Urokinase induces its own expression in Beas2B lung epithelial cells

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Urokinase induces its own expression in Beas2B lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 283: L319–L328, 2002; 10.1152/ajplung.00395.2001.—The urokinase-type plasminogen activator (uPA) interacts with its receptor (uPAR) to promote local proteolysis as well as cellular proliferation and migration. These functions contribute to the pathogenesis of lung inflammation and remodeling as well as the growth and invasiveness of lung neoplasms. In this study, we sought to determine if uPA alters its own expression in lung epithelial cells. Using immunoprecipitation and Western and Northern blotting techniques, we found that uPA treatment enhanced uPA expression in Beas2B lung epithelial cells in a time- and concentration-dependent manner. The induction of uPA expression is mediated through its cell surface receptor uPAR and does not require uPA enzymatic activity. The amino-terminal fragment of uPA, lacking the catalytic domain, is sufficient to induce uPA expression. The serine protease plasmin and the protease inhibitor aprotinin failed to alter uPA-mediated uPA expression, whereas α-thrombin potentiated the response. Pretreatment of Beas2B cells with a tyrosine kinase inhibitor, herbimycin, suggests that activation of tyrosine kinase(s) is involved in the uPA-mediated uPA expression. Induction of uPA expression by exposure of lung-derived epithelial cells to uPA is a newly defined pathway by which this protease could influence expression of local fibrinolytic activity and other uPA-dependent cellular responses. A recent study indicates that uPA also interacts with cell surface proteins other than uPAR, suggesting that intracellular signaling could be regulated by interactions between uPA and alternative receptors (22, 26). Stimulation of the lung epithelium by uPA elicits responses via signaling mechanisms that are incompletely characterized at present. Expression of growth factor activity has been attributed to uPA stimulation.

Activation of plasminogen can be facilitated by at least two enzymes: urokinase-type (uPA) and tissue-type plasminogen activators. The former is mainly involved in extravascular proteolysis implicated in stromal remodeling and neoplasia. A defect of uPA-related fibrinolytic activity has been implicated in lung dysfunction associated with acute lung injury in acute respiratory distress syndrome (ARDS) and interstitial lung diseases (16–20). In addition, evidence for involvement of the uPA system in cancer spread has steadily increased, and it now seems beyond reasonable doubt that plasminogen activation is central to this process (15). Along these lines, several model systems provide evidence for a causal role of uPA in metastasis and the growth of solid neoplasms (9, 28, 31). High levels of uPA and its receptor (uPAR) in lung tumors correlate with poor prognosis (35, 36).

uPA-mediated activation of plasminogen is tightly regulated by its high-affinity receptor (uPAR) and two specific and fast-acting inhibitors [plasminogen activator inhibitor (PAI)-1 and PAI-2]. Synthesis of fibrinolytic components (uPA, uPAR, PAI-1, and PAI-2) is regulated by a variety of hormones, growth factors, and cytokines, acting at either the transcriptional or posttranscriptional levels (9, 29, 30, 37, 45, 52). Binding of uPA to uPAR enhances cell-surface plasminogen activation severalfold compared with that of fluid phase uPA (9, 31). Aside from facilitating plasmin formation, uPA is also involved in cellular functions such as migration, adhesion, signaling, and mitogenesis. Most of these biological activities are dependent on the association of uPA with uPAR, but uPA is reported to generate intracellular signals by either uPAR-dependent (3, 8, 9, 10, 27, 50) or -independent (26) mechanisms. A recent study indicates that uPA also interacts with cell surface proteins other than uPAR, suggesting that intracellular signaling could be regulated by interactions between uPA and alternative receptors (22, 26).

Stimulation of the lung epithelium by uPA elicits responses via signaling mechanisms that are incompletely characterized at present. Expression of growth factor activity has been attributed to uPA stimulation.

EXTRACELLULAR PROTEOLYTIC ENZYMES such as urokinase (uPA) and metalloproteases have been implicated in the pathogenesis of lung inflammation and the growth of lung tumors. In both situations, these proteases promote remodeling of the transitional stroma via the breakdown of basement membranes and extracellular matrix proteins, including fibrin (9, 28, 31). Plasmin, a serine protease, is involved in the remodeling of the extracellular matrix during tissue degradation, either directly or through activation of latent collagenases.
uPA bound to its receptor also activates intracellular signaling through unknown protein(s). uPAR overexpression by tumor cells, in turn, appears to activate several tyrosine kinases (3, 14, 25, 33). It is noteworthy that the signaling pathways activated by uPA/uPAR seem to be the same pathways that induce their own expression. This information suggests the hypothesis that uPA might regulate its own expression in lung epithelial cells, a possibility that has not, to our knowledge, been previously explored. The potent growth factor effects of uPA further prompted us to evaluate this possibility. We now report a new paradigm in which uPA expression by lung epithelial cells is autoregulated. We used Beas2B lung-derived epithelial cells as a model system to characterize the responsible mechanism.

**METHODS**

**Materials.** Culture media, penicillin, streptomycin, fetal calf serum (FCS) were purchased from Gibco BRL laboratories (Grand Island, NY); tissue culture plastics were from Becton Dickinson Lab Ware (Lincoln Park, NJ). α-Thrombin, heparin, bovine serum albumin (BSA), ovalbumin, Tris-base, aprotagin, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), silver nitrate, ammonium persulfate, and phenol/chloroform were obtained from Eastman Kodak (Rochester, NY). We used the Imubind uPA/pcRII-TOPO kit (product no. 894) from American Diagnostica to measure uPA antigen concentrations.

**Cell cultures.** Human bronchial epithelial cells (Beas2B) were obtained from ATCC. These cells were maintained in LHC-9 and RPMI 1640 medium containing 10% heat-inactivated FCS, 1% glutamine, and 1% antibiotics as previously described (46).

**Total protein extraction and immunoprecipitation.** The cells were grown to confluence and were then serum starved overnight with RPMI-methionine-free media. The cells were treated with or without various agents for indicated times in methionine-free media supplemented with [35S]methionine (46). The conditioned media (CM) were collected, and the cells were washed with PBS. The cells were suspended in lysis buffer (10 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1% Triton X-100, 15% glycerol, 1 mM Na3VO4, 1 mM NaF, 1 mM EDTA, 1 mM PMSF, and 3–10 μg aprotinin/100 ml). The cell lysates (CL) were prepared using three cycles of freezing and thawing. Total protein from CL and CM (200 μg) in 500 μl of lysis buffer was initially cleared for nonspecific binding by immunoprecipitation with nonspecific mouse IgG followed by protein-A agarose. The supernatant was further immunoprecipitated with uPA or β-actin monoclonal antibody, and the immune complexes were separated on SDS gel electrophoresis and autoradiograph to measure uPA in the cell lysate and CM.

**Western blotting.** The proteins isolated from Beas2B CL and CM 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, hybridized overnight with uPA or antiphosphotyrosine monoclonal antibody in the same buffer at 4°C, and washed, and uPA proteins were detected by enhanced chemiluminescence. The membranes were stripped with β-mercaptoethanol and subjected to Western blotting with β-actin monoclonal antibody.

**Plasmid construction.** Plasmid uPA/pcRII-TOPO was obtained by polymerase chain reaction amplification of a human lung cDNA library. The cDNA corresponding to the coding region (1.3 kb) was subcloned to pCRII-TOPO vector (Invitrogen), and the sequence of the clones was confirmed by nucleotide sequencing. The uPA insert was released by HindIII or XbaI, purified on 1% agarose gels, extracted with phenol/chloroform, and used as cDNA probe for Northern blotting.

**Random priming of uPA cDNA.** The full-length template of uPA was released with HindIII or XbaI, purified on 1% agarose gels, and labeled with 32P with the use of a Rediprime labeling kit (Amersham Pharmacia Biotech). Passage through a Sephadex G-25 column removed unincorporated radioactivity. The specific activity of the product was 8 × 10^6 counts · min⁻¹ · μg⁻¹.

**Nuclear run-on transcription activation assay.** Cells grown to confluence in two T182 flasks were serum starved overnight in RPMI-BSA media. The cells were later treated with PBS or uPA (1 μg/ml) for 12 h at 37°C and then washed with ice-cold PBS. The cells were then resuspended in 0.4 ml of lysis buffer (10 mM HEPES buffer pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml each leupeptin and aprotinin, and 0.5 mg/ml benzamidine). The cells were homogenized and incubated on ice for 1 h to release the nuclei. The nuclear pellet was subjected to sucrose-gradient centrifugation by careful layering on top of 7.5 ml of sucrose buffer (1.3 M) followed by centrifugation at 10,000 g for 15 min at 4°C. The nuclear pellet was resuspended in 200 μl of lysis buffer. The nuclei (10–20 × 10⁶) were added to the transcription reaction in the presence of 250 μCi of [32P]UTP at 30°C for 30 min. 32P-labeled nuclear RNA was isolated with TRI reagent, and unincorporated radioactivity was removed by repeated precipitation and washing with cold ethanol. RNA pellets were dissolved in 40 μl of RNase-free water. Recombinant plasmid DNA (10 μg/50 ml) was denatured by heating at 95°C in 0.25 M NaOH/5 mM EDTA solution for 5 min and chilling on ice. Denatured DNA solution was spotted on a nitrocellulose membrane with a slot-blot apparatus under vacuum. The membranes were vacuum-baked and prehybridized overnight at 42°C in a hybridization buffer (50 mM HEPES, pH 7.0, 50% formamide, 4 × SSC, 2 × Denhardt’s solution, 2 mM EDTA, 0.1% SDS, 0.2% Sarkosyl, 0.1 mg/ml salmon sperm DNA). 32P-labeled RNA (10 × 10⁶ counts · min⁻¹ · ml⁻¹) was added to the hybridization solution, and the membranes were incubated in a hybridization oven for 48 h. The filters were washed with 2–0.1 × SSC with in between RNase digestion, air-dried, and exposed to X-ray film.

**uPA mRNA assessment by Northern blotting.** A Northern blotting assay was used to assess the level of uPA mRNA. Beas2B cells grown to confluence were serum starved overnight in RPMI-BSA media and treated with two-chain human recombinant uPA for varying times (0–24 h) in the same media. Total RNA was isolated with TRI reagent, and RNA

**Nuclear run-on transcription activation assay.** Cells grown to confluence in two T182 flasks were serum starved overnight in RPMI-BSA media. The cells were later treated with PBS or uPA (1 μg/ml) for 12 h at 37°C and then washed with ice-cold PBS. The cells were then resuspended in 0.4 ml of lysis buffer (10 mM HEPES buffer pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml each leupeptin and aprotinin, and 0.5 mg/ml benzamidine). The cells were homogenized and incubated on ice for 1 h to release the nuclei. The nuclear pellet was subjected to sucrose-gradient centrifugation by careful layering on top of 7.5 ml of sucrose buffer (1.3 M) followed by centrifugation at 10,000 g for 15 min at 4°C. The nuclear pellet was resuspended in 200 μl of lysis buffer. The nuclei (10–20 × 10⁶) were added to the transcription reaction in the presence of 250 μCi of [32P]UTP at 30°C for 30 min. 32P-labeled nuclear RNA was isolated with TRI reagent, and unincorporated radioactivity was removed by repeated precipitation and washing with cold ethanol. RNA pellets were dissolved in 40 μl of RNase-free water. Recombinant plasmid DNA (10 μg/50 ml) was denatured by heating at 95°C in 0.25 M NaOH/5 mM EDTA solution for 5 min and chilling on ice. Denatured DNA solution was spotted on a nitrocellulose membrane with a slot-blot apparatus under vacuum. The membranes were vacuum-baked and prehybridized overnight at 42°C in a hybridization buffer (50 mM HEPES, pH 7.0, 50% formamide, 4 × SSC, 2 × Denhardt’s solution, 2 mM EDTA, 0.1% SDS, 0.2% Sarkosyl, 0.1 mg/ml salmon sperm DNA). 32P-labeled RNA (10 × 10⁶ counts · min⁻¹ · ml⁻¹) was added to the hybridization solution, and the membranes were incubated in a hybridization oven for 48 h. The filters were washed with 2–0.1 × SSC with in between RNase digestion, air-dried, and exposed to X-ray film.
Time-dependent induction of uPA by uPA. Because we previously found that lung carcinoma-derived and nonmalignant lung epithelial cells differentially express uPA in vitro (42), we initially sought to extend this work and determine whether uPA induces its own expression in Beas2B cells, a nonmalignant lung epithelial cell line. We treated the cells with the HMW, two-chain form of uPA for varying lengths (0–24 h) of time in the presence of [35S]methionine. Total proteins from CL as well as CM were used for immunoprecipitation. We measured uPA expression at the cell surface by immunoprecipitation with an anti-uPA antibody followed by autoradiography. Because ELISA or Western blot cannot differentiate between uPA produced by Beas2B cell from added uPA, we chose to assess induction of endogenous uPA by semiquantitative densitometry of [35S]-labeled endogenous uPA by immunoprecipitation and SDS-PAGE. The data in Fig. 1 demonstrate that uPA induces its own expression in Beas2B cells in a time-dependent manner. The induction starts between 3 and 12 h after the addition of uPA, and maximal induction was achieved at 12–24 h. We also found similar results by Western blotting (data not shown). In preliminary experiments, we determined that basal levels of uPA or uPA-mediated uPA expression were virtually the same in LHC-9- vs. RPMI-cultured Beas2B cells (data not shown).

To determine whether uPA induction of uPA expression is due to contaminating lipopolysaccharides (LPS) present in the HMW uPA preparation, we first measured the LPS content using the Limulus amebocyte lysate ELISA method and found that this uPA preparation contains very negligible amounts (1 pg/ml) of LPS. To determine whether the LPS content of the preparation could account for the induction of uPA, we next treated Beas2B cells with the same concentration, 1 pg/ml, as well as a tenfold excess, 10 pg/ml of LPS, and then measured uPA expression by Western blotting as we described in Western blotting. We found that these concentrations of LPS failed to induce uPA expression (data not shown), indicating that the induction of uPA by the uPA preparation we used could not be attributable to LPS contamination.

uPA mRNA expression by uPA in lung epithelial cells. Having determined that uPA mediates time-dependent uPA protein expression by Beas2B cells, we next wanted to confirm that the increased expression of uPA is attributable to increased expression of uPA mRNA. We measured the steady-state levels of uPA mRNA in uPA-treated normal epithelial cells by Northern blot analysis using a uPA cDNA probe. As shown in Fig. 2, uPA induces uPA mRNA in Beas2B cells, and the induction is observed as early as 3 h after the treatment. However, maximum accumulation of uPA mRNA is achieved between 6 and 24 h. These data confirm that uPA mediates increased uPA expression by Beas2B cells as determined by immunoprecipitation. The level of uPA mRNA was quantitated by densitometric scanning and normalized against β-actin loading controls. As shown in Fig. 2B, resting Beas2B cells express a small amount of uPA mRNA. After uPA treatment, the level of uPA mRNA increased by 3 h and continued to increase over 24 h.

Transcriptional activation of uPA, run-on transcription experiments. Nuclear run-on experiments demonstrated that treatment of Beas2B cells with uPA for
12 h did not induce substantial de novo RNA synthesis (Fig. 3A).

*Decay of uPA mRNA.* Having confirmed that uPA mRNA is increased by uPA treatment in Beas2B cells, we next wanted to determine whether the increased uPA mRNA expression in these cells was due to increased stability of uPA mRNA. To address this possibility, we treated Beas2B cells stimulated with or without uPA for 12 h with DRB for varying lengths of time to inhibit ongoing transcription. We isolated total RNA from these cells and then analyzed it by Northern blotting using a 32P-labeled uPA cDNA as shown Fig. 3B. The uPA mRNA half-life was 1.5 h. However, uPA treatment stabilized the mRNA over 24 h. These data demonstrate that exogenous uPA stabilizes the uPA message.

*Effect of uPA concentration on uPA mRNA and protein expression.* We next treated Beas2B cells with varying amounts (0–3 μg/ml, 0–55.5 nM) of uPA for 24 h and then measured uPA expression by immunoprecipitation. The data shown in Fig. 4A indicate that uPA induced uPA expression by Beas2B cells in a concentration-dependent manner. The induction effect is apparent at concentrations as low as 250 ng/ml (4.6 nM) of uPA. Maximum uPA expression was observed at 1,000 ng/ml (18.5 nM) of uPA. These data suggest that the induction of uPA in Beas2B cells by uPA is concentration dependent. We also found a similar concentration-dependent increase in uPA mRNA expression between 100 and 1,000 ng/ml (1.85–18.5 nM) of added uPA (Fig. 4C).

*Effects of phosphatase and phosphotyrosine kinase inhibitors on uPA-mediated uPA induction.* To determine whether uPA-mediated uPA expression involves cellular signaling, we pretreated Beas2B cells with herbimycin A separately or in combination with uPA. As shown in Fig. 5, herbimycin A alone did not induce uPA expression (Fig. 5A, lane 3). However, herbimycin, a phosphotyrosine kinase inhibitor, abrogated uPA-mediated uPA expression by Beas2B cells (Fig. 5A, lane 4). We also found that uPA induced tyrosine phosphorylation of several cytoplasmic proteins (data not shown). We could not detect basal uPA expression by metabolic labeling and immunoprecipitation and, therefore, attempted to do so by Western blotting of uPA antigen, which was likewise unsuccessful. We therefore used ELISA analyses to determine the effects of the inhibitors on basal expression of uPA by Beas2B cells. Using this approach, we found that herbimycin inhibits the basal level of uPA in Beas2B cells as well as uPA-treated cells (0.99 ng/ml, basal to 0.36 ng/ml and 0.25 ng/ml, respectively). We also confirmed that sodium orthovanadate decreased basal and uPA-mediated cellular uPA expression (0.99 ng/ml to 0.55 ng/ml).

Fig. 2. Time-dependent induction of uPA mRNA by uPA treatment of Beas2B cells. The cells were treated as described in Fig. 1. Total RNA (20 μg/lane) was isolated using TRI reagent, separated by agarose-formaldehyde gel electrophoresis, and subjected to Northern blotting with 32P-labeled uPA and β-actin cDNAs (A). The integrated densitometric data from 3 separate experiments are shown (B).

Fig. 3. Effect of uPA on the rate of transcription and decay of uPA mRNA in Beas2B cells. A: nuclei isolated from Beas2B cells treated with or without uPA for 12 h as described in Fig. 2 were subjected to the transcription reaction in the presence of [32P]UTP at 30°C for 30 min. 32P-labeled nuclear RNA was hybridized with uPA cDNA immobilized on nitrocellulose membrane. β-Actin and pcDNA3 cDNAs were used as positive and negative loading controls, respectively. B: effect of uPA on uPA mRNA stability. The Beas2B cells were treated with PBS or uPA for 12 h, after which 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (10 μg/ml) was added for various periods of time. uPA mRNA was analyzed by Northern blotting.
and 0.29 ng/ml), suggesting that this inhibitor might somehow increase release of uPA from the cell surface, as demonstrated in the immunoprecipitation experiments. By immunoprecipitation, pretreatment of cells with uPA and sodium orthovanadate (a tyrosine phosphatase inhibitor) increased uPA expression in the CM of Beas2B cells (Fig. 5).

**Effect of ATF and LMW uPA on uPA expression.** We next treated Beas2B cells with either the ATF, uPAR binding or LMW, catalytic fragment of uPA to determine whether the induction of uPA in Beas2B cells by uPA is mediated by receptor occupancy. As shown in Fig. 6A (lanes 3–4), ATF alone or in combination with uPA induced uPA expression. However, the LMW frag-
ment of uPA, which does not bind to uPAR, failed to induce uPA expression (lane 5).

Effect of uPA enzymatic activity on uPA-induced expression of uPA. We assessed the requirement of uPA enzymatic activity in the induction of uPA by two independent experiments. In the first experiment, as shown in Fig. 7A, treatment of Beas2B cells with anti-uPA monoclonal antibody alone did not induce uPA expression, but preincubation of uPA with the antibody did not abrogate its ability to induce uPA expression. In another experiment, pretreatment of cells with B-428 (an inhibitor of uPA) (4) or PAI-1 did not inhibit uPA-mediated uPA induction (Fig. 7A). These data confirm that uPA catalytic activity is not required for uPA-mediated induction of uPA in these cells.

Role of uPAR in uPA-mediated uPA expression. To determine whether uPA interacts with Beas2B cell uPAR to induce uPA, we pretreated Beas2B cells with selected agents that block uPAR for 2 h and then treated the cells with uPA for 24 h. As shown in Fig. 7B, uPAR antibody alone did not induce uPA expression, and pretreatment of Beas2B cells with uPAR antibody followed by stimulation with uPA inhibited uPA expression (Fig. 7B). To otherwise confirm that uPA-mediated uPA expression involves uPAR, we treated Beas2B cells with A5 compound (a uPAR agonist) alone (4) or in combination with uPA. As shown in Fig. 7B, A5 alone did not induce uPA expression but inhibited expression of uPA induced by uPA treatment. In a third approach, we removed uPAR from the cell surface by treating with phosphatidylinositol-phospholipase C (PI-PLC) and then tested to see whether uPA would stimulate uPA expression. It is known that uPAR is a glycosyl phosphatidylinositol (GPI)-linked protein and that PI-PLC completely removes GPI-linked protein, including uPAR from the cell surface (4, 41, 44). PLC treatment inhibited uPA-mediated uPA expression (Fig. 7B). In a separate Western blotting experiment, we confirmed that PI-PLC completely cleaved uPAR from the cell surface (data not shown), providing further evidence that the effect is mediated by the association of uPA with uPAR at the cell surface.

Effect of proteases and a protease inhibitor on uPA-mediated uPA expression. We inferred that selected proteases known to be expressed in remodeling of the extracellular matrix might influence uPA-induced uPA expression. We therefore treated Beas2B cells with uPA in the presence of aprotinin. As shown in Fig. 8, aprotinin alone did not induce uPA expression, nor did it interfere with uPA-induced uPA expression. Plasmin added alone or in combination with uPA likewise failed to alter uPA expression. We also treated Beas2B cells with α-thrombin, another serine protease, separately or in combination with uPA. α-Thrombin alone modestly induced uPA expression, and induction of uPA was additive in the presence of both α-thrombin and uPA (Fig. 8).

DISCUSSION

uPA/plasmin-mediated proteolysis is critical for cellular migration and tissue remodeling in inflammation, tumor propagation, and metastasis (5, 51). uPA is also essential for pericellular proteolysis and is localized to the leading edge of migrating cells (51). Interactions between uPA and uPAR can facilitate cellular movement, which could contribute to remodeling of the lung in ARDS or the interstitial lung diseases (16, 19, 20). The interaction between uPA and uPAR at the cancer cell surface also appears to be a critical event in the pathogenesis of neoplastic growth and metastasis, mediating tissue remodeling, tumor cell invasion, adhesion, and proliferation (9, 38). Tumor cell invasion is also facilitated by saturation of uPAR with either exogenously supplemented uPA or overexpressed uPA (1, 34). In addition, the binding of uPA to uPAR mediates cell proliferation in several cell types, including nonmalignant lung epithelial cells, lung carcinoma-derived cells, and mesothelioma cells (43, 44). Regulation of the uPA-uPAR system is therefore germane to the
pathogenesis of disparate pathological conditions, including lung injury and neoplasia.

Lung carcinomas are among the solid tumors that express increased amounts of uPA and its receptor (6, 15, 21, 32, 39). uPAR is a high-affinity receptor for uPA, but its affinity and molecular size vary in different cell types due to glycosylation. The more aggressively tumor cells behave in vivo, the more uPA and uPAR are generally expressed (21). The binding of uPA to uPAR at cancer cell surfaces is therefore considered as one of the critical events in the metastatic process, as suppression of cellular invasiveness results after treatments by PAIs, anti-uPA antibodies, or uPA-like surrogate compounds that bind to uPAR (7). uPAR is also expressed at the surface of nonmalignant lung epithelial cells, which also elaborate uPA, and thereby contribute to the regulation of fibrinolytic activity in the alveolar compartment (19, 43). On the basis of such observations, we sought to elucidate novel mechanisms by which uPA is expressed and regulated in lung epithelial cells.

Regulation of uPA expression involves both transcriptional and posttranscriptional aspects. In previous studies, we found that a posttranscriptional pathway influences levels of uPA mRNA in lung cancer-derived cell lines and nonmalignant lung epithelial cells (42). Similar findings were previously reported in PMA- and translation inhibitor-treated porcine renal epithelial cells (2, 52). Cytokines that occur in the setting of acute lung injury or in the tumor microenvironment increase uPA expression by this mechanism. The identification of a newly identified posttranscriptional mechanism by which the lung epithelium regulates uPA suggested the possibility that other novel pathways might likewise influence expression of this protease.

We previously observed that specific binding capacity for uPA and cell surface uPAR expression was increased by PMA, epidermal growth factor, LPS, transforming growth factor-β, and tumor necrosis factor (TNF)-α in different lung epithelial cell lines (41).

To extend these observations, we analyzed the effect of uPA itself on uPA and uPA mRNA expression using Beas2B cells as a model lung epithelial cell system.

![Fig. 7. Effect of inhibitors of uPA activity and inhibitors of uPAR on uPA-mediated uPA expression by Beas2B cells. A: effect of uPA inhibitors on uPA-mediated uPA expression. Confluent cells were treated with or without B-428 (0.02 mM), an anti-uPA monoclonal antibody (PAAb, 2 µg/ml), or plasminogen activator inhibitor (PAI-1, 4 µg/ml) for 2 h followed by uPA (1 µg/ml) for 24 h at 37°C in basal medium containing [35S]methionine. Total proteins from CL as well as CM were isolated and subjected to immunoprecipitation. The immune-complex proteins were separated on 8% SDS-polyacrylamide gel and autoradiographed. B: role of uPAR in uPA-mediated uPA induction. Confluent cells were treated with or without anti-uPAR antibody (RAb, 2 µg/ml), A5 compound (1 µg/ml), and phosphatidylinositol phospholipase C (PI-PLC, 10 U/ml) for 2 h followed by uPA (1 µg/ml) for 24 h at 37°C as described in A. Cellular proteins were immunoprecipitated with an anti-uPA antibody to determine cellular expression of uPA under these conditions.](http://ajplung.physiology.org/)

![Fig. 8. Effect of proteases and protease inhibitors on uPA-mediated uPA induction in Beas2B cells. Confluent cells were treated with or without aprotinin (Apr), plasmin (Pla), or α-thrombin (Thr). One microgram per ml of Apr, Pla, and Thr alone for 2 h was followed by uPA (1 µg/ml) for 24 h at 37°C. The cellular proteins were subjected to immunoprecipitation with anti-uPA and anti-β-actin monoclonal antibodies.](http://ajplung.physiology.org/)
uPA strongly and rapidly induced cell surface uPA expression and secretion, predicated on a rapid increase in the cellular level of uPA mRNA. PMA and TNF-α increase the rate of transcriptional activation of uPA (S. Shetty, unpublished results). We also found that uPA mRNA was posttranscriptionally regulated in these cells (42). The cause of the increase of uPA mRNA could therefore be due to increased stability as well as gene transcription.

uPA is mitogenic for small airway epithelial and H-157 cells (4, 41). These cells also express readily detectable amounts of uPA (42), which might act as an autocrine mitogen through association with uPAR at the cell surface. We previously showed that the binding of uPA to its receptor was mitogenic for MS-1 and MeT5A cells and that proliferation of these cells could be blocked by antisense oligonucleotides directed against uPAR (46, 47). Using Beas2B lung epithelial cells, we now show that uPA-mediated uPA protein expression occurs, and the effect is dependent on the uPA concentration (0–18.5 nM) to which the cells are exposed; at concentrations of exogenous uPA of ≤1,000 ng/ml, endogenous uPA and uPA mRNA are induced. With exogenous uPA stimulation ≥1,000 ng/ml, uPA protein expression continued to increase, while uPA mRNA declined to basal levels, raising the possibility that the response could involve translational processing. This effect is consistent with uPA-uPAR interaction kinetics (44). The range of concentrations tested in this study is also likely to be of physiological relevance. Because uPAR is a high-affinity receptor for uPA and is expressed by lung epithelial cells, including the Beas2B cell line and small airway epithelial cells (44), the concentration of uPA localized to the cell surface may be very high. These cells are also capable of expressing uPA (42), which could increase the local concentrations of uPA to which the cells are exposed. We previously reported that concentrations of uPA antigen in bronchoalveolar lavage (BAL) of patients with ARDS reached 12–16 ng/ml at 3, 7, or 14 days after recognition of the syndrome (18). Because BAL represents a very dilute form of the true epithelial lining fluid under these circumstances (18), it is quite conceivable that the range of uPA concentrations we used to stimulate the cells approaches that to which epithelial cells are exposed in the alveolar compartment in evolving acute lung injury. In lung carcinomas, the concentrations of uPA in tumor tissue are markedly increased, further supporting the possibility that lung epithelial cells in these circumstances could be exposed to high concentrations of uPA (12, 35, 36). Although we cannot be certain as to the precise concentrations of uPA to which lung epithelial cells are actually exposed in vivo, our observations suggest the likelihood that the concentrations we used in this study are of physiological relevance.

The uPA-mediated uPA expression in Beas2B cells is uPAR dependent and proteolysis independent. The uPA induction process also appears to involve activation of tyrosine kinases because herbimycin inhibits both basal as well as uPA-mediated uPA expression. We found that sodium orthovanadate also inhibited basal and uPA-mediated cellular expression of uPA, further suggesting the possibility that this inhibitor effected the release of uPA from the cell surface. Together, the data indicate that the induction process is complex and likely involves additional intermediate steps. The difference in uPA expression at the cell surface and in CM could also be influenced by differences in the rate of turnover of uPA-uPAR-PAI-1 complex internalization by Beas2B cells under diverse treatment conditions. This is particularly relevant because these cells express all these components (46). The sustained effect of uPA on both the protein and the mRNA level suggests that the specific message sent by tyrosine kinase(s) has a long half-life or that uPA prolongs the half-life of uPA mRNA. Alternatively, uPA stimulation of Beas2B cells may also induce synthesis of growth factors or cytokines, which in turn may induce increases in the uPA mRNA level. The mechanisms responsible for the effect of uPA on uPA mRNA levels are now under investigation. In our study, pretreatment of Beas2B cells with PI-PLC for 2 h followed by uPA treatment for 24 h inhibited uPA expression.

Although the mechanism by which uPA induces uPA in Beas2B cells is at present unknown, it is clearly not mediated via generation of plasmin, because exposure of cells to aprotinin or treatment with plasmin inhibitors or induces uPA expression. One possibility is that uPA, through association with uPAR, may activate a transmembrane protein to initiate signals (10, 13, 14, 50). We also used α-thrombin as a positive control to compare the magnitude of uPA-mediated uPA expression, since α-thrombin was found to induce uPA in human cerebral microvascular endothelial cells (40). Our results indicate that α-thrombin similarly induces uPA expression in Beas2B cells. However, unlike the effect of α-thrombin on uPA-mediated uPAR expression (44), α-thrombin augmented uPA-mediated expression of uPA by Beas2B cells. In a related vein, α-thrombin has been shown to induce uPA and PAI-1 in endothelial cells and interleukin-6 production in endothelial (49) and epithelial cells (48).

In summary, we confirmed that uPA stimulates expression of uPA by cultured Beas2B lung epithelial cells. This newly identified pathway is, to our knowledge, the first description of the ability of uPA to regulate its own expression in epithelial cells. This pathway, if operative in vivo, could contribute to the maintenance of uPA-related alveolar fibrinolytic capacity in the absence of disease. In acute or chronic lung injury, this pathway might serve to counter the fibrinolytic defect that occurs in alveolar lining fluids under these conditions (49). In epithelium-derived malignancies such as lung cancer, cellular invasiveness could be potentiated by this mechanism. It is therefore conceivable that this novel pathway might contribute to the regulation of uPA-dependent fibrinolysis and other epithelial cell responses germane to lung injury or neoplasia.
REFERENCES


38. Rabban SA, Mazar AP, Bernier SM, Haq M, Bolivar I, Henkin J, and Golzman D. Structural requirements for the
growth factor activity of the amino-terminal domain of uroki-

39. Schmitt M, Janicke F, and Graeff H. Tumor-associated fibrin-
olysis: the prognostic relevance of plasminogen activators uPA
and tPA in human breast cancer. Blood Coagul Fibrinolysis 1:

40. Shatos MA, Orfeo T, Doherty JM, Penar PL, Collen D, and
Mann KG. α-Thrombin stimulates urokinase production and
DNA synthesis in cultured human cerebral microvascular endo-

41. Shetty S and Idell S. Posttranscriptional regulation of uroki-
nase receptor gene expression in lung carcinoma and malignant
mesothelioma cells in vitro. Mol Cell Biochem 199: 189–200,
1999.

42. Shetty S and Idell S. Posttranscriptional regulation of uroki-
nase mRNA: identification of a novel mRNA-binding protein in

43. Shetty S and Idell S. Posttranscriptional regulation of the
fibrinolytic system in lung and pleural disease. Res Adv Pathol 1:

44. Shetty S and Idell S. Urokinase induces expression of its own

45. Shetty S, Kumar A, and Idell S. Posttranscriptional regula-
tion of urokinase receptor mRNA: identification of a novel uroki-
nase receptor mRNA binding protein in human mesothelioma

46. Shetty S, Kumar A, Johnson A, Pueblitz S, and Idell S.
Urokinase receptor in human malignant mesothelioma cells: role
in tumor cell mitogenesis and proteolysis. Am J Physiol Lung

47. Shetty S, Kumar A, Johnson AR, and Idell S. Regulation of
mesothelial cell mitogenesis by antisense oligonucleotides for the

48. Sower LE, Froelich CJ, Carney DH, Fenton JW II, and
Klimpel GR. Thrombin induces IL-6 production in fibroblasts

49. Stern DM, Bank I, Nawroth PP, Cassimeris J, Kisiel W,
Fenton JW II, Dinarello C, Chess L, and Jaffe EA. Self-
regulation of procoagulant events on the endothelial cell surface.

50. Tang H, Kerins DM, Hao Q, Inagami T, and Vaughn DE.
The urokinase-type plasminogen activator mediates tyrosine
phosphorylation of focal adhesion proteins and activation of
mitogen activated protein kinase in cultured endothelial cells.

51. Vassalli JD, Sappino AP, and Belin D. The plasminogen

52. Ziegler A, Knsel J, Fabbro D, and Nagamine Y. Protein
kinase C down-regulation enhances cAMP-mediated induction
of urokinase-type plasminogen activator mRNA in LLC-PK1 cells.