The PCPH Oncoprotein Antagonizes the Proapoptotic Role of the Mammalian Target of Rapamycin in the Response of Normal Fibroblasts to Ionizing Radiation

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ABSTRACT

Exposure of normal mouse fibroblasts (MEF3T3) to ionizing radiation (IR) resulted in a dose-dependent increase of mTOR mRNA and protein levels and the shuttling of the mTOR protein from its normal, predominantly mitochondrial location to the cell nucleus. The same IR doses that activated mTOR induced the phosphorylation of p53 on Ser18 (mouse equivalent to human Ser15) and the subsequent transcriptional activation of PUMA, a known proapoptotic p53-target gene, and promoted apoptosis involving increased overall caspase activity, caspase-3 activation, cleavage of poly(ADP-ribose) polymerase (PARP) and classic protein kinase C (PKC) isoforms, and DNA fragmentation. The proapoptotic role of mTOR in this process was demonstrated by the fact that rapamycin, a mTOR inhibitor, blocked p53 Ser18 phosphorylation, the induction of PUMA, and all other apoptosis events. Furthermore, the proapoptotic function of mTOR was also antagonized by the expression in MEF3T3 cells of the PCPH oncoprotein, known to enhance cell survival by causing partial ATP depletion. Tetracyclin (Tet)-regulated expression of oncogenic PCPH, or overexpression of normal PCPH, blocked both phosphorylation and nuclear shuttling of mTOR in response to IR. These results indicate that alterations in PCPH expression may render tumor cells resistant to IR, and perhaps other DNA-damaging agents, by preventing mTOR activation and signaling.

INTRODUCTION

Nuclear DNA damage is a well-known trigger of the so-called intrinsic pathway of cellular apoptosis, which involves mitochondrial membrane permeabilization, cytochrome c release, and the activation of the proteolytic caspase cascade (1, 2). Several enzymes (ATM, ATR, DNA-PK) involved in sensing DNA damage, double-strand breaks in particular, are members of the PIKK family (3). These enzymes initiate signaling pathways that converge in the tumor suppressor p53 (4). Phosphorylation of specific p53 residues (5, 6) has enzymes initiate signaling pathways that converge in the tumor suppressor p53 (4). Phosphorylation of specific p53 residues (5, 6) has been identified as a key determinant of the final cellular response (cell cycle arrest or apoptosis) to DNA damage.

Recent reports have shown that another member of the PIKK family, the mTOR (also known as FRAP or RAFT; Ref. 7), likewise plays an important role in a p53-dependent, mitochondria-mediated apoptotic process that is initiated independently of DNA damage: the formation of synctia by the fusion of cells expressing the HIV-1 Env gene with cells expressing the CD4/CXCR4 receptor complex (8, 9). In this system, apoptosis occurs after nuclear translocation of mTOR and mTOR-mediated phosphorylation of p53 on Ser15 (p53Ser15), a residue known to be phosphorylated also in response to DNA-damaging agents (10, 11). Evidence for the direct involvement of mTOR in apoptosis through p53Ser15 phosphorylation was based on the observations that: (a) compared with unfused cells, mTOR was the only kinase out of a large panel of proteins analyzed whose expression was up-regulated on synctia formation; (b) mTOR coprecipitated with p53; and (c) rapamycin, a mTOR-specific inhibitor, prevented phosphorylation of p53Ser15 and the execution of the apoptotic process (8).

A universal proapoptotic role of mTOR, however, has not been conclusively established to date. An independent line of evidence suggests that the mTOR pathway may be in fact inactivated by DNA-damaging agents before the commitment stages of apoptosis, in a caspase-independent manner (12). Treatment of Swiss 3T3 and Rat-1 cells with etoposide, cisplatin, or mitomycin-C resulted in the dephosphorylation of two known mTOR substrates involved in the regulation of translation: the p70S6 kinase and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Although formal demonstration of mTOR inactivation is lacking, these findings suggest that mTOR may be a cellular context-dependent, pleiotropic regulator of apoptosis (13). Indeed, an antiapoptotic role has also been described for mTOR (14). Consistent with this notion, the inhibition of mTOR has been shown to sensitize human tumor cells to fractionated radiation therapy in nude mouse xenografts (15).

The PCPH gene was first identified as an oncogene activated in primary Syrian hamster fetal cells initiated with 3-methylcholanthrene (16). The PCPH gene is conserved from yeast to human cells, being expressed in a broad range of tissue types (17). However, expression of the PCPH protein is limited to a few histological locations during mouse embryo development. Most recently, we showed that expression of PCPH is frequently altered in human tumor cells and solid tumors (18, 19) and that the characteristic pattern of PCPH alterations in human mammary tumor cells was reproducible in a rat model for mammary carcinogenesis (20), strongly suggesting the possible involvement of PCPH in human cancer development. Previous studies from our laboratory established that increasing cellular resistance to apoptosis is an important component of the transforming activity of the PCPH oncogene (21, 22). This represents a major functional difference between the PCPH proto-oncogene and the oncogene, because expression of the proto-oncogene provided only a marginal protection to various apoptotic agents, including IR and chemotherapeutic drugs. We described recently (21) that mouse fibroblasts ectopically expressing the PCPH oncoprotein contained lower intracellular ATP concentrations than did control cells and that their resistance to apoptosis could be reversed by ATP replenishment. Because PCPH has intrinsic AMP dephosphohydrolase activity (23), our data indicated that partial ATP depletion mediated the stress-survival function of the PCPH oncoprotein, most likely by decreasing...
phosphate donor availability for stress-induced phosphorylation cascades. To evaluate the role of mTOR in the apoptotic response to DNA damage, we studied changes in mTOR expression, phosphorylation, and localization in mouse embryo fibroblasts (MEF3T3) exposed to increasing IR doses and correlated them with changes in molecular determinants of the intrinsic apoptotic pathway. We used rapamycin to inhibit mTOR-related parameters and Tet-regulated PCHP expression to modulate the radiation susceptibility of MEF3T3 cells to apoptosis. Results demonstrated a proapoptotic role for mTOR and showed that this mTOR function was antagonized by the expression of oncogenic PCHP or overexpression of normal PCPH, which blocked mTOR phosphorylation and nuclear shuttling. These results demonstrate a key role for mTOR in the response to IR and indicate that alterations in PCPH expression may increase the resistance of tumor cells to IR, and perhaps other DNA-damaging agents, by preventing mTOR activation and signaling.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, Antibodies, and General Reagents. Mouse MEF3T3 cells, a line derived from Swiss 3T3 embryo fibroblasts, were purchased from BD Clontech (Palo Alto, CA) and maintained in DMEM (Biofluids, Rockville, MD) supplemented with 10% Life Technologies, Inc. donor calf serum (Invitrogen, Grand Island, NY), 100 μg/ml G418, and 1% Life Technologies, Inc. penicillin/streptomycin, at 37 °C, in an atmosphere of 5% CO2 and 95% air. Polyclonal antibodies against mTOR, phospho(Ser2448) mTOR, p53, phospho(Ser 6, 9, and 15) p53, Bax, caspase 9, caspase 3 and PKC isoforms α and βII were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-PUMA was purchased from Abcam (Cambridge, United Kingdom). Oligonucleotide primers were from Bio-Synthesis (Lewisville, TX). Rapamycin was purchased from Sigma (St. Louis, MO) and Dox from BD Clontech. The polyclonal anti-PCPH antisera (no. 1892) was raised in rabbits (Bio-Synthesis) by immunization with a synthetic peptide corresponding to amino acid residues 109–122 of the mouse PCPH protein. The anti-PARP polyclonal antisera, which recognizes intact PARP and its small (M, 25,000) caspase cleavage product, was described previously (24).

Cell-permeable, pan-caspase inhibitor Z-VAD-FMK was purchased from Promega Corp. (Madison, WI).

Irradiation and Apoptosis Assays. All of the irradiations were carried out using a J.L. Shepherd (San Fernando, CA) Mark 1 130Co irradiator, delivering total doses of 2.5, 5.0, or 10.0 Gy, at 1.93 Gy/min. Apoptosis was evaluated by four methods: cell viability determinations, PARP cleavage, caspase processing, and TUNEL assays. Cell viability was determined by trypan blue exclusion: Cells were suspended in 0.04% trypan blue in PBS, placed on a hemocytometer, and counted under the microscope (25).

PARP cleavage and caspase processing were studied by Western analyses with specific antibodies. Caspase activity was measured using the CaspACE Assay PARP cleavage and caspase processing were studied by Western analyses with a 2700 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) and consisted of 35 amplification cycles. Denaturation was performed at 94 °C for 20 s; annealing was at 58 °C for mTOR and 64 °C for PUMA; and extension at 72 °C for 45 s. As a routine housekeeping reference, conditions for actin amplification were always identical to those used for the transcript under investigation. PCR products were resolved on 1.5% agarose gels and quantified using the Molecular Analyst Macintosh data analysis software and a Bio-Rad (Hercules, CA) Image Analysis System. Amplification products were purified using the QiAquick PCR Purification kit (Qiagen) according to the manufacturer’s instructions, and sequenced using an ABI Prism 310 system (Perkin-Elmer).

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) and the lysates were centrifuged at 13,000 × g at 4 °C for 30 min. Protein content in the supernatants was determined with the BCA Protein Assay system (Pierce, Rockford, IL). Proteins (30 μg) in cell extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.2% Tween 20, membranes were incubated at 4 °C overnight with the different antibodies. Blots were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000) and the peroxidase activity was analyzed with the ECL chemiluminescence substrate system (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence. Cells grown on two-chamber slides were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature and were rinsed and permeabilized with 0.4% Triton X-100 in PBS for 30 min. Permeabilized cells were then incubated for 30 min at room temperature with 10% donkey serum in PBS to block nonspecific binding. After thorough rinsing with PBS, cells were incubated for 1 h at 37 °C with anti-mTOR antibody, followed by 30 min at 37 °C with FITC-coupled antirabbit antibody. Cells were rinsed again with PBS and twice with distilled water and were mounted in Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA), which provided nuclear fluorescence counterstaining, and were visualized with a Nikon E600 fluorescence microscope. Mitochondria were stained by incubating the cells with the MitoTracker red (Molecular Probes, Eugene, OR) for 30 min at 37 °C. Appropriate controls were maintained by substituting the primary antibody with normal donkey and/or rabbit serum to check for nonspecific binding.

Establishment of Tet-Regulated PCHP Expression Systems. The DNA sequences encompassing the ORFs of the normal and oncogenic PCPH cDNAs (22) were amplified by PCR using primers that created an EcoRI site immediately upstream of the translation initiation codon and a BamHI site immediately downstream from the translation termination TGA codon. The upper primer (CCGAAATTCCTGACCATCCCTGTCGCG) was the same in both cases, whereas the lower primer CTGAGTAACTAGCCGATTCGAG- GCCCAG was used to amplify the normal ORF and CTGGATCATCAATCCAAATATCAAGTAA was used to generate constructs with oncogenic PCHP. Amplified fragments were gel-purified using the Sephaglass BandPrep kit (BD Pharmingen, San Diego, CA) and were directionally cloned into the corresponding site of BD Clontech’s Tet-On 3G system. The resulting plasmids, PCPH ORFs under the translational control of the PmCMV+ operator, which contains the Tet-responsive element (TRE) immediately upstream of the minimal cytomegalovirus (CMV) promoter. The pTRE-PCPH and pTRE-mt-PCPH plasmids were then cotransfected into MEF3T3 Tet-Off cells with pTK-Hyg, a plasmid that confers hygromycin resistance, to allow the selection of stable transfectedants. MEF3T3 Tet-Off cells are Swiss 3T3 fibroblasts that constitutively express the Tet-controlled transactivator (TA), which will interact with and activate the PmCMV+ promoter on Dox removal from the culture medium. Transfections were performed using the GenePORTER 2 reagent (GTS Inc., San Diego, CA). A number of individual clones derived from each transfection
Mitochondria Are the Primary Subcellular Location of mTOR in MEF3T3 Cells. Stimulation of mTOR has been reported to result in its translocation from the cytoplasm to the nucleus (34). We wanted to investigate the possibility that IR stimulation of mTOR would cause a similar phenomenon. However, there are several contradictory reports on the localization of mTOR. Some researchers reported a cytoplasmic localization with a large portion of mTOR ascribed to mitochondria (35), whereas others described a predominantly nuclear localization for mTOR (36). These differences cannot be explained simply by the fact that different cell types were examined in each case, because both groups included mouse embryo fibroblasts in their studies. Therefore, we first performed immunofluorescence experiments to ascertain the localization of mTOR in MEF3T3 cells. We compared the localization pattern of mTOR, visualized with a FITC-conjugated secondary antibody, with the distribution of nuclear (DAPI) and mitochondrial Mitotracker (CMXRos) staining in unirradiated or exponentially growing MEF3T3 cells. Merging of the three staining patterns (Fig. 2) clearly demonstrated that mTOR is localized in the cytoplasm and absent from the cell nuclei. Results also showed that mTOR is predominantly associated with mitochondria, although a small proportion of mTOR may be either solubile or associated, perhaps transiently, with other compartments in the cytoplasm.

IR Induces Nuclear Translocation of mTOR. Similar localization studies were carried out on MEF3T3 cells after IR exposure. Results showed that IR induced the shuffling of mTOR from the cytoplasm to the nucleus (Fig. 3). Mock-irradiated MEF3T3 cells, which were TUNEL negative (Fig. 3B) and had no signs of nuclear abnormalities (Fig. 3C), showed the typical cytoplasmic, preferentially mitochondrial, localization (Fig. 3A). After 7-Gy irradiation (24 h), mTOR staining was detectable only in the nuclei (Fig. 3D) of cells that were already weakly TUNEL positive (Fig. 3E) and showed signs of nuclear condensation (Fig. 3F). At later times, overtly apoptotic, strongly TUNEL-positive cells, undergoing a complete disorganization of their cytoplasmic and nuclear compartments, showed a widespread, diffuse staining for mTOR (data not shown). These results

Fig. 1. Effect of IR on the expression of mTOR in normal mouse fibroblasts. Exposure of MEF3T3 cells to IR resulted in (A) a dose-dependent increase in mTOR mRNA levels, as determined by semiquantitative RT-PCR, and (B) the concomitant rise in the levels of total and phosphorylated mTOR (p-hmTOR) protein, as determined by Western blot analyses with the antibodies indicated. The letter C, control unirradiated cells. Histograms (right panels), the densitometric evaluation of results from RT-PCR and Western hybridization analyses. Data represent the mean from three similar experiments; bars, ±SD. * P ≤ 0.05 for test group versus actin absorbance.
demonstrated that, similar to mitogenic stimulation (34), IR exposure results in the translocation of mTOR into the nucleus.

**Rapamycin Blocks IR-Induced Apoptosis.** To determine whether mTOR activation was an effector in the apoptotic pathway or a consequence of the apoptotic process, we examined the effect of rapamycin, a mTOR-specific inhibitor (37, 38), on the response of MEF3T3 cells to IR exposure. Although the addition of rapamycin (10 ng/ml in DMSO) to mock-irradiated controls produced a slight decrease in cellular viability (Fig. 4A, Control), rapamycin treatment enhanced the survival of cells exposed to IR (Fig. 4A). The magnitude of this cell-survival-enhancing effect increased with the IR dose used. The protective effect of rapamycin was also evidenced by its ability to

![Fig. 2. Mitochondria are the primary location of the mTOR protein in normal murine fibroblasts. Exponentially growing MEF3T3 cells were immunostained with anti-mTOR antibody using a FITC-conjugated secondary antibody (mTOR panel), or stained with DAPI or CMXRos to identify the nuclear and mitochondrial compartments. Merging of the three images (MERGE panel) identified mitochondria as the primary subcellular localization for the mTOR protein.](image)

![Fig. 3. IR induces mTOR nuclear translocation. In agreement with data shown in Fig. 2, immunofluorescence analyses showed a preferential cytoplasmic (mitochondrial) location for mTOR in unirradiated MEF3T3 cells (A) which were TUNEL negative (B) and contained intact nuclei (C). On the contrary, mTOR was detected exclusively in the nucleus (D) after exposure to IR (7 Gy) in cells that were clearly TUNEL positive (E) and showed signs of nuclear condensation (F).](image)
block the increase in caspase activity induced by IR exposure (Fig. 4B). Indeed, rapamycin was as efficient in blocking caspase activation as the cell-permeable, pan-caspase inhibitor Z-VAD-FMK (Fig. 4B, 10 Gy /H11001). In agreement with its inhibitory effect on IR-induced caspase activation, rapamycin treatment prevented the proteolytic activation of caspase 3 and the apoptosis-induced cleavage of cellular proteins (39, 40) such as PARP and PKC (Fig. 4C). Overall, these results showed that the inhibition of mTOR blocked IR-induced apoptosis, thus demonstrating that mTOR is a positive effector in the process.

**mTOR Mediates p53 Phosphorylation and the Subsequent Transcriptional Activation of the BH3-Only, Proapoptotic Protein PUMA.** Results from studies in other experimental systems suggested that mTOR may exert its proapoptotic activity by mediating, or even directly carrying out, the phosphorylation of p53 on Ser15 (8, 9). Because this is a residue known to be phosphorylated in response to DNA damage (5, 6) and because our data showed that mTOR is a positive effector of IR-induced apoptosis, we examined the possibility that mTOR may mediate p53 phosphorylation at Ser18, the equivalent amino acid residue in murine p53 (41), in the response of MEF3T3 cells to IR. Western blot analyses of total extracts from mock-irradiated cells and cells exposed to various IR doses with antibodies anti-p53 and antiphospho(Ser15) p53 (which also recognizes the equivalent modification in mouse p53) showed that, even though IR exposure did not remarkably modify the total content of p53 in the cells, there was a significant increase in the level of p53 phosphorylated at the Ser18 residue (Fig. 5A) on IR treatment. The direct dependence of this phosphorylation event on mTOR was demonstrated by the fact that it was abrogated by inhibiting mTOR with rapamycin. The extent of the inhibition of p53 phosphorylation on Ser18 caused by rapamycin correlated with its survival enhancing effect relative to the IR doses (Fig. 4A): the higher the IR dose, the more stringent the inhibitory effect of rapamycin on p53 Ser18 phosphorylation (Fig. 5A).

It is well established that, in response to DNA damage, p53 contributes to cell survival by regulating cell cycle progression and apoptosis (42). Phosphorylation events at various amino acid residues within the NH2-terminal transactivation domain of p53 determine the nature of the response to DNA damage, by inducing or repressing several genes that regulate cell cycle arrest, DNA repair and/or apoptosis (43). In the apoptotic process, p53 up-regulates the transcription of target genes encoding proapoptotic proteins. Several of these downstream products have been identified (44–48). Accordingly, we tested the possibility that p53Ser18 phosphorylation may result in the induction of p53 target products such as Bax (49), Noxa (47), PUMA (44, 45, 48), or other proteins (50, 46) that may mediate the release of cytochrome c from mitochondria and the activation of the caspase 9/3 cascade (51). RT-PCR and Western hybridization analyses demonstrated that the levels of Bax, which is expressed in MEF3T3 cells, were not increased and that the expression of Noxa was not induced by IR treatment (data not shown). However, IR exposure caused the

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**Fig. 4. Inhibition of mTOR protects MEF3T3 cells from IR-induced apoptosis.** The protective effect of rapamycin (black bars) relative to cells maintained without the mTOR inhibitor (gray bars) was demonstrated by its ability to: increase cell survival after exposure to increasing IR doses (A), prevent caspase activation (B) as efficiently as the caspase-specific inhibitor Z-VAD-FMK (10+Inh Lane in B), and block the cleavage of caspase 3, PARP, and PKC (C). The arrow in panel C, the PARP cleavage product identified by the polyclonal antiserum used in this study. R, rapamycin; the letter C, control unirradiated cells.

**Fig. 5. mTOR mediates p53 phosphorylation (php53) and expression of PUMA.** The inhibitory effect observed in the presence of rapamycin demonstrated that mTOR mediated (A) the IR-induced phosphorylation of p53 on Ser15, as demonstrated by Western blot (WB) analyses with the indicated antibodies and (B) the subsequent transcriptional activation (RT-PCR) and increased protein levels (WB) of the proapoptotic p53 target PUMA. C, control unirradiated cells.
transcriptional up-regulation of PUMA and the subsequent increase in the cellular content of its protein product (Fig. 5B). That this phenomenon was also dependent on mTOR activation was evidenced by the fact that it was inhibited by treatment with rapamycin (Fig. 5B). Overall, these results demonstrate that mTOR exerts its proapoptotic role in response to IR-induced DNA damage by mediating the phosphorylation of p53 at Ser15 and the subsequent transcriptional activation of a gene product such as PUMA, recently described as essential for p53-mediated apoptosis (52).

Expression of the PCPH Oncoprotein Prevents IR-Induced mTOR Phosphorylation and Nuclear Translocation and Protects from IR-Induced Apoptosis. Because it was previously reported that mTOR is an ATP sensor and that mTOR signaling is influenced by the intracellular concentration of ATP (53), we examined whether the cellular ATP content could modulate the proapoptotic role of mTOR in response to IR. To accomplish that, we established MEF3T3 cells that expressed the PCPH oncprotein (22), the cell survival function of which is determined by its ability to significantly decrease cellular ATP levels because of its intrinsic ATP diphosphohydrolase activity (21, 23). Taking into consideration that normal MEF3T3 cells do not express the PCPH gene, and to prevent unwanted toxicity effects, we used a Tet-regulated promoter to control the PCPH oncprotein expression in MEF3T3 cells (54). The use of a Tet-off system allowed us to control the levels of PCPH expression by modifying the Dox concentration in the culture medium. As a control, the same system was used for the expression of normal PCPH, known to have only minor effects on the cellular ATP content and marginal cell survival consequences (21, 22). MEF3T3 cells expressing an enzymatically inactive form of the PCPH oncprotein obtained by site-directed mutagenesis within its ATP-binding region were used as a second control for these experiments. A number of clones transfected with either normal or oncogenic PCPH were characterized for promoter leakiness and the extent of transcriptional stimulation on induction. For unknown reasons, all of the clones derived from normal PCPH showed some level of leakiness, even in the presence of high Dox concentrations, whereas all of the clones derived from oncogenic PCPH were tightly regulated. Two clearly inducible clones (Fig. 6A) expressing normal (pA3.1 clone) or oncogenic (mt1A.1 clone) PCPH were selected for further characterization.

Exposure to IR of cells induced to express the PCPH oncprotein (Fig. 6C, mt1A.1-Dox) resulted, as expected, in levels of apoptosis markedly lower than those observed in uninduced controls (Fig. 6C, mt1A.1+Dox) or in untransfected MEF3T3 cells (Fig. 6C, Control). This was particularly evident at the highest IR dose used (10 Gy). Total extracts of uninduced and induced, unirradiated or irradiated, mt1A.1 cells were prepared 24 h after irradiation. Western analyses of these samples with anti-mTOR and antiphospho-mTOR showed that, although expression of the PCPH oncprotein did not seem to affect the total amount of mTOR protein, it had a dramatic inhibitory effect on mTOR phosphorylation (Fig. 6B, −Dox panels), which was rendered undetectable even in unirradiated controls (Fig. 6B, −Dox, Lane C). Furthermore, expression of the PCPH oncprotein completely prevented the translocation of mTOR into the nucleus on irradiation (Fig. 7A). To explore the possibility that this behavior may be attributable to the clonal origin of the mt1A.1 cells rather than being an effect of the expression of the PCPH oncprotein, we tested whether mTOR translocated into the nucleus in uninduced mt1A.1 cells, which do not express the PCPH oncprotein (Fig. 6A). Results showed that, although uninduced mt1A.1 cells were less efficient than the original MEF3T3 cell population in translocating mTOR to the nucleus on irradiation, most of the mTOR signal was still detectable in the nucleus of irradiated mt1A.1 cells (Fig. 7G). These results confirmed that expression of the PCPH oncprotein was indeed responsible for the complete blockade of the translocation of mTOR into the nucleus in induced, irradiated mt1A.1 cells (Fig. 7A). When the same type of experiments were performed with cells maintained in the presence of Dox concentrations that would induce the expression of levels of normal PCPH similar to those achieved for the PCPH oncprotein in the absence of Dox, the effects of the expression of normal PCPH on both mTOR phosphorylation and nuclear translocation were only marginal (data not shown). Even when the removal of Dox resulted in levels of normal PCPH about 20-fold greater than those of the PCPH oncprotein (Fig. 6A), the effects of this overexpression on mTOR phosphorylation and nuclear translocation were lower in magnitude than those caused by induction of the PCPH oncprotein (Figs. 6B and 7A): overexpression of normal PCPH did not cause a complete blockade of mTOR phosphorylation (data not shown) and, consistent with its effect on mTOR phosphorylation, overexpression of normal PCPH allowed only a partial translocation of mTOR into the nucleus (Fig. 7D). The fact that expression of enzymatically inactive PCPH oncprotein had no detectable effect on the normal role of mTOR in the response to IR exposure (data not shown) demonstrated that the inhibitory effect of the expression of the PCPH oncprotein (or the overexpression of the normal PCPH protein) on mTOR phosphorylation and nuclear translocation was indeed mediated by its ATP diphosphohydrolase activity, and strongly suggested that phosphorylation is a requirement for the shuttling of mTOR to the nucleus.

Fig. 6. Effect of expression of the PCPH oncprotein on the response of murine fibroblasts to IR and mTOR status. Clonal Tet-off lines were developed for the conditional expression of normal (pA3.1) or mutant (mt1A.1) PCPH proteins on removal of Dox from the culture medium (A). Compared with uninduced (+Dox) controls, Western analyses with the indicated antibodies revealed that induction of mutant PCPH expression (mt1A.1 cells, −Dox), completely prevented mTOR phosphorylation (B, −Dox panel) and rendered the cells more resistant to IR (C). The letter C, control unirradiated cells.
Overall, results described above identified mTOR as an IR-responsive gene, the expression of which is up-regulated on IR treatment (Fig. 1). In fact, in our experimental system, the mTOR gene product closely fulfills the requirements described (55) for a DNA damage sensor: to be located near DNA and to react to DNA damage. Our results show that, on exposure to a DNA damaging agent, mTOR mRNA, protein, and phosphorylation levels are up-regulated (Fig. 1) and that the mTOR protein moves from its normal mitochondrial position in the cytoplasm (Fig. 2) to a location near the DNA in the nucleus (Fig. 3), in which it exerts its reaction by performing a prominent role in the apoptotic response of normal fibroblasts to IR.

Rapamycin inhibition of the downstream cascade of IR-induced apoptotic events (phosphorylation of p53Ser18, PUMA expression, caspase activation, cleavage of PARP, and PKC) conclusively demonstrated their dependence on mTOR activity (Figs. 4 and 5). It is possible that, similar to its role in controlling cell size (56), mTOR may be influenced by and react to changes taking place in the cell nucleus as a whole, rather than on the DNA itself. For instance, mTOR-mediated p53Ser18 phosphorylation during HIV-induced syncytial apoptosis has been shown not to take place until a dramatic event such as nuclear fusion (karyogamy) has been completed (9). Further investigation is required to explore a possible role of mTOR in this context.

Our results demonstrating that mTOR is activated by IR in normal, Swiss 3T3-derived, murine MEF3T3 fibroblasts, and that it mediates IR-induced apoptosis, contrast with previous reports describing that exposure of Rat-1 and, especially, Swiss 3T3 cells to other DNA damaging agents (i.e., etoposide, mitomycin C, and cisplatin) caused the inactivation of translational regulators linked to mTOR signaling (12). Interestingly, it remains unclear why the inhibition of mTOR with rapamycin in these two cell types still delayed caspase-3 activation and etoposide-induced apoptosis (12). Although clonal heterogeneity between cell lines kept in different laboratories may be a cause of the apparent disparity with our cellular system, it is also possible that activation of mTOR and stimulation of its proapoptotic activity may be an effect specific to the type of damage caused by IR. This possibility is currently under investigation.

Our finding that, in response to IR, mTOR mediates the transcriptional activation of p53 agrees with published reports (8, 9), although there are specific mechanistic differences with other experimental systems. IR exposure of normal fibroblasts resulted in the exclusive phosphorylation of p53 at Ser18, whereas additional residues (i.e., mouse equivalent to human Ser6 and Ser9) are simultaneously phosphorylated in response to IR in other cell types (5). Moreover, in contrast to other systems in which mTOR also plays a proapoptotic role (8, 13) and Bax is reported to be the main downstream target of phosphorylated p53Ser15/18 (8, 9, 13, 57), mTOR-mediated p53 transactivation resulted in the exclusive up-regulation of PUMA in IR-exposed MEF3T3 cells. This result also contrasts with reports that PUMA is induced along with Bax in response to IR in primary murine thymocytes (44). Whether the exclusive induction of PUMA is an IR-specific response of murine fibroblasts remains to be elucidated. Similarly, additional experiments are required to conclusively dem-

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**DISCUSSION**

Overall, results described above identified mTOR as an IR-responsive gene, the expression of which is up-regulated on IR treatment (Fig. 1). In fact, in our experimental system, the mTOR gene product closely fulfills the requirements described (55) for a DNA damage sensor: to be located near DNA and to react to DNA damage. Our results show that, on exposure to a DNA damaging agent, mTOR mRNA, protein, and phosphorylation levels are up-regulated (Fig. 1) and that the mTOR protein moves from its normal mitochondrial position in the cytoplasm (Fig. 2) to a location near the DNA in the nucleus (Fig. 3), in which it exerts its reaction by performing a prominent role in the apoptotic response of normal fibroblasts to IR.

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**Fig. 7. Expression of the PCPH oncprotein blocks mTOR nuclear translocation after IR exposure.** Tet-off mt1A.1 cells were induced by Dox removal (A) or maintained in the presence of Dox (G), and the location of the mTOR protein was visualized by immunofluorescence after IR exposure (7 Gy). Induction of high levels of expression of normal PCPH (D) resulted in a partial blockade of mTOR translocation. Nuclei were visualized by DAPI staining (B, E, H). Phase-contrast images of the cells are also shown (C, F, I).
Nevertheless, our results on the inhibitory effect of rapamycin demonstrate whether mTOR directly carries out the phosphorylation of p53 at Ser18. Nevertheless, our results on the inhibitory effect of rapamycin on the phosphorylation of mTOR (Fig. 5A) and the localization of mTOR neighboring p53 in the nucleus (Fig. 3), together with published reports of coprecipitation of mTOR with p53 (8), strongly support the notion that mTOR, or a closely associated kinase, phosphorylates p53.

The fact that expression of the PCPH oncoprotein, or overexpression of normal PCPH, antagonizes the effect of IR exposure on mTOR phosphorylation (Fig. 6) and nuclear translocation (Fig. 7) is consistent with reports indicating that mTOR function is influenced by the intracellular ATP content (53). Furthermore, our results strongly support the notion that phosphorylation is a key determinant for the nuclear translocation of mTOR. It is also noteworthy that the extent of the inhibitory effect elicited by PCPH is comparable with that of rapamycin. This and the complete blockade of mTOR phosphorylation observed in cells expressing oncogenic PCPH indicate that the PCPH oncoprotein has a greater specificity for the mTOR kinase than for other stress-related kinases such as JNK and p38, previously reported to be inhibited only partially, or not inhibited at all, by the PCPH oncoprotein in murine fibroblasts and other cell types (21, 58).

Fig. 8 depicts a scheme of the antagonistic interaction between mTOR and PCPH in the response of MEF3T3 cells to IR. This antagonism may have clinical implications for tumor management, more specifically, for the outcome of radiotherapeutic treatments. Results from our laboratory have shown that PCPH expression is frequently deregulated in tumor cell lines (19) and in animal (20) and human (18) tumor development. It is possible that alterations in PCPH expression may render tumor cells resistant to IR, and perhaps other DNA-damaging agents, by preventing mTOR activation and signaling.

The nature of the mechanism by which the increased mTOR protein content induced by IR is maintained in a phosphorylated state remains unknown at this time. A novel pathway in which the mitogenic content induced by IR is maintained in a phosphorylated state remains closely associated kinase, phosphorylates p53.

However, the fact that the generation of PA by PLD is not sensitive to ATP depletion (62), whereas mTOR phosphorylation is, suggests that an alternative kinase must be responsible for mTOR activation. Nevertheless, treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 did not prevent IR-induced apoptosis (data not shown), indicating that the highly conserved upstream PI3K-AKT activators (33) do not play a role in mTOR activation in this system.

It is important to emphasize that, although our results demonstrate that rapamycin prevented IR-induced apoptosis in normal mouse cells, inhibition of mTOR with rapamycin has been reported to sensitize human tumor xenografts in nude mice to fractionated radiation therapy (15). Our results conceptually agree with recently proposed therapeutic uses of kinase inhibitors (63, 64) and provide the basis for additional studies to determine whether this differential effect of rapamycin on the IR response of normal and tumor cells may be clinically useful; the integration of mTOR inhibition with apoptosis-inducing radiotherapy and, perhaps, chemotherapy protocols may enhance the killing of cancer cells and spare normal cells at the same time.

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The PCPH Oncoprotein Antagonizes the Proapoptotic Role of the Mammalian Target of Rapamycin in the Response of Normal Fibroblasts to Ionizing Radiation

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