus oligonucleotide, and this activity was increased in nuclear extracts from cells expressing the activated *ras* or *raf* onco-

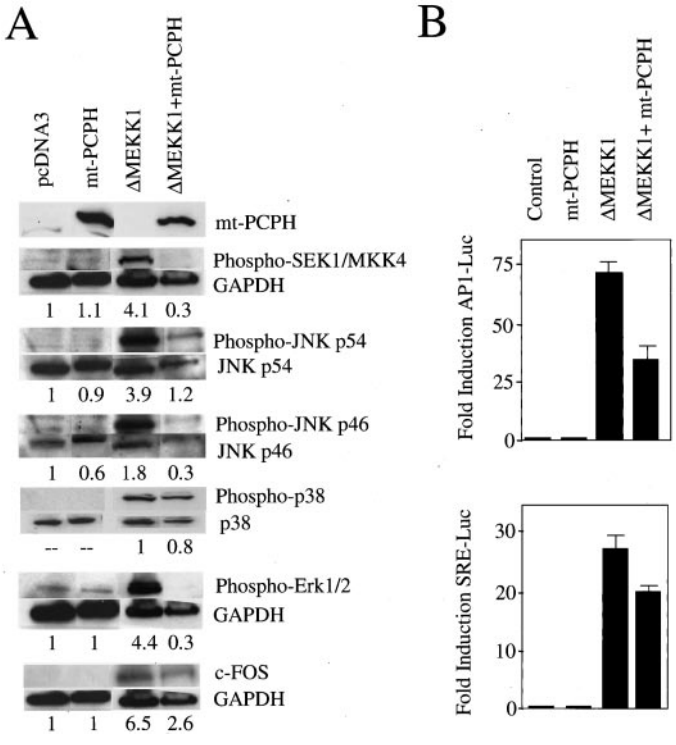

Fig. 1. Inhibition of stress-induced signaling and gene expression by mt-PCPH. NIH 3T3 cells were transfected with pcDNA3 alone or with vectors encoding mt-PCPH or ΔMEKK1, as indicated. *A*, cells were subjected 24 h after transfection to immunoblot analysis with antibodies to the indicated proteins. Relative phosphoprotein abundance was determined by densitometry and is shown below relevant panels. *B*, cells were also transfected with the reporter plasmids pAP1-Luc or pSRE-Luc and, after 48 h, assayed for luciferase activity. Data are expressed as fold induction relative to cells transfected with the reporter plasmid and pcDNA3 alone (*Control*).

Table 1 *Comparison between the effects of wild-type PCPH and mt-PCPH on stress-response signaling, cellular ATP content, and cisplatin-induced apoptosis in NIH3T3 cells*[a]

	pcDNA3 control			Normal PCPH			PCPH oncoprotein		
	Alone	+ΔMEKK	+TPA	Alone	+ΔMEKK1	+TPA	Alone	+ΔMEKK1	+TPA
SEK1 activation	1.0	4.1	ND[b]	1.2	3.2	ND	1.1	0.3	ND
p54 JNK activation	1.0	3.9	ND	1.4	4.0	ND	0.9	1.2	ND
p46 JNK activation	1.0	1.8	ND	1.2	1.9	ND	0.6	0.3	ND
p38 activation		1.0	ND		0.9	ND		0.8	ND
ERK 1/2 activation	1.0	4.4	ND	0.9	3.4	ND	1.0	0.3	ND
AP1 transactivation	1.0	76.4	11.8	1.2	68.6	9.1	1.1	36.2	4.9
SRE transactivation	1.0	24.7	16.5	0.9	19.3	13.6	1.3	4.4	7.2
c-FOS activation	1.0	6.5	3.5	1.0	5.4	3.1	1.0	2.6	1.9
ATP content	100%	ND	ND	95 ± 1.1%	ND	ND	75 ± 2.6%	ND	ND
CP apoptosis	30–46%	ND	ND	28–42%	ND	ND	14–21%	ND	ND

[a] Data are expressed as fold increase relative to values for cells transfected with pcDNA3 vector, unless otherwise indicated. See text for further details.
[b] ND, not determined; CP, cisplatin.

genes (Fig. 2*A*). Expression of mt-PCPH resulted in marked decreases in both basal and stimulated DNA-binding activity; the inhibitory effect of mt-PCPH was only partial, however, and appeared more pronounced in unstimulated cells. Competition with unlabeled AP-1 consensus oligonucleotide confirmed the specificity of the protein-DNA complexes.

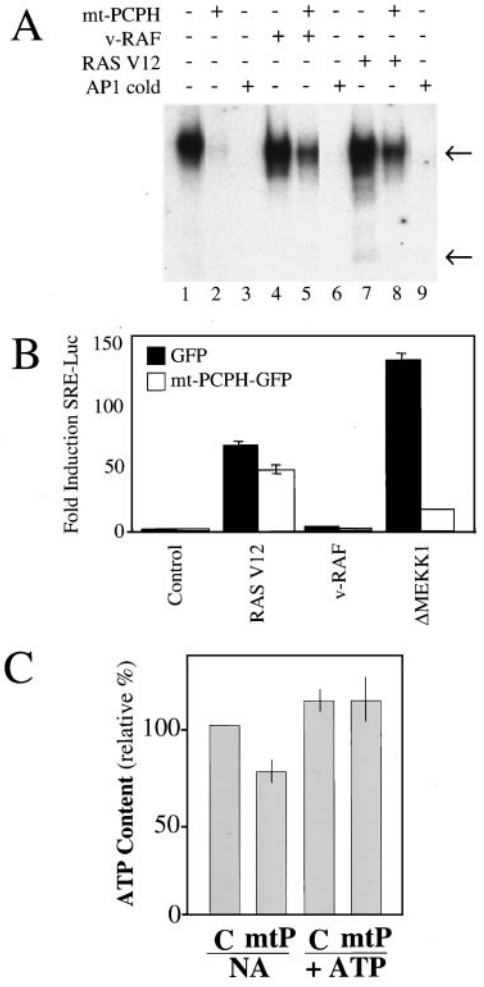

Fig. 2. Expression of mt-PCPH inhibits AP-1 and SRE function and depletes intracellular ATP. *A*, nuclear extracts prepared from cells 48 h after transient transfection with vectors encoding the indicated combinations of mt-PCPH, v-RAF, and RAS^{V12} were subjected to EMSA analysis with a ^{32}P-labeled AP-1 consensus oligonucleotide. Unlabeled oligonucleotide was included in the reaction mixture as a competitor as indicated. *Arrows*, the major DNA-protein complexes. *B*, stably transfected cells expressing either GFP alone or GFP-tagged mt-PCPH were also transfected with pSRE-Luc and vectors for RAS^{V12}, v-RAF, or ΔMEKK1 and, after 48 h, assayed for luciferase activity. *C*, NIH 3T3 cells stably transfected with pcDNA3 (*C*) or pcDNA3-*mt-PCPH* (*mtP*) were incubated for 48 h in the absence (*NA*) or presence of 50 μM ATP, after which the intracellular ATP content was determined. Data are expressed relative to the value for cells transfected with pcDNA3 and incubated in the absence of ATP and are means of triplicates from three independent experiments; *bars*, SD.

To determine whether the observed inhibitory effects of mt-PCPH were attributable to the transient, relatively high levels of mt-PCPH expression attained early (up to 72 h) after transfection, we examined the extent of SRE-mediated trans-activation in NIH 3T3 cells stably transfected with pcDNA3 encoding either GFP alone or mt-PCPH tagged at its NH_2 terminus with GFP. The cells were also transfected with pSRE-Luc, and vectors for RAS^{V12}, v-RAF, or ΔMEKK1 and luciferase activity were determined 48 h after transfection. The PCPH oncoprotein inhibited the increases in SRE-dependent luciferase activity induced by RAS^{V12}, v-RAF, or ΔMEKK1 (Fig. 2*B*). The observed inhibitory actions of mt-PCPH on stress-response signaling were thus not the result of an artifact of transient transfection.

The PCPH Oncoprotein Induces Depletion of Intracellular ATP. Our present and previous (8) data demonstrate that mt-PCPH inhibits various protein kinases that participate in mitogenic and stress-activated signaling pathways and, consequently, also inhibits the activation of transcription factors and the expression of early-response genes such as c-*Fos*. These inhibitory effects are unlikely to be mediated by direct interaction between mt-PCPH and each individual kinase. Moreover, expression of mt-PCPH had no effect on the steady-state abundance of any of the kinases that it inhibited (Fig. 1*A*; Ref. 8). We therefore investigated the possibility that mt-PCPH inhibits kinase activity by limiting the availability of the phosphate donor for these reactions, which for most kinases is ATP (14). This notion is also consistent with the intrinsic ATP diphosphohydrolase activity of mt-PCPH (4).

We first determined the intracellular ATP content of exponentially growing, early-passage NIH 3T3 cells stably transfected with either pcDNA3-*mt-PCPH* or the empty vector. Cells expressing mt-PCPH contained ~25% less ATP than did the control cells (Fig. 2*C*). As has been demonstrated in other systems (15), the ATP content of NIH 3T3 cells expressing mt-PCPH was restored to normal, or slightly above normal, levels by supplementing the culture medium with 50 μM ATP and maintaining this concentration for at least 48 h (Fig. 2*C*).

ATP Replenishment Restores Stress Sensitivity and Stress-Response Signaling. We next investigated the effect of ATP replenishment on the sensitivity of NIH 3T3 cells expressing mt-PCPH to cisplatin-induced apoptosis. Treatment with cisplatin (10 μg/ml) for 48 h induced apoptosis in ~38% of control cells but in only ~17% of cells expressing mt-PCPH (Fig. 3*A*). This inhibition of apoptosis by mt-PCPH was abolished in cells incubated in the presence of exogenous ATP. Supplementation with extracellular ATP also restored the sensitivity of cells expressing mt-PCPH to nutritional deprivation or ionizing radiation (data not shown), indicating that partial ATP de-

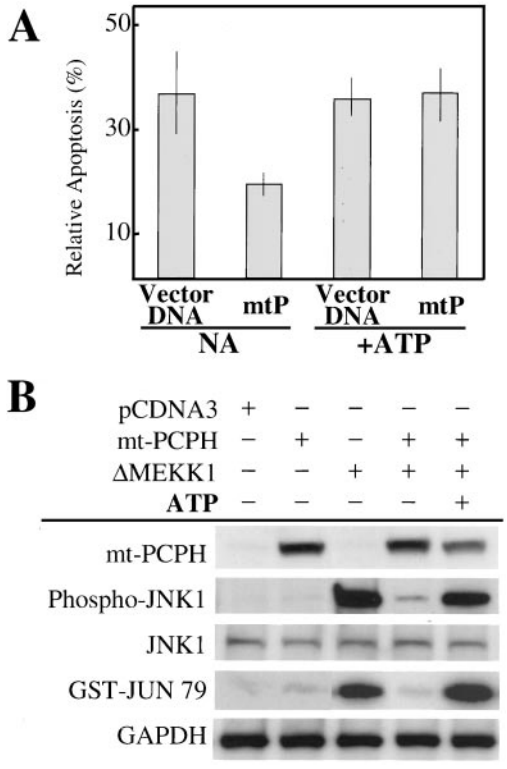

Fig. 3. Exogenous ATP restores sensitivity to cisplatin-induced apoptosis and stress signaling in cells expressing mt-PCPH. *A*, cells were incubated in the absence or presence of ATP as in Fig. 2*C* as well as with cisplatin (10 μg/ml) for 48 h, after which the percentage of apoptotic cells was determined by flow cytometry. Data are mean of triplicates from three independent experiments; *bars*, SD. *B*, cells transfected with the indicated constructs were incubated for 48 h in the absence or presence of 50 μM ATP and were then subjected to immunoblot analysis with antibodies to the indicated proteins. Cell lysates were also assayed for c-JUN kinase activity *in vitro* with [γ-^{32}P]ATP and a glutathione *S*-transferase fusion protein containing residues 1–79 of c-JUN (*GST-JUN 79*) as substrate. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

pletion was responsible for the original development of resistance to these stimuli.

We then determined the effect of exogenous ATP on the inhibition by mt-PCPH of ΔMEKK1-induced activation of the JNK signaling pathway in transfected NIH 3T3 cells. The ΔMEKK1-induced activation of JNK1 as well as the ability of JNK to phosphorylate c-JUN *in vitro* were fully restored in cells expressing mt-PCPH by culture in the presence of ATP (Fig. 3*B*). Given that ATP is required early in the commitment to apoptosis (16), our results suggest that mt-PCPH promotes the survival of cells exposed to stressors by reducing the intracellular concentration of ATP to levels below those required for optimal stress signaling and apoptosis (17), a mechanism of action not demonstrated previously for an oncogene product. Consistent with this notion, an enzymatically inactive form of mt-PCPH generated by site-directed mutagenesis of the ATP binding region failed to reproduce any of the effects of the intact oncoprotein described above (data not shown).

Expression of Wild-Type PCPH Has a Minimal Impact on Cell Survival. Wild-type PCPH also possesses ATPase activity *in vitro* (4, 8), but its overexpression does not promote stress resistance in NIH 3T3 cells (3). To investigate this apparent discrepancy, we repeated all of the experiments described above with NIH 3T3 cells transfected with pcDNA3-*PCPH*, which encodes the wild-type protein (3). Ectopic expression of the normal PCPH protein had either no effect (activation of p54 or p46 JNK) or an inhibitory effect markedly less than that of mt-PCPH (activation of SEK1 or ERK1/2, induction of c-FOS, or trans-activation mediated by AP-1 or SRE) on stress signaling (Table 1). Similarly, expression of wild-type PCPH resulted in a reduction in cellular ATP content of only ~5% (compared with the 24% decrease observed with mt-PCPH). Furthermore, wild-type PCPH did not confer resistance to cisplatin-induced apoptosis. These observations are thus consistent with our proposal that the marked depletion of cellular ATP induced by expression of mt-PCPH is responsible for the increase in cell survival observed on treatment with cisplatin.

The intracellular concentration of ATP affects both the susceptibility of cells to the induction of apoptosis (or necrosis) by various agents (17) and the set point of signal transduction pathways (18). The existence of a threshold ATP concentration below which commitment to apoptosis is not possible has been proposed, although this proposal remains controversial. However, various components of the apoptotic machinery are differentially affected by changes in the cellular concentrations of nucleoside di- and triphosphates (17, 19). The reduction in the cellular ATP concentration caused by wild-type PCPH may thus not be sufficient to affect the induction of apoptosis, whereas mt-PCPH likely reduces the ATP concentration to a level below the required threshold for apoptotic death. The nucleoside triphosphate diphosphohydrolase activities of wild-type PCPH and mt-PCPH are similar *in vitro* (3, 4, 8). It therefore remains unclear why these proteins exhibit markedly different effects on the intracellular ATP concentration. However, the subcellular localizations of the two proteins appear to differ (data not shown), so that they might have access to different ATP pools. It is also possible that the two proteins possess different affinities for ATP *in vivo*.

Our data suggest that detection of mt-PCPH in human tumors might facilitate optimization of therapy. Treatment with adenosine or nucleoside analogues might thus sensitize tumor cells to standard radio- or chemotherapy. Alternatively, given that the ATP content of tumor cells expressing mt-PCPH is presumably already reduced, it might be possible to reduce it further by recently proposed protocols (20) and thereby to induce necrotic cell death.

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