Urokinase Expression by Tumor Suppressor Protein p53

A Novel Role in mRNA Turnover

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Lung carcinoma (H1299) cells deficient in p53 (p53^{-/-}) express large amounts of urokinase-type plasminogen activator (uPA) protein and uPA mRNA, and exhibit slower degradation of uPA mRNA than that of p53-expressing nonmalignant Beas2B human airway epithelial cells. Expression of p53 protein in H1299 cells, upon transfection with p53 cDNA, suppressed basal as well as uPA-induced expression of uPA protein in both conditioned media and cell lysates, and decreased the level of steady-state uPA mRNA primarily due to increased uPA mRNA turnover. Inhibition of p53 expression by RNA silencing (siRNA) in Beas2B cells enhanced basal and uPA-mediated uPA protein and mRNA expression with stabilization of uPA mRNA. Purified p53 binds to the uPA mRNA 3' untranslated region (UTR) in a sequence-specific manner and endogenous uPA mRNA associates with p53 protein isolated from Beas2B cytosolic extracts. p53 binds to a 35-nucleotide uPA 3'UTR sequence and insertion of this sequence into β -globin mRNA accelerates degradation of otherwise stable β -globin mRNA. These observations confirm a new role for p53 as a uPA mRNA binding protein that down-regulates uPA mRNA stability and decreases cellular uPA expression.

Keywords: post-transcriptional regulation; urokinase-type plasminogen activator; p53; mRNA stability; RNA binding protein

Plasminogen activation by tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA) is mainly responsible for intravascular and extravascular thrombolysis, respectively (1). Plasmin generated through cleavage of plasminogen by uPA facilitates tissue remodeling either directly by extracellular matrix degradation or through activation of latent matrix metalloproteinases. The involvement of uPA in remodeling of the extracellular matrix in acute and chronic lung injury, repair (1, 2), and lung neoplasia (3–5) has become increasingly clear during the past decade.

uPA, a 50-kD glycoprotein, is secreted by lung epithelial cells as an inactive proenzyme single chain form that is activated by plasmin and other proteases (6). Lung epithelial cells also express cell surface uPA receptor (uPAR), which is a glycolipid-anchored receptor (7). Receptors for uPA, uPAR, and plasminogen allow proximate cell surface activation of plasminogen. Binding of uPA to uPAR activates several cellular signaling intermediaries involved in cell proliferation, migration, adhesion, and invasion (8). uPA induces both uPA and uPAR

CLINICAL RELEVANCE

The current study describes the underlying mechanism involved in lung epithelial cell death during injury such as acute respiratory distress syndrome or acute lung injury.

expression through post-transcriptional stabilization of respective mRNAs (7, 9).

uPA is an important mediator of tumor progression and metastasis and increased expression of uPA has been shown in many nonmalignant and malignant cells (9). Similarly, interaction of uPA with uPAR facilitates cell adhesion, chemotaxis, cell viability, and proliferation (10–13). Lung epithelial cell apoptosis is subject to regulation by uPA (13), and uPA-mediated remodeling of extracellular matrix is operative in the setting of diverse lung diseases such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, or a variety of interstitial lung diseases (2). These diseases are characterized by lung epithelial cell apoptosis that promotes development of progressive fibrosis, ultimately leading to respiratory failure.

Recent observations indicated cross-talk between uPA-mediated remodeling of extracellular matrix and viability or proliferation of lung epithelial cells (13, 14). We recently provided evidence that links these two important phenomena. We found that uPA modulates lung epithelial cell apoptosis/proliferation through expression of p53 (13). Loss of p53 function due to deletion or mutation (14–17) and expression of elevated levels of uPA by tumor cells (9) as well as autoinduction and proliferative responses by uPA in several cell types (9, 18) prompted us to test the inference that uPA–p53 cross-communication regulates uPA expression and downstream pathophysiologic functions of uPA in lung epithelial cells. To our knowledge, this area has not been previously explored. We now demonstrate a newly identified uPA–p53 cross-talk that regulates uPA expression and influences cellular fibrinolytic capacity and viability. We show, for the first time, that p53 is a sequence-specific mRNA binding protein that destabilizes uPA mRNA and expression of uPA by lung epithelial or carcinoma cells.

MATERIALS AND METHODS

Cell Cultures

Human bronchial epithelial (Beas2B) cells and p53-deficient human lung non-small cell carcinoma (H1299) cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA). These cells were maintained in LHC-9 medium and Roswell Park Memorial Institute (RPMI) 1640 medium, as previously described (13, 19).

uPA Analysis

Beas2B cells or H1299 cells grown to confluence were treated with phosphate-buffered saline (PBS) or amino-terminal fragment (ATF) of uPA, which is known to induce endogenous uPA (20) in serum-free media. The proteins from conditioned media (CM) and cell lysates (CLs)

(Received in original form November 9, 2007 and in final form April 3, 2008)

This work was supported by National Heart, Lung, and Blood Institute grants R01 HL071147 and Project 2 of P01 HL62453.

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Am J Respir Cell Mol Biol Vol 39, pp 364–372, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0406OC on April 3, 2008

Internet address: www.atsjournals.org

were analyzed for the expression of uPA protein by either immunoblotting or immunoprecipitation using anti-uPA monoclonal antibody as previously described (9, 21). Alternatively, these cells were treated with PBS or uPA in the presence of ^{35}S -labeled methionine. Total protein from CM and CLs was subjected to immunoprecipitation using anti-uPA monoclonal antibody. ^{35}S -labeled uPA was detected by autoradiography as described earlier (9).

Plasmid Construction and cDNA Labeling

Previously cloned uPA and p53 cDNAs were individually subcloned in *Hind* III and *Xba* I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA) and the nucleotide sequences of the clones were confirmed by sequencing. Full-length templates of uPA or p53 cDNAs were labeled with ^{32}P -labeled 2'-deoxycytidine 5'-triphosphate (dCTP) using a rediPrime labeling kit (GE Healthcare, Buckinghamshire, UK).

Northern Blotting of uPA or p53 mRNA

A Northern blotting assay was used to assess the level of uPA or p53 mRNA. Beas2B or H1299 cells were treated with PBS or uPA for 12 hours in RPMI media. Total RNA was isolated using total RNA isolation (TRI) reagent. RNA (20 μg) was subjected to Northern blotting using ^{32}P -labeled uPA or p53 cDNA probe as described earlier (7). The intensity of the bands was measured by densitometry and normalized against that of β -actin. uPA mRNA stability was assessed by transcription chase experiments. In these experiments, cells stimulated with PBS or uPA for 12 hours were treated with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) to inhibit ongoing transcription, after which total RNA was isolated at specific time points. uPA mRNA was measured by Northern blot as described above.

Nuclear Run-On Transcription Activation Assay

Confluent cells grown in two T182 flasks were serum-starved overnight in RPMI media. The cells were later analyzed for uPA mRNA synthesis using the transcription activation assay as described earlier (21).

Transfection of p53-Deficient H1299 Cells with p53 cDNA

To confirm that p53 regulates uPA expression, p53 cDNA was cloned into an eukaryotic expression vector pcDNA3.1. H1299 cells were transfected with vector cDNA or vector DNA harboring p53 cDNA using LipofectAMINE (Invitrogen), and stably transfected H1299 cell lines were created as described earlier (7). The cells were treated with PBS or uPA and the effect of p53 expression on basal and uPA-mediated p53, uPA protein, and mRNA expression was analyzed as described above. The effect of p53 expression on basal and uPA-mediated uPA mRNA synthesis was determined by run-on transcription. uPA mRNA stability was assessed by transcription chase experiments using DRB to inhibit ongoing transcription, after which total RNA was isolated at specific time points. uPA mRNA was measured by Northern blotting as described above.

Inhibition of p53 Expression by RNA Silencing in Beas2B Cells

The Beas2B cells grown to 70% confluence were treated with non-specific siRNA or p53-specific siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA) for 36 hours. The cells were then treated with PBS or uPA, and the expression of p53 or uPA protein and mRNA was analyzed by Western or Northern blotting. The effect of p53 inhibition on basal and uPA-mediated uPA mRNA synthesis or decay was determined as described above.

In Vitro Transcription

Linearized plasmids containing human uPA mRNA transcriptional templates of uPA cDNA were transcribed *in vitro* with T_7 or Sp_6 polymerase (Ambion, Austin, TX). The uPA mRNA transcripts were synthesized according to the manufacturer's protocol except that 50 μCi of ^{32}P -uridine triphosphate (UTP) was substituted for unlabeled UTP in the reaction mixture as described earlier (19).

Molecular Cloning, Expression, and Purification of p53

The recombinant p53 (rp53) protein in glutathione S-transferase (GST) fusion protein form was expressed using pGEX-2TK prokaryotic ex-

pression vector and purified on a glutathione-sepharose column. The bound GST fusion protein was treated with 2 ml of a thrombin digestion buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl_2) containing 5 units/ml bovine thrombin as described earlier. The rp53 protein was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed by Coomassie staining and Western blotting using anti-p53 monoclonal antibody to check the purity of the preparation.

Gel Mobility Shift Assay

The binding activities of rp53 protein were tested by gel mobility shift assay using uniformly ^{32}P -labeled transcripts corresponding to the uPA coding region (CDR) or the 3'-untranslated region (UTR) as described earlier (19). Samples were then separated by electrophoresis on 5% native polyacrylamide gel, dried, and autoradiographed at -70°C using Kodak X-ray film (Carestream Health Inc., Rochester, NY).

Whole Cell Extraction and Immunoprecipitation of RNA-Protein Complex

To confirm the direct interaction of p53 protein with uPA mRNA *in vivo*, we cross-linked Beas2B cells treated with PBS and uPA (50 ng/ml or 1 $\mu\text{g}/\text{ml}$) with formalin as described before (22). The cytosolic extracts were immunoprecipitated using nonspecific mouse IgG followed by monoclonal antibody against human p53 protein in the presence of RNase inhibitor and ribosomal RNA for 1 hour at room temperature. The immune complexes were later precipitated using protein A/G-agarose, and the agarose beads were washed three times with lysis buffer. Total RNA was isolated from the immune complexes using TRI reagent and associated uPA mRNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers in the presence of ^{32}P -labeled dCTP. The PCR products were later identified by nucleotide sequencing of corresponding nonradioactive bands or Southern blotting using ^{32}P -labeled uPA cDNA (21, 23).

Competitive Inhibition by Sense and Antisense mRNA or Polyribonucleotides

The rp53 protein was incubated with various amounts (0–125-fold excess) of unlabeled sense or antisense uPA 3'UTR mRNA at 30°C for 30 minutes and then treated with RNase T1 and heparin as described above, and the reaction mixtures were run on 5% native gels, dried, and autoradiographed. In separate experiments to determine the specificity of the RNA-protein complex, rp53 protein was pretreated with a molar excess of ribonucleotide poly(A), (C), (G), or (U) for 30 minutes at 30°C before the ^{32}P -labeled uPA mRNA and RNase T1 steps.

Effects of SDS and Proteinase K

The rp53 protein was treated with SDS (0.1%) or proteinase K (2.5 mg/ml) for 30 minutes at 37°C before addition of ^{32}P -labeled uPA mRNA. The reaction mixtures were subjected to RNase T1 and heparin digestion as described above, after which the complexes were resolved on 5% native gels that were then dried and exposed to X-ray film at -70°C . In separate experiments, ^{32}P -labeled uPA 3'UTR mRNA was predigested with RNase T1 (50 units) for 30 minutes at 37°C before undergoing gel mobility shift assay using rp53 protein.

Determination of p53 Binding Site on uPA mRNA 3'UTR

uPA 3'UTR cDNA fragments of various sizes were synthesized by PCR amplification of a full-length uPA cDNA template. These deletion fragments were cloned into pcDNA3.1 and transcribed *in vitro* in the presence of ^{32}P -labeled UTP. ^{32}P -labeled deletion transcripts were subsequently used as probes for gel mobility shift and ultraviolet cross-linking studies to localize the rp53 protein binding sequence on uPA 3'UTR mRNA.

Construction of β -Globin/uPA Chimeric Message

Two 35-bp DNA fragments, one corresponding to the rp53 binding sequence from uPA 3'UTR cDNA and the other a control nonbinding cDNA, were prepared by PCR. Each of these cDNA fragments was inserted into the 3'UTR of the full-length human β -globin cDNA. The

chimeric β -globin/uPA 3'UTR cDNA constructs were cloned into the *Hind* III-*Xba* I site of a eukaryotic expression vector, pcDNA3.1. The orientations and sequences of the chimeric clones were verified by sequencing. Beas2B cells were transfected with the prepared chimeric plasmid constructs by lipofection using LipofectAMINE, and transient transfectants were grown in culture flasks. Total RNA was isolated at various time points after inhibiting the transcription by DRB. Chimeric β -globin/uPA mRNA was then measured by Northern blotting using 32 P-labeled cDNA. The half-life of the mRNA at each interval was determined by densitometry, normalized to the β -actin control mRNA of the samples, and subsequently compared with the densitometric values of samples determined at the 0-hour baseline of each experiment.

Effect of p53 Binding uPA mRNA 3'UTR Sequence on uPA Expression

The H1299 cells expressing vector cDNA or p53 cDNA were treated with p53 binding uPA mRNA 3'UTR or control non-p53 binding sequence for 48 hours. uPA expression was determined by Western blotting.

RESULTS

Differential Expression of uPA Protein and mRNA by p53-Deficient Lung Carcinoma Cells and Lung Airway Epithelial Cells

We recently found that uPA at concentrations greater than 250–1,000 ng/ml (5–20 nM) induces its own expression through a process that involves stabilization of its mRNA (9). However, at concentrations of 500 ng/ml or higher, p53 expression in these cells is invariably abolished (13). To confirm that p53 influences uPA expression, we initially analyzed nonmalignant Beas2B as well as malignant large cell lung carcinoma H1299 cells for p53 expression by Western blotting. H1299 lung carcinoma cells behave differently from normal lung epithelial cells. However, lack of p53 and expression of stable uPA mRNA make these cells an ideal cell line to determine if p53 is involved in the regulation of uPA expression. Beas2B cells are SV40 transformed, nonmalignant bronchial epithelial cells; nonetheless, their pattern of uPA and p53 expression and their response to uPA stimulation were comparable to that of primary human small airway epithelial cells or primary bronchial epithelial cells (7, 13). It is still likely that Beas2B cells behave differently from

normal lung epithelial cells. As shown in Figure 1A, Beas2B cells expressed low levels of p53 protein. We earlier reported that uPA regulates Beas2B cell p53 expression in a biphasic manner with maximal induction at a low uPA (~ 1 nM) concentration and its total suppression at a higher uPA (>10 nM) concentration (13). Therefore, we treated p53-expressing Beas2B and p53-deficient H1299 cells with 1 nM (50 ng/ml) of uPA to induce maximum p53. ATF of uPA mimics full-length uPA in inducing both p53 and uPA expression, and the uPA antibody that we used in Western blots recognizes the C-terminal domain of uPA. Therefore, we substituted ATF for full-length uPA in some of the experiments to avoid interference by the added uPA from quantifying the synthesized uPA (9, 13, 20). In selected experiments, we also tested Beas2B and H1299 cells with full-length uPA in the presence of 35 S-labeled methionine and analyzed the metabolically labeled endogenous uPA by immunoprecipitation and autoradiography. The response of the full-length uPA was comparable to that of ATF. H1299 cells neither expressed p53 nor responded to uPA stimulation. Nevertheless, Northern blotting analysis indicated that uPA failed to alter expression of p53 mRNA in Beas2B cells and p53 mRNA likewise was not detected in H1299 cells treated with or without uPA (data not shown). Furthermore, the CLs and CM of these two cell types were used to determine if uPA is altered due to the differences in their basal levels of p53 by Western blotting. As shown in Figure 1B, both CM and CLs of Beas2B and p53-deficient large cell carcinoma cells contained uPA, and uPA treatment (1 μ g/ml) increased uPA's own expression in both the cell types. However, p53-deficient cells expressed at least 10-fold more basal uPA, which was further induced by uPA versus Beas2B cells. A similar increase in expression of uPA mRNA in p53-deficient cells was also observed and uPA exerted an additive effect in p53-deficient cells compared with Beas2B cells (Figure 1C). This observation led us to speculate that p53 could be involved in the auto-induction of uPA.

Effects of Overexpression or Inhibition of p53 on uPA Protein and mRNA Expression

To confirm that p53 regulates uPA-induced uPA expression in lung epithelial or carcinoma cells, we expressed p53 in p53-

Figure 1. Regulation of urokinase-type plasminogen activator (uPA) protein and mRNA expression by p53 in lung epithelial cells. (A) Expression of p53 protein by lung-derived cells. Lysates of Beas2B (p53^{+/+}) and H1299, p53-deficient non-small cell carcinoma (p53^{-/-}) cells treated with phosphate-buffered saline (PBS) or amino-terminal fragment (ATF) of uPA (50 ng/ml) for 24 hours were subjected to Western blotting using anti-p53 antibody. The same membrane was stripped and developed with an anti- β -actin antibody for equal loading. (B) Increased uPA expression in p53-deficient cells. Total proteins from conditioned media (CM) and cell

lysates (CL) of p53^{+/+} and p53^{-/-} cells treated with PBS or 1 μ g/ml of uPA for 24 hours in the presence of 35 S-labeled methionine were subjected to immunoprecipitation using anti-uPA antibody followed by β -actin antibody for equal loading. The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. (C) RNA isolated from p53^{+/+} and p53^{-/-} cells treated with PBS or uPA (1 μ g/ml) for 12 hours was subjected to Northern blotting using 32 P-labeled uPA cDNA and 32 P-labeled β -actin cDNA. Experiments were repeated three times and representative data are shown.

deficient H1299 cells by transfecting either vector (pcDNA3.1) cDNA alone or p53 cDNA cloned in pcDNA3.1. Stable cell lines were generated by antibiotic selection and analyzed for p53 expression by Western blotting using anti-p53 monoclonal antibody. Cells transfected with p53 cDNA expressed p53 protein in H1299 cells, whereas empty pcDNA3.1 vector cDNA-transfected cells failed to express p53 (Figure 2A). Treatment of p53 cDNA-transfected cells with 1 μ g/ml of either full-length uPA or its amino-terminal uPAR binding fragment (ATF) reduced p53 expression (data not shown), confirming its regulation at the level of p53 translation (13). These cells were next treated with PBS or uPA (1 μ g/ml) in the presence of 35 S-labeled methionine, and endogenous uPA expression was analyzed by immunoprecipitation using anti-uPA antibody followed by β -actin antibody for equal loading as previously described. As shown in Figure 2B, pcDNA3.1-transfected p53-deficient cells expressed more uPA both in the CM and CLs, and uPA stimulation further increased the expression in these cells. However, pcDNA3.1/p53 DNA-transfected H1299 cells suppressed basal uPA expression and decreased the ability of uPA to induce its own expression compared with vector cDNA-transfected cells. We next analyzed the effects of p53 restoration on uPA mRNA level by Northern blotting and found that cells transfected with pcDNA3.1/p53 cDNA inhibited uPA mRNA expression compared with cells transfected with vector cDNA alone (Figure 2C). These results confirm the inference that p53 directly affects uPA expression in H1299 cells.

To extend these findings, we next assessed the effect of silencing of p53 expression in nonmalignant Beas2B cells. Western blotting analysis showed that treatment of p53 SiRNA in Beas2B cells inhibited both basal and uPA-mediated p53 expression by more than 95% compared with control non-

specific SiRNA-treated cells (data not shown). We next analyzed the basal and uPA-induced uPA expression in SiRNA-treated cells by immunoprecipitation of 35 S-labeled uPA. As shown in Figure 2D, Beas2B cells treated with nonspecific SiRNA expressed minimal basal levels of uPA, and uPA treatment enhanced cellular uPA expression by several fold, whereas p53 SiRNA treatment increased basal uPA expression and uPA augmented the effect in these cells. We also confirmed that the inhibition of p53 expression induced basal uPA mRNA expression and that uPA treatment further increased uPA mRNA compared with control SiRNA-treated Beas2B cells (Figure 2E). These results demonstrate that p53 directly regulates uPA expression.

Effect of p53 on Rates of uPA mRNA Synthesis and Degradation

p53 Binds to DNA promoter sequences and regulates transcription of p53 target genes (15, 24). Kunz and colleagues (25) showed that the uPA promoter lacks a p53 response element but p53 represses uPA transcription. Therefore, we tested the inference that p53 affects the rate of uPA mRNA transcription in p53-deficient cells. Nuclei from p53-deficient cells transfected with (or without) vector or p53 cDNA in pcDNA 3.1 were isolated and the nuclear extracts were subjected to run-on transcription to determine the rate of uPA mRNA synthesis. As shown in Figure 3A, p53 expression failed to affect rate of uPA mRNA synthesis. Because uPA induces uPA expression through post-transcriptional stabilization of uPA mRNA (9), we suspected that p53 might likewise control uPA expression at the level of uPA mRNA turnover. We therefore treated naive p53-deficient cells or p53-deficient cells transfected with vector

Figure 2. The protein p53 inhibits urokinase-type plasminogen activator (uPA) expression in Beas2B cells. (A) Cells lacking p53 ($p53^{-/-}$) were transfected with vector cDNA (pcDNA3.1) or p53 cDNA (p53) in pcDNA3.1. Stably transfected cell lines expressing vector cDNA or p53 cDNA were analyzed for p53 expression by Western blotting as described in Figure 1A. (B) $p53^{-/-}$ Cells transfected with vector pcDNA3.1 or Wt. p53 cDNA in pcDNA3.1 were treated with phosphate-buffered saline (PBS) or amino-terminal fragment (ATF) (1 μ g/ml). The conditioned media (CM) and cell lysates (CL) were subjected to Western blotting using an anti-uPA antibody. (C) RNA isolated from $p53^{-/-}$ cells transfected with vector cDNA or p53 cDNA treated with PBS or uPA (1 μ g/ml) for 12 hours was subjected to Northern blotting using 32 P-labeled uPA cDNA and 32 P-labeled β -actin cDNA. (D) Beas2B cells transfected with control nonspecific silencing RNA (NspSiRNA) or p53 SiRNA were treated with PBS or 1 μ g/ml of uPA for 24 hours in methionine-deficient medium containing 35 S-methionine. The CM and CL were subjected to immunoprecipitation using anti-uPA and anti- β -actin antibodies as described in Figure 1B. (E) Beas2B cells transfected with control SiRNA or p53 SiRNA were treated with PBS or uPA (1 μ g/ml) for 12 hours. The total RNA isolated from these cells was subjected to Northern blotting using 32 P-labeled uPA cDNA and β -actin cDNA. The figure shown is a representation of four independent experiments.

control nonspecific silencing RNA (NspSiRNA) or p53 SiRNA were treated with PBS or 1 μ g/ml of uPA for 24 hours in methionine-deficient medium containing 35 S-methionine. The CM and CL were subjected to immunoprecipitation using anti-uPA and anti- β -actin antibodies as described in Figure 1B. (E) Beas2B cells transfected with control SiRNA or p53 SiRNA were treated with PBS or uPA (1 μ g/ml) for 12 hours. The total RNA isolated from these cells was subjected to Northern blotting using 32 P-labeled uPA cDNA and β -actin cDNA. The figure shown is a representation of four independent experiments.

Figure 3. Effect of p53 on urokinase-type plasminogen activator (uPA) mRNA expression. (A) Effect of p53 on uPA mRNA synthesis by lung epithelial cells. Naive H1299 cells (p53^{-/-}) or H1299 cells transfected with vector cDNA (pcDNA3.1) or p53 cDNA were switched to serum-free media for 12 hours. Nuclei isolated from H1299 cells were subjected to the transcription reaction in the presence of ³²P-labeled uridine triphosphate (UTP) at 30°C for 30 minutes. ³²P-labeled nuclear RNA was hybridized with uPA cDNA immobilized on nitrocellulose membrane. β-Actin and plasmid University of California (PUC)18 cDNAs were used as positive and negative loading controls, respectively. The data represent the findings of three independent analyses. (B) Expression of wild-type (Wt) p53 in non-small cell lung carcinoma cells lacking p53 (p53^{-/-}) destabilizes uPA mRNA. Naive p53^{-/-} cells (p53^{-/-}) or stable p53^{-/-} cells overexpressing vector cDNA

(pcDNA3.1) or p53 cDNA (p53 cDNA) were subjected to transcription chase experiment by treating with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (20 μg/ml). The level of uPA mRNA was measured by Northern blotting at 0–12 hours. Experiments were repeated four times. (C) Inhibition of p53 expression enhances both basal and uPA-mediated stabilization of uPA mRNA. Beas2B cells transfected with control nonspecific (Nsp) silencing RNA (SiRNA) or p53 SiRNA were treated with PBS or uPA (1 μg/ml) for 12 hours. Ongoing transcription was inhibited by treatment with DRB and the decay of uPA mRNA was analyzed for 0–12 hours by Northern blotting. β-Actin mRNA expression is shown for equal loading. Densitometric scanning of individual bands is shown as a line graph, where mRNA level at 0 hours for each treatment is calculated as 100%. The experiments were repeated at least four times.

alone or vector harboring p53 cDNA with DRB to inhibit ongoing transcription, and the decay of uPA mRNA was analyzed over time by Northern blotting. H1299 cells that expressed p53 produced unstable uPA mRNA (with half-life [t_{1/2}] < 3 h) versus untransfected (t_{1/2} ~ 12 h) or vector cDNA (t_{1/2} ~ 12 h)-treated p53-deficient cells (Figure 3B). These results indicate that p53 regulates uPA mRNA expression at the post-transcriptional level by mRNA stabilization.

To confirm that p53 affects uPA mRNA turnover in non-malignant lung epithelial cells, Beas2B cells were treated with control SiRNA and p53 SiRNA to suppress basal p53 expression. Because Beas2B cells express low levels of basal uPA and respond to uPA stimulation (Figure 1C), both control and p53 SiRNA-transfected cells were treated with PBS or uPA for 12 hours to induce maximum uPA mRNA (7). Ongoing transcription was inhibited and the decay of uPA mRNA was determined by Northern blotting. We found that uPA mRNA is relatively unstable (t_{1/2} < 3 h) in control SiRNA-treated Beas2B cells and uPA treatment stabilized uPA mRNA. However, inhibition of p53 by p53 SiRNA enhanced both basal (t_{1/2} > 6 h) and uPA-mediated (t_{1/2} > 6 h) stabilization of uPA mRNA (Figure 3C). These results confirm that p53 directly regulates uPA mRNA stability in Beas2B cells.

Identification of p53 as uPA mRNA Binding Protein

Post-transcriptional regulation of mRNA in general and uPA mRNA in particular involves its interaction with specific mRNA binding proteins. We therefore hypothesize that p53 binds to uPA mRNA and regulates its degradation. To test this

possibility, we expressed a recombinant p53–GST fusion protein (rp53) in a prokaryotic system and affinity purified the rp53 protein using glutathione sepharose column chromatography. Purification (>95%) of rp53 was confirmed by Western blotting using an anti-p53 monoclonal antibody (data not shown). Next, we tested the ability of rp53 to interact with the uPA mRNA CDR and 3'UTR by gel mobility shift assay. As shown in Figure 4A, rp53 failed to bind the ³²P-labeled uPA mRNA CDR, whereas it formed a specific RNA–protein complex with the uPA mRNA 3'UTR. To confirm that p53 likewise binds to uPA mRNA *in vivo*, we immunoprecipitated lysates from Beas2B cells treated with PBS or uPA (50 ng or 1 μg/ml) with anti-p53 antibody, and p53-associated uPA mRNA was amplified by RT-PCR. As shown in Figure 4B, uPA mRNAs were detected in Beas2B CLs immunoprecipitated using anti-p53 antibody; the uPA mRNA is absent in the immune complex of control nonspecific IgG-treated samples, indicating the specificity of the p53–uPA mRNA interaction.

Specificity of p53–uPA 3'UTR mRNA Interaction

We next examined the specificity of the p53–uPA mRNA 3'UTR interaction by a series of cold competition experiments. Preincubation of rp53 protein with a molar excess of unlabeled uPA 3'UTR sense transcripts resulted in dose-dependent inhibition of the p53–³²P-labeled uPA mRNA 3'UTR interaction (Figure 4C), whereas addition of the same amount of unlabeled antisense transcript failed to interfere with p53 binding to the ³²P-labeled uPA mRNA 3'UTR (Figure 4D). Pretreatment of p53 with a molar excess of homopoly(A), (C), (G), or (U)

Figure 4. The protein p53 binds to urokinase-type plasminogen activator (uPA) 3' untranslated region (UTR) mRNA. (A) The recombinant p53 protein (rp53) expressed in the prokaryotic system was incubated with the 32 P-labeled uPA mRNA coding (CDR) or 3'UTR, and the mRNA–p53 complexes were analyzed by gel mobility shift assay on 5% nondenaturing polyacrylamide gel electrophoresis. Lanes: uPA mRNA was incubated with rp53 protein (p53) or buffer alone (free probe [Fp]). Arrow indicates RNA–protein complex. (B) Beas2B cells treated with varying amounts (0–1 µg/ml) of uPA for 24 hours were lysed and the lysates were immunoprecipitated using anti-p53 monoclonal antibody (p53 mAb) or nonspecific mouse IgG (NSp mlgG). The p53 protein–associated uPA mRNA was detected by reverse transcriptase–polymerase

chain reaction (PCR) using 32 P-labeled 2'-deoxycytidine 5'-triphosphate (dCTP) and verified by nucleotide sequencing of the corresponding nonradioactive PCR product. Experiments were repeated at least three times. Competitive inhibition of p53–uPA mRNA interaction using unlabeled uPA mRNA sense and antisense transcripts to determine the specificity of p53–uPA mRNA 3'UTR interaction. p53 Protein was incubated with 32 P-labeled probe uPA 3'UTR mRNA in the presence of 0- to 125-fold molar excess of unlabeled sense (C) or antisense (D) transcript. (E) Effects of polyribonucleotides, proteinase K, and sodium dodecyl sulfate (SDS) on uPA mRNA interaction with the binding protein. p53 Protein incubated with a 125-fold excess of unlabeled sense, poly(A), (C), (G), and (U); proteinase K (2.5 mg/ml); and 0.1% SDS for 30 minutes at 30°C. 32 P-labeled uPA mRNA probe was added, and the mixture was digested with RNase T1 and analyzed by gel mobility shift assay. (F) rp53 Protein was incubated with 200-fold molar excess of p53 consensus sequence (5' prom) or 32 P-labeled uPA 3'UTR mRNA predigested with RNase T1 before exposure to p53 (RNase T1). Experiments are representations of four independent analyses.

ribonucleotides likewise failed to affect the p53–uPA 3'UTR mRNA interaction (Figure 4E), indicating that p53 binds to specific nucleotide sequences on uPA mRNA 3'UTR. Treatment of p53 with proteinase K or SDS abolished its ability to interact with uPA mRNA 3'UTR. Last, a molar excess of unlabeled p53 promoter binding DNA consensus sequence did not prevent binding of p53 to the uPA mRNA 3'UTR, indicating that the p53 transactivation domain is independent of the uPA mRNA binding region (Figure 4F).

Identification of the p53 Binding Sequence on uPA 3'UTR mRNA and Determination of Its Function in uPA mRNA Turnover and uPA Expression

To identify the specific p53 binding sequence(s) on uPA mRNA 3'UTR, we made a series of PCR-based overlapping deletions of uPA cDNA. These cDNA fragments were subcloned into pcDNA 3.1, and 32 P-labeled deletion transcripts were prepared by *in vitro* transcription. These transcripts were then individually tested for p53 binding by gel mobility shift assay. As shown in Figure 5, p53 protein specifically bound to a 35-nt (nt 1585–1620) sequence present on the uPA mRNA 3'UTR.

To determine if the p53 binding 35-nt uPA 3'UTR sequence contains information for message stability, we inserted this sequence into the β -globin cDNA (Figure 6A) and the chimeric β -globin–uPA 3'UTR cDNA containing the 35-nt p53 binding sequence (Figure 6B, C3) was subcloned into the eukaryotic expression vector pcDNA3.1. We also prepared chimeric β -globin–uPA 3'UTR cDNA containing a control 35-nt (nt 780–

815) non-p53 binding sequence (Figure 6B, C4). Beas2B cells were then transfected with chimeric β -globin–uPA 3'UTR cDNA constructs as well as β -globin cDNA alone. The decay of chimeric β -globin–uPA 3'UTR mRNA was determined by Northern blotting after inhibiting ongoing transcription. Figure 6C shows that insertion of the p53 binding 35-nt (C3) uPA 3'UTR sequence destabilized β -globin mRNA (Figure 6C, C3). Insertion of a similarly sized control non-p53 binding sequence (C4) failed to alter the stability of β -globin mRNA (Figure 6C, C4), and β -globin mRNA was likewise quite stable (data not shown), indicating that the p53 binding 35-nt uPA 3'UTR sequence contains regulatory information that controls uPA mRNA stability.

We then directly tested the effect of the p53 binding 35-nt (Figure 6B, C3) uPA mRNA 3'UTR sequence on uPA expression. H1299 cells transfected with vector cDNA or p53 cDNA were treated with 35-nt (Figure 6B, C3) p53 binding or control 35-nt (Figure 6B, C4) sequences for 48 hours and the conditioned media were analyzed for uPA expression. As shown in Figure 6D, treatment of H1299 cells with the 35-nt p53 binding but not the 35-nt control sequence reversed the inhibitory effect of p53. These results demonstrate that the p53 interaction with the 35-nt uPA mRNA (Figure 6B, C3) 3'UTR sequence regulates uPA expression.

DISCUSSION

uPA-mediated plasminogen activation is involved in pericellular proteolysis, remodeling of extracellular matrix, and a variety

Figure 5. Identification of the p53 protein binding sequence on urokinase-type plasminogen activator (uPA) 3' untranslated region (UTR) mRNA. (A) Deletion map indicating the p53 protein binding site on uPA mRNA. Pst1, restriction site of enzyme Pst I. (B) Recombinant p53 (rp53) protein expressed in *E. coli* was affinity purified. The rp53 was incubated with 32 P-labeled uPA mRNA full-length coding region (CDR-FL; 1) or full-length 3'UTR (3'UTR-FL; 2) or 3'UTR deletion transcripts (3'UTR-DL; 3–9), and the RNA–protein complex was analyzed by gel mobility shift assay. Arrow indicates the uPA mRNA–p53 protein complex. The 32 P-labeled uPA mRNA probe (Fp) is incubated with buffer alone. Data are representative of four independent analyses.

of nonproteolytic functions, such as proliferation, adhesion, migration, cell signaling, and gene expression (7, 12, 13, 26–28). These functions of uPA may contribute to remodeling of the lung, which occurs in acute respiratory distress syndrome or the interstitial and neoplastic lung diseases (29–32).

The tumor suppressor protein p53 is induced and activated in response to different forms of stress and regulates cellular repair or apoptosis. In addition, p53 normally transactivates a variety

of target genes that contain one or more p53 binding sites in their promoter regions (19, 32–35). Stabilization and activation of p53 are achieved by protein–protein interactions (e.g., MDM2 ubiquitin ligase) (36) and post-translational modification such as phosphorylation, acetylation, and sumolation. In most tumor cells, the p53 function is reduced or abolished, which results in uncontrolled proliferative potential in these cells. Inhibition of p53 function is caused by either lack of p53 ex-

Figure 6. Determining the destabilizing function of p53 binding uPA 3' untranslated region (UTR) mRNA sequence. (A) Structure of β -globin/uPA 3'UTR chimeric mRNA. The p53 protein binding 35-nucleotide (nt) sequence corresponding to nt 1585 to 1620 of uPA 3'UTR cDNA (C3) and a control sequence of similar size corresponding to the nonbinding region (C4) were inserted into the 3'UTR of β -globin cDNA. The chimeric β -globin/uPA 3'UTR cDNA was subcloned into pcDNA3.1. (B) Nucleotide sequence of p53 binding region nt 1585–1620 (C3) or nonbinding control sequence (C4). (C) Decay of β -globin/uPA 3'UTR chimeric mRNA. Beas2B cells were transfected with the chimeric β -globin/uPA 3'UTR gene containing the 35-nt (nt 1585 to 1620) p53 binding sequence (C3) or nonbinding control sequence (C4) in pcDNA3.1. Total RNA was isolated at different time intervals after treatment with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) as described above (Figure 3B) and analyzed the level of chimeric mRNA by Northern blotting using a 32 P-labeled probe. (D) Effect of p53 binding uPA

mRNA 3'UTR sequence on uPA expression. Stably transfected H1299 cells overexpressing p53 cDNA in pcDNA 3.1 were transfected with non-specific control (C4, NSP) or p53 binding (C3, p53) uPA mRNA 3'UTR sequences. The conditioned medium samples were analyzed for uPA expression by Western blotting using an anti-uPA antibody. Data are representative of at least four independent analyses.

pression or inactive mutation of the p53 gene. Altered p53 expression may elicit epithelial cell transformation and control the potential of these cells to degrade the extracellular matrix. Most tumor cells that lack p53 exhibit increased cell surface plasminogen activation and uPA-induced proliferation, migration, and invasiveness (37–39). We therefore sought to unravel the mechanism by which uPA and p53 interact and to determine how p53 and uPA expression in lung epithelial and carcinoma cells is regulated by uPA.

p53 suppresses cell surface fibrinolysis by inhibition of uPA-mediated plasminogen activation via induction of plasminogen activator inhibitor (PAI)-1 expression through promoter transactivation. The PAI-1 promoter exhibits a p53 binding sequence (25). Unlike PAI-1 promoter, the uPA promoter lacks a p53 binding site. However, p53 represses uPA expression most likely through TATA-binding protein-associated factors (25).

uPA induces its own expression in a concentration-dependent manner (9, 20), and at a low concentration of approximately 1 nM, uPA induces p53. However, at concentrations greater than 10 nM, uPA inhibits cellular p53 expression. The process involves both Ser15 phosphorylation of p53 as well as MDM2-mediated p53 ubiquitination (13). Our findings indicate the existence of an inverse relationship between p53 and uPA expression in lung epithelium-derived cells, which is contradictory to the earlier reports in which both p53 and uPA levels are decreased in breast cancer cells (40–41). This discrepancy could be due to tissue origin and tumor metastasis stages. These observations support the possibility for cross-talk between p53 and uPA expression. However, a direct link between p53 and uPA expression has been lacking. Here we show for the first time that expression of the uPA gene is regulated by p53. Our results indicate that basal uPA expression is elevated in p53^{-/-} cells and uPA further increases uPA expression.

We recently reported that maximum uPA expression was observed when lung epithelial cells were treated with a uPA concentration greater than 10 nM (500 ng/ml). We also found that uPA regulates p53 expression in a concentration-dependent manner and that uPA induces its own expression at a concentration at which p53 expression is totally suppressed. Unlike uPA-mediated Beas2B cell p53 expression (13, 23), uPA and uPAR levels are induced by inhibition of protein tyrosine phosphatase and suppressed by blockade of tyrosine kinase activity (7, 9). Furthermore, β_1 -integrin activation by treatment with anti- β_1 -integrin antibody as well as overexpression of protein tyrosine phosphatase SHP2 alters p53 expression (13). Hence, it is likely that signal transduction through these proteins could contribute to uPA expression. We now show that cells lacking p53 express elevated levels of uPA. We interpret the results to indicate that p53 affects uPA expression and that dose-dependent induction of uPA in lung epithelial cells is due to inhibition of p53. The role of p53 in uPA expression is further supported by the fact that restoration of p53 in p53^{-/-} cells suppressed basal uPA expression. Interestingly, uPA failed to induce uPA expression in these cells. Furthermore, induction of basal uPA expression as well as the added effect of uPA in p53-inhibited (SiRNA-treated) cells shows that p53 regulates cellular uPA expression.

Although the uPA promoter lacks the p53 binding sequence, it has previously been reported that p53 represses uPA mRNA synthesis (25). Our results show that expression of p53 in p53^{-/-} cells failed to induce uPA mRNA synthesis, indicating that inhibition of uPA mRNA synthesis by p53 may not be responsible for the observed effect. This interpretation is further supported by our earlier observation that uPA stabilizes uPA and uPAR mRNA (7, 9). We recently found that p53 inhibits cell surface uPAR expression and uPA-mediated lung cancer

cell proliferation (42), which suggests that induction of uPAR may be in part due to suppression of p53-mediated uPA mRNA destabilization. Decay of uPA mRNA in H1299 cells transfected with p53 cDNA further demonstrates the involvement of the role of p53 in the destabilization of uPA mRNA.

We hypothesized that p53 directly interacts with uPA mRNA to regulate its stability. We confirmed this assumption by showing that rp53 interacts with the uPA mRNA 3'UTR by gel shift assay. Coprecipitation of uPA mRNA with p53 from Beas2B CLs substantiated the existence of such an interaction *in vivo*. The p53–uPA mRNA 3'UTR complex was resistant to both RNase A and T1 digestion. The specificity of p53 interaction with the uPA mRNA 3'UTR was assessed by self-competition experiments in which a labeled sense probe was successfully competed by its unlabeled analog. An unlabeled antisense probe failed to compete for specific binding. Furthermore, addition of a molar excess of a homopolyribonucleotide poly(A), (C), (G), or (U) had no effect on p53 protein binding to ³²P-labeled uPA mRNA 3'UTR, indicating that p53 binding requires unique sequence. The specificity of p53 binding to the uPA 3'UTR mRNA is also supported by the finding that pretreatment of p53 by proteinase K or SDS or predigestion of uPA mRNA probe with RNAase A and RNAase T1 totally abolished the p53–uPA mRNA 3'UTR complex.

Deletion experiments showed that rp53 specifically binds to a 35-nt uPA mRNA 3'UTR sequence and that the binding region is located upstream of the 66-nt 30-kD uPA mRNA binding protein (mRNABp) binding sequence. Our studies indicate that there is no other p53 binding sequence in uPA CDR or 3'UTR other than this 35-nt region, which corresponds to the uPA cDNA sequence from 1585 to 1620. We found that insertion of the 35-nt p53 binding sequence into β -globin mRNA destabilized the chimeric transcript in Beas2B cells, indicating that the p53 binding sequence contains information for mRNA destabilization. Transfection of cells with this sequence induced uPA expression in p53-expressing H1299 cells by competing with endogenous mRNAs, further confirming that the p53 interaction with the 35-nt uPA mRNA 3'UTR sequence destabilizes uPA mRNA. Our observations show that p53 interacts with the 35-nt uPA mRNA destabilizing element and that increased expression of uPA occurs in cells where expression of wild-type p53 is reduced, delineating a novel regulatory mechanism. Transfection of p53 inhibits uPA expression through down-regulation of uPA mRNA. In all, these studies demonstrate a novel function of p53 as an mRNA binding protein that regulates the expression of uPA mRNA by altering its stability. The pathophysiologic consequences of this pathway on clinical lung diseases, including acute lung injury, pulmonary fibrosis, or lung neoplasia, are currently unclear. However, these diverse diseases are characterized by disordered epithelial viability and disordered alveolar fibrin turnover (2). Our results strongly suggest that this newly identified p53-mediated regulatory function could play a role in epithelial cell survival and uPA expression in these contexts.

In summary, we confirmed that the p53–uPA mRNA 3'UTR interaction regulates uPA mRNA stability in lung epithelial cells. To our knowledge, this newly identified pathway is the first description of the capability of p53 to interact with uPA mRNA and control its expression at the post-transcriptional level. This pathway adds a new dimension to the p53 functional repertoire and represents a new link between viability of lung epithelial cells and pericellular proteolysis, as occurs in neoplastic transformation, growth, and spread of lung neoplasms. This pathway may likewise contribute to the regulation of uPA-dependent responses of the lung epithelium in the context of lung injury and repair.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank M. B. Harish, Brad Low, and Janet Harris for technical assistance.

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