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TOR Kinase and Ran Are Downstream From PI3K/Akt in H_2O_2-Induced Mitosis

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Abstract Hydrogen peroxide (H_2O_2) activates signaling cascades essential for cell proliferation via phosphatidylinositol-3-kinase (PI3K) and Akt. Here we show that induction of mitogenic signaling by H_2O_2 activates sequentially PI3K, Akt, mammalian target of rapamycin (mTOR), and Ran protein. Akt activation is followed by signaling through the mTOR kinase and upregulation of Ran in primary type II pneumocytes, a cell type implicated in the development of lung adenocarcinoma. Pretreatment of the cells with wortmannin, a specific inhibitor of PI3K, or rapamycin, a specific inhibitor of mTOR kinase, prevented H_2O_2-increased mitosis. H_2O_2-induced Akt ser-473 phosphorylation and upregulation of Ran protein were prevented by wortmannin but not by rapamycin, indicating that PI3K is upstream of Akt and mTOR is downstream from Akt. Overexpression of myr-Akt or Ran-wt in type II pneumocytes increased Akt ser-473 phosphorylation and mitosis in a catalase-dependent manner, indicating that H_2O_2 is essential for Akt and Ran signaling. These results indicate that H_2O_2-induced mitogenic signaling in primary type II pneumocytes is mediated by PI3K, Akt, mTOR-kinase, and Ran protein. J. Cell. Biochem. 91: 1293–1300, 2004. © 2004 Wiley-Liss, Inc.

Key words: mitosis; signaling; Akt; mTOR; Ran

INTRODUCTION

Reactive oxygen species (ROS) participate as second messengers in mitogenic signal transduction [Esposito et al., 2003]. Hydrogen peroxide (H_2O_2) has been shown to increase phosphorylation of tyrosine kinase receptors in the absence of growth factor binding, thereby inducing activation of downstream signaling molecules including Akt (protein kinase-B) [Esposito et al., 2003]. Akt activation by H_2O_2 is mediated by phosphatidylinositol-3-kinase (PI3K) [Shaw et al., 1998; Esposito et al., 2003] and promotes cell survival [Datta et al., 1999] and proliferation [Jin et al., 2003].

Cell proliferation requires cell growth, however, proliferation and growth are two distinct processes [Schmelzle and Hall, 2000]. Cell proliferation refers to the orderly progression through the cell cycle resulting in cell division and increase in cell number and is controlled by cyclin dependent kinase(s) (Cdks) [Radisavljevic and Gonzalez-Flecha, 2003]. Cell growth refers to an increase in cell mass as a result of enhanced synthesis of proteins and other macromolecules [Malumbres and Barbacid, 2001; Prober and Edgar, 2001; Tapon et al., 2001; Volarevic and Thomas, 2001] and is controlled by the mammalian target of rapamycin (mTOR) kinase [Schmelzle and Hall, 2000]. Mitogenic stimuli induce protein synthesis by several mechanisms including ribosome biogenesis, increased translational rates, and enhanced uptake of amino acid. mTOR-kinase integrates signals from mitogens and nutrients to control protein synthesis and therefore cell growth. Inhibition of mTOR kinase using rapamycin, a natural immunosuppressive product and anti-neoplastic drug, prevents increased protein synthesis, cell growth, and proliferation in response to mitogens [Hidalgo and Rowinsky, 2000; Schmelzle and Hall, 2000; Pyronnet and Sonenberg, 2001; Rohde et al., 2001] and nutrients [Crespo and Hall, 2002].

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Nucleocytoplasmic trafficking at the appropriate time as well as proper spindle pole assembly are also critical regulatory steps in the control of cell proliferation [Percipalle et al., 1999]. However, their possible regulation by oxidants has not been extensively investigated. Ran, a small guanine nucleotide triphosphatase (GTPase) required for nucleocytoplasmatic transport and for the induction of spindle formation [Smith et al., 2002], has been implicated in cell cycle control and post-mitotic nuclear assembly during cell proliferation [Dasso, 2001]. Ran stimulates spindle assembly presumably by releasing spindle assembly factors such as Target Protein for *Xenopus* kinesin-like protein 2 (TPX2) [Tsai et al., 2003] and NuMA from the importin-α and -β complex [Gruss et al., 2001] by regulating the balance of microtubule-motor activities [Wilde et al., 2001]. Nuclear import and export proceed through nuclear pore complexes and can occur along a great number of distinct pathways mediated by importins. Many of these pathways cooperate with the Ran system to regulate the interactions with their cargoes [Dasso, 2001].

We have recently shown that H_2O_2 orchestrates a complex signaling network regulating S phase entry, nuclear trafficking, and spindle pole formation through activation of Cdk2, importin-α, and NuMA and leading to increases in type II pneumocyte proliferation using the catalase/glucose oxidase system [Radisavljevic and Gonzalez-Flecha, 2003]. This system is created by catalase/glucose oxidase, where catalse scavenges present H_2O_2 that is already present and glucose oxidase generate new H_2O_2 at non-toxic levels which induces increased cell proliferation and G2/M phase, maintains apoptosis at low level as in normal untreated cells, and increased H_2O_2 levels at elevated physiological concentrations, which trigger many signaling processes for cell proliferation and cell growth. However, use of glucose oxidase only cause increased apoptosis, decreased cell proliferation, G2/M phase, and a 15-fold increased level of H_2O_2 above normal. So, the catalase/glucose oxidase system represents excellent system to study signaling processes during cell proliferation [Radisavljevic and Gonzalez-Flecha, 2003]. In this study, we used the same system to investigate the mechanisms of neoplastic cell growth in type II pneumocytes. Specifically, we studied the signaling pathway initiated by PI3K to control Akt and its downstream targets mTOR and Ran, in the H_2O_2-induced mitosis.

MATERIALS AND METHODS

Materials

Glucose oxidase, catalase, wortmannin, and rapamycin were purchased from Calbiochem (San Diego, CA). Elastase was from Worthington Biochem. Co. (Lakewood, NJ) and Dulbecco's modified Eagle's medium (DMEM) and vitamins from Cellgro (Herndon, VA). Polyclonal antibodies against phosphorylated Akt ser-473 and against non-phosphorylated Akt were from Cell Signaling (Beverly, MA). Ran polyclonal goat antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated rabbit polyclonal secondary antibodies were from Cell Signaling (Beverly, MA), and anti-goat plolyclonal secondary antibodies from Santa Cruz Biotech (Santa Cruz, CA).

Constructs

Full-length Akt cDNA (pLNCX-HA-Akt, myr-Akt) construct and the Akt mutant cDNA (HA-Akt-K179M construct, carrying a lysine 179 to methionine site mutation) were generated as previously described [Radisavljevic et al., 2000]. The pQE-Ran-his-tagged wild-type and mutant pQE-Ran-Q69L-his-tagged cDNA were a gift from Dr. Karsten Weis (University of California, Berkeley, CA) [Nachury et al., 2001].

Cell Culture

Primary alveolar type II pneumocytes were isolated from pathogen-free Sprague Dawley rats (180–220 g) as previously described [Dobbs, 1990; Gonzalez-Flecha et al., 1996]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) and the heart and lungs were rapidly removed. Lungs were perfused via the pulmonary artery, lavaged and incubated with elastase solution (30 U/ml) for 20 min at 37°C. The tissue was then minced and filtered through sterile filters of 120 and 20 μm nylon mesh. Type II cells were purified by differential adherence to IgG-coated plates and suspended in DMEM containing 10% fetal bovine serum, 1% vitamins, 2 mM glutamine, 40 mg/ml gentamicin, 100 U/ml penicilin, and 100 μg/ml streptomycin. Viability of the final preparation, assayed by exclusion

of Trypan blue, was higher than 90%. Type II pneumocytes purity, evaluated after Papanicolaou staining, was higher than 95%. Cells were plated at $0.2 \times 10^6/cm^2$ and grown at 37°C with 5% CO_2 in supplemented DMEM. After 24 h, adherent cells (20% of the original inoculum) were washed with DMEM to remove unattached cells and switched to 1% fetal bovine serum. Type II pneumocytes (30–50% confluency) were treated with 300 nM catalase or catalase plus 20 mU/ml glucose oxidase as previously described [Radisavljevic and González-Flecha, 2003]. Wortmannin (100 nM) or rapamycin (50 nM) were added to the culture prior to the addition of glucose oxidase.

Immunoblotting

Total cell lysates (100 μg protein/lane) were electrophoresed in SDS–8% polyacrylamide gels and transfered to polyvinylidene-difluoride membranes (BioRad, Hercules, CA). After blocking with 5% non-fat milk in TBS-Tween (50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the membranes were probed with the indicated primary antibodies and horseradish peroxidase-conjugated affinity-purified secondary antibodies. Proteins were visualized with an enhanced chemiluminescence detection system (Enhanced Luminal Reagent NEN, Life Science Products, Inc., Boston, MA). Primary and secondary antibodies were used at dilutions of 1:1,000 and 1:20,000, respectively.

Transfection

Type II pneumocytes were transiently transfected with pLNCX-HA-Akt (myr-Akt cDNA construct), HA-Akt-K179M (Akt-mutant cDNA constructs), control vectors pLNCX, or pQE using the LipofectAMINE protocol (GIBCO Life Technologies, Inc., Carlsbad, CA) [Radisavljevic et al., 2000]. Transfection efficiency (~50–55%) was assessed by co-transfection with green fluorescent protein GFP-pLNCX or GFP-pQE and detection of positive green fluorescent cells 24 h after transfection. Cells were harvested and analyzed by Western blotting for Akt, Ran expression, and Akt phosphorylation or for cell cycle progression.

Flow Cytometry

Type II pneumocytes were harvested by trypsinization for cell cycle analysis. Cells were fixed in 70% ethanol, washed in 30% ethanol for 2 min, pelleted and resuspended in 0.05% BSA/PBS for cell cycle analysis. Cellular DNA was stained with propidium iodide (50 μg/ml) in the presence of 40 μg/ml RNA-ase for 30 min at room temperature and the DNA content per cell was analyzed by flow cytometry (Coulter-EPICS-ELITE-ESP flow cytometry system, Miami Lakes, FL). Cell cycle phases (G1, S, and G2/M) were detected and analyzed using the Advanced DNA Multy Cycle Analysis Software (Phoenix flow systems, San Diego, CA).

RESULTS

mTOR-Kinase and Ran Are Downstream From Akt in H_2O_2-Induced Mitosis

To delineate the signaling pathway by which H_2O_2 increases mitosis, we determined the effect of H_2O_2 treatment (catalase/glucose oxidase system) on Akt ser-473 phosphorylation, Ran expression, and number of type II cells in G2/M phase. H_2O_2 significantly ($P < 0.0001$) increased the number of type II pneumocytes in G2/M phase, Akt ser-473 phosphorylation, and the expression of Ran protein (Fig. 1). Increased number of type II cells in G2/M phase was followed by significant ($P < 0.001$) increase in S phase from 2 ± 1% SD (catalase only) to 16 ± 1% SD (catalase/glucose oxidase) in comparison with non-treated cells 8 ± 2% SD of S phase. Wortmannin, a specific inhibitor of PI3K, prevented H_2O_2-induced mitosis, increase in Akt ser-473 phosphorylation, and in Ran protein expression, indicating that PI3K activation is an early event in this pathway. Rapamycin, a specific inhibitor of mTOR kinase, significantly ($P < 0.0001$) decreased H_2O_2-induced mitosis and Ran expression but had no effect on H_2O_2-induced phosphorylation of Akt ser-473, indicating that mTOR is downstream of Akt in this mitogenic signaling pathway (Fig. 1).

H_2O_2 Signaling Via Akt

To determine the time course of increase in Akt ser-473 phosphorylation H_2O_2, we examined phosphorylation of Akt ser-473 in cells treated with catalase or catalase plus GO at the indicated times (Fig. 2). H_2O_2-induced phosphorylation of Akt was already observed at 15 min and reached maximum values at 60 min. Akt phosphorylation started to decline at 70 min (Fig. 2).

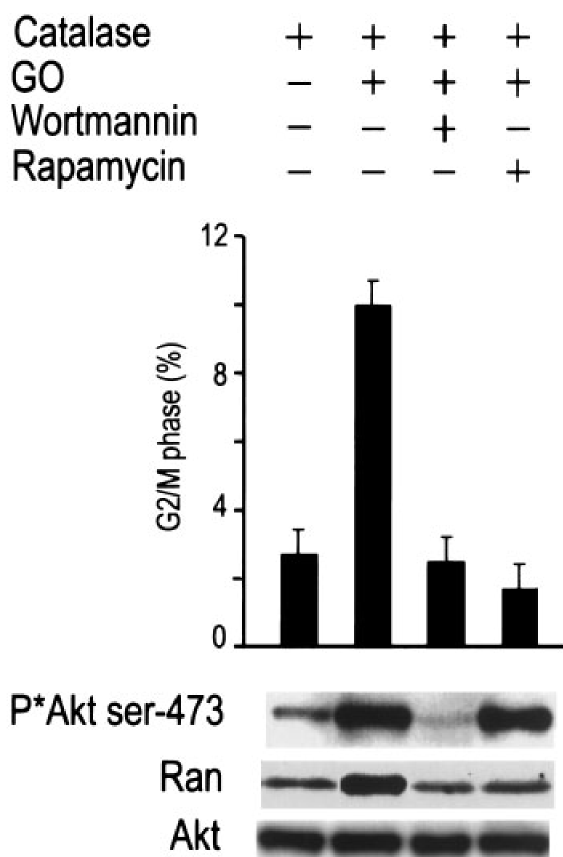

Fig. 1. mTOR kinase is downstream from PI3K/Akt in H_2O_2-induced mitosis. Primary cultures of type II pneumocytes were treated with 300 nM catalase, or catalase plus 20 mU/ml glucose oxidase (GO) in the presence or absence of the PI3K inhibitor wortmannin, or the mTOR inhibitor rapamacyn. Cell cycle was determined 20 h after treatment. Results are expressed as the mean ± SD of six independent experiments. Treatment with GO caused significant ($P < 0.0001$) increase in the number of cells in G2/M phases. Wortmannin (100 nM) or rapamycin (50 nM) effectively prevented H_2O_2-induced mitosis. Total cell lysates were prepared and analyzed by Western blot for phosphorylated (P*) Akt ser-473, Ran, and Akt protein expression.

H_2O_2 Signaling Via Ran

To characterize the time course of the effect of H_2O_2 on Ran protein expression, type II pneumocytes were stimulated with H_2O_2 using catalase/glucose oxidase and Ran protein expression was followed for 20 h. H_2O_2 increased Ran protein expression starting at 5 h and was maintained for 20 h (Fig. 3).

Myr-Akt and Ran-wt cDNA Increase ser-473 Phosphorylation in a Catalase-Dependent Manner

To confirm the finding that Akt and Ran are regulated by H_2O_2 (Fig. 1), we transfected type II pneumocytes with myr-Akt (Fig. 4) or with Ran-wt (Fig. 5) and examined the effect of catalase on Akt phosphorylation and Ran protein expression. Transfected myr-Akt increased Akt ser-473 phoshorylation and total Akt, as well as Ran protein expression (Fig. 4). Transfected Ran-wt consistently increased Akt ser-473 phosphorylation (Fig. 5) indicating that increased protein expression of Ran can regulate Akt phosphorylation. Treatment of both myr-Akt and Ran-wt transfected cells with 300 nM catalase effectively prevented increased Akt phosphorylation, indicating that H_2O_2 is an important mediator in this signaling pathway, because when H_2O_2 is scavenged by catalase in medium, there is no activation of tyrosine kinase receptors by H_2O_2 and there is no activation of Akt and downstream targets mTOR and Ran.

Myr-Akt and Ran-wt Increase Mitosis in a Catalase-, Wortmanin-, Rapamycin-Dependent Manner

To confirm the involvement of Akt and Ran in H_2O_2-induced mitosis, we analyzed the number of type II pneumocytes in G2/M phase after

Fig. 2. H_2O_2 signaling via Akt. Primary type II pneumocytes were treated with 300 nM catalase or catalase plus 20 mU/ml glucose oxidase (GO). Akt ser-473 phosphorylation was already detectable 15 min after addition of GO and reached maximum values at 60 min. No change was observed in total Akt. Akt phosphorylation was analyzed by Western blot. The data shown in this figure is representative of three experiments.

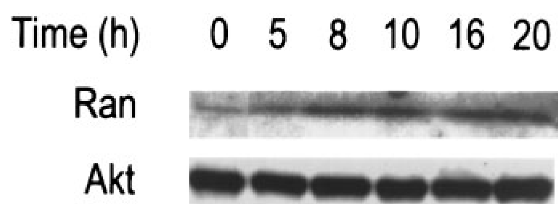

Fig. 3. H_2O_2 signaling via Ran. Primary type II pneumocytes were treated with 300 nM catalase or catalase plus 20 mU/ml glucose oxidase (GO) as in Figure 1. Upregulation of Ran was observed at 5 h and maintained for 20 h. Ran expression was analyzed by Western blot. The data shown in this figure is representative of three experiments.

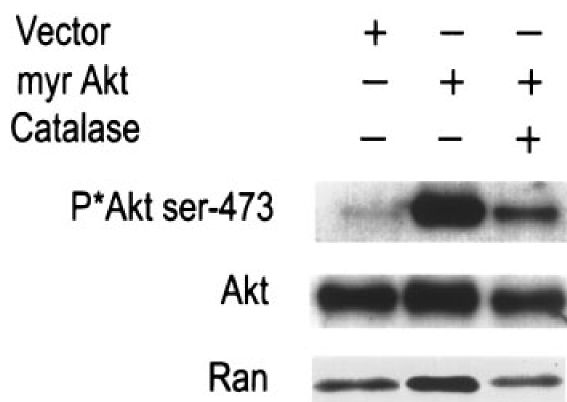

Fig. 4. Myr-Akt increases Akt ser-473 phosphorylation in a catalase-dependent manner. Primary type II pneumocytes were transfected with constitutively active myr-Akt. Total cell lysates were analyzed for Akt ser-473 phosphrorylation and total Akt 24 h after transfection. Constitutively active myr-Akt increased Akt ser-473 phosphorylation. Increased phosphorylation was prevented by catalase (300 nM). The data shown in this figure is representative of three experiments.

transfection with myr-Akt or Ran-wt (Fig. 6). We found that myr-Akt and Ran-wt significantly ($P < 0.0001$) increased the number of type II cells in G2/M phase. Mutant forms of both proteins showed no effect on G2/M phase. Catalase significantly ($P < 0.0001$) prevented the increase in the number of type II cells in G2/M phase induced by myr-Akt or Ran-wt, indicating that H_2O_2 is essential for Akt and Ran signaling. Wortmannin (PI3K inhibitor) significantly ($P < 0.0001$) prevented myr-Akt or Ran-wt induced mitosis confirming participation of PI3K in this pathway. Rapamycin (mTOR inhibitor) also ($P < 0.0001$) prevented the increase in the number of type II cells in G2/M phase induced by myr-Akt or Ran-wt in type II pneumocytes, confirming participation of mTOR in this pathway (Fig. 6).

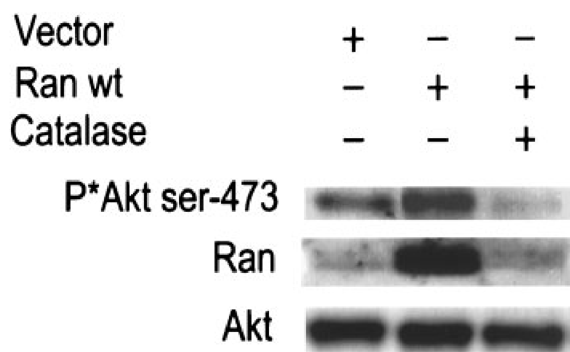

Fig. 5. Ran-wt increases phosphorylation of Akt ser-473 in a catalase-dependent manner. Primary type II pneumocytes were transfected with Ran wt. Total cell lysates were analyzed for Akt ser-473 phosphorylation, total Akt protein, and Ran protein expression 24 h after transfection. Ran-wt induced Akt ser-473 phosphorylation. Increased phosphorylation was prevented by catalase (300 nM). The data shown in this figure is representative of three experiments.

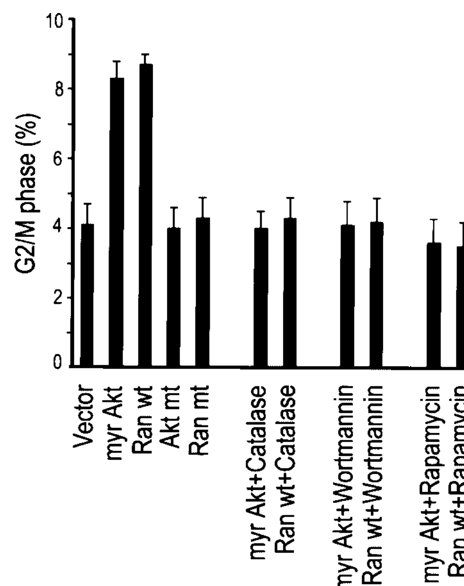

Fig. 6. Ran-wt and myr-Akt increase mitosis in catalase-, wortmannin-, rapamycin-dependent-manner. Primary type II pneumocytes were transfected with constitutively active myr-Akt or Ran-wt, and treated with catalase (300 nM), wortmannin (100 nM), or rapamycin (50 nM) 3 h after transfection. After transfection (24 h), cells were harvested and analyzed for cell cycle position using flow cytometric analysis of DNA content. Myr-Akt or Ran-wt significantly ($P < 0.001$) increased the number of cells in G2/M phase. Mutants of both proteins did not affect mitosis. Catalase, wortmannin, or rapamycin prevented increase of the type II cells in G2/M phase induced by transfected myr-Akt or Ran-wt. Bars represent mean $\pm$ SD of six experiments.

DISCUSSION

There is evidence that H_2O_2 signals through PI3K and Akt. However, the propagation of these signals towards downstream signaling molecules, such as mTOR and Ran, are unknown. In this study, we show that PI3K/Akt activates downstream mTOR-kinase and Ran protein during H_2O_2-dependent mitogenic signaling in type II pneumocytes.

Akt is a crucial mediator in signaling pathways leading to cell survival [Datta et al., 1999] and cell proliferation [Jin et al., 2003]. Akt is activated after binding of growth factors to their receptors [Radisavljevic et al., 2000; Testa and Bellacosa, 2001], after activation of tyrosine kinase receptors by ROS, or after activation of the tyrosine kinase Src by ROS [Esposito et al., 2003]. Diverse arrays of physiological

stimuli can induce Akt activity primarily through PI3K [Testa and Bellacosa, 2001]. PI3K generated phospholipids, phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-triphosphate, regulate Akt activity by direct binding to the pleckstrin homology domain. Akt is then translocated from the cytoplasm to the inner surface of the plasma membrane, where it can be phosphorylated at thr-308/309, in the kinase activation loop, and ser-473/474, in the carboxyl-terminal tail. Thr-308 and ser-473 are phosphorylated mostly in response to extra-cellular stimuli [Alessi et al., 1996; Andjelkovic et al., 1996]. Thr-308/309 phosphorylation is necessary for Akt activation, and ser-473/474 phosphorylation is required for maximal activity. In agreement with previous results in tumor cell lines [Shaw et al., 1998; Datta et al., 1999; Esposito et al., 2003], we show here that H_2O_2 triggers phosphorylation of Akt ser-473. As in these previous reports [Shaw et al., 1998; Esposito et al., 2003], our data show that activation of Akt by oxidative stress is preceded by PI3K activation (Fig. 1).

There are two known pathways controlling cell growth: one is regulated by PI3K and Akt and responds to growth factors and oxidants [Shaw et al., 1998; Datta et al., 1999; Esposito et al., 2003], and the second responds to nutrient supply and is controlled by mTOR [Schmelzle and Hall, 2000]. Here we show that mTOR-kinase mediates H_2O_2-induced mitosis and that mTOR is downstream of PI3K/Akt (Fig. 1). Our results show that mTOR is integrated into the signaling pathway induced by activation of PI3K/Akt by ROS and leading to increased mitosis of type II pneumocytes. TOR kinase is activated by amino acids and high ATP concentrations, suggesting that mTOR might be a nutrient and metabolism sensor [Dennis et al., 2001]. Since the intracellular concentrations of ROS are higher in metabolically active and proliferating tumor cells [Gonzalez-Flecha and Demple, 2000], it is possible that H_2O_2 mediates activation of mTOR by ATP or nutrients. Our results showing that mTOR activation is necessary for ROS-induced mitosis support this possibility.

TOR is a phosphatidylinositol kinase (PIK)-related kinase that controls cell growth and proliferation in response to nutritional cues [Long et al., 2002]. PIK-related kinases are involved in diverse cellular functions, such as control of cell growth, cell cycle, and DNA damage checkpoints, and recombination and maintenance of telomere length. Dysfunction of the PIK-related kinases results in a wide spectrum of severe diseases ranging from cancer to immunodeficiency [Keith and Schreiber, 1995]. Therefore, our findings could be relevant for the understanding of the mechanisms leading to hyperplasia of type II pneumocytes in association with pulmonary oxidative stress induced by oxygen therapy [Bowler and Crapo, 2002].

We have previously shown that H_2O_2 controls two central regulators of the cell cycle; the spindle formation regulator NuMA (nucleus-mitotic apparatus protein), and the regulator of nucleocytoplasmatic trafficking importin-α [Radisavljevic and Gonzalez-Flecha, 2003]. In this study, we show that H_2O_2 also induces upregulation of another nucleocytoplasmatic transporter, the Ran protein. Increased Ran protein expression was observed at 5 h and maintained for 20 h after H_2O_2 stimulation. These data are in agreement with the two known functions of Ran in mammalian cells, namely, as nucleocytoplasmatic transporter and spindle assembly factor [Smith et al., 2002]. Upregulation at 5 h would be most likely related to the transporting function, whereas continuous expression for up to 20 h would be most probably related to spindle formation [Smith et al., 2002]. Our data show for the first time that Ran is controlled by ROS through the PI3K/Akt/mTOR pathway during mitosis.

We also observed that overexpression of Ran in type II pneumocytes increased Akt ser-473 phosphorylation suggesting that Akt is regulated by Ran protein, and that overexpression of Akt increased Ran expression, suggesting that coordination between these two proteins may be crucial for cell survival and cell proliferation of type II pneumocytes. Ran protein is known to interact with several cell cycle related kinases. Ran-GTP promotes the interaction between TPX2 and the *Xenopus* Aurora A kinase, Eg2 leading to phosphorylation and increased kinase activity of Eg2 [Tsai et al., 2003]. Also, Ran-binding protein Ran-BPM (Ran-BP9) can interact with MET, a receptor protein-tyrosine kinase (RPTK) for hepatocyte growth factor (HGF) [Wang et al., 2002a], and with homeodomain-interacting protein kinase 2 (HIPK2) [Wang et al., 2002b].

Ran-mediated GTP hydrolysis is the main driving force for cargo-transport across the nuclear pore complex. This process is mediated

by a catalytic loop made of tyr-39, lys-23, and glu-69 of Ran [Seewald et al., 2002]. Our results show that a mutant form of Ran, carrying a mutation in the catalytic loop, could not increase mitosis indicating that the catalytic loop is important for the progression of mitosis. Furthermore, we show here that catalase suppressed Ran-induced mitosis suggesting that H_2O_2 signaling regulates Ran catalytic activity.

Our data show that H_2O_2-induced mitosis is mediated by activation of PI3K, which precedes activation of the downstream targets Akt, mTOR kinase, and Ran. These observations may be relevant for our understanding of the mechanisms involved in the development and progression of the type II pneumocytes hyperplasia or lung adenocarcinoma.

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