We show here that the interaction between the uroki-
nase-type plasminogen activator and its receptor, which
plays a critical role in cell invasion, is regulated by
heparan sulfate present on the cell surface and in the
extracellular matrix. Heparan sulfate oligomers show-
ing a composition close to the dimeric repeats of heparin
(glucosamine-NSO₂₆-O-SO₃)-iduronic acid(2-O-SO₃)ₙ n =
5 and n > 5, where iduronic acid may alternate with
glucuronic acid, exhibit affinity for urokinase plasmi-
ogen activator and confer specificity on urokinase/
urokinase receptor interaction. Cell surface clearance of
heparan sulfate reduces the affinity of such interac-
tion with a parallel decrease of specific urokinase bind-
ing in the presence of an unaltered expression of recep-
tor. Transfection of human urokinase plasminogen
activator receptor in normal Chinese hamster ovary fi-
broblasts and in Chinese hamster ovary cells deficient
for the synthesis of sulfated glycosaminoglycans results
in specific urokinase/receptor interaction only in non-
defective cells. Heparan sulfate/urokinase and receptor/
urokinase interactions exhibit similar K_d values. We
concluded that heparan sulfate functions as an adaptor
molecule that confers specificity on urokinase/receptor
binding.

The urokinase receptor (u-PAR) binds urokinase-type plas-
minogen activator (u-PA) by specific interactions with u-PAR
domain 1 and vitronectin (VN) by interactions with a site
within u-PAR domains 2 and 3, the affinity of these bindings is
increased when u-PAR is occupied by (pro)-u-PA (1). u-PAR
domains 2 and 3 may also reversibly bind an enlarging series of
integrins (including β₁, β₂, and β₃ integrins). The interaction of
integrins with u-PAR modulates the affinity of the integrin for
its corresponding ligand. Some experiments indicate that in-
teraction with u-PAR reduces the affinity of the associated
integrin for certain ligands, whereas in other experimental
setups the affinity of the interaction between integrin and
ligand increases after binding of u-PAR to the integrin (2). Both
interactions of u-PAR-associated integrins with extracellular
matrix (ECM) and the activation of a cell surface-associated
proteolytic cascade by u-PAR-bound u-PA (1, 3) are required for
invasion of cells within ECM in processes such as cancer and
angiogenesis (1, 4). Therefore, the regulation of u-PAR expres-
sion and u-PAR affinity for u-PA on the cell surface is critical
for the regulation of cell invasion. Activation of certain cellular
oncogenes leads to a higher expression of some of the com-
ponents of the u-PA/u-PAR system (5). Many hormones, growth
factors, cytokines, and tumor promoters regulate the expres-
sion of the system at the transcriptional level (6, 7). A tran-
scriptional activation of the u-PAR gene has been observed in
invasive colon cancer cells (8), and a post-transcriptional regu-
lation of u-PAR mRNA by integrin engagement was reported
in leukocytes (9).

Few studies deal with the post-translational regulation of
the u-PA/u-PAR system. The internalization and rapid degra-
dation of u-PAR-bound u-PA after complex formation with
plasminogen activator inhibitors (10) was the first described
mechanism regulating u-PAR exposure and u-PAR-bound
u-PA. On the cell surface u-PA is present in two forms as
follows: a three-domain form (domain 1 + 2 + 3), which exhib-
its u-PA-binding properties, and a two-domain form (domain 2
+ 3), lacking domain 1 and therefore u-PA binding activity. The
two-domain form is the product of u-PA or plasmin cleavage
of the three-domain form. Such cleavage may represent a post-
translational mechanism exploited by proteinases to control
their own activity on cell surface (11). Moreover, the level of
glycosylation modulates u-PA affinity for its ligand (12). u-PA
has a significant affinity for heparin (HP)-like glycosaminogly-
cans (GAGs), namely heparan-sulfate (HS) (13), which is one of
the most ubiquitous molecules present on cell surface, ECM
and basement membrane (14) in the form of proteoglycans
(PG). u-PA does not contain the typical HP-binding consen-
sus sequence reported by Cardin and Weintraub (15), although it
seems to bind to HP through the kringle domain of its amino-
terminal fragment (ATF), where several basic amino acid res-
dues are found (16–19). There is also evidence that the u-PA
B-chain and the u-PA catalytic site participate in u-PA/HP
interactions (19, 20). Moreover, injection of HP in mice in-
creases the fibrinolytic activity in the plasma euglobulin frac-
tion by an increase of u-PA protein levels, probably following
u-PA elution from endothelial cells (21). A recent paper by
Brunner et al. (22) has reported that sulfated GAGs enhance
tumor cell invasion in vitro by stimulating plasminogen activ-
ation both in the fluid phase and on the cell surface.
We report here that HS promotes the interaction of u-PA with u-PA, thereby providing an efficient post-translational mechanism by which the pericellular environment may regulate u-PA activity on the cell surface and the subsequent degradation of anatomical barriers, which is required for cell invasion. We show that a form of HS, characterized by a hexosamine and uronic acid composition very close to that of HP, exhibits affinity for u-PA and that in the absence of HS a specific u-PA/u-PA interaction does not occur. Such activity is expressed by a sequence of more than 5 repeats of uronic acid-glucosamine. These data suggest that HS functions as an adaptor molecule allowing the interaction between u-PA and u-PAR.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Cell Transfections with the Human u-PAR Construct, and Incorporation of Radioactive Precursors—*Human skin fibroblasts (HSF) from skin biopsies and Chinese hamster ovary fibroblasts (CHO K1) were grown as monolayers at 37 °C, in 175-cm² dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM t-glutamine, and penicillin/streptomycin. The pgsA 745 clone of CHO fibroblasts, which lacks the xylosyltransferase that initiates GAG synthesis (13), was obtained from Dr. J. D. Ede-Siok and the University of Alabama at Birmingham. Both parental CHO and the pgsA clone were stably cotransfected with Okayama-Berg vector (23) containing u-PAR cDNA (24) and pGDSV2 plasmid, conferring neomycin resistance (kindly provided by Dr. Y. Tsujimoto, University of Osaka, Japan), using the calcium phosphate technique. Incorporation of radioactive precursors was performed in low sulfate and low leucine medium for 48 h with [35S]leucine (20 μCi/ml) and [35SO₄]²⁻ (50 μCi/ml) (both from Amersham Pharmacia Biotech).

**Extraction and Purification of Sulfated PG—**After incubation with radioactive precursors, the culture medium was decanted and the cell monolayer washed 3 times with 20 ml of ice-cold phosphate-buffered saline (PBS)/dish. Cells were extracted overnight at 4 °C with 4 ml guanidinium chloride (GdmCl) and 1% Triton X-100, containing proteinase inhibitors (10 mM N-ethylmaleimide, 1 mM diisopropylphospho-flouridate, 10 mM EDTA). The extract was centrifuged at 30,000 × g for 30 min, and the supernatant was subjected to isopycnic density gradient centrifugation in CsCl. The extract was adjusted to a density of 1.35 g/ml in 4 M GdmCl, 1% Triton X-100, and centrifuged at 30,000 × g for 60 min. The resulting pellets were dissolved in distilled water (27). Sulfate-labeled GAGs were then incubated with bacterial mutants—

## Preparation of GAGs from Cell Cultures—After incubation for 48 h with 50 μCi/ml [35SO₄]²⁻, cell monolayers were washed twice with 20 ml/dish of phosphate-buffered saline and removed from the culture dish by trypsinization. The trypsinate and the cells were incubated overnight at 37 °C with Pronase (Calbiochem, B grade) and papain (Merck) at a final concentration of 1 mg/ml each. After addition of trichloroacetic acid (5% final concentration), the mixture was kept at 4 °C for 60 min and centrifuged 10 min at 15,000 × g. The sulfated GAG side chains were precipitated by adding 8 ml of ice-cold 6 M GdmCl and 1% Triton X-100, containing proteinase inhibitors (10 mM N-ethylmaleimide, 1 mM diisopropylphospho-flouridate, 10 mM EDTA). The extract was centrifuged at 30,000 × g for 30 min, and the supernatant was subjected to isopycnic density gradient centrifugation in CsCl. The extract was adjusted to a density of 1.35 g/ml in 4 M GdmCl, 1% Triton X-100, and centrifuged at 30,000 × g for 60 min. The resulting pellets were air-dried and then solubilized in 200 μl of the appropriate buffer prior to digestion with chondroitinase ABC from Proteus vulgaris or heparitinase from Flavobacterium heparinum (Seikagaku Kogyo Co., Tokyo, Japan). Either the ethanol precipitates of crude preparations or the digested samples were used in affinity chromatography experiments.

**Preparation of GAGs from Cell Cultures—**After incubation for 48 h with 50 μCi/ml [35SO₄]²⁻, cell monolayers were washed twice with 20 ml/dish of phosphate-buffered saline and removed from the culture dish by trypsinization. The trypsinate and the cells were incubated overnight at 37 °C with Pronase (Calbiochem, B grade) and papain (Merck) at a final concentration of 1 mg/ml each. After addition of trichloroacetic acid (5% final concentration), the mixture was kept at 4 °C for 60 min and centrifuged 10 min at 15,000 × g. The sulfated GAG side chains were precipitated by adding 2.5 volumes of cold ethanol. The samples were maintained for 24 h at 4 °C, centrifuged at high speed, and washed twice with ethanol, and the pellet was dissolved in distilled water (27). Sulfate-labeled GAGs were subjected to affinity chromatography as such or after treatment with chondroitinase ABC or heparitinase.

**Treatment of Cells with Sodium Chlorate or GAG Lyases—**HSF or CHO monolayers were incubated with 30 mM sodium chloride for 12 days to inhibit ATP sulfurylase and hence the production of 3'-phosphoadenosine 5'-phosphosulfate, the active sulfate donor for sulfotransferases (28). To control the effectiveness of such a treatment, parallel cell monolayers were incubated in the presence of [35SO₄]²⁻ (50 μCi/ml) added on days 1 and 5 and then processed for GAGs extraction as described above. Sulfate-labeled GAGs were then incubated with bacterial heparitinase (2.5 units/ml, 12 h, 37 °C, pH 6.2). Sulfate-labeled HS degradation products were analyzed by gel filtration on Sepharose 6B. Chlorate-treated cell monolayers were used for binding studies with [125I]u-PA. For some experiments, HSF or CHO monolayers were treated with heparitinase or chondroitinase ABC. Cell monolayers were incubated with 2.5 units/ml F. heparinum heparitinase (Seikagaku Kogyo Co. Ltd., Japan) or 1 unit/ml of P. vulgaris chondroitinase ABC (EC 4.2.2.4) (Seikagaku Kogyo Co Ltd., Japan) in PBS containing 1 mg/ml bovine serum albumin (BSA) for 2 h at room temperature. At the end of incubation cells were washed twice with cold PBS and used for binding experiments.

**Preparation of HS Subfamilies and HS Oligomers from HP By-products—**HP by-products from beef lung (HS) were supplied by Glaxo Operations Ltd. (Runcorn, UK) and were fractionated according to Rowden et al. (29) and Fransson et al. (30). Briefly, crude material (1 g) was treated with alkaline copper sulfate to remove dermatan sulfate. The soluble material was fractionated with ethanol as the calcium salt to remove chondroitin sulfate. GAGs that precipitated between 18 and 36% (v/v) ethanol were recovered and fractionated as quaternary ammonium complexes with cetylpyridinium chloride. The complexes were dissociated and dissolved by increasing concentrations of NaCl. The following fractions were obtained: HS-1 (0.2–0.4 M NaCl), HS-2 (0.4–0.6 M NaCl), HS-3 (0.6–0.8 M NaCl), and HS-4 (0.8–1.2 M NaCl). After the addition of 0.6 M NaCl, HS-3 (0.6–0.8 M), HS-4 (0.8–1.0 M), and HS-5 (1.0–1.2 M). The resulting pellets were subjected to isopycnic density gradient centrifugation in CsCl. The extract was adjusted to a density of 1.35 g/ml in 4 M GdmCl, 1% Triton X-100, and centrifuged at 30,000 × g. The sulfated GAG side chains of HS-4 were eluted from molecular standards commercially available (Amersham Pharmacia Biotech). The sulfated GAG side chains of HS subfamilies and oligomers from HS-4 were used in ligand binding competition experiments on cell monolayers and in affinity chromatography.

**Iodination of u-PA and Soluble u-PAR, u-PAR Binding Studies, u-PAR Assay, and Phospholipase C Treatment of Cell Monolayers—**Human two-chain u-PA was purified by affinity chromatography on Sepharose CL-4B (Amersham Pharmacia Biotech) substituted with p-amino-benzenesulfonic acid (34), as described previously (35). u-PA was pure on polyacrylamide gel electrophoresis performed after the purification step (not shown). Binding studies were performed as described previously (36), using [125I]-u-PA iodinated with IODO-GEN (Pierce), with a specific activity of 25 μCi/μg. Briefly, confluent monolayers of cells cultured in 24-well multiple plates (Nunc-Intermed, Denmark), maintained in serum-free conditions for 24 h after reaching confluence, were washed 4 times with ice-cold PBS. One ml of ice-cold PBS containing increasing amounts of [125I]-u-PA was then added to each well. Each ligand concentration was added in triplicate wells, and the plates were incubated on ice for 60 min. After 4 washes of the unbound radioactivity with ice-cold PBS, the monolayers were solubilized with 0.3 ml of NaOH 1.0 M and counted in a LKB-Minigamma Counter. Specific binding was determined by measuring the difference of cell-bound radioactivity in the presence and absence of 5 μg of unlabeled u-PA. The results of binding experiments were analyzed by the Scatchard plots to obtain both the receptor number and the Kd values of receptor/ligand interaction. Before binding, the cells were also subjected to acid wash to uncouple endogenous u-PA from u-PAR (37). Low molecular weight (33,000 Da)
u-PA (LMW u-PA), a kind gift of Dr. M. L. Nolli (Istituto di Ricerche Center, Varese, Italy) was also iodinated and used in affinity chromatography experiments (specific activity, 23 µCi/µg). A soluble recombinant form of human u-PA (su-PA), a gift from Dr. U. H. Weidle (Roche Molecular Biochemicals), was iodinated with the same method, for use in affinity binding experiments (specific activity, 28 µCi/µg). The partially periodate-oxidized chains were dialyzed and then coupled to CNBr-activated Sepharose—u-PAR (internal u-PAR) and of the GPI-PLC incubation medium (external u-PAR), which was released into the culture medium of a CHO cell line transfected with the GPI-defective human u-PAR. Cell monolayers were treated with GPI-specific phospholipase C (GPI-PLC) as described (36) to cleave the GPI anchor linking u-PAR to the cell surface. The quantity of receptor was then determined on aliquots of the cell lysate of GPI-PLC-treated cells (internal u-PAR) and of the GPI-PLC incubation medium (external u-PAR).

Affinity Chromatography—Various HS subfamilies were prepared from bovine lung HP by-products as described above (29, 30); HP from beef intestinal mucosa was a kind gift from Laboratorio Derivati Organici (Vercelli, Italy); DS was obtained as the copper salt from the same procedure on the material of the same source (beef lung) and then transformed into the sodium salt; HA was from human umbilical cord; C6S was from shark cartilage, and C4S from bovine trachea was of commercial origin (Sigma). GAGs were immobilized on Sepharose 4B (Amersham Pharmacia Biotech) activated with CNBr and substituted with adipic acid dihydrazide, as previously described (38); C4S, C6S, and HA were partially periodate-oxidized for 5 min at 37 °C in 0.02 M NaIO₄, 0.05 M sodium phosphate buffer, pH 7.0; HP, various HSs, and DS were oxidized in 0.02 M NaIO₄, 0.05 M formate buffer, pH 3.0, at 4 °C for 5 min. Both reactions were stopped by the addition of 10% mannitol. The partially periodate-oxidized chains were dialyzed and then coupled to adipic acid-substituted agarose. The resulting aldimines were stabilized by reduction with NaBH₄. The incubation mixture (5 mg of GAGs/ml agarose) was washed in distilled water, and amino sugars were measured in an amino acid analyzer. The binding efficiency was 90%. Affinity chromatographies were performed at room temperature on columns (10 × 150 mm) containing 12 ml of gel. The columns were equilibrated with 0.1 M Tris/HCl buffer, pH 7.9, containing 0.1% BSA. Either one of 125I-labeled u-PA or su-PA (10 ng in 1 ml of the same buffer) was applied and drained into the column. 5 ml (void volume) was then eluted to distribute the ligand throughout the whole bed of the gel. 2 h later the column was washed with the same buffer at a rate of 40 ml/cm²·h⁻¹. 5 column volumes (50 ml) were eluted, until radioactivity decreased to the background value. Elution was then performed with a linear gradient of 0.0–1.5 M NaCl in 0.1 M Tris/HCl buffer, pH 7.9, containing 0.1% BSA, at a flow rate of 40 ml/cm²·h⁻¹. The slope of the gradient was checked by conductivity measurements. Affinity chromatographies of labeled cell PGs and GAGs were performed on u-PA or u-PA-substituted CNBr-activated Sepharose 4B, derivatized with the ligands according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Gel loading and washing were as described above, but the amount of gel was 6 ml (10 × 75 mm column), and the flow rate 20 ml/cm²·h⁻¹. The same flow rate was used in NaCl gradient elution, which was performed with the same buffer described above.

RESULTS

Affinity Chromatography of u-PA and u-PA on GAGs—derivatized Sepharose—Fig. 1a shows the binding profile of 125I-u-PA on HP (Sepharose-HP) (a) or with subfamilies of HS with increasing degrees of sulfation (b); HS-1 (filled black circles); HS-2 (filled black squares); HS-3 (empty squares); HS-4 (empty circles). The columns were equilibrated with 0.1 M Tris/HCl buffer, pH 7.9, containing 0.1% BSA. 1 ml of 125I-labeled ligand (1.5 ng/ml, corresponding to about 75 × 10⁵ cpm) was applied and drained into the column, and the affinity chromatography was performed as described under “Experimental Procedures.” The whole volume of each fraction (1 ml) was counted. The recovery of radioactivity was 87% for Sepharose-HP and ranged from 79 to 84% for various HS subfamilies; NaCl gradient was evaluated by measuring conductivity of each fraction. c shows u-PA elution from Sepharose-HS-4 in the presence of 100 µg each of HS-4-derived oligomers added with u-PA at the moment of gel loading. Also in these experiments the recovery of the applied radioactivity (75 × 10⁵ cpm) ranged from 78 to 84% for various oligomers.

NaCl, whereas low sulfated HS-1/3 released u-PA at NaCl concentrations below 0.5 M (Fig. 1b). Fig. 1c shows u-PA affinity chromatography on HS-4-substituted agarose, where 125I-u-PA binding was competed with HS-4-derived oligomers. The ligand (10 ng of 125I-u-PA) was preincubated with 10 µg of each HS-4 degradation product at 37 °C for 1 h before application to the affinity gel and then loaded on the top of the column, as described under “Experimental Procedures.” Affinity binding patterns clearly indicate that only oligomers with n > 5 (namely oligomers with more than five dimeric repeats of uronic acid and glucosamine) were able to compete efficiently with u-PA/
HS-4 binding (Fig. 1c). When LMW $^{125}$I-u-PA was used as ligand in affinity chromatographies, no binding was measured (data not shown). These results indicate that u-PA/GAGs interactions mainly occur through a sequence present on the u-PA ATF, which is not present in LMW u-PA. No binding was observed when $^{125}$I-su-PAR was used in affinity chromatography experiments on GAG-substituted gels (data not shown), indicating that the receptor does not exhibit any affinity for HS and other GAGs.

Affinity Chromatography of Labeled Cellular PGs and GAGs on u-PA-substituted Agarose—Fig. 2a shows the isopycnic density gradient centrifugation in CsCl, 4 M GdmCl, 1% Triton X-100 of a crude dual-labeled PG preparation from HSF monolayers. Highly sulfated fractions (p = 1.45–1.3 g/ml) were pooled, dialyzed against 4 M GdmCl, and chromatographed on Sepharose CL-2B (Fig. 2b). Fractions containing highly sulfated material were pooled as indicated in Fig. 2b and ethanol-precipitated, to be used in affinity chromatography experiments. The crude dual-labeled PG preparation was affinity chromatographed on u-PA-substituted agarose (Fig. 3a). The elution pattern showed that highly sulfated material elutes at 1 M NaCl. Fig. 3b shows that chondroitinase ABC treatment, able to cleave C4S, C6S, DS, and HA, leaves unaltered the PG/u-PA affinity, which is completely abolished by heparitinase treatment, that specifically degrades HSs. These results indicate that neither the protein nor the chondroitin-sulfate moiety affect PG/u-PA affinity, which relies solely on u-PA-HS side chain interactions. To confirm these observations, purified $^{35}$S-labeled GAGs extracted from cultured HSF were chromatographed on the same gels. The undigested GAGs mixture eluted at 1.0 M NaCl (Fig. 3c), whereas the treatment with heparitinase abolished the binding, which was unaffected by chondroitinase ABC treatment (Fig. 3d), thus giving further support to the data indicating HS as the only u-PA-binding GAG. Affinity chromatography of PGs and GAGs on u-PAR-substituted agarose gave negative results (not shown), thus confirming data obtained with $^{125}$I-u-PA on GAGs-substituted columns.

Binding of u-PA on Cells Treated with Sodium Chlorate and Heparitinase—In 12-day cultures in the presence of 30 mM sodium chlorate, HSF showed a decrease of sulfated GAGs, as shown in Fig. 4a. We were unable to obtain the complete inhibition of sulfated GAGs expression either by increasing sodium chlorate concentration to 60 mM or by extending the treatment. Similar results were obtained with heparitinase treatment. The amount of u-PAR, determined by enzyme-linked immunosorbent assay in control, untreated cells and following each treatment, did not show appreciable differences: 4.0 ± 0.5 ng of u-PA/10$^6$ cells under control conditions (n = 6); 4.1 ± 0.7 and 4.15 ± 0.7 ng/10$^6$ cells after treatment with heparitinase (n = 8) or sodium chlorate (n = 8), respectively. Since the chemical change produced in membrane PGs may have caused a change in the traffic of u-PAR between the inside and outside of the cell, we also measured the selective release of membrane u-PAR by phospholipase C cleavage (36) to exclude that chloride caused a change in the external display of functional u-PAR on the cell surface. In untreated control cells, about 60% u-PAR (2.5 ± 0.4 ng/10$^6$ cells) was released from the cell membrane. Similar values were obtained in heparitinase-treated HSF (2.6 ± 0.4 ng/10$^6$ cells) and in chlorate-treated HSF (2.5 ± 0.5 ng/10$^6$ cells), indicating that the treatments did not produce a redistribution of u-PAR between the inside and the outside of the cell. On the contrary, the Scatchard elaboration of ligand binding studies on sodium chlorate and heparitinase-treated cells (Fig. 4b) indicated a 30% reduction of bound u-PA and a parallel decrease of u-PA/u-PAR affinity. The values of the slope of the regression lines were $-4.0 \times 10^{-7}$ (with a confidence interval (CI) $-5.9 \times 10^{-7}$–$1.2 \times 10^{-7}$, and a $K_d$ of $1.0 \times 10^{-9}$ M) in control untreated HSF and $-1.5 \times 10^{-7}$ (CI, $-2.5 \times 10^{-7}$–$5.2 \times 10^{-8}$, and a $K_d$ of $2.7 \times 10^{-9}$ M) in chlorate-treated cells. The slopes were compared by the Student’s t test for parallelism, which gave a $p = 0.05$, indicating that the two lines diverged significantly. The number of bound u-PA molecules/cell varied from 61.4 ± 9 × 10$^3$ in control HSF to 41.0 ± 7 × 10$^3$ in chlorate-treated HSF. Following heparitinase treatment, the slope of the regression line was $-1.9 \times 10^{-7}$, and $p = 0.08$, indicating that the lines of the Scatchard plot of control and heparitinase-treated HSF did not diverge significantly (not shown in the figure). Nevertheless, the number of bound u-PA molecules/cell was 44.6 ± 11 × 10$^3$. Binding experiments were performed on cell monolayers pre-washed with acidic buffer, to release u-PAR-bound u-PA, as described.
FIG. 4. Effect of chlorate on sulfate incorporation and u-PA binding. a, HSF were cultured in the absence (filled circles) or in the presence (empty circles) of 30 mM chlorate and incubated with $^{35}$SO$_4$, as described under “Experimental Procedures.” After 12 days HSF were processed for GAGs extraction. Sulfate-labeled ethanol-precipitable material was digested with heparitinase for 12 h at 37 °C, pH 6.2, and analyzed by gel filtration on Sepharose 6B to check low molecular weight HS degradation products released upon heparitinase treatment. The gel was loaded with $12.0 \times 10^3$ 35S cpm, and about 89.5% cpm were recovered, in 0.9-ml fractions. b, typical Scatchard plots of $^{125}$I-u-PA ligand binding assay on control HSF (filled circles) and chlorate-treated HSF (open circles). The experiments shown in this figure were performed with a $^{125}$I-u-PA preparation with a specific activity of 20 mCi/mg. 10 data points for each binding curve were obtained ($n = 10$), where each point indicates the difference between total and nonspecific radioactivity bound to cell monolayers. Each point is the result of the difference between the mean of 3 different determinations in 3 different wells for each condition (total cell-bound radioactivity and nonspecific radioactivity). Each plot also shows the 95% CI, the confidence interval.

FIG. 3. Affinity chromatography of PGs and GAGs from HSF monolayer cultures, on Sepharose u-PA. Before application to affinity columns, aliquots of double-labeled ethanol-precipitated PGs, pooled from Sepharose Cl 2B as described in Fig. 2, were solubilized in distilled water ($40 \times 10^3$ 35S cpm were applied). a, chromatography of PGs (only $^{35}$S labeling is shown) on Sepharose-u-PA (filled squares). Each 1-ml fraction was counted, and 84% of total applied $^{35}$S radioactivity was recovered. A Sepharose-albumin gel was used as negative control (open circles). b, affinity pattern of PGs to u-PA-substituted gels following chondroitinase ABC treatment (filled squares, 53% recovery of total applied $^{35}$S radioactivity) and affinity after heparitinase treatment (open squares, 9.25% recovery of total applied $^{35}$S radioactivity). c, affinity chromatography of purified GAGs ($^{35}$S-labeled) on Sepharose u-PA (15.0 $\times 10^3$ 35S cpm applied, 79.3% recovered). d, affinity chromatography of purified labeled GAGs on Sepharose-u-PA, following chondroitinase ABC treatment (filled squares, recovery 54.2%) and heparitinase treatment (open squares, recovery 14.2%).
TABLE I  
u-PAR determination and distribution in parental and transfected clones of CHO fibroblasts  

| Clone          | Total u-PARa | External u-PARb |  
|----------------|--------------|----------------|  
| CHO K1 parental | Undetectable |                |  
| CHO K1 clone 3   | 49.3 ± 2.8   | 31.7 ± 1.5 (64.3%) |  
| CHO K1 clone 5   | 98.0 ± 4.6   | 63.4 ± 4.0 (64.8%) |  
| CHO K1 clone 6   | 147.3 ± 9.2  | 91.5 ± 4.8 (62.0%) |  
| CHO K1 clone 9   | 80.5 ± 4.0   | 49.8 ± 2.5 (62.0%) |  
| CHO K1 clone 11  | 210.0 ± 8.6  | 126.2 ± 6.2 (60.0%) |  
| CHO K1 clone 12  | 120.0 ± 5.0  | 78.5 ± 4.4 (65.4%) |  
| CHO pgsA parental | Undetectable |                |  
| CHO pgsA clone 1 | 50.6 ± 2.3   | 32.2 ± 1.6 (63.7%) |  
| CHO pgsA clone 2 | 42.4 ± 2.2   | 28.3 ± 1.5 (66.8%) |  
| CHO pgsA clone 4 | 95.0 ± 5.2   | 61.1 ± 3.0 (64.3%) |  
| CHO pgsA clone 6 | 250.1 ± 13.5 | 152.3 ± 9.1 (61.0%) |  
| CHO pgsA clone 7 | 135.0 ± 7.4  | 88.6 ± 5.1 (65.6%) |  
| CHO pgsA clone 8 | 234.2 ± 12.0 | 162.1 ± 8.8 (69.2%) |  

a Measured on aliquots of cell lysate (values represent the mean of four determinations ± S.D.).  
b Measured on aliquots of the incubation medium, after treatment of cells with GPI-PLC, as described in the text (values represent the mean of four determinations ± S.D.).  
c Values in parentheses represent the percentage of GPI-PLC removable u-PAR with respect to total u-PAR.

under “Experimental Procedures” (37). The decrease of u-PA binding and the decrease of its affinity for u-PAR may be likely related to the presence of small amounts of HS on the cell membrane or in the ECM, still able to favor a specific u-PA/u-PAR interaction. No variation of u-PA binding was observed after treatment of cell monolayers with chondroitinase ABC from P. vulgaris. In the case of heparitinase treatment the decrease of binding could depend on the release of ECM-associated FGF-2, which is known to induce fibroblasts to secrete u-PA (39) that, in turn, could saturate free u-PA on the cell surface. Thus, some experiments with heparitinase were performed in the presence of anti-FGF-2 antibodies to prevent interaction of FGF-2 with its receptor. The results of u-PA binding were similar to those observed in the absence of antibodies (not shown).

u-PAR Assay and u-PA Binding in CHO Cells Transfected with u-PAR—Wild type CHO fibroblasts do not express human u-PAR (Table I). Both parental CHO cell clones (GAGs expressing CHO K1 and mutant CHO pgsA 745 which do not express any GAG) were stably transfected with human u-PAR cDNA by the method of calcium phosphate. Many clones were obtained, expressing human u-PAR at various extents, as reported in Table I. To check whether u-PAR was expressed on the cell surface or within the transfected cells, CHO monolayers were treated with GPI-PLC, as described (36), to cleave the GPI anchor linking u-PAR to the cell surface. u-PAR determination was then performed in aliquots of the GPI-PLC incubation medium (external u-PAR). Internal u-PAR was calculated as the difference between total u-PAR and the GPI-PLC-removable fraction. Reproducible results were obtained, indicating that u-PAR was mainly expressed on the external cell surface of transfected cells. Table I also shows u-PAR distribution measured in all the transfected clones of CHO K1 and CHO pgsA. Typical 125I-u-PA binding assays were performed on both parental clones and on all the clones obtained by u-PAR transfections. The Scatchard analysis of binding data (shown in Fig. 5 for 5 parental and 5 GAGs-defective clones of human u-PAR transfected CHO fibroblasts) indicated that only transfected wild type CHO K1 cells were able to bind specifically and saturably u-PA (u-PAR number and Kd value of u-PAR/u-PAR interaction are reported in the figure legends, together with the statistical analysis of data), whereas mutant transfected CHO pgsA could bind u-PA only unspecifically (Fig. 5, a and b). No specific u-PA binding was obtained in mutant CHO pgsA when binding assays were performed in the presence of externally added beef lung HS-4 (0.1–10 mg/ml) (data not shown). This observation may indicate that only solid phase-associated HS (cell surface-associated or ECM-entrapped) can confer specificity on u-PA/u-PAR interaction, as further addressed under the “Discussion.”

Contribution of Surface HS to u-PA-specific Binding in CHO Cells—Since HS shows u-PA affinity, we planned experiments to evaluate the contribution of surface HS to the total specific binding. We therefore performed 125I-u-PA ligand binding on parental CHO that express HS, but not receptors for human u-PA, and on parental CHO transfected with human u-PAR (CHO K1 clone 11), extensively treated with GPI-PLC to clear u-PA from the cell membrane. In binding experiments only specific binding was evaluated, namely the difference between total round 125I-u-PA and nonspecific 125I-u-PA (that is the binding that occurs in the presence of an excess of unlabeled u-PA, added to the binding solution containing increasing amounts of labeled ligand), as previously described (37). In both cases the Scatchard analysis of binding data revealed the presence of specific u-PA-binding sites different from u-PAR. The Kd of the interaction was −1.9 nM, the same order of magnitude of u-PA/u-PAR affinity observed in these cells and reported in the literature (7). The number of specific cell-associated u-PA molecules/cell was 9.4 ± 2.3 × 103. Parallel parental CHO K1 monolayers were also treated with chondroitinase ABC or with heparitinase and then subjected to u-PA binding. The treatment with chondroitinase ABC did not show appreciable variations of u-PA binding (8.7 ± 3.0 u-PA molecules/cell; n = 4). Following heparitinase treatment, the Scatchard plots of binding data did not allow us to calculate specific binding (not shown). The same experiments, performed with parental GAGs-defective mutants CHO pgsA 745 or the same clones transfected with human u-PAR extensively treated with GPI-PLC, did not reveal the presence of any specific binding.

Activity of HS-4, HS-4 Oligomers on u-PA Binding to u-PAR-transfected CHO Cells—To compete for specific u-PA binding to cell surface HS, binding assays were performed by addition to cell monolayers of fixed amounts of 125I-u-PA in the presence of increasing concentrations of either one of the following compounds: HS-4 or HS-4 fragments corresponding to oligomers with the general formula GlcN−HexUA−GlcNm−R, as described under “Experimental Procedures.” Fragments with n = 1–4 and >5 were used. Experiments were performed on HSF and on CHO K1 clone 11 that express sulfated GAGs and human u-PAR. Fig. 6a shows that in CHO cells the addition of exogenous HS-4 decreased u-PA binding in a dose-dependent fashion. Only HS-4-derived oligomers with n > 5 were able to displace efficiently u-PA in binding competition experiments in the same cell line (Fig. 6b). The same experiments, performed in HSF monolayers, gave similar results (not shown).

DISCUSSION

In this study, we demonstrated that fibroblast HS, the most ubiquitous HP-like GAG, strongly affects the interaction of u-PA with its cellular receptor u-PAR. Such an activity is mediated by affinity interactions of HS with u-PA, which occurs through binding of u-PA to a sequence of more than 5 repeating dimeric units of a highly sulfated copolymeric HS, rich in HP-like repeats with the following general formula: GlcN−NSO3(6-OSO3)x−IdoUA(2-OSO3)y, where IdoUA may alternate with GlcUA. The binding that resulted between u-PA and u-PAR was reinforced with regard to both the affinity and the number of interacting molecules. In either the presence (treatment of cells with sodium chlorate or with heparitinase)
FIG. 5. Scatchard plots of binding data in parental and GAGs-defective CHO fibroblasts. All the transfected CHO clones were subjected to ligand binding experiments. 10 data points for each binding curve were obtained (\( n = 10 \)), where each point indicates the difference between total and nonspecific radioactivity bound to cell monolayers. As reported in the legend to Fig. 4, each point is the result of the difference between...
or the absence (CHO cells deficient for the synthesis of sulfated GAGs) of low amounts of HS in the extracellular environment, specific u-PAR saturation by exogenously added u-PA was reduced or completely absent. On this basis, we suggest that extracellular HS (cell-associated or ECM-associated) could serve at least two functions in the regulation of u-PA/u-PAR interaction as follows: (a) as an adaptor molecule, which promotes the interaction of u-PA with its receptor; (b) as a source of a ready-to-use u-PA, an optimal site to endow rapidly u-PAR with its specific ligand. In our experience, and on the basis of all the data reported in the literature on a large variety of cell lines, the Scatchard analysis of u-PA/u-PAR binding data is in agreement with a model of a single class of receptors. By taking into consideration the close similarity between the $K_d$ values of u-PA/u-PAR and of the u-PA/HS interaction (both in the range of 1.5–2.0 nM), the impossibility of identifying two different binding sites makes sense. Moreover, the capacities of u-PA/u-PAR and of u-PA/HS compartments are also similar.

The structural background of the HS-binding capacity of u-PA, which does not contain the typical HP-binding motif (XBBXXB and/or XBBBXXB, where B is the probability of a basic residue and X is a hydrophobic residue) (15), is not well known. Stephens et al. (18) have shown that a synthetic decapeptide Arg$^{52}$-Trp$^{62}$ from the kringle sequence of the u-PA ATF, rich in basic residues, is effective in competitive assays of u-PA binding to HP and dextran sulfate. Therefore, the u-PA kringle is endowed with affinity for polyanion binding. Highly sulfated copolymeric HS-4, rich in HP-like repeats, may provide an optimal substrate for such interactions.

u-PA binding to the wild type CHO cells transfected with human u-PA was strongly inhibited by exogenously added HS-4 or decasaccharides and larger HS-4 saccharides. However, no specific binding of u-PA was observed in mutant CHO pgsA cells transfected with human u-PA when binding was performed in the presence of exogenously added HS-4. Therefore, we suggest that only solid phase-associated HS can confer specificity to the u-PA/u-PAR interaction. We think that this apparent discrepancy may be explained on the basis of the privileged location of membrane- or ECM-associated HS. Indeed, on the cell surface and within the ECM, HP-like repeats of HS have the chance to interact not only with u-PA, but also with $\beta_1$ integrins (40–44), leukocyte integrin Mac-1 (45), fibronectin and laminin (46), collagens (47), and VN (48). Many of these proteins are essential components of the focal adhesions (49), where also u-PAR has been shown to be preferentially located (50). The resulting multimolecular complex may possibly cooperate to increase the affinity of solid phase-associated HS for u-PA. Such cooperations are likely to be favored for liquid phase HS and HS-u-PA complexes, as also suggested by the high concentrations of HS-4 or its decasaccharides and larger saccharides required for the displacement of about 50% u-PA specifically bound to CHO cells. There is the additional possibility that soluble HS-u-PA complexes are unable to bind the receptor, as described for soluble GAGs-chemokines complexes (51).

Interest in HS PGs stems from the increasing evidence of the functional implications of their interactions with growth factors and ECM molecules (52). Of particular importance is their role in the retention of growth factors in the ECM (53) and their participation as coreceptors at the cell surface together with tyrosine kinase FGF receptors (54–56). A possible explanation for the failure of FGF to signal in the absence of HS is that the receptor binding cannot occur. The sulfate residues, which may be present in four different positions of the GAG backbone, have been shown to determine the specificity in HS/proteins interaction (57). All the members of the FGF family, which consists of nine structurally related polypeptides, bind HP and HS with relatively high affinity and are thus referred to as “heparin-binding growth factors.” Since also u-PA is endowed with an intrinsic growth factor activity in many cell lines (7), interacts with HS, and requires HS to bind u-PAR, we propose to enclose it among “heparin-binding growth factors.”

The mean of 3 different determinations in 3 different wells for each condition (total cell-bound radioactivity and nonspecific radioactivity). Each plot also reports b, the slope of the regression line, and 95% CI, its confidence interval. The following values of p (which indicates the probability to obtain the measured value of b, given a theoretical slope = 0) were obtained: for parental CHO K1: clone 5, p < 0.0005 ($K_d = 1.5 \times 10^{-5}$ M; $17.8 \times 10^3$ molecules of bound u-PA/cell); clone 6, p = 0.004 ($K_d = 2.4 \times 10^{-5}$ M; $3.96 \times 10^3$ molecules of bound u-PA/cell); clone 9, p = 0.071 ($K_d = 2.0 \times 10^{-5}$ M; $15.3 \times 10^3$ molecules of bound u-PA/cell); clone 11, p = 0.083 ($K_d = 4.0 \times 10^{-5}$ M; $53.4 \times 10^3$ molecules of bound u-PA/cell); clone 12, p = 0.010 ($K_d = 1.8 \times 10^{-5}$ M; $33.5 \times 10^3$ molecules of bound u-PA/cell). For GAGs-defective CHO pgsA: clone 1, p = 0.485; clone 3, p = 0.048; clone 4, p = 0.005; clone 5, p < 0.0005; clone 6, p = 0.005. To compare b values of CHO K1 and of CHO pgsA, the two-sample Wilcoxon rank-sum test for unpaired samples has been used. The results indicated a significant difference between the medians of the slopes of the two groups: p = 0.0062.

FIG. 6. u-PA binding in normal CHO cells transfected with human u-PAR with HS-4 (a) and with HS-4 oligomers (b). 125I-u-PA (100 ng/ml) was incubated for 60 min with confluent CHO monolayers in the absence and in the presence of cold u-PA to obtain the specific binding, as described in the text (control conditions). Parallel triplicate wells were incubated in the same conditions in the presence of increasing amounts of HS-4 (a) or HS-4 oligomers (b), and the specific binding was calculated as usual. For HS-4 oligomers the symbols are as follows: up triangle, n = 1; down triangle, n = 2; diamond, n = 3; square, n = 4; circle, n = 5 and n > 5. Each value represents the mean ± S.D. of 6 determinations.
The importance of HS/u-PA interaction is likely to be many fold as follows: 1) regulation of functional interactions of u-PA/u-PA with ECM. At the level of focal contacts of cultured cells, u-PA and u-PA localize with vinculin at the intracellular side with and VN and other ECM components at the extracellular side, thereby connecting ECM with the cytoskeleton (for a review see Ref. 58). Interaction of u-PAR domains 2 and 3 with VN, is 10-fold enhanced upon u-PAR interaction with native u-PA or u-PA derivatives devoid of catalytic activity (59). VN, in turn, binds to integrin receptors αβ3 or αβ5, and the simultaneous interactions between u-PAR/u-PA, VN, and integrins are required for cell spreading and migration, which also requires cell detachment mediated by u-PA bound to u-PAR domain 1. A particular form of HS PG (syndecan 4) is selectively localized at focal contacts in several cell lines, including fibroblasts (for a review see Ref. 52). Since HS is indispensable for the specific binding between u-PA and u-PA, one may infer that HS is essential for u-PA-dependent cell spreading and migration. It is possible that the reported stimulation of tumor cell invasion into fibrin gels by HS and HP (22) relies on HP-like GAGs-dependent promotion of u-PA/u-PA interaction. It is noteworthy that in melanomas a predominance of HS PG at the cell surface is a marker of a more aggressive phenotype (60). 2) Coreceptors of u-PA at the cell surface are therefore able to promote transduction of u-PAR/u-PA interaction. Some evidence indicates that u-PA is structurally and functionally coupled with a variety of integrins. Such coupling inhibits the adhesive properties of integrins and stimulates u-PA-dependent adhesion to VN (61). There are indications that the u-PAR/u-PAR/PAI-1 system modulates the affinity of integrins for their ECM ligands also by generating intracellular signals (62). Since u-PA is a GPI-anchored protein, the only possible mechanism of transducing u-PA/u-PAR interaction relies on the presence of an “adapter” protein able to sense u-PAR/u-PA binding and to transfer the signal to other membrane transduction effectors. In some cell lines the multipolar cell surface u-PA transducing apparatus seems to be localized at the level of specific sites of cell membrane, referred to as caveolae, microinvaginations of the plasma membrane whose role is unknown (63). The most specific protein of these specialized areas of membrane is caveolin, which is in close contact with u-PA (64), so that it can be coprecipitated with anti-u-PA antibodies. This suggests that caveolae promote plasmin generation by recruitment of u-PA and u-PA within a very restricted area of the cell membrane (65). Within caveolae, caveolin oligomerizes with integrins and co-clusters with GPI-anchored proteins (66). Interestingly, such GPI-anchored proteins include u-PA and a particular HS PG (67), whose structural characteristics suggest association with caveolar-signaling components, as extensively discussed by Mertens et al. (68). Caveolin is phosphorylated on tyrosine residues and physically interacts with a number of signal transduction effectors, including G proteins, Ras, and receptor tyrosine kinases (7). By regulating u-PAR/u-PA interaction, HS may also promote caveolin-generated signaling. Taken together, our results and other observations suggest that the GAG moiety of HS PG, by regulating the affinity of u-PA for its cellular receptor, has the possibility to post-translationally regulate cell surface plasmin generation-dependent events, such as u-PA-dependent invasion and plasmin generation-independent ones (recently reviewed in Ref. 7), involving signal transduction upon u-PAR/u-PA interaction. In this respect, the activity of HS in the u-PA system does not substantially differ from the activity of HP-like molecules in the regulation of the activity of FGF-2. Indeed, the GAG moiety of HS PG is necessary for binding of FGF-2 to its high affinity receptor by induc-
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