Urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR) act in concert to stimulate cytoplasmic signaling machinery and transcription factors responsible for cell migration and proliferation. Recently we demonstrated that uPA activates the Janus kinase/signal transducers and activators of transcription (Stat1) signaling in human vascular smooth muscle and endothelial cells. However, the important question whether other transcription factors of the Stat family, in addition to Stat1, are involved in the uPAR-related signaling has not been addressed. In this study, we demonstrate that Stat4 and Stat2, but not Stat3, Stat5, or Stat6, are rapidly activated in response to uPA. We demonstrate further that Stat4 and Stat2 rapidly and transiently translocate to the cell nucleus where they bind specifically to the regulatory DNA elements. Analysis of Stat complexes formed in response to uPA revealed a Stat2-Stat1 heterodimer, which lacks p48, a DNA-binding protein known to combine with Stat1-Stat2. This new uPA-induced Stat2-Stat1 heterodimer binds to GAS (the interferon-γ activation site) distinct from the interferon-stimulated response element to which the p48 protein containing complexes generally bind. We conclude that uPA activates a specific and unusual subset of latent cytoplasmic transcription factors in human vascular smooth muscle cells that suggests a critical role of uPA in these cells.

A variety of cytokines, growth factors, and polypeptide hormones use the Janus kinases (Jak)/signal transducers and activators of transcription (Stat) pathway to regulate expression of specific genes (1, 2). Activated via receptor-associated Jak5, Stat proteins can form homo- or heterodimers in which the phosphotyrosine of one partner binds to the SH2 domain of the other (3). Activated Stat dimers translocate then to the cell nucleus where they bind to specific DNA sequences leading to transcriptional activation of target genes (4). The ability of individual receptors to activate overlapping but distinct sets of Stat complexes contributes to their signal specificity. Another important level of specificity in Stat signaling is based on the unique sequence recognition by each homo- or heterodimer that is formed from activated Stat monomers (5).

Stats were first described as components of interferon signaling triggered via the activation of the cytokine receptor superfamily (1). However, a substantial body of evidence has recently accumulated suggesting that Stats are also involved in transducing signals initiated by other receptors, such as growth factors receptor tyrosine kinases (5), G-protein-coupled receptors (6), and glucocorticoid receptors (7).

The urokinase-type plasminogen activator receptor (uPAR) is a multifunctional protein responsible for several processes such as direction of cell-surface proteolysis in space and time, regulation of cellular adhesion, cell migration, and proliferation (see Refs. 8 and 9). uPAR possesses a high signaling capacity and can induce transmembrane signaling leading to the activation of different signal transduction pathways within the cytoplasm and transcriptional apparatus (10–17). However, a molecular basis for the biochemical events involved in the uPAR-mediated signaling leading to gene expression is still incompletely understood.

Recently we have demonstrated that in human vascular smooth muscle (VSMC) and endothelial cells, uPAR is associated with two kinases of the Janus family, Jak1 and Tyk2 (18, 19). uPAR activation by its ligand, urokinase-type plasminogen activator (uPA), leads to the activation of these kinases, which, in turn, provide the activation of Stat1 and its subsequent translocation to the nucleus. uPAR association with the Jak1/Stat1 pathway was also shown in human kidney epithelial tumor cells (20). However, the question of how and to what extent other Stats contribute to VSMC uPA responsiveness or whether Stat1 is the only Stat protein involved in the uPAR-induced intracellular signaling has yet not been addressed.

Besides formation of homodimers, activated Stat1 can form heterodimers in combination with Stat3 or Stat2. The Stat1-Stat3 heterodimer binds to the interferon-γ activation site (GAS), whereas the Stat1-Stat2 heterodimer associates with DNA-binding protein p48 to form the transcription factor ISGF3, which recognizes an interferon-stimulated response element (ISRE) present in many promoters activated by interferon-α/β (see Refs. 3 and 21). Stat4, Stat5, and Stat6 have yet to be shown to form heterodimeric complexes, although the highly related isoforms of some of these Stats form heterodimers (7, 22).

In this study, we investigated the responsiveness of various Stat proteins to ligand-induced activation of uPAR in human VSMC. We demonstrate that, in addition to Stat1, Stat2 and Stat4 are activated in response to uPA. Upon uPA stimulation,
FIG. 1. uPA-induced tyrosine phosphorylation of Stat2 and Stat4. The cells were treated with 1 nM uPA for different times and lysed as described under “Experimental Procedures.” Stat proteins were immunoprecipitated from uPA-activated cells using antibodies (Ab) to individual Stats, and the immunoprecipitates (IP) were then subjected to SDS-PAGE and Western blotting with anti-(P)-Tyr antibody (upper panels). Quantification of the results by densitometry is shown below each panel.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were of high quality commercial grade and were purchased from Sigma, Amersham Pharmacia Biotech, Merck, or Serva (Heidelberg, Germany). Radiochemicals were obtained from NEN Life Science Products, and chemiluminescent signal enhancers were from Tropix, Inc. (Bedford, MA) and NEN Life Science Products. Aqua-Poly/ Mount mounting media was purchased from Polysciences, Inc. (War- rington, PA). Oligonucleotides were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), T4 polynucleotide kinase was purchased from Stratagene, and poly(dI-dC) and NAP-5 Sephadex G-25 DNA grade columns were from Amersham Pharmacia Biotech.

**Antibodies**—Mono- and polyclonal anti-phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), and mono- and polyclonal antibodies for Stat proteins were from Santa Cruz Biotechnology, Inc. and Transduction Laboratories. Highly phosphospecific polyclonal antibodies for Stat1 (Tyr701) and Stat3 (Tyr705) were purchased from Transduction Laboratories. Highly phosphospecific polyclonal antibodies for Stat2 (Tyr701) and Stat4 (Tyr705) were provided by Quality Controlled Biochemicals (Hopkinton, MA). The monoclonal p48 (ISGF3) antibody was purchased from Transduction Laboratories, and polyclonal antibody was from Santa Cruz Biotechnology, Inc. Alexa 488-conjugated anti-mouse IgG was purchased from Molecular Probes, Inc. (Eugene, OR), and Cy2-conjugated anti-mouse IgG was from Jackson Immuno-Research Laboratories (West Grove, PA).

**Cell Culture**—Human vascular smooth muscle cells from coronary artery were obtained from Clonetics (San Diego, CA). The cells were grown in SmGM2 medium and then treated with 1 nM uPA as described below.

**Tyrosine Phosphorylation, Western Blotting, and Stripping**—Subconfluent and serum-starved VSMC were washed twice with HEPES/NaCl buffer (10 mM HEPES, pH 7.5, 150 mM NaCl) and were treated with 1 nM uPA (Sigma) at 37 °C for 5–30 min. Cells were then subjected to SDS-PAGE and Western blotting with anti-(P)-Tyr antibody (upper panels). Quantification of the results by densitometry is shown below each panel.

Both Stats are tyrosine-phosphorylated and translocate to the nucleus where they bind to specific DNA elements. Analysis of Stat complexes formed in response to uPA revealed a Stat2-Stat1 heterodimer that lacks p48, a DNA-binding protein known to combine with Stat1-Stat2. This new uPA-induced Stat2-Stat1 heterodimer binds to the GAS element distinct from ISRE to which the p48 protein-containing complexes generally bind.
pH 7.4, 5 mM MgSO₄, 2 mM dithiothreitol, and protease inhibitors). A solution of 1% Nonidet P-40 was added to a final concentration of 0.1%. The pellets were incubated on ice for 40 min and homogenized by 30 strokes in a Teflon glass Dounce homogenizer. The homogenates were adjusted to 1.4 M sucrose by the addition of 2.1 M sucrose in buffer A. 8 ml of this suspension were transferred to each centrifuge tube and laid between 1 ml of 2.1 M and 2 ml of 0.8 M sucrose in buffer A. The tubes were filled up with 1 ml of 0.25 M sucrose in buffer A and centrifuged at 100,000 × g for 65 min at 4 °C in a swinging bucket rotor. The pellets containing the nuclei were resuspended in a small volume of buffer B (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitors). The samples were used immediately or aliquoted and stored at −80 °C.

EMSA was performed for 30 min at room temperature in a volume of 20 µl containing 0.5 µg of nuclear protein extracts, 40 ng of poly(dI-dC), 4 µl of 5× binding buffer (1× binding buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) with or without 50- or 100-fold excess of cold competitor or of unrelated competitor, and a radiolabeled probe (3 × 10⁴ cpm). In supershift EMSA, nuclear extracts were incubated with 2 µg of experimental or isotypic control antibody prior to the addition of a ³²P-labeled probe. DNA-protein complexes were separated on a 5% polyacrylamide gel in Tris-glycin buffer (50 mM Tris, 0.4 M glycine, 2 mM EDTA).

The following double-stranded oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. and were used in this study: GAS/ISRE, 27 bp (catalog no. 2537); Stat4, 27 bp (catalog no. 2569); Stat1, 25 bp.

Fig. 2. uPA-induced nuclear translocation of Stat2. A subconfluent VSMC monolayer was treated with 1 nM uPA at 37 °C for indicated periods of time, fixed, and stained using anti-Stat2 antibody and corresponding Alexa-488-conjugated secondary antibody as described under “Experimental Procedures.”
Fig. 3. uPA-induced nuclear translocation of Stat4. The subconfluent VSMC monolayer was treated with uPA, fixed, and stained as described in the legend to Fig. 2. Monoclonal anti-Stat4 antibody was used for staining.

Results

uPA Induces Tyrosine Phosphorylation of Stat2 and Stat4 and Their Nuclear Translocation—Cytoplasmic extracts of uPA-treated or -untreated VSMC were precipitated using one of six (anti-Stat1, Stat2, Stat3, Stat4, Stat5, and Stat6) antibodies, separated by SDS-PAGE, and examined for phosphotyrosine incorporation into the proteins of Stats size range. Reprobing of stripped blots with antibodies to individual Stat proteins was used for their identification in the immunoprecipitates. In addition to the previously observed activation of Stat1 (18, 19), uPA induced tyrosine phosphorylation of Stat2 and Stat4 (Fig. 1, A, B, and C) but not Stat3, Stat5, or Stat6 (Fig. 1D, shown for Stat3). The kinetics of this activation peaked relatively fast after 8–10 min of stimulation for both Stats and decreased after 15 min of stimulation. The phosphorylation of Stat4 remained at this basal level, whereas Stat2 showed a second reversible uPA-induced peak of tyrosine phosphorylation after 20 min of stimulation.

To determine whether uPA-induced activation of Stat2 and Stat4 results in their translocation to the cell nucleus, we resorted to laser scanning confocal microscopy to analyze the subcellular localization of both Stats. As shown in Figs. 2 and 3, uPA-activated Stat2 and Stat4 translocated efficiently into the nucleus. The kinetics of this translocation were very close to the kinetics of their tyrosine phosphorylation in response to uPA. Five to ten min of cell activation resulted in predominantly nuclear staining for both Stats, which declined after 13–15 min, although Stat2 demonstrated again a second response phase peaking after 20 min of cell stimulation.

uPA Induces DNA Binding Complexes Which Contain Stat2 and Stat4—In EMSA using nuclear extracts from untreated cells and stimulated with uPA, we used two oligonucleotide probes, the GAS/ISRE probe, which serves as binding sites for various activated Stats, and the GAS-like probe possessing a high binding specificity for Stat4 (Stat4 gel shift oligonucleotide) (23). Complex formation with these probes was examined by EMSA using nuclear extracts from untreated cells and stimulated with uPA cells (Fig. 4). uPA induced the formation of at least one prom-
Urokinase Induces Formation of Stat Complexes

Fig. 4. uPA-induced nuclear complexes contain Stat1, Stat2, and Stat4. Nuclear extracts of VSMC were prepared after uPA treatment for different periods of time, as indicated. EMSA was performed with the GAS/ISRE (A) or Stat4 probe (B). Solid arrows indicate the position of the protein-DNA complex. For cold competition EMSA experiments, a 100-fold molar excess of unlabeled GAS/ISRE, Stat4 probe competitor, or an unrelated competitor (AP-1 or NFκB element sequence) was included as indicated. Inhibition or supershifting of protein-DNA complexes containing Stat1, Stat2, and Stat4, respectively, were achieved by adding specific antibodies (Ab) to the binding reaction, as indicated. Open arrows on the right denote supershifted Stat2- and Stat4-containing complexes.

Urokinase Induces Formation of Stat Complexes—In response to stimulation, tyrosine phosphorylated Stat1 and Stat2 multimerize to form either Stat1 homodimers that bind GAS sequence through an intrinsic DNA binding domain or Stat1-Stat2 heterodimers that bind ISRE sequences through the DNA binding domain of p48 (3–5). Because uPA induces activation of both Stat1 and Stat2, the binding activity attributable to the Stat1-Stat2 complex was determined in EMSA using nuclear extracts of VSMC prepared after uPA treatment for different periods of time, as indicated. EMSA was performed with the GAS/ISRE (A) or Stat4 probe (B). Solid arrows indicate the position of the protein-DNA complex. For cold competition EMSA experiments, a 100-fold molar excess of unlabeled GAS/ISRE, Stat4 probe competitor, or an unrelated competitor (AP-1 or NFκB element sequence) was included as indicated. Inhibition or supershifting of protein-DNA complexes containing Stat1, Stat2, and Stat4, respectively, were achieved by adding specific antibodies (Ab) to the binding reaction, as indicated. Open arrows on the right denote supershifted Stat2- and Stat4-containing complexes.
extracts and a Stat1-specific GAS oligonucleotide probe. Fig. 5C shows that uPA induced DNA binding activity in a biphasic manner; the first peak of complex formation was observed after 5 min of stimulation, and the second one was observed after 15 min. Antibodies used in our study that specifically recognize Stat1 and Stat2 shifted this complex when added to the DNA binding reaction demonstrating the presence of the Stat1 and Stat2 proteins in the complex.

DISCUSSION

In this study, we demonstrate that, in addition to Stat1, Stat2 and Stat4 are activated in response to uPA in human VSMC. Upon uPA stimulation, both Stats are tyrosine-phosphorylated and translocate to the nucleus where they bind to DNA elements. Analysis of Stat complexes formed in response to uPA revealed a new Stat2-Stat1 heterodimer that lacks p48 protein and binds to the GAS DNA sequence.

Seven different Stat proteins have been identified raises the question about the mechanisms underlying transcriptional specificity of cytokine and growth factor signaling. In part, this specificity might be explained by a different pattern of activation of each Stat family member. Moreover, particular Stats are activated differently in response to growth factors and cytokine depending upon the cell type (5). However, the most important level of specificity in Stat signaling is achieved via selective interactions of individual Stats upon complex formation that allow specific binding of these complexes to DNA consensus sequences (3, 4, 21). Recent findings revealed also that Stats bound to a distinct pattern of adjacent sites, none of which bear a close resemblance to the high affinity sequence identified by the random selection method (25).

Our finding that physiological concentration of uPA is able to induce Stat4 activation, such as tyrosine phosphorylation, nuclear translocation, and binding to the DNA Stat4-specific GAS element, is, to our knowledge, the first indication that there is at least one more natural ligand for the Stat4 protein beyond...
interleukin-12. Interleukin-12 is known to be unique in inducing activation of Stat4 and the subsequent formation of protein-DNA complexes in human natural killer cells, T helper cells, and lymphocytes (26–28). However, expression of Stat4 in myeloid cells developing spermatogonia (29) and vascular smooth muscle cells2 suggested that other Stat4-activating natural ligands might exist. In response to interleukin-12, the Stat4 GAS binding homodimer is generally induced (25). However, there are some findings demonstrating that at least two additional Stat4-containing complexes do exist. Thus, Stat4-Stat3 and Stat4-Stat1 heterodimers have been found in some cells (26–28), although the level of Stat3 phosphorylation was minimal and the significance of this finding is unclear. Based on our results from coimmunoprecipitation, tyrosine phosphorylation, and the reactivity with antibodies in EMSA it is likely that uPA induces in VSMC complexes containing Stat4 only. However, we cannot rule out that some unknown Stat-unrelated proteins might also be a component of these complexes or that uPA might induce binding of these Stat4-containing complexes to other DNA sequences distinct from GAS.

Our study aiming at the uPA-induced Stat2 activation clearly demonstrates that Stat2 undergoes tyrosine phosphorylation, nuclear translocation, and DNA binding in response to uPA. Notably, these processes revealed biphasic kinetics, peaking first at 5–8 min of activation and then, after some decrease, again at 15–20 min. Interestingly, a similar biphasic activation of Stat1 and Stat2 was shown in rat cardiomyocytes activated by angiotension (30). However, when the GAS/ISRE probe was used in our EMSA experiments, the time course of complex formation was different, peaking only once at 10 min, which confirms our previous data about the uPA-induced Stat1-GAS/ISRE binding (18, 19). These results also suggest that the use of the GAS probe favors the binding of a Stat2-containing complex in a biphasic manner. Experiments exploring a composition of a uPA-induced Stat2-containing complex, such as coimmunoprecipitation, tyrosine phosphorylation, cross-linking, gel-shift, and supershift analyses, revealed the formation in human VSMC of a novel uPA-inducible factor consisting of Stat1 and Stat2. Stat1 protein coimmunoprecipitated in these experiments with Stat2 demonstrated the same uPA-induced biphasic activation of tyrosine phosphorylation, as Stat2 did. These data indicate that both components of the heterodimer are activated simultaneously in response to uPA. Of particular interest is the notion that the Stat1-Stat2 heterodimer does not include p48 and binds to the GAS sequence. At least, under our experimental conditions we did not determine p48 after Stat1-Stat2 coimmunoprecipitation or after cross-linking. Use of anti-p48 antibody in EMSA also did not reveal any effect. These data point to a revision of the standard model where it has been assumed that all three subunits are required and the significance of this finding is unclear. Based on our results from coimmunoprecipitation, tyrosine phosphorylation, and the reactivity with antibodies in EMSA it is likely that uPA induces in VSMC complexes containing Stat4 only. However, we cannot rule out that some unknown Stat-unrelated proteins might also be a component of these complexes or that uPA might induce binding of these Stat4-containing complexes to other DNA sequences distinct from GAS.

The biological significance of uPA-inducible Stat1-Stat2 complexes, as well as the functional role of uPA-related Jak/Stat activation, is difficult to assess, and it needs further intensive study. However, it is obvious that uPA activates a specific and unusual subset of latent cytoplasmic transcription factors in human VSMC that suggests a critical role of uPA in these cells, which might confer specificity of a rapid and specific biological response.

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