

Urokinase Receptor Is Associated with the Components of the JAK1/STAT1 Signaling Pathway and Leads to Activation of This Pathway upon Receptor Clustering in the Human Kidney Epithelial Tumor Cell Line TCL-598*

(Received for publication, December 12, 1996, and in revised form, September 4, 1997)

Yuri Koshelnick‡, Monika Ehart‡, Peter Hufnagl‡, Peter C. Heinrich§, Bernd R. Binder‡¶

From the ‡Department of Vascular Biology and Thrombosis Research, University of Vienna, A-1090 Vienna, Austria and the §Institute of Biochemistry, RWTH Aachen, Germany

The urokinase-type plasminogen activator (uPA) binds to cells via a specific receptor attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Despite the lack of a transmembrane domain, the urokinase receptor (uPAR) is capable of transducing extracellular signals affecting growth, migration, and adhesion. Several Tyr kinases of the *src* family as well as $\beta 1$, $\beta 2$, and $\beta 3$ integrins were found to be associated with the uPAR. We found that in the human kidney epithelial line TCL-598, also components of the JAK1/STAT1 signal transduction pathway including gp130, are associated with uPAR as revealed by coimmunoprecipitation and are co-localized in caveolae. Upon clustering of uPA-uPAR complex by a monoclonal antibody, JAK1 associates with uPAR, which in turn leads to STAT1 phosphorylation, dimerization, specific binding to DNA, and gene activation. To prove the dependence of STAT1 activation on the uPAR, TCL-598 cells were treated with sense and antisense uPAR oligonucleotides. In antisense-treated cells in which uPAR expression was reduced to less than one third, activation of STAT1 by the clustering antibody was abolished while STAT1 activation by interferon- γ was unaffected. Therefore, in this cell line, uPA-uPAR also utilizes the JAK1/STAT1 pathway for signaling, and gp130 might be the transmembrane adapter for this signal transduction pathway.

The urokinase-type plasminogen activator (uPA)¹ is a serine protease that promotes pericellular proteolysis during cell migration, angiogenesis, wound healing, and tumor invasion (1–5). In addition, binding of uPA to the specific receptor (uPAR) could induce numerous intracellular signaling events. These include serine phosphorylation of cytokeratins (6); tyrosine phosphorylation of a 38-kDa protein (7); association of uPAR

with several tyrosine-kinases of the *src* family (p60^{src}, p53/p56^{lyn}, p56/p59^{hck}, p59^{fgfr}) (8, 9); $\beta 1$, $\beta 2$, and $\beta 3$ -integrins (8, 10, 11); mechanical coupling to the cytoskeleton (12); *de novo* synthesis of diacylglycerol (13); cAMP formation (14); activation of inositol phosphate turnover; induction of Ca²⁺ influx; release of Ca²⁺ from intracellular stores (15); and *c-fos* gene expression (16). uPAR was found to form stable complexes with integrins and modulate their signaling function in cell adhesion and migration (8, 10). uPAR is a glycosylphosphatidylinositol (GPI) anchored glycoprotein, predominantly localized in plasmalemmal vesicles and rich in glycosphingolipids, sphingomyelin, and cholesterol. These vesicles, termed “caveolae” (17) contain caveolin, actin and actin-binding proteins, GPI-anchored proteins, a Ca²⁺ pump, the protein kinase C and GTP-binding proteins (18–20), EGF-stimulated Ras/Raf-1 (21, 22), and proteins known to interact with the phosphorylated PDGF receptor (23). Based on this evidence, it was hypothesized that caveolae may serve as signaling organelles (24).

We could show that clustering of uPA-uPAR by a monoclonal antibody directed against the protease domain of uPA stimulates migration and proliferation of the human epithelial kidney tumor cell line TCL-598 (25) and induces tyrosine phosphorylation of several intracellular proteins (19). JAK1 kinase was found to be associated with the receptor upon receptor clustering (26). It was the aim of the present study to analyze whether, in addition to JAK1, other components of the JAK/STAT signaling pathway are also associated with uPAR and whether this pathway is in fact activated by uPA-uPAR clustering. We found that two other components of the JAK/STAT pathway are associated with uPAR, namely the transmembrane protein gp130 and a STAT1 protein. All components of the JAK/STAT pathway together with uPAR are co-localized in Triton X-100-insoluble membrane fractions. Upon activation of uPAR by clustering, JAK1-kinase associates with the receptor, and the STAT1-protein is phosphorylated, dimerized, excluded from the cytosol, and binds to a specific DNA site. This activation of STAT1 was clearly dependent on uPAR, as cells treated with uPAR antisense oligonucleotide show no detectable STAT1, activation upon uPAR clustering while STAT1 activation via IFN- γ was intact.

EXPERIMENTAL PROCEDURES

Materials—Chemicals and reagents were obtained from the following sources: phenylmethylsulfonyl fluoride (PMSF), Triton X-100, EDTA, dithiothreitol (DTT), MES, NaF, Na₃VO₄, aprotinin, leupeptin, and protein A-Sepharose were from Sigma; Tris, glycerol, and Nonidet P-40 were from ICN Biomedicals Inc.; disuccinimidyl suberate (DSS) was obtained from Pierce (Rockford, IL); human IFN- γ was obtained from Endogen (Woburn, MA); poly(dI-dC) was from Pharmacia Biotech Inc., Sweden; and uPAR sense (5'-GACATGGGTCACCCGCGCT-GCTG-3') and antisense (5'-CAGCAGCGCGGGTGACCCATGTC-3')

* This work was supported by Grants F509 and 10049 from the Austrian Fund for the Promotion of Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Vascular Biology and Thrombosis Research, University of Vienna, Schwarzschanerstrasse 17, A-1090 Vienna, Austria. Tel.: 43-1-40480 230; Fax: 43-1-4087500; E-mail: bernd.binder@univie.ac.at.

¹ The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; GPI, glycosylphosphatidylinositol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; DSS, disuccinimidyl suberate; IFN- γ , interferon- γ ; IL, interleukin; PBS, phosphate-buffered saline.

phosphorothioate oligodeoxynucleotides and oligonucleotide probe used for gel shift assay (mutated *sis*-inducible element of the human *c-fos* promoter) (5'-GATCCGGGAGGGATTTACGGGAAATGCTG-3', 3'-GC-CCTCCCTAAATGCCCTTTACGACTTAA-5') were from Vienna Bio-Center, Austria. Glutamine, penicillin, streptomycin, fungizone, and trypsin-EDTA solution were obtained from BioWhittaker (Walkersville, MD); supplemented calf serum was from Hyclone Laboratories (Logan, Utah); prestained molecular weight markers and polyacrylamide gel electrophoresis reagents were from Bio-Rad Laboratories; and ECL reagent was obtained from Amersham Corp.

Antibodies—The following antibodies were used for immunoprecipitation/immunodetection. Guinea pig anti-human soluble gp130 was kindly provided by Dr. K. Yasukawa, Tosoh Corporation; rabbit anti-STAT1 was kindly provided by Dr. T. Decker, Vienna University; and rabbit anti-Tyr(P) was obtained by immunization of a rabbit with keyhole limpet hemocyanine-Tyr(P) conjugates. All other antibodies were purchased commercially: rabbit anti-uPAR (American Diagnostica, Greenwich, CT), rabbit anti-caveolin (Transduction Laboratories, Lexington, KY), mouse-anti-phosphotyrosine (clone 4G10), rabbit anti-JAK1, rabbit anti-mouse STAT1- α (p91) C-terminal and rabbit anti-STAT3 (Upstate Biotechnology Inc., Lake Placid, NY), horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Amersham, UK), or anti-guinea pig antibodies (Accurate Chemical and Scientific Corp.).

Cell Culture—The human kidney epithelial tumor cell line TCL-598 has been characterized previously (27, 28) and was a gift from Prof. Bachmayer from the Sandoz Research Institute (Vienna, Austria). Cells were routinely cultured in RPMI 1640 (Sigma) supplemented with 5% supplemented calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 0.25 units/ml fungizone and passaged using trypsin-EDTA solution. Confluent TCL-598 cells were incubated for 24 h in serum-free medium and stimulated with monoclonal antibodies directed against either the protease domain of uPA (scuPA8), which induces clustering of uPA-uPAR complex, or against the EGF homology domain of uPA (scuPA35) as a control.

Treatment of Cells with uPAR Antisense Oligonucleotide—TCL-598 were grown to approximately 70% confluency and treated with 3 μ M uPAR antisense oligonucleotide or uPAR-sense oligonucleotide (as a control) for 24 h in serum-free medium.

Localization of Components of Signal Transduction Pathways—Triton-insoluble complexes were obtained as described (29); briefly, confluent cells (1.5×10^8) were incubated for 24 h in serum-free RPMI 1640, washed with serum-free medium, and afterward incubated for 20 min at 37 °C either with 1.5 μ g/ml scuPA8 or 1.5 μ g/ml scuPA35. Controls were run without antibodies. After incubation, cells were washed with ice-cold MBS buffer (25 mM MES, pH 6.5, and 150 mM NaCl), containing 50 mM NaF and 1 mM Na₃VO₄ and lysed in MBS buffer containing 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 1 mM PMSF, 2 mM EDTA, and 50 μ g/ml aprotinin. Lysates of the cells were subjected to centrifugation for 20 h at 100,000 $\times g$ on a sucrose density gradient ranging from 40 to 5% sucrose, the starting material being made 40% in sucrose. Fractions were collected from the top to the bottom of the gradient and analyzed by dot blotting for the presence of caveolin with appropriate antibodies. The fractions enriched with caveolin (15–20% sucrose) were combined and further analyzed for presence of uPAR, clustering antibody, Tyr(P), JAK1, and STAT1 using appropriate polyclonal antibodies by Western blotting.

Association of Components of the Signal Transduction Pathway with the uPAR—Proteins associated with uPAR were analyzed by immunoprecipitation of cell lysates with anti-uPAR antibody followed by Western blotting. Cells (5×10^6) were incubated for 10 min with scuPA8 or scuPA35 (control), washed in ice-cold PBS, and lysed for 30 min at 4 °C in 20 mM Tris buffer (pH 8.0) containing 138 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 2 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 50 μ g/ml aprotinin, and 50 μ g/ml leupeptin. Lysates were centrifuged at 11,000 $\times g$ for 15 min. The supernatant was diluted 4 times in the same buffer and used for overnight immunoprecipitation at 4 °C with 3 μ g of antibodies. Samples were incubated for an additional 2 h with 50 μ l of slurry protein A-Sepharose, briefly centrifuged at 11,000 $\times g$, washed 3 times in ice-cold lysis buffer, and boiled 5 min with 50 μ l of reducing Laemmli sample buffer (30). Immunoprecipitates were separated by 7.5% SDS-PAGE, blotted onto nitrocellulose, and detected with the appropriate antibodies followed by horseradish peroxidase-conjugated antibodies and ECL.

Analysis of Activation of the JAK1/STAT1 Pathway—Phosphorylation of STAT1 upon uPAR stimulation was analyzed by anti-Tyr(P) (4G10) Western blotting of anti-STAT1 immunoprecipitates obtained from cells, stimulated with scuPA8 or scuPA35. For analysis of STAT1, dimerization cells were stimulated for 20 min with scuPA8 or scuPA35,

washed with ice-cold PBS, and lysed. Cell lysates were then incubated for 20 min with 1 mM DSS, blocked with 0.5 mM NH₄OH, and used for immunoprecipitation and Western blotting with anti-STAT1 antibodies. Cytosol fractions of TCL-598 cells were obtained in lysis buffer without Nonidet P-40 by three freezing-thawing cycles of cells in liquid nitrogen and centrifugation at 11,000 $\times g$ for 60 min.

Preparation of Nuclear Extracts—TCL-598 were conditioned in serum-free medium with or without addition of 3 μ M uPAR-sense oligonucleotide or uPAR-antisense oligonucleotide for 24 h and stimulated with scuPA8 (1.5 μ g/ml) or IFN- γ (200 units/ml) for 1 h. Cells were washed with PBS, harvested with a cell scraper, and collected by centrifugation (5 min at 1500 rpm). Cells were resuspended in lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na₃VO₄, left on ice for 20 min, and homogenized with 50 strokes in a Dounce homogenizer. Nuclei were collected by centrifugation (10 min at 13,000 rpm), resuspended in hypertonic buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na₃VO₄, and after addition of 1/9 volume of 4 M (NH₄)₂SO₄ in 50 mM HEPES, pH 7.9, extracted for 30 min at 4 °C. Nuclear extracts were dialyzed against 50 volumes of buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na₃VO₄ and stored at -70 °C.

Electrophoretic Mobility Shift Assay—The oligonucleotide probe was labeled by incubation of 2.5 pmol of double-stranded oligonucleotide with overhanging ends with 5 units of Klenow enzyme, 0.5 mM dCTP, dGTP, and dTTP each, and 20 μ Ci of [α -³²P]dATP (3000 Ci/mmol) in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithioerythritol. Nuclear extracts (5 μ g) were incubated in buffer containing 20 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT in a final volume of 20 μ l with 2 μ g of poly(dI-dC) and 10⁶ cpm of labeled oligonucleotide for 30 min at room temperature. Control assays for unspecific binding were performed by addition of 2 pmol of unlabeled oligonucleotide probe; supershift assays for detection of STAT1 or STAT3 binding were performed by addition of a rabbit antibody to STAT1 or STAT3 at a final dilution of 1:200. The oligonucleotide probe used for all assays was the *sis*-inducible element of the human *c-fos* promoter mutated in a way to allow equal recognition of STAT1 and STAT3. After DNA-protein complex formation, samples were separated on 5% polyacrylamide gels at 150 V for 2 h; the running buffer was 0.5 \times Tris-borate-EDTA. The gels were dried onto Whatman No. 3MM paper and autoradiographed overnight at -70 °C.

RESULTS

Analysis of Sucrose Density Gradient Fractions—Sucrose density fractions of TCL-598 lysates were firstly analyzed for caveolin using dot blot technique. Caveolin-enriched low density fractions of a sucrose gradient (15–20% of sucrose) were collected and further analyzed by Western blotting. Using a set of specific antibodies, we could find that uPAR, caveolin, JAK1, and STAT1 are co-localized within the detergent-insoluble low density fractions of a sucrose gradient. After treatment of cells with scuPA8, the amount of uPAR, clustering antibodies (IgG), and JAK1 in these fractions was increased while the amount of STAT1 found in the respectively treated cells was lower (Fig. 1). Specific Tyr phosphorylation of 75–90 kDa and 130 kDa proteins was increased upon uPA-uPAR clustering. Levels of caveolin (Fig. 1) were not significantly changed after uPA-uPAR clustering. Treatment of the cells with the other anti-uPA antibody (scuPA35), which interferes with binding of uPA to uPAR and does neither induce receptor clustering nor activation of the cells, did not significantly alter protein distribution in these high molecular weight complexes. In all cases, Western blot analysis revealed the expected molecular weights of the proteins analyzed; the two protein bands reacting with anti-Tyr(P) antibody upon uPAR clustering corresponded in their mobility seen in the Western blot to JAK1 protein (high molecular weight band) on the one hand and to one of the two bands of STAT1 (lower molecular weight band) on the other hand.

Analysis of Proteins Co-immunoprecipitated with uPAR—As several components of the JAK/STAT pathway were found co-localized in detergent-insoluble fractions, an association of

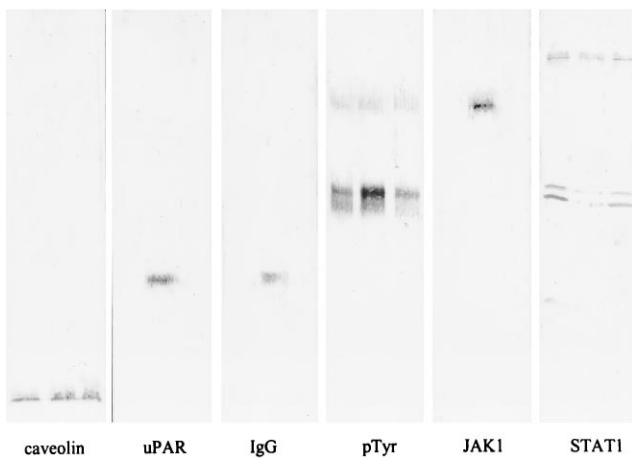


FIG. 1. Western blot analysis of low density sucrose fractions. Detergent-insoluble low density cell fractions enriched in caveolin were prepared as described under "Experimental Procedures." TCL-598 cells were stimulated either with clustering scuPA8 antibody (*middle lanes*) or non-clustering scuPA35 antibody (*right lanes*) for 20 min. *Left lanes*, control, non-stimulated cells. Sucrose fractions enriched in caveolin were analyzed by Western blotting for caveolin, uPAR, mouse IgG, Tyr(P), JAK1, and STAT1 using appropriate polyclonal antibodies.

these components with uPAR was analyzed by co-immunoprecipitation with a monoclonal antibody against uPAR. Precipitated proteins were detected by Western blotting with anti-JAK1, anti-STAT1, and anti-gp130 antibodies (Fig. 2). While JAK1-kinase was associated with uPAR predominantly after receptor clustering with scuPA8 (Fig. 2A), the STAT1 protein was found in the immunoprecipitates also without activation of the cells by scuPA8, and its amount was decreased after receptor clustering (Fig. 2B). In addition, gp130, the transmembrane adapter linking the receptors of the interleukin-6 family to the JAK1/STAT1 pathway, was found to be associated with uPAR in the resting cells and to be increased after receptor clustering (Fig. 2C). Incubation of cells with the non-clustering scuPA35 antibody did not induce changes in proteins co-precipitated with uPAR. These data are consistent with the relative changes in the respective proteins seen in the sucrose density gradient fractions and indicate that the JAK1/STAT1 signaling pathway is not only co-localized with uPAR in caveolae but is in fact associated with this receptor.

Analysis of STAT1 Activation—As we have found that the level of STAT1 protein in the caveolin-containing low density fractions as well as in the co-precipitates was decreased upon stimulation with scuPA8 (Figs. 1 and 2B) and was not affected by scuPA35, we tried to analyze a possible activation of the JAK1/STAT1 signaling pathway by uPA-uPAR clustering. As revealed by immunoprecipitation with anti-STAT1 antibodies and detection with anti-Tyr(P)-antibodies, phosphorylation of the STAT1-protein was induced in a time-dependent manner (Fig. 3A). To prove whether STAT1 phosphorylation actually led to dimerization, we analyzed STAT1-protein interactions using the bifunctional chemical cross-linker disuccinimidyl suberate (DSS). We could show that 5 min after uPA-uPAR clustering, STAT1 appeared in cell lysates also in a high molecular weight complex (greater than 300 kDa); after 20 min; it was detected to about 50% in a form corresponding to a STAT1 dimer (Fig. 3B). Fig. 3C shows changes of STAT1 amount in the cytosolic fraction of TCL-598. One h after stimulation with scuPA8, levels of STAT1 in the cytoplasm were significantly decreased. STAT1 activation was confirmed by electrophoretic mobility shift assay utilizing the mutated *sis*-inducible element of the human *c-fos* promoter as an oligonucleotide probe. This probe allows equal recognition of STAT1 and STAT3. As can be

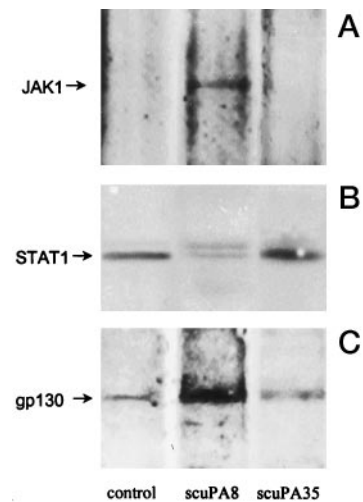


FIG. 2. Association of components of the JAK/STAT signaling pathway with uPAR. Immunoprecipitation of cell lysates was performed with anti-uPAR antibodies bound to protein A-Sepharose as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto nitrocellulose membrane, and detected for JAK1, STAT1, and gp130. Cells were stimulated for 10 min with scuPA8 (*middle lanes*) or with scuPA35 (*right lanes*). *Left lanes*, controls without antibodies.

seen in Fig. 4, the clustering antibody induced an increase in the intensity of the shifted bands already seen in the controls (*left lanes*); a STAT1-specific antibody induced a supershift (*right lanes*) while an anti-STAT3 antibody did not (data not shown). In the presence of an excess of unlabeled oligonucleotide, the shifted bands were quenched (*middle lanes*). Treatment of the cells with IFN- γ induced specific activation of STAT1 as revealed by shift and supershift assays. However, the position of bands was different in IFN- γ -treated cells as compared with cells treated with scuPA8. Since some shift was already seen in control cells, we wanted to analyze the specificity of STAT1 activation via clustering of the urokinase receptor by using cells treated with uPAR antisense oligonucleotide. In these cells, the amount of uPAR expressed was reduced to less than 1/3 (data not shown), and in fact, neither in the controls nor in cells treated with clustering antibody were any specific bands seen (Fig. 4B). IFN- γ , however, induced the same band shift as in untreated cells. Utilizing a sense oligonucleotide as a further control, a band shift pattern comparable with that in untreated cells was revealed both for the control as well as scuPA8 and IFN- γ -treated cells (Fig. 4C).

DISCUSSION

In this report, we can show that in the TCL-598 cell line components of the JAK/STAT pathway are associated with uPAR and participate in signal transduction. The JAK1/STAT1 signaling pathway is commonly linked to the receptors of the IL-6-related cytokine subfamily (31–34), IFN- γ , or polypeptide growth factor receptors (35–38), respectively. While members of the IL-6 family such as leukemia inhibitory factor, oncostatin M, and IL-11, as well as the GPI-anchored ciliary neurotrophic factor (CNTF), utilize gp130 as a common signal transducer. STAT1 activation by IFN- γ is mediated through a cytosolic tyrosine motif, Tyr-440, of the IFN- γ receptor (39). Growth factors, such as insulin, EGF, bFGF, and PDGF induce activation of STAT proteins independently of gp130 and, partially, independently of JAK. On the other hand, gp130 is not only associated with receptors for cytokines but was also found to form complexes with tyrosine kinases hck, fes, btk, and tec (40–42). This indicates that JAK/STAT as well as gp130 participate in differently regulated signal transduction pathways.

FIG. 3. Activation of STAT1 upon clustering of uPAR with scuPA8. *A*, time course of tyrosine-specific STAT1 phosphorylation; *B*, cross-linking of STAT1 with bifunctional chemical cross-linker DSS; *C*, detection of STAT1 in the cytosol. TCL-598 cells were stimulated with scuPA8 for indicated times and lysed as described under "Experimental Procedures." For analysis of tyrosine phosphorylation of STAT1 (*A*), anti-STAT1 immunoprecipitates were detected with monoclonal 4G10 anti-Tyr(P) antibodies. To analyze dimerization of STAT1 (*B*) and exclusion of STAT1 from the cytosol (*C*), anti-STAT1 immunoprecipitates were probed with anti-STAT1 antibodies.

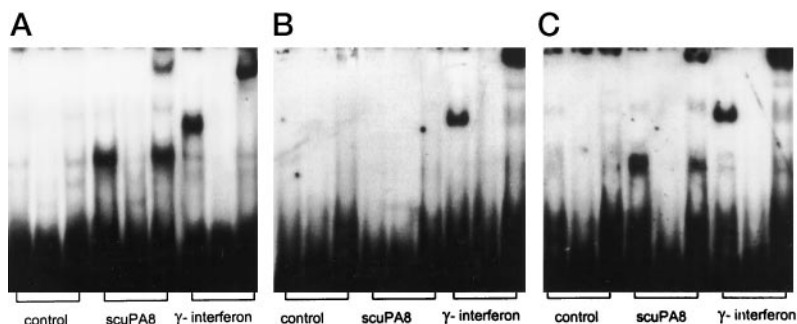
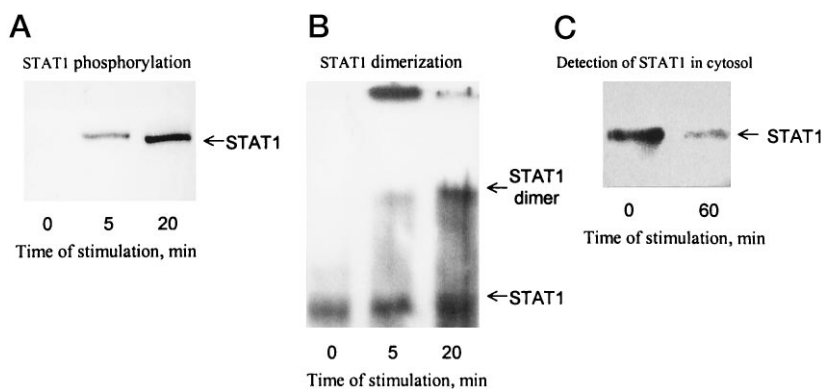


FIG. 4. Activation of STAT1 protein upon uPAR activation with clustering antibody. Electrophoretic mobility shift assay was performed as described under "Experimental Procedures." TCL-598 cells were incubated for 24 h in serum-free medium (*A*) or in serum-free medium containing uPAR antisense oligonucleotide (*B*) or uPAR sense oligonucleotide (*C*) and stimulated with scuPA8 or IFN- γ for 20 min. *Left lanes*, labeled oligonucleotide probe; *middle lanes*, competitive inhibition with unlabeled oligonucleotide probe; *right lanes*, supershift with anti-STAT1 antibody.

We found that, in the resting state, gp130 as well as STAT1 protein form a complex with uPAR, and JAK1 associates with uPAR after stimulation with the clustering antibody. This indicates that, in addition to pathways mentioned above, gp130/JAK1/STAT1 participate also in signal transduction via uPAR. In this context, it is of interest that the *src* family kinase *hck*, which associates with gp130, was also found to be associated with uPAR in a monocytic cell line THP-1 (9).

Upon clustering of uPAR by the scuPA8 antibody, JAK1 associates with the receptor, but STAT1 protein seems to dissociate. Dissociation of STAT1 from the receptor coincides with its phosphorylation and dimerization. In case of STAT1 activation via receptors of the IL-6 family, the initial step of signal transduction is homo or heterodimerization of receptors followed by activation and phosphorylation of multiple JAK family kinases and STAT proteins. In case of uPAR activation, clustering seems to substitute receptor dimerization leading to STAT1 activation.

Upon phosphorylation, STAT1 is translocated from the cytosol to the nucleus and binds to a specific site on the DNA inducing gene activation. This is made evident by electrophoretic mobility shift assays using an oligonucleotide representing the *sis*-inducible element (SIE) present in the *c-fos* gene promoter (Fig. 4). The addition of an anti-STAT1 antibody induced a supershift of part of the oligonucleotides while an anti-STAT3 antibody did not. The position of the shifted band upon scuPA8 clustering was different as compared with IFN- γ used as a positive control; furthermore, in the case of IFN- γ , the supershift induced by anti-STAT1 antibody was almost complete. This indicates that in the case of uPAR, binding of STAT1 to the *sis*-inducible element might not occur via a homodimer but via a complex with other proteins. This shift was specific because it could be quenched by an excess of unlabeled oligonucleotide. As can be seen from Fig. 4A, some gel shift was present also in cells not exposed to scuPA8 as a clustering

antibody. This indicates that activation of the STAT1 signaling might already occur in the untreated cells. Such activation could be mediated by pro-uPA secreted by the cells and might occur also in the absence of the clustering antibody. To address this question and to further prove that STAT1 activation induced by clustering antibody is specific for uPAR, we used cells treated with an anti-uPAR oligonucleotide. Cells made deficient in uPAR (*panel B*) failed to show any bandshift regardless of whether cells were treated with scuPA8 or not, whereas the bandshift induced with the IFN- γ could still be seen. In the sense control (*panel C*), scuPA8 induced a bandshift and antibodies against STAT1 induced a respective supershift, indicating that the effect seen in the cells treated with uPAR antisense oligonucleotide was specific for quenching the uPAR. It is of interest that the proliferation rate of TCL-598 treated with antisense uPAR oligonucleotide was significantly decreased (data not shown).

In the TCL-598 cell line, all components of the JAK1/STAT1 pathway including uPAR are localized in detergent-insoluble caveolin-containing complexes. All changes in associated proteins seen upon immunoprecipitation are also reflected by the relative content of the respective proteins in these fractions; upon receptor clustering, the amount of JAK1 is increased while STAT1 decreases. Therefore, activation of the JAK1/STAT1 pathway seems to take place in conjunction with caveolae. In fact, localization of uPAR in caveolae was already reported by us and others (19, 43), and other potential activators of JAK1/STAT1, such as insulin, EGF, bFGF, and PDGF were also found to be localized in caveolae (21–23). Therefore, it is likely that caveolae participate in signal transduction. However, this is the first evidence that components of JAK1/STAT1 pathway can be co-localized in caveolae together with the uPAR.

The data presented in this report indicate for the first time that components of the JAK1/STAT1 pathway are associated

with uPAR, localized in caveolin-containing detergent-insoluble fractions, and participate in the signal transduction processes mediated by uPA-uPAR clustering in the TCL-598 cell line. Apparently, uPA-uPAR/antibody complexes form clusters that are taken up into caveolae. Such clustering of uPAR should also lead to clustering of the associated gp130 and association of JAK1-kinase with these complexes. JAK1-kinase would then be activated in a fashion similar to that in the interleukin 6 pathway and in turn would activate STAT-proteins. It is likely that upon activation STAT1 is released from these high molecular weight caveolae fractions, phosphorylated, and dimerized; then STAT1 translocates to the nucleus and causes gene activation. The results obtained by the gel-shift assay in sense and antisense uPAR-treated cells further indicate that activation of the JAK1/STAT1 pathway in this cell line occurs also to some extent under resting conditions. This basal "autocrine" activation of the uPAR pathway might further be responsible for basal cell proliferation as already described by us several years ago for a human melanoma cell line (44).

This hitherto not described association of uPA-uPAR with the JAK1/STAT1 signal transduction pathway, in addition to other signaling systems, points toward a general modifier role of uPA-uPAR in regulation of cell activity. uPA-uPAR might be linked to several intracellular signal transduction pathways depending on other receptors activated simultaneously rather than to a single specific pathway. The gp130 found in this cell line, as the likely linker between the GPI-linked uPAR outside the cell and the JAK/STAT signal transduction pathway inside the cell, is not necessarily the general adapter molecule for uPAR signaling but might serve as the adapter for that specific pathway. Therefore, uPAR might not only utilize different signal transduction pathways but also different adapter molecules. Such diversity of signal transduction pathways was also found for integrins (10, 45, 46) and might be a general phenomenon for adhesion molecules (47) modifying functional activities of cells.

Acknowledgment—We are grateful to T. Nardelli for technical assistance in preparing the artwork.

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