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P53 is Transported Into the Nucleus Via an Hsf1-Dependent Nuclear Localization Mechanism

Qiang Li and Jesse D. Martinez*

Arizona Cancer Center, University of Arizona, Tucson, Arizona

Abstract

Loss of p53 function can occur through disruption of its ability to localize to the nucleus. Previously we showed through characterization a set of mutant cell lines that lacked the ability to import p53 into the nucleus that nuclear translocation of p53 appeared to be mechanistically different from that of the SV40 T-antigen (SV40TAg). Here we extend that work by examining nuclear importation of p53 and SV40TAg using both in vivo and in vitro assays for nuclear localization. We show that disruption of microtubule polymerization using colchicine suppresses nuclear localization of p53 but not of SV40TAg. We also show, for the first time, that the heat shock transcription factor (Hsf1), is required for establishment of the microtubule network in cells and for nuclear localization of p53. In contrast, SV40TAg does not interact with polymerized microtubules suggesting that it is transported into the nucleus through an alternative mechanism. Interestingly, lacking of Hsf1 expression and suppressing Hsf1 by siRNA also made cells more resistant to the cytotoxic effects of paclitaxel. Hence, loss of Hsf1 activity not only suppressed p53 function, but also led to reduced sensitivity to killing by drugs that target microtubules.

Keywords

p53; nuclear localization; microtubules; heat shock factor; Hsf1

INTRODUCTION

The p53 tumor suppressor is a sequence specific transcription factor that performs its function by activating a variety of genes involved in multiple cellular activities, the two most important being cell cycle arrest and apoptosis [1]. As a consequence of p53's function as a transcription factor, its localization to the nucleus is essential for its function and exclusion from the nucleus is one of the mechanisms that results in loss of p53 tumor suppressor activity. Consistent with this cytoplasmic sequestration of p53 is observed in neuroblastomas and a subset of colorectal and breast cancers and is an indicator of poor clinical prognosis [2-4]. This suggests that disruption of p53 nuclear importation can eliminate its tumor suppressor activity and it suggests that the mechanism that controls trafficking into the nucleus may itself be disrupted during tumorigenesis.

Nuclear importation of p53 is made possible by virtue of a bipartite nuclear localization signal positioned near the c-terminal end of the protein [5,6]. Importation requires association between p53 and importin alpha [7] and transport along the microtubule cytoskeleton through the actions of the microtubule motor protein dynein [8]. However, p53

accumulates in the nucleus of cells that have been subjected to genotoxic stress because of decreased export from the nucleus as well as increased importation [9]. This results in activation of p53 and induction of p53-mediated gene expression including the induction of two prototypical p53 responsive genes, Mdm2, and p21 [9].

Surprisingly, although the dynamics of associations between p53 and other regulatory proteins that occur as it is transported into the nucleus have been well studied, there is little information regarding the characteristics of the p53 nuclear importation pathway itself. Here, we show that the p53 nuclear importation pathway requires a functional heat shock transcription factor (Hsf1).

MATERIALS AND METHODS

Cell Culture and Reagents

A1-5 is a rat fibroblast cell line transfected with the temperature-sensitive murine p53^{val135} gene [10]. MDBK cells were obtained from American Type Cell Culture Collection (ATCC, Manassas, VA). All cell lines were maintained in complete DMEM medium consisting of 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD). MDBK and A1-5 cells were incubated at 37°C and in an atmosphere containing 5% CO₂ unless otherwise noted. ALTR12 cell lines were maintained under the same conditions as A1-5 cells except that they were normally incubated at 32°C. AFT024 cells were kindly provided by Dr. Felicia Goodrum (University of Arizona, BIO5 Institute, Tucson, AZ).

Antibodies specific for p53 (PAb421) and to SV40 T-antigen (SV40TA_g) (PAB419) were kindly provided by Dr. Arnold Levine. Anti-α-tubulin, anti-P21, anti-Mdm2, and anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Hsf1 (SPA-950) antibodies were from Assay Designs/StressGen (Ann Arbor, MI).

Preparation of the EGFP Fusion Proteins and In Vitro Nuclear Import Assay

The preparation of GST-SagNLS-EGFP and GST-p53BNLS-EGFP fusion proteins were described previously [11]. The preparation of A1-5 cytosols and in vitro nuclear import assays were performed as described previously [12]. Assays in the absence of cytosol were conducted as a negative control.

Microtubule Cosedimentation Assay

Microtubule cosedimentation assay were performed according to the methods of Donkor et al.[13] In brief, A1-5 or ALTR12 cells were washed first in PBS and then in PEM-1 (0.1 mM Pipes, pH 7.6, 1 mM EGTA, 1 mM MgSO₄). Cells were resuspended in cold swelling solution (1 mM EGTA, pH 7.6, 1 mM MgSO₄) and homogenized after addition of protease inhibitors cocktail. After addition of Pipes (final concentration 0.1 M) and centrifugation for 5 min at 700 g, the supernatant was aspirated and supplemented with DTT (0.5 mM), GTP (1 mM), and paclitaxel (20 µM) and incubated at 37°C for 2 min. The mixture was centrifuged at 48,000 g for 30 min at 4°C onto a sucrose cushion (10% sucrose in BRB80X: 80 mM Pipes pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 1 mM GTP, 5 µM paclitaxel, and protease inhibitors cocktail). The pellet was resuspended in BRB80X and again centrifuged at 45,000 g for 15 min at 20°C. The resulting pellet was boiled in SDS sample buffer. As a control, the same experiment was performed with the following modifications: the supernatant after the first centrifugation step was supplemented with DTT (0.5 mM) only and the mixture incubated at 4°C for 30 min. High-speed centrifugations were then performed as described but omitting both GTP and paclitaxel in BRB80X solutions. The final pellet was boiled in SDS sample buffer and analyzed by SDS-PAGE.

In Vitro Toxicology Viability Assay, MTT-based

The cell viability was determined by the MTT (3-(4, 5-dimethylthiazole)-2, 5-diphenyltetrazoliumbromide) assay. Briefly, 10^4 cells/well were seeded in 96-well plates and allowed to attach overnight. The concentrations of paclitaxel were 0.1, 0.5, and 1, 5 $\mu\text{mol/L}$, respectively. The 0.1% dimethyl sulfoxide (DMSO) treated cells were set as negative controls. After treatment for 48 h, 10 μl of MTT (5 mg/ml) was added to each well and the cells were then returned to a 37°C incubator for 4 h. After the incubation period, the resulting formazan crystals were dissolved with 200 μl of DMSO for 15 min. To ensure complete dissolution of formazan crystals, the solution was gently pipetted up and down, and the absorbance was then read using a Max 200 microplate spectrophotometer at a wavelength of 570 nm. All experiments were performed in triplicate.

siRNA Transfection

A1-5 cells were grown in antibiotics free DMEM medium supplemented with 10% FBS to be ~60% confluent. Cells were then transfected twice with the rat Hsf1 siRNA pool (D-081010-00, Dharmacon), or negative control siControl Non-targeting siRNA#1 (Dharmacon) respectively. The DharmaFECT1 transfection reagent was used for transfection following the manufacturer's protocol (Dharmacon, Lafayette, CO). The final siRNA concentration was 20 nM for each transfection. 48 h after the last transfection, cells were collected and subjected to further analyses.

Indirect Immunofluorescence and Western Blotting

For immunofluorescence, cells were plated on coverslips 18 h prior to use. Next the cells were processed and analyzed as described previously [14]. Western blot analysis was performed according to standard procedures using PVDF membranes (Millipore, Bedford, MA). Signals were detected by the chemiluminescence method using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

RESULTS

Nuclear Importation of the Temperature Sensitive p53^{val135} Protein Requires an Intact Microtubule Cytoskeleton

Previously we characterized a set of cell lines, called ALTR cell lines, in which p53^{val135} nuclear localization was defective [11] and then subsequently showed that in one of those lines, the ALTR12 line, exclusion of p53 from the nucleus was due to loss of the Hsf1 transcription factor [15]. While using a digitonin permeabilized cell, in vitro nuclear importation system to characterize these lines, we noted that cells that did not support nuclear translocation of a p53NLS-containing substrate, but did support nuclear localization of a SV40TAgNLS-containing substrate suggesting the existence of distinct nuclear importation mechanisms.

The report that nuclear importation of p53 requires a microtubule cytoskeleton network [8] prompted us to examine the relationship between p53 and microtubules in ALTR12 cells. To begin, we first confirmed that nuclear translocation of the p53^{val135} temperature sensitive mutant in parental A1-5 cells required an intact microtubule cytoskeleton. A1-5 cells were exposed to the microtubule disruptors colchicine and nocodazole, or to the actin filament disruptors cytochalasin D and the cells incubated either at 37°C or shifted to 32°C (Figure 1A). As expected, the p53 in A1-5 cells not treated with a drug collected in the nucleus after shifting the incubation temperature to 32°C. However, incubating with either colchicine or nocodazole resulted in cytoplasmic localization of p53 even when the cells were incubated at 32°C. Quantitation of cells with nuclear p53 showed nearly 90% of the cells with p53 in the nucleus. However, this was reduced to about 10% in cells treated with colchicine or

nocodazole (Figure 1B). Notably, p53 nuclear localization was not affected by the actin filament disruptors cytochalasin D.

For comparison, we tested whether colchicine had any effect on nuclear localization of the SV40TAg. The AFT024 cell line, which is stably transfected with a temperature sensitive mutant SV40TAg were incubated with colchicine [16]. As shown in Figure 1C, nuclear accumulation of SV40TAg was observed at 32°C. However, this was reduced to about 10% after shifting the incubation temperature to 37°C for 16 h in control cells. Importantly, SV40TAg nuclear localization still occurred in cells treated with colchicine (Figure 1D).

Activation of p53 leads to induction of p21 and mdm2 expression, and loss of p53 function also leads to loss of p53-mediated induction of these two proteins [1]. Consequently we tested whether disruption of microtubule polymerization had any effect on induction of p21 and mdm2 in A1-5 cells incubated at 32°C (Figure 2). As shown, pre-treatment with either colchicine or nocodazole suppressed the expression of both p21 and Mdm2 induced by temperature shift. As expected cytochalasin D had no effect on p21 and Mdm2 induction. Hence, the induction of nuclear localization of the p53^{val135} protein by temperature shift required microtubule function, which also compromised its ability to activate gene expression.

Nuclear Importation Promoted by the SV40TAgNLS Does Not Require a Microtubule Cytoskeleton

Previously we utilized an in vitro nuclear importation assay using a p53 nuclear localization signal containing substrate and showed that while nuclear importation of the GFP-p53NLS was compromised in some ALTR cells, nuclear importation of the GFP-SV40TAgNLS containing substrate was supported by cytosol from all of the cells [11]. This suggested two distinct nuclear importation pathways. Hence, we tested the effect of microtubule disruptors on the in vitro nuclear importation of these two substrates. We used two different cell lines, A1-5 and MDBK, in our in vitro assays. The cells were grown on coverslips and pre-treated with colchicine or nocodazole and then permeabilized with digitonin as described in Materials and Methods Section. Visualization of the GFP-p53NLS substrates showed that pre-treating A1-5 or MDBK with microtubule disruptors suppressed importation of the substrate protein (Figure 3A) and that this treatment resulted in a drop from 80% of the cells with nuclear localized substrate to only 10% (Figure 3B). Moreover, the result was the same regardless of whether A1-5 or MDBK cells were used. In contrast, pre-treatment with colchicine or nocodazole had no effect on the nuclear importation of the GFP-SV40TAgNLS (Figure 4). These results suggest that intact microtubule structure is required for nuclear importation mediated by the p53-NLS but not by the SV40-NLS.

P53 Association With Microtubules is Defective in the ALTR12

Characterization of ALTR12 cells, which were derived by mutagenizing A1-5 cells, demonstrated that nuclear importation of p53 was defective in these cells and that this defect could be attributed to loss of the Hsf1 transcription factor [15]. Previously, Giannakakou et al. [8] demonstrated that p53 cosedimented with microtubules, a property that was indicative of a functional p53 nuclear importation pathway. Since p53 nuclear importation was defective in ALTR12 cells, we tested for p53 association with microtubules using microtubule cosedimentation. As shown in Figure 5A, paclitaxel caused α -tubulin polymerization and p53 cosedimented with it in extracts from parental A1-5 cells. However, in addition to less α -tubulin sedimentation, no p53 cosedimented with α -tubulin in paclitaxel treated ALTR12 extracts even though α -tubulin protein levels were the same as that seen in parental A1-5 cells. Importantly, cosedimentation of p53 with polymerized α -tubulin was restored in ALTR12 cells that were stably transfected with a constitutively

active Hsf1 (Figure 5A, ALTR12/Hsf1). These results suggested that Hsf1 may be important for p53 to associate with microtubules. We next examined the interaction between p53 and α -tubulin to determine whether coprecipitation of p53 with α -tubulin was due to a genuine protein-protein interaction or to trapping (Figure 5B). As can be seen α -tubulin immunoprecipitated with p53 in all three cell lines. However, the amount of p53 bound with α -tubulin was reduced in ALTR12 cells. Interestingly, Hsf1 was also found to cosediment with α -tubulin in paclitaxel treated A1-5 and ALTR/ActHsf1 extracts (Figure 5A). However, Hsf1 did not co-immunoprecipitate with p53 (Figure 5B), suggesting that Hsf1-bound α -tubulin and p53-bound α -tubulin are two distinct protein complexes.

We next tested whether SV40TAg cosedimented with polymerized microtubules. Extracts were prepared from AFT024 cells which express SV40TAg and treated them with paclitaxel as described above (Figure 5C). We found that SV40TAg did not cosediment with polymerized α -tubulin even though SV40TAg did accumulate in the nucleus (Figure 1C). Taken together these results suggest that nuclear localization of p53 occurs through a mechanism requiring microtubules and that this is distinct from the importation mechanism utilized by SV40TAg.

ALTR12 Cells Have an Abnormal Microtubule Structure and are Resistant to Paclitaxel-Mediated Cytotoxicity

We previously showed that Hsf1 was not expressed in ALTR12 cells [15]. Hence, to determine whether Hsf1 is involved in the regulation of microtubule polymerization, we examined the microtubule network in these cells by staining the cells with a FITC- α -tubulin antibody (Figure 6A). Visualization of the anti-microtubule staining showed that the microtubule network is more disorganized and that the network structure as seen in A1-5 cells is nearly absent in ALTR12 cells. However, the microtubule network is restored to normal in ALTR12 cells that were stably transfected with constitutively active Hsf1. Hence, loss of Hsf1 leads to a disrupted microtubule structure in vivo. Our observation that the addition of paclitaxel to extracts from ALTR12 cells resulted in less α -tubulin sedimentation, suggested that these cells might be resistant to paclitaxel since this drug acts by interfering with the normal function of microtubule breakdown. Hence, we speculated that the ALTR12 might be less sensitive than their parental A1-5 cells in their sensitivity to paclitaxel. To test this, we incubated A1-5 cells, ALTR12 cells, and ALTR12/Hsf1 cells to paclitaxel and survival quantified using the MTT assay (Figure 6B). We found that ALTR12 cells were highly resistant to killing by paclitaxel, whereas both parental A1-5 and ALTR12/Hsf1 cells were quite sensitive. We confirmed that the paclitaxel sensitive cells were non-viable by trypan blue staining and found that paclitaxel treatment resulted in a marked increase in non-viable cells (Figure 6C).

Since paclitaxel is known to induce p53 activation [17], we tested the effect of Hsf1 on p53 activation by examining its nuclear localization, phosphorylation, and capacity to induce p21. As expected, paclitaxel treatment induced p53 nuclear accumulation in A1-5 and ALTR12/ActHsf1 cells, but not in ALTR12 cells (Figure 7A and B). Quantitation of cells with nuclear p53 showed nearly 50% of the cells with p53 in the nucleus in paclitaxel treated A1-5 or ALTR12/ActHsf1 cells. However, paclitaxel had no apparent effect on p53 nuclear localization in ALTR12 cells. Western blotting indicated that paclitaxel resulted in the phosphorylation p53 on serine 15 and the induction of p21 expression when cells had a functional Hsf1 (Figure 7C). These results suggested that Hsf1 is required for p53 activation in response to paclitaxel.

Suppression of Hsf1 Expression by siRNA Affects p53 Association with Microtubules and Sensitivity to Paclitaxel

To confirm the above results, we utilized an hsf1 siRNA to knock down Hsf1 expression in A1-5 cells and then performed a microtubule cosedimentation assay on extracts prepared from these cells. As can be seen knockdown of Hsf1 using siRNA resulted in decreased paclitaxel-induced cosedimentation of p53 with α -tubulin (Figure 8A). This is consistent with the hypothesis that Hsf1 plays a role in regulation of the association of p53 with microtubule. As expected, the Hsf1 siRNA caused a dramatic reduction in Hsf1 protein. In addition, we found that knocking down Hsf1 expression also resulted in reduced cytotoxicity in cells treated with paclitaxel (Figure 8B). Hence, Hsf1 is required for p53 association with polymerized α -tubulin.

DISCUSSION

The primary observation in this work is that p53 and SV40TAg are transported into the nucleus through distinct nuclear localization pathways. SV40TAg is imported into the nucleus through a pathway that is not compromised by microtubule disruptors. In contrast, nuclear importation of p53 is abolished by colchicine and nocodazole both in vitro and in vivo, consistent with the observation that p53 is transported along the microtubule cytoskeleton during its importation into the nucleus [8]. Importantly, the p53-specific importation mechanism requires that Hsf1 be functional. Hsf1 is the major regulator of the heat shock response which is implicated in protecting organisms from heat and other stresses such as heavy metals, small molecule chemical toxicants, infection, and oxidative stress [18]. Recently, Hsf1 was reported to contribute to cell resistance to genotoxic stress caused by doxorubicin [19] suggesting a protective role of Hsf1 against genotoxic stress. Given Hsf1's key role in the response of cells to various stresses we propose that Hsf1 mediates a stress responsive nuclear importation mechanism. This is consistent with p53's role as a stress response signaling protein. Hence, it appears that activation of p53 is mediated through a pathway that is regulated by Hsf1 and that this results in the transportation of p53 into the nucleus.

A novel observation in this work is that a functional Hsf1 is required for microtubule polymerization. This is supported by the fact that the quantity of α -tubulin sedimentation induced by paclitaxel in ALTR12 extract was less than that observed in extracts from A1-5 or ALTR12/Hsf1 cells; the siRNA suppression of Hsf1 protein also resulted in decreased α -tubulin sedimentation; and the microtubule network in ALTR12 cells is less organized than that in A1-5 and ALTR12/ActHsf1 cells. Since heat shock proteins such as Hsp90, Hsp70, TCP-1, and other small heat shock proteins can bind to microtubules and affect polymerization [20], it is possible that Hsf1 influences microtubule polymerization by regulating the expression of heat shock proteins. However, as Hsf1 also cosediments with microtubules, it is also possible that Hsf1's association with microtubules may affect microtubule stabilization. Importantly, elimination of Hsf1 activity as occurred in ALTR12 cells or by using siRNA to knockdown the protein resulted in an inability of p53 to associate with microtubules. Co-immunoprecipitation also showed a decrease in p53 association with tubulin in ALTR12 cells. These results indicated that Hsf1 plays a role in p53 nuclear localization by facilitating p53 association with microtubule. It was shown that the N-terminus of p53 which includes MDM2 binding domain is the region that associates with microtubules [8]. Hence, we speculate that Hsf1 may somehow disrupt MDM2 binding and thus increase p53 association with microtubules. However, no detectable Hsf1 interaction with p53^{Val135} was showed in co-immunoprecipitation experiments (Figure 5B), and Mdm2 bound with p53^{Val135} only at permissive temperature (Supplementary data) making it difficult to test this hypothesis in our model cell line. P53 is known to interact with hsp90/hsp70-based chaperone machinery [21]. Components of the hsp90/hsp70 machinery such as

hsp90, hsp70, and FKBP52 are important for regulating p53 nuclear import [22-24], protecting p53 protein from degradation by the MDM2-dependent ubiquitin-proteasome pathway of proteolysis [25], and p53 association with microtubule [23]. Hence, Hsf1 might be expected to affect p53 association with microtubule via regulating Hsp90/Hsp70 chaperone machinery.

Interestingly, inactivation of Hsf1 resulted in cells that were less sensitive to the cytotoxicity of paclitaxel. ALTR12 cells which lack the Hsf1 protein or cells in which Hsf1 was reduced using a siRNA were considerably more resistant to paclitaxel. Examination of p53 activation in response to paclitaxel showed that paclitaxel induces p53 nuclear translocation, phosphorylation (Ser15), and p21 expression in parental A1-5 and ALTR12/ActHsf1 cells but not in ALTR12 cells, suggesting an effect of Hsf1 on p53 activation. Since paclitaxel has been reported to induce apoptosis in both p53-dependent [26] and - independent [27] manner, it be that it is the loss of Hsf1 rather than the failure of p53 activation that accounts for the reduced cytotoxicity seen in Hsf1 deficient cells. Hence, the functional status of Hsf1 may be an important determinant of tumor cell sensitivity to paclitaxel.

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Abbreviations

Hsf1	heat shock transcription factor
SV40TAg	SV40 T-antigen
DMSO	dimethyl sulfoxide

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Figure 1.

Nuclear importation of p53 is inhibited in A1-5 cells by microtubule filament disruptors. (A) A1-5 cells were grown on coverslips at 37°C and then treated with DMSO (control), colchicine, nocodazole, or cytochalasin D for 30 min at 37°C. Subsequently, incubation was continued at either 37°C or 32°C for additional 2 h. The cells were then fixed and stained for p53 localization using Pab421 antibody. (B) The fraction of cells with nuclear localized p53 was determined for the samples in panel A and graphed. Bars represent the average percentage of cells $\pm$ SE exhibiting nuclear localized p53 calculated from examining ~500 cells in random microscope fields. The average is from three separate experiments. (C) AFT024 cells were grown on coverslips at 32°C overnight and either transferred to 37°C incubator or left at 32°C for an additional 16 h. The cells were then fixed and stained for SV40Tag localization using Pab419 antibody. (D) The fraction of cells with nuclear localized SV40Tag was determined for the samples in panel C and graphed. Bars represent the average percentage of cells $\pm$ SE exhibiting nuclear localized SV40Tag calculated from examining ~500 cells in random microscope fields. The average is from three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

Microtubule disruptors also inhibit p53-mediated induction of p21 and Mdm2. A1-5 cells at 70% confluence were pre-treated with either DMSO, colchicine, nocodazole, or cytochalasin D at the concentrations indicated for 30 min at 37°C. Subsequently, the cultures were further incubated for an additional 6 h at 37°C or at 32°C and then the cells harvested and analyzed by Western blotting for the presence of p21 or Mdm2. β -actin was used as a loading control.

Figure 3.

Microtubule filaments disruptors inhibited in vitro nuclear importation of GST-p53BNLS-EGFP. (A) A1-5 and MDBK cells were either not treated or treated with colchicine or nocodazole for 30 min. The cells were then permeabilized with digitonin and then incubated with GST-p53BNLS-EGFP substrate for 45 min at 30°C in the presence or absence of A1-5-derived cytosol. Subcellular localization of GST-p53BNLS-EGFP was visualized using fluorescent microscopy. (B) The number of cells with nuclear localized GST-p53BNLS-EGFP was determined by counting ~500 cells in random microscope fields. The bars represent the percentage of cells exhibiting nuclear localized substrate $\pm$ SE. The values graphed are averaged from three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Microtubule disruptors do not inhibit nuclear importation of GST-SV40TagNLS-EGFP. (A) A1-5 and MDBK cells were either not treated or pre-treated with colchicine or nocodazole for 30 min. The cells were then permeabilized with digitonin and then incubated with GST-SV40TagNLS-EGFP for 45 min at 30°C in the presence or absence of A1-5-derived cytosol. Subcellular localization of GST-SV40TagNLS-EGFP was visualized using fluorescent microscopy. (B) The number of cells with nuclear localized GST-SV40TagNLS-EGFP was determined by counting ~500 cells in random microscope fields. The bars represent the percentage of cells exhibiting nuclear localized substrate $\pm$ SE. The values graphed are from three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5.

p53 but not SV40TAg cosediments with polymerized tubulin. (A) A1-5, ALTR12 and ALTR12/Acthsf1 cells at 80% confluence were harvested and extracts prepared. Polymerization of tubulin was induced using paclitaxel as described in Material and Methods Section. The total amount of α -tubulin, p53, Hsf1, and β -actin was determined in the starting extracts (cell extract) and the amount of α -tubulin, p53, Hsf1, and β -actin that cosedimented in extracts that were either untreated (paclitaxel -) or treated (paclitaxel +) with paclitaxel using the appropriate antibodies. (B) A1-5, ALTR12, and ALTR12/Acthsf1 cells were grown to 80% confluency, harvested, and cell lysates prepared and p53 immunoprecipitated using Pab421 antibody. Mouse IgG was used as a control. The immunoprecipitates were examined by Western blotting using anti- α -tubulin and anti-Hsf1 antibody. Input represented 10% of cell lysates used in the co-IP experiment. The p53 antibody (Pab421) alone was included as a negative control. (C) AFT024 cells at 80% confluence were harvested and extracts prepared. Polymerization of tubulin was induced using paclitaxel as described in Material and Methods Section. The total amount of α -tubulin and SV40TAg was determined in the starting extracts (cell extract), the polymerized and sedimented tubulin (paclitaxel +), and in sedimented material from extracts that were not treated with paclitaxel (paclitaxel -) using the appropriate antibodies.

Figure 6.

Loss of Hsf1 function results in disrupted microtubule network and decreased sensitivity to paclitaxel-mediated cell killing. (A) A1-5, ALTR12, and ALTR12/ActHsf1 cells were grown on coverslips overnight and stained for tubulin by an anti-tubulin antibody (FITC- α -tubulin). (B) A1-5, ALTR12, or ALTR12/ActHsf1 cells were treated with increasing concentrations of paclitaxel for 48 h and cell viability was determined using the MTT assay. The values graphed are the average absorbance at 570 nm compared to that of no paclitaxel control $\pm$ SE from three independent experiments. (C) A1-5, ALTR12, or ALTR12/ActHsf1 cells were treated with increasing concentrations of paclitaxel for 48 h and the number of surviving cells was determined using Typan Blue staining. The values graphed are the average surviving fraction compared to that of no paclitaxel control $\pm$ SE from three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 7.

Loss of Hsf1 function results in inhibition of p53 activation in response to paclitaxel. (A) A1-5, ALTR12, and ALTR12/ActHsf1 cells were grown on coverslips overnight and then treated with DMSO (control), or 0.1 μ M paclitaxel (paclitaxel) for 2 h at 37°C. The cells were then fixed and stained for p53 localization using Pab421 antibody. Typical results are shown. (B) The fraction of cells with nuclear localized p53 was determined for the samples in panel A and graphed. Bars represent the average percentage of cells $\pm$ SE exhibiting nuclear localized p53 calculated from examining ~500 cells in random microscope fields. The average is from three separate experiments. (C) A1-5, ALTR12, or ALTR12/ActHsf1 cells were grown to 70% confluence and then pre-treated with either DMSO (paclitaxel -), or 0.1 μ M paclitaxel (paclitaxel +) for 6 h at 37°C and then the cells harvested and analyzed by Western blotting for the presence of p21, phospho-p53 (Ser15) or p53 (PAb421). Beta actin was used as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 8.

Suppression of Hsf1 expression inhibited cosedimentation of p53 with polymerized tubulin and reduced sensitivity to paclitaxel-mediated cell killing. (A) A1-5 cells were either mock transfected (Mock) or transfected with either a control siRNA (siCTR) or with an anti-Hsf1 siRNA. Forty-eight hours after transfection extracts were prepared and tubulin polymerized using paclitaxel as described in Figure 5. The total amount of Hsf1, p53, α-tubulin, and β-actin was determined in the starting extracts (cell extract), the polymerized and sedimented tubulin (paclitaxel +), and in sedimented material from extracts that were not treated with paclitaxel (paclitaxel -) using the appropriate antibodies. (B) A1-5 cells were grown on 96-well plate overnight and transfected with Mock, siCTR, or Hsf1 siRNA as described in Material and Methods Section. Forty-eight hours after transfection, cells were treated with increasing concentrations of paclitaxel for 48 h. Cell death was determined using the MTT assay. The values graphed are the average absorbance at 570 nm compared to that of no paclitaxel control $\pm$ SE from three independent experiments.