

Regulation of Urokinase Receptor Expression by Phosphoglycerate Kinase

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Post-transcriptional regulation represents a major mechanism by which eukaryotic gene expression is regulated through *cis-trans* interactions that serve as signals for rapid alterations of messenger RNA (mRNA) stability. Regulation of urokinase-type plasminogen activator receptor (uPAR) mRNA involves the interaction of a uPAR mRNA coding region sequence with a 50 kD uPAR mRNA binding protein. We purified this protein from human bronchial epithelial (Beas2B) cells and identified it as phosphoglycerate kinase (PGK). We cloned PGK cDNA by polymerase chain reaction and expressed the recombinant PGK protein, which specifically bound the uPAR mRNA coding region by gel mobility shift and Northwestern blotting. We also confirmed a direct interaction of PGK protein with uPAR mRNA by immunoprecipitation. Overexpression of PGK in uPAR-overproducing H157 lung carcinoma cells resulted in decreased cytoplasmic uPAR mRNA and cell surface uPAR protein expression. Reduced uPAR mRNA expression involved decreased stability of the uPAR mRNA. Decline in ³H-thymidine incorporation and migration occurred in H157 cells transfected with PGK cDNA. These results demonstrate that PGK regulates uPAR expression at the post-transcriptional level.

Degradation of extracellular matrix proteins by serine proteinases and metalloproteinases has been implicated in the pathogenesis of several diseases, including lung injury and lung neoplasia (1–3). These proteinases influence inflammatory cell traffic or tumor cell invasiveness via the degradation of basement membranes and extracellular matrix (4–7). Plasmin, a serine protease, is generated via the action of plasminogen activators such as urokinase (uPA) or tissue plasminogen activator (tPA). Plasmin can influence tissue remodeling either directly or through activation of latent collagenases. uPA plays a pivotal role in extravascular proteolysis and is implicated in stromal remodeling in acute and chronic lung injury (8–10) and in tumor metastasis (7). During the last decade, evidence for the involvement of the uPA system in lung injury and repair or lung neoplasia (1) has steadily increased, and it now seems beyond reasonable doubt that uPA-dependent plasminogen activation and interaction with its receptor, uPAR, is central to these processes. Because many biological activities of uPA depend on association with uPAR, this receptor plays a central role in localized uPA-mediated plasminogen activation at the cell surface. Increased expression of uPA or uPAR has, for example, been inversely correlated with prognosis in lung cancer (11, 12). Interaction of uPA with its cellular receptor

also promotes cellular signaling, which can thereby influence the course of lung inflammation or cancer. Better understanding of the specific pathways that regulate uPAR expression is therefore germane to the pathogenesis of lung injury or the spread of lung neoplasms.

The expression of a number of proteins is regulated by specific and rapid decay of their transcripts. The steady state of any mRNA in turn reflects a balance between its synthesis and lability. Among the various mechanisms that regulate mRNA stability, control of mRNA decay is a potentially important determinant of the level of gene expression in eukaryotic cells. Synthesis of uPAR is regulated by a variety of hormones, growth factors and cytokines either at the transcriptional or posttranscriptional level (13–20). Both uPA and uPAR, as well as plasminogen activator inhibitor-1 and -2 are expressed by lung epithelial cells (21–23), and it is now clear that post-transcriptional regulation contributes to the regulation of uPA, uPAR, and plasminogen activator inhibitor-1 by these cells (16, 22–26). The responsible mechanisms involve *cis-trans* interactions that involve the 3' untranslated region (UTR) and, in some cases the coding region of the specific mRNA. The identity of most of the regulatory proteins that govern post-transcriptional regulation of these proteins, including uPAR, remains obscure at present.

Expression of uPAR controls several cellular functions, including epithelial cell adhesion, signaling, and mitogenesis, because most of the biological activities of uPA are dependent on its association with the uPAR (4, 13, 27–33). The expression of uPAR by the lung epithelium is tightly regulated during normal physiologic processes and is disordered in lung injury or lung cancer. It is noteworthy that squamous cell carcinoma is characterized by elevated uPAR expression, which is associated with increased proliferation as well as migration compared with other lung carcinoma cells (24, 34). We therefore postulated a novel strategy to further elucidate how post-transcriptional regulation of uPAR expression is achieved. In these studies, we purified a cytoplasmic protein from cultured human bronchial epithelial cells (Beas2B) lung airway epithelial cells that specifically interacts with the uPAR coding region mRNA and identified this protein as phosphoglycerate kinase (PGK). Subsequently, we cloned this cDNA and expressed the recombinant protein in bacteria and eukaryotic cells. Both these preparations specifically bound to the uPAR mRNA coding region. Overexpression of PGK protein in H157 lung squamous carcinoma cells reduced cell surface uPAR expression as well as uPAR-mediated cellular functions at the post-transcriptional level. Immunochemical analyses confirm that PGK is the uPAR mRNA binding protein (uPAR mRNA_Bp) we previously described.

Materials and Methods

Material

Culture media, penicillin, streptomycin, and fetal calf serum (FCS) were purchased from GIBCO BRL Laboratories (Grand Island, NY); tissue culture plastics were from Becton Dickinson Labware (Lincoln Park, NJ). Herbimycin was from Calbiochem (La Jolla, CA) and bovine serum albumin (BSA), ovalbumin, Tris-base, aprotinin, dithiothreitol,

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Abbreviations: human bronchial epithelial cells, Beas2B; bovine serum albumin, BSA; fetal calf serum, FCS; isopropylthio- β -galactoside, IPTG; messenger RNA, mRNA; plasmid complementary DNA, pcDNA; phosphoglycerate kinase, PGK; urokinase-type plasminogen activator, uPA; urokinase-type plasminogen activator receptor, uPAR; uPAR mRNA binding protein, uPAR mRNA_Bp; untranslated region, UTR; sodium dodecylsulfate, SDS; sodium dodecylsulfate-polyacrylamide gel, SDS-PAGE; saline sidum citrate, SSC.

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phenylmethylsulfonyl fluoride, and ammonium persulfate were from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bisacrylamide, and nitrocellulose were from Bio-Rad Laboratories (Richmond, CA). Anti-uPAR antibody was obtained from American Diagnostics (Greenwich, CT). XAR X-ray film was purchased from Eastman Kodak (Rochester, NY).

Cell Cultures

Beas2B and lung squamous cell carcinoma (H157) cells were maintained in LHC-9, and RPMI 1,640 medium containing 10% heat-inactivated FCS, 1% glutamine, and 1% antibiotics as previously described (30).

Plasmid Construction

Plasmid uPAR/pBluescript was obtained from the ATCC. The human uPAR mRNA template containing a complete sequence of uPAR cDNA (nucleotides -16 to 1,144) from uPAR pBluescript was subcloned to *Hind*III and *Xba*I sites of pRC/CMV (Invitrogen, Carlsbad, CA) and the sequences of the clones were confirmed by sequencing.

In Vitro Transcription

Linearized plasmids containing the human uPAR mRNA transcriptional templates of uPAR cDNA were transcribed *in vitro* with T₇ or Sp₆ polymerase (Ambion, TX). The uPAR mRNA transcripts were synthesized according to the supplier's protocol except that 50 μ Ci of [³²P]UTP was substituted for unlabeled UTP in the reaction mixture. Passage through a Sephadex G-25 column removed unincorporated radioactivity. The specific activities of the product were 4.9×10^8 cpm/ μ g.

Purification of the uPAR mRNA^{Bp} from Beas2B Cells

Beas2B cells grown to confluence were serum-starved overnight in RPMI media. The cells were collected by treating with trypsin/EDTA, washed using ice-cold HBSS. The cells were lysed in lysis buffer on ice for 1 h, after which the lysate was centrifuged at 15,000 rpm for 15 min. The clear supernatant was used as a cytosolic extract. This cytosolic extract was further fractionated to 0–50%, 50–80%, and 80–100% by ammonium sulfate fractionation. These fractions were later subjected to uPAR mRNA binding activity by gel mobility and Northwestern assays. Protein extracts from 80–100% ammonium sulfate fractionation were loaded onto a heparin sepharose column, and bound proteins were eluted with 100 ml 0–1,000 mM linear gradient of NaCl. Fractions were desalted by dialysis and subjected to uPAR mRNA binding analysis as described above. Active fractions were equilibrated with 1.7 M ammonium sulfate and loaded onto a phenyl sepharose column. The bound proteins were eluted from the phenyl sepharose column using an ammonium sulfate reverse gradient and used for uPAR mRNA binding after dialysis. The positive fractions were concentrated using polyethylene glycol and loaded onto a Mono-Q column connected to a FPLC system. Bound proteins were eluted by a NaCl gradient, after which positive fractions were pooled and loaded onto Mono-Q columns for a second time, then collected in an identical fashion.

Peptide Sequencing

Active fractions containing RNA-binding activity were separated on sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE), then transferred to a PVDF membrane. Tryptic peptides were obtained from protein blotted onto ProBlott membranes (Applied Biosystems, Foster City, CA). Peptides were purified by reverse-phase HPLC and sequenced. The N-terminal sequence was determined and this sequence was used for peptide Blast searches. The longest amino acid peptide resulted in 83% homology to PGK.

PCR Cloning

PGK cDNA was generated by PCR using poly (A) RNA from Beas2B cells and cloned to pcDNA3.1D/V5-His-Topo vector. Sequence and orientations were confirmed by nucleotide sequencing. Alternatively, we also cloned this cDNA to a pET plasmid vector and transfected into BL21 gold. The protein expression was induced by isopropylthio- β -galactoside (IPTG), and native PGK fusion protein was affinity purified by Ni-NTA-His column.

Transfection of H157 Cells

The H157 cells were transfected with or without PGK cDNA in pcDNA3.1D/V5-His-TOPO or empty vector by lipofection, as we described earlier (34). The stable cell lines were generated by antibiotic selection after which the cells were cultured in large amounts and expression was confirmed by Western blotting using an anti-V5 antibody. The recombinant fusion proteins were affinity purified by passing the lysate through a Nickel column after which it was subjected to uPAR mRNA binding studies by gel mobility shift and Northwestern assay as described below.

Gel Mobility Shift Assay

Eluates purified from Nickel column of lysates of H157 cells, transfected with PGK, or vector cDNA were incubated with 2×10^4 cpm of ³²P-labeled transcript in a mixture containing 15 mM KCl, 5 mM MgCl₂, 0.25 mM dithiothreitol, 12 mM Hepes (pH 7.9), 10% glycerol, and *Escherichia coli* transfer RNA (tRNA) (200 ng/ μ l) in a total volume of 20 μ l at 30°C for 30 min. The reaction mixture was treated with 50 U of RNase T₁ or A and incubated at 37°C for 30 min. To avoid nonspecific binding, 5 mg of heparin per milliliter was added, and the mixture was incubated at room temperature for an additional 10 min. Samples were separated by electrophoresis on a 5% native polyacrylamide gel with 0.25 \times tris-borate-EDTA running buffer. The gels were dried and developed by autoradiography at -70°C.

Northwestern Assay

Alternatively, we confirmed the molecular weight and uPAR mRNA-protein interaction by Northwestern assay. Protein eluates from the Nickel column were separated on 8% SDS-PAGE, then blotted to a nitrocellulose membrane. The membrane was blocked with gel shift buffer containing 1% BSA and 20 μ g ribosomal RNA for 1 h. The membrane was replaced with fresh buffer containing ³²P-labeled uPAR mRNA (2×10^5 cpm/ml) and incubated for an additional 1 h at room temperature. The membrane was later washed three times with 50 ml of gelshift buffer for 10 min each, air-dried, and exposed to X-ray film. The membrane was later stripped and developed by Western blotting using a β -actin monoclonal antibody as described above to control for equal loading. In separate experiments, we performed gel mobility shift and Northwestern assays using ³²P-labeled uPAR CDR and 3'UTR to confirm the specificity of their interaction with PGK.

Whole-Cell Extraction and Immunoprecipitation of RNA-Protein Complex

To confirm the direct interaction of PGK with uPAR mRNA *in vivo*, we cross-linked Beas2B or H157 cells with ultraviolet irradiation on ice for 30 min at 2,500 μ J. The cytosolic extracts were prepared by lysing the cells in lysis buffer (25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). These extracts were immunoprecipitated using polyclonal antibody against PGK in the presence of RNase inhibitor and ribosomal RNA for 1 h at room temperature. The immune complexes were later precipitated using protein-Ag agarose, and the agarose beads were washed three times with lysis buffer. Total RNA was isolated from the immune complex and uPAR was amplified by RT-PCR using specific primers. The PCR products were later identified by Southern blotting using ³²P-labeled uPAR cDNA.

Total Cellular Membrane Extraction and Western Blotting

Polyclonal stable H157 cells as well as stable cell lines of PGK or vector transfected cells grown to confluence were serum starved overnight with RPMI-glutamine media containing 0.5% BSA. The cells were washed with phosphate-buffered saline. Receptor bound uPA was removed by glycine-HCl treatment as described earlier (29, 30). We used SDS gel electrophoresis and Western blotting to measure functional uPAR at the cell surface. Membrane proteins isolated as described earlier (29, 30) from the cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 1% BSA in wash buffer for 1 h at room temperature followed by overnight hybridization with uPAR monoclonal antibody in the same buffer at 4°C, washed, and uPAR proteins were detected by enhanced chemiluminescence (ECL) as described earlier (34).

Random Priming of uPAR cDNA

The full-length template of uPAR was released with Hind III or Xba I, purified on 1% agarose gels and labeled with ^{32}P dCTP using a rediPrime labeling kit (Amersham, Arlington Heights, IL). Passage through a Sephadex G-25 column removed unincorporated radioactivity. The specific activity of the product was 6×10^8 cpm/ μg .

Northern Blotting of uPAR mRNA

A Northern blotting assay was used to assess the level of uPAR mRNA. H157 cells grown to confluence were serum-starved overnight in RPMI-BSA media. Total RNA was isolated using total RNA isolation reagent. RNA (20 μg) was isolated on agarose/formaldehyde gels. After electrophoresis, the RNA was transferred to Hybond N⁺ according to the instructions of the manufacturer. Prehybridization and hybridization was done at 65°C in NaCl (1 M)/ sodium dodecylsulfate (SDS) (1%) and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA. Hybridization was performed with a uPAR cDNA probe (1 ng/ml) labeled to $\sim 6 \times 10^8$ cpm/ μg of DNA overnight. After hybridization, the filters were washed twice for 15 min at 65°C, with: 2 \times saline sodium citrate (SSC), 1% SDS; 1 \times SSC, 1% SDS, and 0.1% SSC, 1% SDS, respectively. The membranes were next exposed to X-ray film at -70°C overnight. The intensity of the bands was measured by densitometry and normalized against that of β -actin. We also measured the stability of uPAR mRNA in these cell lines by transcription chase experiments as we described earlier (16).

DNA Synthesis

H157 and stable cell lines transfected with PGK cDNA in pcDNA3.1 or vector alone were grown to subconfluence in 24-well plates. The cells were serum starved overnight in RPMI medium containing 0.5% BSA. ^3H -thymidine (1 $\mu\text{Ci}/\text{ml}$, 20.3 mmol Ci) was later added to the same media and incubated for an additional 6 h. The cells were washed with ice-cold phosphate-buffered saline three times and five washes with ice-cold 5% TCA. The cells were lysed in 0.2 N NaOH and the incorporated ^3H -thymidine was then counted using a scintillation counter.

Cell Migration/Invasion Assay

Cell migration assays were performed using modified Boyden Chambers with 6.5 mm diameter, 10 μm thickness, porous (8.0 μm) polycarbonate membrane separating two chambers (Transwell; Costar, Cambridge, MA). Cells were added at a final concentration of 10,000 cells to upper chamber in RPMI (10% FCS) media. The cells were incubated for 72 h at 37°C. At the end of the assay the cells from the upper and lower chamber were detached by trypsin/EDTA. The cells which migrated to the lower chamber and those remaining in the upper chamber were counted using a Coulter counter. The percentage of cells in the upper and lower chambers were calculated based on the total number of cells present in upper and lower chambers. The invasion/migration ratio was calculated as a ratio of the percent cells in the lower chamber versus the upper chamber, as we previously described (24, 25).

Results

Purification of uPAR Coding Region mRNA Binding Protein

To purify the uPAR coding region mRNA binding protein from Beas2B cells, we used conventional chromatography combined with gel mobility shift and Northwestern assay to track uPAR mRNA binding activity. As shown in Figure 1, the purified protein formed a specific complex with the uPAR mRNA coding region transcript. The same purification fraction was separated by SDS-PAGE and developed with silver staining, which showed a major species with an approximate molecular weight of 50 kD. The mobility pattern was consistent with the uPAR CDR binding activity seen on Northwestern assay indicating that this protein species interacts with uPAR mRNA. The N-terminal end of the purified protein was microsequenced, revealing a protein species of 50 kD demonstrating 83% homology to phosphoglycerate kinase (PGK) (35).

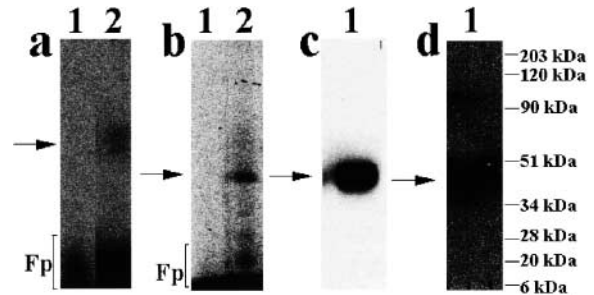


Figure 1. Purification and analysis of uPAR mRNABp in Beas2B cells. (a) Gel mobility shift assays of purified uPAR mRNABp interaction with ^{32}P -labeled uPAR mRNA coding region transcript (lane 1, free probe; lane 2, purified uPAR mRNABp). (b) Corresponding samples were subjected to UV cross-linking assays (lane 1, free probe; lane 2, purified uPAR mRNABp). (c) The purified uPAR mRNABp was separated on SDS-PAGE, electroblotted to nitrocellulose membrane and developed by Northwestern blot assay (lane 1). (d) Silver staining of purified uPAR mRNABp preparation from Beas2B cells (lane 1). Fp = free probe, arrow indicates the uPAR mRNABp.

Cloning and Expression of PGK

Primers corresponding to entire PGK coding region were designed for PCR cloning. A 1,200-nt clone was amplified from Beas2B cells and this fragment was subjected to a directional cloning using the pENTER directional TOPO cloning kit (Invitrogen). The PCR product was sequenced to confirm orientation. This was later subcloned in a eukaryotic expression vector pcDNA3.1 D/V5-His-TOPO as well as prokaryotic expression pET vector. The constructs were transfected to eukaryotic (H157) and prokaryotic (BL21) cells respectively. Polyclonal stable cell lines were created by treating the transfected cells with genitecin (G418), and these cells were cultured in 150 \times 25 mm culture dishes. The cell lysates were prepared and native fusion proteins were purified using a Ni-NTA-His column (Qiagen, Valencia, CA). The native proteins were later subjected to gel mobility shift (Figure 2) and Northwestern (Figure 3) assays to confirm uPAR mRNA binding activity. We also confirmed the expression of the fusion protein by Western blotting using an anti-V5-monoclonal antibody (Figure 3C). In BL21 cells, the native PGK protein was induced by IPTG. PGK expressed in both eukaryotic (Figures 2a and 3a) and prokaryotic (Figures 2b and 3b) cells showed uPAR mRNA binding activity by gel mobility and Northwestern assays.

PGK Interaction with uPAR mRNA *In Vivo*

We confirmed direct *in vivo* interaction of PGK protein with Beas2B uPAR mRNA by combined immunoprecipitation-RT-PCR (Figure 4a). The results indicate that PGK protein interacts with uPAR mRNA in Beas2B cells and that this interaction is diminished in uPAR overexpressing H157 cells (24, 34).

To confirm the specificity of the PGK-uPAR mRNA interaction, we incubated purified PGK with uPAR mRNA transcripts containing the full-length coding sequence or a 110-nt 3'UTR uPAR mRNA sequence containing the AU-rich determinant. The results indicate that PGK bound the coding region transcript (Figure 4b). Similarly pretreatment of PGK with proteinase K or SDS reversed its interaction with uPAR mRNA (data not shown) further supporting the specificity of the interaction.

Effect of PGK on Cell Surface uPAR Expression

Because PGK specifically interacted with uPAR mRNA we next wanted to determine whether this interaction regulates cell surface uPAR expression. To confirm our hypothesis that it does,

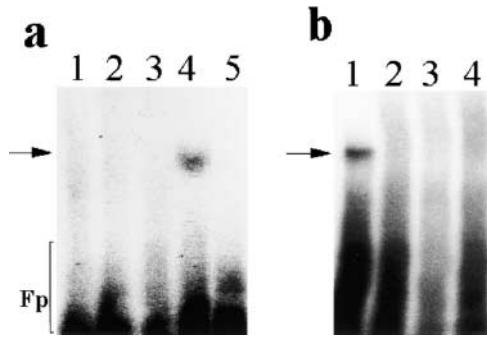


Figure 2. Urokinase receptor mRNA binding activity of recombinant PGK by gel mobility shift assay. (a) uPAR mRNA binding activity demonstrated by gel mobility shift assay. Five micrograms of each protein were used. (a) Lane 1 = free probe, protein eluate of cytosolic extracts of nontransfected H157 cells (lane 2) or H157 cells transfected with pcDNA 3.1 alone (lane 3) or PGK cDNA in pcDNA 3.1 (lane 4) purified on a Nickel column. Recombinant PGK protein purified from H157 cells in the presence of a 200-fold molar excess of unlabeled uPAR CDR mRNA (lane 5). (b) PGK protein purified from prokaryotic expression vector using Nickel column. Bacterial lysates from IPTG induced BL21 cells transfected with PGK cDNA in pET vector (lane 1), recombinant PGK purified from BL21 in the presence of 200-fold molar excess of unlabeled uPAR mRNA (lane 2). IPTG induced BL21 bacterial lysates eluted from the Nickel column (lane 3). Lane 4 = free probe. Arrow indicates uPAR mRNA-PGK complex.

we cultured stable H157 cells transfected with empty vector alone or PGK cDNA in eukaryotic expression vector pcDNA3.1 separately in culture dishes. We also used H157 cells without any transfection as controls. As shown in Figure 5a, transfection of PGK cDNA reduced cell surface uPAR expression compared with vector transfected or control naïve H157 cells. These data strongly suggest the likelihood that PGK regulates expression of cell surface uPAR expression in H157 cells via regulation of the levels of cellular uPAR mRNA. To confirm this impression, we next determined the effect of PGK on uPAR mRNA expression in these cells. As shown in Figure 5b, H157 cells overexpress-

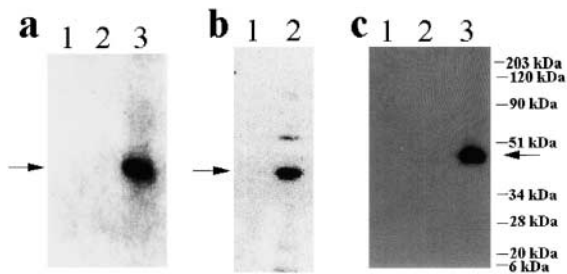


Figure 3. Assessment of uPAR mRNA binding activity and molecular weight of PGK. (a) The proteins prepared as described in Figure 2 were separated on SDS-PAGE, then transferred to a nitrocellulose membrane. The membranes were later developed by Northwestern assay using 32 P-labeled uPAR mRNA coding region transcript and autoradiography. Protein eluate of cytosolic extracts of untransfected H157 cells (lane 1) or H157 cells transfected with pcDNA 3.1 alone (lane 2) or PGK cDNA in pcDNA3.1 (lane 3) purified on a Nickel column. (b) PGK protein purified from a prokaryotic expression vector using Nickel column. IPTG induced BL21 bacterial lysates eluted from the Nickel column (lane 1). Bacterial lysates from IPTG induced BL21 cells transfected with PGK cDNA in pET vector (lane 2). (c) The corresponding membrane shown in Figure 3a was stripped and developed using anti-V5 monoclonal antibody. Arrow indicates PGK.

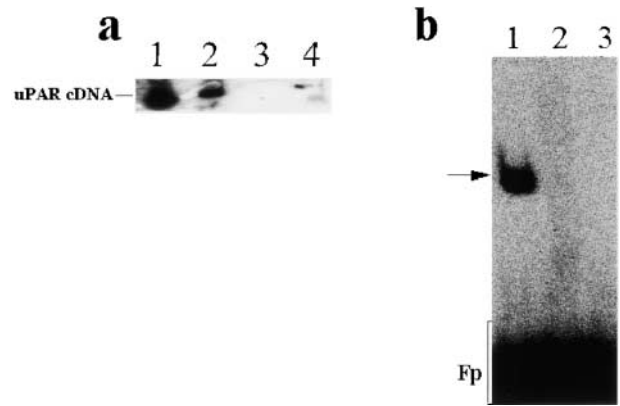


Figure 4. Demonstration of PGK interaction with uPAR mRNA in Beas2B and H157 cells. (a) Coprecipitation of uPAR mRNA with PGK protein. Cytosolic extracts of Beas2B cells or H157 cells were immunoprecipitated with PGK-specific antibody in the presence of RNase inhibitor. Total RNA associated with PGK immune complex was isolated using total RNA isolation reagent; uPAR mRNA was amplified by RT-PCR using specific primers. The uPAR PCR products were developed by Southern blotting using 32 P-labeled uPAR cDNA. As a positive control, uPAR cDNA was used as template for PCR amplification (lane 1). uPAR cDNA was amplified from the PGK-immune complex isolated from the cytosolic extracts of Beas2B (lane 2) or H157 cells (lane 4). A negative control without template cDNA is also illustrated (lane 3). (b) Gel mobility shift assay to assess the specificity of the binding of PGK protein to various uPAR mRNA transcripts. The purified protein was incubated with full uPAR coding region transcript (lane 1), AU-rich region of uPAR 3'UTR (lane 2) or probe alone (lane 3). The arrow indicates the RNA-protein complex. The data illustrated are representative of three independent experiments.

ing PGK cDNA inhibited uPAR mRNA expression compared with control H157 cells or H157 cells transfected with empty vector. These data show that PGK-mediated inhibition of cell surface uPAR expression is due to decreased uPAR mRNA expression.

Effect of PGK on Regulation of uPAR mRNA Expression

We next wanted to determine whether PGK-mediated inhibition of uPAR mRNA expression in H157 cells was due to decreased mRNA synthesis or enhanced mRNA degradation. We therefore cultured these cell lines in T170 flasks and isolated intact nuclei, which were then subjected to nuclear run-on assays (34). The

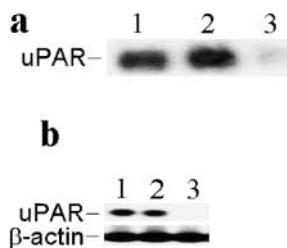


Figure 5. Inhibition of uPAR and mRNA expression by PGK in H157 cells. (a) Urokinase receptor expression in PGK transfected H157 cells. Control H157 cells (lane 1) or stable H157 cells transfected with pcDNA 3.1 alone (lane 2) or PGK cDNA in pcDNA 3.1 (lane 3) were grown to confluence. The membrane proteins isolated from these cells were separated on 8% SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were subjected to Western blotting using a urokinase receptor monoclonal antibody. (b) Urokinase receptor mRNA expression in PGK transfected H157 cells. H157 cell lines as mentioned in Figure 5a were grown to confluence in RPMI 1640 media with or without G418. Total RNA was isolated and uPAR mRNA was measured by Northern blot using 32 P-labeled uPAR cDNA. The data illustrated are representative of four independent experiments.

results of nuclear run-on assays indicate that there is no discernable change in the rate of uPAR mRNA transcription in PGK cDNA transfected cells (Figure 6a). Because PGK binds to uPAR mRNA, we inferred that PGK probably regulates uPAR expression at the post-transcriptional level. To confirm this inference, we next treated confluent H157 cells with 5,6-dichloro-1 β -D-ribofuranosylbenzamidazole (10 μ g/ml) to inhibit ongoing transcription and analyzed uPAR mRNA expression by Northern blotting. As shown Figure 6b, uPAR mRNA is quite stable in H157 cells with a half-life of 6–8 h. Conversely, uPAR mRNA was degraded much faster in PGK overexpressing cells.

Effect of PGK on Proliferation and Migration/Invasion

Because cellular proliferation of H157 cells is influenced by differences in uPAR expression (24,32), we assessed the effect of PGK overexpression on the rate of proliferation of H157 cells, as assessed by 3 H-thymidine incorporation. 3 H-thymidine uptake by subconfluent monolayers of cells transfected with vector alone or PGK was compared with control H157 cells. As shown in Figure 7a, PGK overexpression predictably inhibited 3 H-thymidine uptake by at least 50% in H157 cells compared with vector transfected or control H157 cells. Having confirmed that PGK regulates proliferation of H157 cells, these cells were lastly studied in invasion/migration assays, which are likewise influenced by cellular uPAR expression (24, 25). As shown in Figure 7b, PGK overexpressing cells exhibited decreased (at least 40%) migration compared with control cells used in the assays.

Discussion

The interaction between uPA and uPAR at the cancer cell surface appears to be a critical determinant of the pathogenesis of neoplastic growth and metastasis, mediating tissue remodeling, tumor cell invasion, adhesion, and proliferation (5, 36). Because many biological activities of uPA depend on association with its receptor, uPAR plays a central role in localizing uPA-mediated

plasminogen activation and cellular signaling (1). Regulation of this receptor could thus influence the broad range of uPA activity and its effects on cellular pathophysiology. In addition, outcome studies in cancer patients have shown that high levels of either uPA and uPAR in tumors correlate with poor prognosis (11, 12). The control of uPAR expression is therefore clinically relevant in the pathogenesis of solid tumors such as lung carcinomas.

We previously reported that uPAR is overexpressed in squamous lung cell carcinoma (H157) cells compared with normal lung epithelial (Beas2B or small airway epithelial) cells (11, 12, 24, 34). Overexpression of uPAR appears to increase the invasiveness of lung carcinomas, which could strongly influence their clinical behavior. Epithelial-derived tumor cell invasion is facilitated by saturation of uPAR with either exogenously supplemented uPA or overexpressed endogenous uPA (35–42). Expression of uPAR by lung nonmalignant lung epithelial and carcinoma cells is now known to be controlled both at transcriptional and post-transcriptional levels by a variety of hormones and cytokines (14–16, 18). At the present time, mechanisms that regulate uPAR expression in these cells at the post-transcriptional level are incompletely understood, even though our earlier study confirmed that uPAR expression was regulated at this level (24, 26, 34). We now extend these observations to further elucidate regulatory mechanism in these cells.

Regulation of uPAR expression by lung epithelial cells is involved in the pathogenesis of lung inflammation as well as lung cancer. Expression of uPAR at the cell surface is germane to a variety of cellular responses involved in the pathogenesis of either process. The interaction between uPA and uPAR at the cell surface appears to influence neoplastic tissue remodeling, tumor cell invasion, adhesion, and proliferation (1, 4, 8, 9, 28–33). In addition, the binding of uPA to uPAR mediates cellular proteolysis and cellular proliferation in both nonmalignant and malignant lung epithelial cells as well as pleural mesothelial or mesothelioma cells (21, 26, 29, 30, 32). Post-transcriptional regulation of uPAR

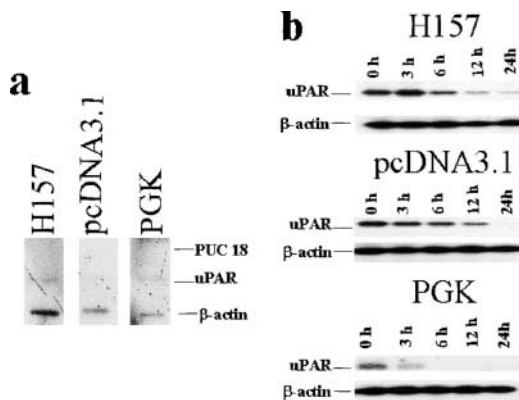


Figure 6. Effect of PGK on the rate of transcription and decay of uPAR mRNA in H157 cells. (a) Nuclei isolated from H157 cell (H157), or stable cell lines transfected with empty vector (pcDNA 3.1) or PGK cDNA in pcDNA 3.1 (PGK) as described above were subjected to the transcription reaction in the presence of 32 P UTP at 30°C for 30 min. 32 P-labeled nuclear RNA was hybridized with uPAR cDNA immobilized on nitrocellulose membrane. β -actin and PUC 18 cDNAs were used as positive and negative loading controls respectively. Results illustrated are representative of triplicate independent experiments. (b) Effect of PGK on uPAR mRNA stability. Ongoing transcription of H157 cells grown to confluence was inhibited by treating the cells with 5,6-dichloro-1 β -D-ribofuranosylbenzamidazole (10 μ g/ml) for 0, 3, 6, 12, and 24 h in the same medium. Total RNA was isolated and uPAR was analyzed by Northern blot.

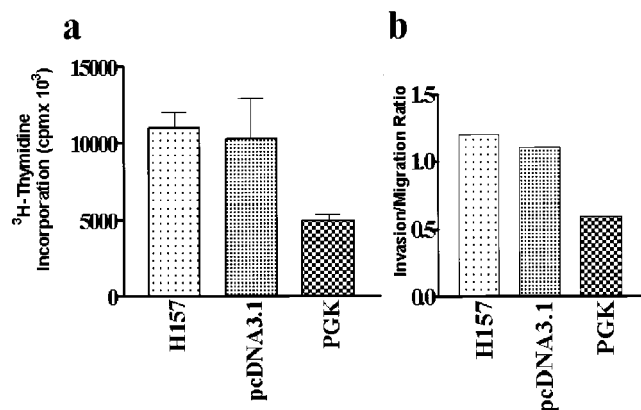


Figure 7. (a) Inhibition of H157 cells 3 H-thymidine uptake by PGK. H157 cell lines were serum starved overnight and treated with 3 H-thymidine for 6 h. The cells were washed and the rate of DNA synthesis was determined by measuring 3 H-thymidine incorporation. The data from seven independent experiments are illustrated. (b) Effect of PGK expression on invasion/migration ratio of H157 cells. The H157 cells grown to confluence were transferred to the upper chamber of Transwell plates for assessment in the invasion/migration assay. The cells were incubated for 72 h at 37°C, the number of cells present in the upper and the lower chambers were counted and the invasion/migration ratios were calculated based on the total number of cells present in both the upper and lower chambers. The mean data from three independent experiments are illustrated.

could therefore influence a wide range of pathophysiologic responses germane to either lung inflammation or lung cancer.

The increased uPAR mRNA stability in lung-derived epithelial and mesothelioma cells correlates with increased uPAR mRNA and cell surface expression of uPAR. There are precedents for this mode of regulation. Lymphocyte engagement, for example, also stabilizes uPAR mRNA, a process that involves AU-rich sequences present in the uPAR 3'UTR (18). The present study is the first to elucidate the role of PGK in the regulatory mechanism that controls uPAR expression at the post-transcriptional level. Our data show that PGK interacts with uPAR mRNA both *in vivo* as well as in cell-free systems. Lower affinity of PGK for endogenous uPAR mRNA in H157 cells (Figure 4a, lane 4) along with the relative overexpression of cell surface uPAR in these cells versus nonmalignant small airway epithelial cells (24, 34) strongly suggests that phosphorylation of PGK may play a major role in regulating its ability to bind uPAR mRNA. These observations are consistent with our recent report (43) that uPAR mRNA stability is increased by phosphorylation of the uPAR mRNA binding protein we now identify as PGK. In addition, PGK regulates the destabilization of uPAR mRNA. Its overexpression decreases uPAR expression at the cell surface and influences pathophysiologic responses that are likewise contingent on the expression of this receptor by H157 lung carcinoma cells.

We used microsequencing to lead us to identify the 50-kD uPAR mRNA-Bp we purified from Beas2B cells as PGK. PGK is a key glycolytic enzyme that catalyzes the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. This protein shares a common nucleotide (either ATP or NAD) binding domain in combination with unique catalytic domains. This observation thus supports a rationale for the RNA binding activity we now ascribe to PGK. This property has previously been attributed to several dehydrogenases and other enzymes shown to have the capacity to bind mRNA sequences. PGK has been shown to possess a wide range of biological activities in addition to its well-characterized role in glycolysis. Our study extends our understanding of the repertoire of this enzyme to the regulation of uPAR mRNA stability and to the regulation of uPAR expression and function at the post-transcriptional level.

Our results provide a novel example of a functional and biochemical chimerism, linking newly recognized mRNA binding by PGK with its previously known glycolytic enzyme activity (44–46). The physiologic relevance of the connection between mRNA decay and glycolysis remains an enigma at this time. The precedent for bifunctional RNA binding protein is the iron-responsive-element binding protein (IRE-Bp), which was found to have aconitase activity; that of an enzyme of the Krebs cycle (47). The 3'AU-rich element binding protein with enoyl-CoA hydratase activity (45) is another precedent for the type of regulatory mechanism we now describe. Indeed, many other proteins involved in the regulation of the turnover and translation of mRNA may serve additional and quite disparate roles, as further exemplified by glutamate dehydrogenase (48), NAD⁺-dependent isocitrate dehydrogenase (49), thymidylate synthase (50), dihydrofolate reductase (51), catalase (52), thiolase (53), glyceraldehyde phosphate dehydrogenase (46), lactate dehydrogenase (54), and tumor suppressor protein P-53 (55).

In summary, we confirmed that PGK regulates uPAR mRNA expression at the post-transcriptional level. This newly recognized pathway involves interaction of PGK with the uPAR mRNA coding region and regulates expression of uPAR at the cell surface. This mechanism represents a pathway by which uPAR-dependent responses of the lung epithelium may be controlled in the context of lung injury and repair, neoplastic transformation, or in the growth and spread of lung neoplasms.

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