Urokinase induces activation of STAT3 in lung epithelial cells

Sreerama Shetty, Gadjipathi N. Rao, Douglas B. Cines, and Khalil Bdeir


1Department of Specialty Care Services, The University of Texas Health Center at Tyler, Texas; 2Department of Pathology and Laboratory Medicine, the University of Pennsylvania, Philadelphia, Pennsylvania; and 3Department of Physiology and the Center for Vascular Biology, University of Tennessee Health Science Center, Memphis, Tennessee

Submitted 9 November 2005; accepted in final form 17 May 2006

Urokinase-type plasminogen activator (uPA) is a serine protease that plays a major role in diverse physiological and pathological processes. Studies from our laboratory have shown that exposure of human lung epithelial cells to uPA induces proliferation. To understand uPA mitogenic signaling events, we sought to elucidate its effects on tyrosine phosphorylation in a human bronchial epithelial cell line (Beas2B). uPA induced tyrosine phosphorylation of several proteins in a time-dependent manner. One of these proteins was identified as the 91-kDa signal transduction activator transcription (Stat)3 moiety. Tyrosine phosphorylation of Stat3 by uPA was time dependent. uPA induced Stat3-DNA binding activity in a time-dependent manner. uPA-induced Stat3 activation does not require uPA catalytic activity, as the uPA amino-terminal fragment alone was as potent as active two-chain uPA (tcuPA) in causing this effect. Single-chain uPA likewise induced tyrosine phosphorylation of Stat3 to a similar extent as intact tcuPA. Plasmin did not alter uPA-induced Stat3 activation. Furthermore, transfection of Beas2B cells with dominant-negative Stat3 blocked uPA-induced DNA synthesis. These results reveal for the first time that the uPA-upAR interaction leads to activation of Stat3, independent of its catalytic activity but dependent on its interaction with its receptor, uPAR, leading to DNA synthesis in lung epithelial cells.

uPA receptor; signal transduction activator transcription-3; tyrosine phosphorylation; fibrinolysis

UROKINASE-TYPE PLASMINOGEN ACTIVATOR (uPA) is a serine protease that catalyzes the conversion of plasminogen to plasmin (7, 22, 24) and has been implicated in the pathogenesis of lung inflammation and the growth of lung tumors. uPA activates pro-hepatocyte growth factor and vascular endothelial growth factor through proteolysis and activates transforming growth factor-β by generating plasmin (25). uPA facilitates remodeling of the transitional stroma via the breakdown of basement membranes and extracellular matrix proteins, including fibrin (7, 8, 22, 24).

In addition to its role in extracellular proteolysis and activation of growth factors, uPA is also involved in the generation of intracellular signals, both independent of and dependent on its interaction with its receptor, uPAR (3, 6, 12, 14, 20, 21, 38). To cite a few of these events, the uPA-uPAR interaction results in tyrosine phosphorylation of a 38-kDa protein in U937 cells (12), activation of protein kinase C in WISH cells (6), diacylglycerol formation in epidermal cells (10), activation of Janus kinase signal transducers and activation of transcription (Jak-stat) signaling in the epithelial cell line TCL-598 (21) and vascular smooth muscle cells (14), Src kinase stimulation in THP-1 and monocyte cells (3), and activation of p125 focal adhesion kinase and p42/44 mitogen-activated protein kinase in endothelial cells (38). These signaling events appear to be involved in uPA-mediated cell adhesion, migration, and proliferation (29).

We previously demonstrated that uPA induces proliferation of lung epithelial cells (31). Earlier work from several laboratories, including ours, indicates a potential role for activation of growth factors in the mitogenic signaling events of uPA (11, 18, 19, 28, 33, 34). However, the mechanism by which uPA induces mitogenesis in these cells is unclear. In this study, we address this gap in current knowledge and now demonstrate that uPA induces tyrosine phosphorylation of signal transduction activator transcription (Stat)3 and thereby its DNA-binding activity via interaction with its receptor uPAR in a manner that does not require its catalytic activity.

EXPERIMENTAL PROCEDURES

Materials. Culture media, penicillin, and streptomycin were purchased from Gibco BRL Laboratories (Grand Island, NY); tissue culture plastics were from Becton Dickinson Labware (Lincoln Park, NJ). Herbimycin A, genestein, bovine serum albumin (BSA), ovalbumin, Tris-base, aprotinin, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and ammonium persulfate (APS) were from Sigma Chemical (St. Louis, MO). Acrylamide, bisacrylamide, and nitrocel lulose were from Bio-Rad Laboratories (Richmond, CA). Recombinant high molecular mass two-chain uPA (tcuPA) was a gift from Dr. Jack Henkin from Abbott laboratories (Abbott Park, IL) and Dr. Andrew Mazar (Attenouon, San Diego, CA). Recombinant single-chain uPA (scuPA), uPA low molecular mass (LMM) fragment, amino-terminal fragment (ATF), growth factor domain (GFD), and kringle domain were prepared in S2 cells as previously described (4). Anti-uPA and anti-uPAR antibodies were obtained from American Diagnostica (Greenwich, CT). Anti-Stat3, anti-phospho-Stat3, anti-phosphotyrosine, anti-Tyk2, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A5 and B428 compounds were a generous gift from Dr. Andrew Mazar (Attenouon). XAR X-ray film was purchased from Eastman Kodak (Rochester, NY).

Cell culture. Human bronchial epithelial cells (Beas2B) were obtained from American Type Culture Collection. These cells were maintained in LHC-9 and 1% antibiotics, as previously described (32). Small airway epithelial cells were obtained from Clonetics (San Diego, CA) and cultured in small airway growth medium. Beas2B cells between passages 2–5, grown to 70–80% confluence, were used in all experiments.

CONTACT}

S. Shetty, Lab C-6, The Univ. of Texas Health Center at Tyler, 11937 U.S. Highway 271, Tyler, TX 75708 (e-mail: sreerama.shetty@uthct.edu).
Total protein extraction and Western blotting. Beas2B cells were grown to confluence and were serum starved overnight with RPMI-1640 media. The cells were then incubated in serum-free media supplemented with 0.5% BSA or the same media supplemented with recombinant human tcuPA or other agents for selected times. The cells were then 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). Cell lysates were prepared using three cycles of freezing and thawing. Proteins from Beas2B cell lysates (50 μg) 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 anti-phospho-Stat3 monoclonal antibody in the same buffer at 4°C. The membrane was then washed, and tyrosine-phosphorylated Stat3 immunoreactive proteins were detected by enhanced chemiluminescence. Membranes were stripped with β-mercaptoethanol and subjected to Western blotting with a monoclonal antibody to total Stat3.

Overexpression of uPA: transfection of Beas2B cells with uPA cDNA. UPA cDNA (2) was subcloned into the eukaryotic expression vector pRc/CMV2 (Invitrogen) containing a cytomegalovirus (CMV) promoter at HindIII/NotI sites. The orientations and sequences were confirmed by nucleotide sequencing. Beas2B cells were transfected with the chimeric plasmid constructs using lipofectamine (Gibco BRL). Stable cell lines were created by treating Beas2B cells with neomycin over 3 mo. Cells carrying plasmid DNA that survived neomycin treatment were scraped from six-well plates and grown in T75 flasks, and the presence of plasmid DNA was confirmed by PCR with the use of specific primers. The overexpression of uPA by cDNA-transfected cells was confirmed by Western blotting of Beas2B cell lysates as well as conditioned media using a uPA monoclonal antibody (32). The effect of endogenous uPA overexpression on tyrosine phosphorylation of Stat3 was then measured by Western blot, as described above.

Overexpression of dominant-negative Stat3 mutant in Beas2B cells. Beas2B cells at 80% confluence were transfected with dominant-negative Stat3 or control vector DNA as described earlier (40). The stable cell lines were generated by antibiotic selection as described above. The cells were later switched to serum-free media for 12 h, followed by stimulation with or without ATF for 12 h. Total and nuclear extracts were prepared, after which phosphorylation and corresponding vector cDNA-transfected controls by Student’s t-test.

RESULTS

uPA induces protein tyrosine phosphorylation of Beas2B cells. We have previously reported that tcuPA induces its own expression in Beas2B cells, a nonmalignant lung epithelial cell line (35), leading to their proliferation (31). tcuPA-induced tyrosine phosphorylation of several proteins with molecular masses ranging from 55 to 150 kDa in a time-dependent manner in Beas2B cells (data not shown). One of the tyrosine-phosphorylated proteins migrated with an apparent molecular mass of 91 kDa. Because tcuPA has been reported to induce the expression of c-fos in many cell types (13), and Stat3 appears to be involved in the regulation of c-fos expression (36), we inferred that the 91-kDa protein that is intensely tyrosine phosphorylated in response to tcuPA is Stat3. To test this inference, Beas2B cells were incubated with tcuPA, after which proteins were immunoprecipitated with anti-Stat3 monoclonal antibody followed by Western blotting with phosphotyrosine antibody. The analysis of anti-Stat3 immunoprecipitants with phosphotyrosine antibodies revealed tyrosine phosphorylation of a protein with a molecular mass of 91 kDa. These results indicate that the tyrosine-phosphorylated 91-kDa protein is Stat3 (data not shown).

To independently confirm this observation, cells were incubated alone or after the addition of tcuPA, and equal amounts of protein were analyzed by Western blotting using phosphospecific Stat3 antibodies. tcuPA induced Stat3 tyrosine phosphorylation in a time-dependent manner, with maximal induction occurring between 8 and 24 h in Beas2B cells (Fig. 1A).
and comparator primary small airway epithelial cells (Fig. 1B). In a separate experiment, we incubated Beas2B cells with IL-6 for varying time periods, and the cell lysates were analyzed for Stat3 activation. As shown in Fig. 1C, IL-6 induced maximum Stat3 phosphorylation ~30 min after exposure. To determine the upstream kinases involved in uPA-induced Stat3 expression, we incubated Beas2B cells with tcuPA and then analyzed the expression and activation of JAK kinases. As shown in Fig. 2A, both expression and tyrosine phosphorylation of only Tyk2 were induced by tcuPA in a time-dependent manner, indicating that uPA-induced Stat3 activation is probably mediated through Tyk2.

To exclude the possibility that contaminating LPS in the high molecular mass uPA (tcuPA) preparation induced tyrosine phosphorylation of Stat3, we first measured the LPS content by the limulus amebocyte lysate ELISA method. The tcuPA preparation contained negligible amounts (~1 pg/ml) of LPS. To independently confirm that the LPS content of the preparation did not account for the phosphorylation of Stat3, we incubated Beas2B cells with 1 pg/ml and 10 μg/ml of LPS and then analyzed phosphorylation of Stat3. As shown in Fig. 2B, LPS at 1 pg/ml failed to induce Stat3 phosphorylation.

To directly confirm that uPA is responsible for Stat3 phosphorylation, we next established uPA-overproducing stable Beas2B cell lines (32). Beas2B cells were transfected with the eukaryotic expression vector pRc/CMV2 containing uPA cDNA or pRc/CMV2, and stable cell lines were selected. We analyzed the Stat3 tyrosine phosphorylation in the stable cell lines by Western blotting. As shown in Fig. 2C, Beas2B cells transfected with uPA cDNA showed a clear increase in Stat3 phosphorylation compared with vector-transfected or nontransfected control cells. Because uPA causes delayed phosphorylation of Stat3, we addressed the possibility that uPA acts indirectly by release of other factors from Beas2B cells. To test this hypothesis, we incubated Beas2B cells with PBS or uPA for 4 h. The condition medium was then removed and added to naive Beas2B cells for 12 h. The cell lysates were analyzed for Stat3 activation by Western blot, as shown in Fig. 2D; the condition media collected from Beas2B cells treated with both PBS and uPA induced activation of Stat3 when added to fresh Beas2B cells. These results indicate that a certain factor(s) or endogenous uPA released from the cells or residual uPA

---

**Fig. 1.** Time-dependent tyrosine phosphorylation of signal transduction activator transcription (Stat)3 in lung epithelial cells. A: confluent Beas2B non-malignant bronchial epithelial cells were incubated with two-chain urokinase-type plasminogen activator (uPA) (tcuPA; 1,000 ng/ml) for 0–24 h at 37°C in basal medium. Total proteins were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-Stat3 antibodies to assess equality of loading. B: primary small airway epithelial (SAE) cells were incubated with PBS or uPA for 24 h in basal medium. Cell lysates were analyzed for total and phospho-Stat3 by Western blotting as described in A. C: Beas2B cells were incubated with IL-6 (20 ng/ml) for 0–24 h at 37°C in basal medium, and lysates were analyzed for phospho- and total Stat3 by Western blotting.

**Fig. 2.** A: time-dependent induction of tyrosine phosphorylation and expression of Tyk2 by uPA in lung epithelial cells. Beas2B cells were incubated with tcuPA (1,000 ng/ml) for 0–24 h at 37°C in basal medium. Total proteins were immunoprecipitated with anti-Tyk2 antibody, the immune complexes were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were developed for total Tyk2 using anti-Tyk2 antibody. Corresponding blots were later stripped and developed with anti-β-actin antibodies to assess the equality of loading. B: Beas2B cells incubated with PBS, uPA (1,000 ng/ml), or LPS (1 pg/ml or 10 μg/ml) for 12 h at 37°C in basal medium. Lysates were analyzed for Stat3 activation by Western blotting. C: overexpression of endogenous uPA induces tyrosine phosphorylation of Stat3. Proteins from cell lysates of untreated Beas2B cells (Beas2B), Beas2B cells transfected with expression vector pRc/CMV alone (pRc/CMV), or Beas2B cells transfected with uPA cDNA in eukaryotic expression vector pRc/CMV (uPA) were assayed for Stat3 phosphorylation. Proteins were separated on 8% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membrane was immunoblotted with an anti-Stat3 antibody. Data illustrated are representative of 3 independent experiments. Bottom: total Stat3 proteins as loading control. D: Beas2B cells were incubated with PBS or uPA for 4 h. Collected conditioned media (CM) (PBS-CM or uPA-CM) were added to fresh Beas2B cells and incubated for 12 h. Lysates were analyzed for Stat3 activation by Western blotting.
remained in the condition media of uPA-treated cells and probably acted upstream of Stat3 activation.

**uPA induces Stat3-DNA binding activity.** We next determined whether tcuPA induces Stat3-DNA binding activity in Beas2B cells. To do so, nuclear extracts were prepared after the cells had been incubated with or without tcuPA. Equal amounts of nuclear proteins from control and tcuPA-treated samples were incubated with $^{32}$P-labeled SIE. The resulting DNA-protein complex was analyzed by gel mobility shift assay. As shown in Fig. 3A, a single DNA-protein complex was observed in Beas2B cells, and it increased in response to tcuPA. Pretreatment of Beas2B cells with cycloheximide, a translational inhibitor, failed to inhibit tcuPA-mediated SIE binding of Stat3, indicating that this event does not require new protein synthesis. The specificity of the DNA-protein interaction was confirmed by a self-competition experiment where pretreatment of nuclear extract with a 100-fold excess of unlabeled SIE completely (Fig. 3B). Pretreatment of nuclear extracts with anti-Stat3 antibody supershifted the DNA-protein complex, indicating the presence of Stat3 (Fig. 3C).

**uPA induces translocation of Stat3 from cytoplasm to the nucleus.** We next sought to determine the capacity of tcuPA to effect translocation of Stat3 from the cytoplasm to the nucleus, followed by its interaction with SIE. We treated Beas2B cells with tcuPA for varying lengths of time, and cytosolic and nuclear extracts were subjected to Western blotting using anti-phospho-Stat3 antibody. Data shown in Fig. 5A indicate that tcuPA induces tyrosine phosphorylation of Stat3 in Beas2B cells in a concentration-dependent manner. The effect is apparent at concentrations as low as 500 ng/ml, and maximum phosphorylation was observed at concentrations of tcuPA between 750 and 1,000 ng/ml. At tcuPA concentrations $>1,000$ ng/ml, tcuPA inhibited phosphorylation of Stat3 protein in Beas2B cells. uPA also induced SIE binding in a concentration-dependent manner similar to its effect on Stat3 phosphorylation (Fig. 5B).

**Effects of tyrosine phosphorylation on uPA-mediated Stat3 phosphorylation.** We then asked whether the pathway proximal to tcuPA-mediated Stat3 phosphorylation involves cellular signaling. To do so, Beas2B cells were incubated with or without herbimycin A or genistein in the presence or absence of nuclear extracts were subjected to Western blotting using anti-P-Stat3 antibody. The same membranes were stripped and developed with an anti-Stat3 antibody to assess equality of loading. B: interaction of P-Stat3 with SIE DNA. Cytosolic and nuclear extracts of Beas2B cells incubated with tcuPA for varying time periods were subjected to gel mobility shift assay using $^{32}$P-SIE DNA.
extracts were subjected to SIE binding by gel mobility shift assay. SIE interaction. Beas2B cells grown to confluence were incubated with varying Stat3 using anti-Stat3 antibody. Anti-P-Stat3 antibody. Membrane was later stripped and developed for total proteins were separated on an 8% SDS-polyacrylamide gel and then were transferred to a nitrocellulose membrane. Membrane was immunoblotted with anti-Stat3 antibody to assess equality of membrane loading. These experiments provide additional evidence that tcuPA-induced Stat3 tyrosine phosphorylation and Stat3-DNA binding activity are mediated by the association of tcuPA with its cell surface receptor, uPAR. Effect of proteases and protease inhibitors on uPA-mediated Stat3 phosphorylation. We next used a dominant-negative mutant to directly assess the effect of tcuPA on tyrosine phosphorylation of Stat3. Beas2B cells were transfected with an empty vector or dominant-negative Stat3 mutant cdNA. The stable cell lines were prepared by antibiotic selection. The cells were then incubated with the ATF of uPA or control media. Cell lysates were subjected to Western blotting using an anti-phospho-Stat3 antibody. As shown in Fig. 9A, ATF induced Stat3 tyrosine phosphorylation in Beas2B cells transfected with or without of tcuPA for 12 h. Both herbimycin A and genistein prevented basal as well as tcuPA-induced Stat3 phosphorylation and SIE-binding activity. In contrast, pretreatment of cells with sodium orthovanadate (a tyrosine phosphatase inhibitor) induced tyrosine phosphorylation of Stat3 and SIE-binding activity (data not shown). Effect of ATF and LMM uPA on phosphorylation of Stat3. We next sought to determine the molecular domains of tcuPA that participate in Stat3 activation by lung epithelial cells. We initially incubated Beas2B cells with either the ATF or the LMM fragment of uPA to determine whether Stat3 phosphorylation in tcuPA-treated Beas2B cells is mediated by receptor occupancy. As shown in Fig. 6A, tcuPA and ATF alone enhanced Stat3 tyrosine phosphorylation. Similarly, single-chain uPA, which has little or no catalytic activity, also induced Stat3 activation (data not shown). LMM uPA, a form of uPA that lacks the receptor-binding domain, failed to phosphorylate Stat3, indicating that its ability to stimulate Stat3 tyrosine phosphorylation requires its interaction with uPAR. Furthermore, the isolated GFD of ATF, but not the isolated uPA kringle domain, induced Stat3 activation (Fig. 6B). Similarly, ATF and GFD induced SIE-binding activity (Fig. 6C).

Effect of uPA enzymatic activity on uPA-induced Stat3 phosphorylation. We next inferred that selected proteases implicated in remodeling of the extracellular matrix might influence tcuPA-induced Stat3 phosphorylation. Plasmin or aprotinin alone or in combination with tcuPA failed to alter tcuPA-mediated Stat3 phosphorylation (Fig. 8A) or its binding to SIE (Fig. 8B).

Fig. 5. A: effect of uPA concentration on phosphorylation of Stat3. Beas2B cells grown to confluence were incubated with varying amounts of tcuPA (0–2,000 ng/ml) for 12 h at 37°C in basal medium containing 0.5% BSA. Total proteins were separated on an 8% SDS-polyacrylamide gel and then were transferred to a nitrocellulose membrane. Membrane was later stripped and developed for total proteins. Membrane was immunoblotted with anti-Stat3 antibody to assess equality of membrane loading. B: effect of uPA concentration on the Stat3-SIE interaction. Beas2B cells grown to confluence were incubated with varying amounts of tcuPA (0–2,000 ng/ml) for 12 h at 37°C in basal medium. Nuclear extracts were subjected to SIE binding by gel mobility shift assay.

Role of uPAR in uPA-mediated tyrosine phosphorylation of Stat3. To independently assess whether the interaction between tcuPA and uPAR is required to induce tyrosine phosphorylation of Stat3 in Beas2B cells, we next preincubated the cells with selected agents that block uPAR, after which the cells were incubated with tcuPA for 12 h. As shown in Fig. 7C, a ligand-blocking uPAR antibody totally inhibited tcuPA-induced Stat3 tyrosine phosphorylation. We also treated Beas2B cells with phosphotidylinositol phospholipase C (PI-PLC) to remove cell surface glycosyl phosphatidylinositol-linked uPAR (21) and then tested the effect of tcuPA on Stat3 tyrosine phosphorylation. PLC treatment inhibited the effect of tcuPA on Stat3 tyrosine phosphorylation. Similarly, both uPAR antibody and PI-PLC treatment blocked Stat3 binding to SIE (Fig. 7D). These experiments provide additional evidence that tcuPA-induced Stat3 tyrosine phosphorylation and Stat3-DNA binding activity are mediated by the association of tcuPA with its cell surface receptor, uPAR.

Effect of proteases and protease inhibitors on uPA-mediated Stat3 phosphorylation. We next used a dominant-negative mutant to directly assess the effect of tcuPA on tyrosine phosphorylation of Stat3. Beas2B cells were transfected with an empty vector or dominant-negative Stat3 mutant cdNA. The stable cell lines were prepared by antibiotic selection. The cells were then incubated with the ATF of uPA or control media. Cell lysates were subjected to Western blotting using an anti-phospho-Stat3 antibody. As shown in Fig. 9A, ATF induced Stat3 tyrosine phosphorylation in Beas2B cells transfected with or without of tcuPA for 12 h. Both herbimycin A and genistein prevented basal as well as tcuPA-induced Stat3 phosphorylation and SIE-binding activity. In contrast, pretreatment of cells with sodium orthovanadate (a tyrosine phosphatase inhibitor) induced tyrosine phosphorylation of Stat3 and SIE-binding activity (data not shown).

Effect of ATF and LMM uPA on phosphorylation of Stat3. We next sought to determine the molecular domains of tcuPA that participate in Stat3 activation by lung epithelial cells. We initially incubated Beas2B cells with either the ATF or the LMM fragment of uPA to determine whether Stat3 phosphorylation in tcuPA-treated Beas2B cells is mediated by receptor occupancy. As shown in Fig. 6A, tcuPA and ATF alone enhanced Stat3 tyrosine phosphorylation. Similarly, single-chain uPA, which has little or no catalytic activity, also induced Stat3 activation (data not shown). LMM uPA, a form of uPA that lacks the receptor-binding domain, failed to phosphorylate Stat3, indicating that its ability to stimulate Stat3 tyrosine phosphorylation requires its interaction with uPAR. Furthermore, the isolated GFD of ATF, but not the isolated uPA kringle domain, induced Stat3 activation (Fig. 6B). Similarly, ATF and GFD induced SIE-binding activity (Fig. 6C).

Effect of uPA enzymatic activity on uPA-induced Stat3 phosphorylation. We next used alternative independent techniques to assess our findings about the role of tcuPA enzymatic activity in the induction of Stat3 tyrosine phosphorylation in Beas2B cells. We first tested the ability of an anti-uPA monoclonal antibody to block Stat3 tyrosine phosphorylation. As shown in Fig. 7A, a monoclonal antibody that blocks tcuPA enzymatic activity alone did not induce Stat3 tyrosine phosphorylation and failed to prevent tcuPA-mediated Stat3 tyrosine phosphorylation. Similarly, pretreatment of tcuPA with plasminogen activator inhibitor-1, an inhibitor of uPA catalytic activity, had no effect on tcuPA-induced Stat3 tyrosine phosphorylation. We also found that the monoclonal antibody developed against uPA catalytic activity and PAI-1 failed to inhibit Stat3 interaction with SIE (Fig. 7B). These data indicate that catalytic activity of tcuPA is not required for its effect on tyrosine phosphorylation of Stat3 and its DNA-binding activity in Beas2B cells.

Fig. 6. Effect of different fragments of uPA on phosphorylation of Stat3. A and B: Beas2B cells grown to confluence were incubated with or without 1 μg/ml each of tcuPA, amino-terminal fragment (ATF), low molecular mass (LMM) fragment, growth factor domain (GFD), or kringle domain (Kringle) of uPA for 12 h at 37°C in basal medium. Total proteins were separated on an 8% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Membrane was immunoblotted with an anti-Stat3 antibody to assess equality of loading. C: corresponding nuclear extracts were subjected to SIE binding by gel mobility shift assay.
vector cDNA. However, overexpression of the dominant-negative Stat3 mutant blocked ATF-induced Stat3 tyrosine phosphorylation. ATF also failed to induce Stat3-DNA (SIE) binding activity in dominant-negative mutant Stat3-overexpressing cells (Fig. 9B), independently confirming that the uPA-uPAR interaction is involved in the tyrosine phosphorylation of Stat3.

Inhibition of uPA-mediated DNA synthesis by Stat3 mutant. TcuPA induced DNA synthesis in Beas2B cells. However, overexpression of a dominant-negative mutant of Stat3 substantially (P < 0.01) reduced the mitogenic capacity of tcuPA compared with vector cDNA-transfected or untransfected cells (Fig. 10).

DISCUSSION

The uPA-uPAR interaction is critical for several key epithelial cell responses including cellular migration and proteolysis. These responses may contribute to remodeling of the lungs in desmoplasia associated with lung malignancies and accelerated fibrosis in acute respiratory distress syndrome and in chronic fibrosis associated with the interstitial lung diseases (16, 17, 37, 39). The interaction between tcuPA and uPAR on the cancer cell surface also appears to be a critical event in the pathogenesis of neoplastic growth and metastasis, mediating tumor cell invasion, adhesion, and proliferation of neoplastic cells (1, 7, 28).

Receptor-bound tcuPA induces cell proliferation in several cell types including nonmalignant lung epithelial cells, lung carcinoma-derived cells, and malignant mesothelioma (33, 34). Tumor cell invasion is often facilitated by a dramatic increase in the production of tcuPA and its receptor (17, 23, 26). A significant level of interest has been generated by the observation that tcuPA transduces signals through interaction with its receptor, even though uPAR lacks intrinsic tyrosine kinase activity, a cytoplasmic tail, and a transmembrane domain (27, 29, 30). In this report, we show that the binding of tcuPA to its receptor (uPAR) activates tyrosine phosphorylation of Stat3 and its binding to the DNA (SIE) element. To our knowledge, this is the first example that activation of Stat3 occurs by the interaction of tcuPA with uPAR and leads to SIE binding. Our results suggest that tcuPA may regulate the transcription of genes that contain SIE-like elements by stimulating tyrosine phosphorylation of Stat3.

It has previously been reported that tcuPA induces c-fos gene expression (13), and our own observations confirmed that...
the expression of c-fos is induced by tcuPA in Beas2B cells (S. Shetty, unpublished results). Tyrosine-phosphorylated Stat3 binds to the c-fos promoter (36). Therefore, we sought to determine whether tyrosine phosphorylation of Stat3 induced by tcuPA is translocated to the nucleus and binds to SIE. There are precedents for the activation of Stat3 in response to tcuPA. As occurs in the case of cell stimulation by cytokines or growth factors, tcuPA induces tyrosine phosphorylation and expression of Tyk2, which leads to the rapid phosphorylation of Stat proteins on tyrosine residues. The phosphorylated Stat proteins immediately translocate to the nucleus and bind to specific SIE-like DNA sequences that, in turn, specifically activate gene expression (9, 15).

We observed that uPA mediates time- and concentration-dependent phosphorylation of Stat3, its translocation to the nucleus, and binding to SIE DNA sequences. Phosphorylated Stat3 appears in the nucleus 3 h after uPA treatment (Fig. 4A), which is much earlier than the Stat3 phosphorylation observed in total cell lysates (Fig. 1A). The difference could be due to differences in total and nuclear protein extraction techniques, and Stat3 activation probably precedes SIE binding. Stat3 binding to SIE has been associated with proliferation of vascular smooth muscle cells (40). Inhibition of Stat3 activation by overexpression of dominant-negative Stat3 inhibited uPA-induced DNA synthesis in Beas2B cells. Inhibition of dominant-negative Stat3 also inhibited platelet-derived growth factor-BB-induced proliferation of vascular smooth muscle cells (40).

Although the serum uPA concentration is considerably lower than the requirement for Stat3 activation, cells secrete higher amounts of uPA during lung inflammation, injury, or transformation. Moreover, because uPA and uPAR are induced by uPA at this concentration (32, 35), and due to its high (sub-nM) affinity for uPA, the local concentration at the lung epithelial cell surface would be expected to be considerably elevated compared with its normal serum concentration. Second, perhaps uPA is required but not sufficient, signaling may be mediated by complexes of uPA/uPAR with low-density lipoprotein receptor-related protein or integrins and induction, and maintenance of sufficient signaling complexes for this delayed response may require higher concentrations for lower-affinity binding.

Similarly, uPA-mediated induction of Jak/Stat signaling and translocation of Stat1 to the nucleus with binding to the DNA sequences have previously been reported to occur in vascular smooth muscle cells (14).

Phosphorylation of Stat3 by tcuPA in Beas2B cells requires binding to uPAR. The LMM fragment of uPA does not reproduce the effect, the effect is recapitulated by the isolated GFD but not the kringle fragment, and the effect is abrogated by PI-PLC and a ligand-blocking anti-uPAR antibody. There are also precedents for this mode of tcuPA-mediated induction of cellular responses. tcuPA is mitogenic in several different cell types. The mitogenic response likewise requires an interaction of tcuPA with uPAR in pleural mesothelioma as well as epidermal tumor cell lines (18, 19, 33). Induction of tyrosine phosphorylation by tcuPA in Beas2B cells clearly is not mediated via generation of plasmin, because exposure of cells to plasmin did not alter the phosphorylation process. Similarly, aprotinin had no affect.

The inhibitory and stimulatory effect of tyrosine kinase and phosphatase inhibitors on Stat3 phosphorylation indicates that the process is regulated by both mechanisms. Alternatively, the possibility that stimulation of Beas2B cells by tcuPA may also induce synthesis of growth factors or cytokines that in turn may
cause tyrosine phosphorylation of Stat3 is highly unlikely, because inhibition of translation by cycloheximide has no effect on Stat3-SIE binding. However, our experiments do not rule out the possibility that certain cytokines released because of uPA treatment induce Stat3 activation. Elucidation of the upstream signaling mechanisms through which tcuPA stimulates tyrosine phosphorylation of Stat3 remains to be determined. However, our results suggest the involvement of Tyk2.

In summary, we now report that tcuPA stimulates tyrosine phosphorylation of Stat3 in human lung airway epithelial cells in vitro. On the basis of these observations, it is conceivable that the uPA-uPAR interaction may trigger activation of this pathway in vivo to direct transduction of extracellular events from the cell surface to the nuclear transcription machinery. The involvement of Stat3 in uPA-uPAR-mediated mitogenic signaling in lung epithelial cells thus strongly suggests its potential role in remodeling. In addition, our work suggests that Stat3 transcriptionally regulated genes play a role in the functional responses of pulmonary epithelial cells to tcuPA in the settings of lung inflammation and remodeling. This pathway may be operative in neoplastic transformation of the lung epithelium, which may be facilitated as a consequence of increased tcuPA expression. This newly identified pathway is, to our knowledge, the first description of the ability of tcuPA to regulate the tyrosine phosphorylation and binding to SIE in any cell type.

ACKNOWLEDGMENTS

We are grateful to Brad Low, Rashmi Shetty, and M. B. Harish for technical assistance and Drs. S. Idell, A. Azghani, and U. R. Pendurthi for helpful discussions.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants R01-HL-071147 and P01-HL-62453 and the University of Pennsylvania Research Foundation.

REFERENCES