*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 7129–7133, July 1994
Biochemistry

# High-affinity urokinase receptor antagonists identified with bacteriophage peptide display

(peptide libraries/cell surface protein/plasminogen activation)

ROBERT J. GOODSON, MICHAEL V. DOYLE, SUSAN E. KAUFMAN, AND STEVEN ROSENBERG*

Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608

*Communicated by Brian W. Matthews, April 13, 1994 (received for review February 18, 1994)*

ABSTRACT **Affinity selection of a 15-mer random peptide library displayed on bacteriophage M13 has been used to identify potent ligands for the human urokinase receptor, a key mediator of tumor cell invasion. A family of receptor binding bacteriophage ligands was obtained by sequentially and alternately selecting the peptide library on COS-7 monkey kidney cells and baculovirus-infected Sf9 insect cells overexpressing the human urokinase receptor. Nineteen peptides encoded by the random DNA regions of the selected bacteriophage were synthesized and tested in a urokinase receptor binding assay, where they competed with the labeled N-terminal fragment of urokinase with $IC_{50}$ values ranging from 10 nM to 10 μM. All of the isolated peptides were linear and showed two relatively short conserved subsequences: LWXXAr (Ar = Y, W, F, or H) and XFXXYLW, neither of which is found in urokinase or its receptor. Competition experiments demonstrated that the most potent peptide, clone 20, prevented binding of bacteriophage displaying the urokinase receptor binding sequence (urokinase residues 13–32). In addition, this peptide blocked other apparently unrelated receptor binding bacteriophage, suggesting overlapping receptor interaction sites for all of these sequences. These results provide a demonstration of bacteriophage display identifying peptide ligands for a receptor expressed on cells and yield leads for the development of urokinase receptor antagonists.**

The migration and invasion of cells are necessary for many normal and pathological processes, including tissue remodeling, embryo implantation, angiogenesis, and tumor cell invasion and metastasis (1–4). Recent reports suggest that these processes require an active cell-surface proteolytic cascade (5, 6). Important components of this cascade are the plasminogen activator/plasmin system, as well as the matrix metalloproteinases (6). The requirement for both protease expression and a cell-surface protease binding protein has been demonstrated most clearly in the case of urokinase plasminogen activator (uPA) and the uPA receptor (uPAR) but has also been recently described for type IV collagenase (7, 8). It has been shown in human colon and breast carcinomas that urokinase is expressed in stromal, fibroblast-like cells and uPAR is expressed on tumor epithelial cells or macrophages, respectively (9, 10). This suggests that a paracrine relationship between uPA and its receptor occurs in these pathological conditions. The *in vitro* observation that human tumor cell invasion is proportional to receptor-bound urokinase, not total urokinase synthesis, further supports the hypothesis that cell-surface protease localization is a key for invasion (11, 12). Other results show that plasminogen activation is more efficient when both uPA and plasminogen are bound on a cell surface, and that cell-surface plasmin is resistant to inhibition by $\alpha_2$-antiplasmin (7). The *in vivo* observation that metastasis of human prostate cancer cells in a nude mouse model is drastically reduced by the expression or administration of a protease-deficient urokinase receptor ligand suggests a role for uPAR antagonists in treating metastatic disease (13).

We have used random peptide bacteriophage display to identify urokinase receptor ligands. Bacteriophage display permits the expression of millions of peptides (14, 15) or proteins (16–18) on the surface of bacteriophage particles, biochemical selection of ligands, and identification by DNA sequencing of the packaged bacteriophage genomes. Peptide ligands for several soluble proteins including streptavidin, concanavalin A, integrins, such as $gpIIb_bIII_a$, and a variety of antibodies have been identified (15, 19–22) as well as antibody fragments that bind to cells (23). We report here the identification and characterization of peptide antagonists with nanomolar affinity for the human uPAR by using a 15-mer peptide library. This extension of bacteriophage peptide display to cell-surface-expressed proteins expands the utility of the method to a wide variety of biologically interesting targets.

## MATERIALS AND METHODS

**Reagents and Strains.** Bacteriophage library construction and bacteriophage growth and isolation were performed as described by Devlin *et al.* (15). The *Escherichia coli* strains H249, a *recA, sup°*, F′ derivative of MM294, and JM103 [F′ *traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15 Δ(*pro-lac*) *supE hsdR endA1 sbcB15 thi-1 strA*λ$^-$] were used for these experiments. Recombinant DNA manipulations were according to Sambrook *et al.* (24); electrocompetent *E. coli* HB101 (Stratagene) were used for subcloning unless otherwise noted. Restriction enzymes were from New England Biolabs; high molecular weight human uPA, plasminogen, and the anti-uPAR monoclonal antibody 3936 were from American Diagnostica (Greenwich, CT). Streptavidin was from Molecular Probes or Sigma, and bovine serum albumin (BSA) was from Sigma. Immulon-2 96-well plates were from Dynatech. The plasmin substrate S-2251 was from Kabi Pharmacia Diagnostics (Piscataway, NJ). Linear synthetic peptides were prepared on an Applied Biosystems model 430A peptide synthesizer using 9-fluorenylmethoxycarbonyl-based chemistry and were purified by reversed-phase HPLC after trifluoroacetic acid cleavage. Alternatively, peptides were obtained from Chiron Mimotopes (Melbourne, Australia). The cyclic uPA peptide encompassing residues 12–32 with Cys-19 changed to Ala was obtained from V. Huebner (Chiron). Bacteriophage-derived peptides were synthesized with free N termini and C-terminal amides and were characterized by amino acid analysis using the Pico-tag method of Waters. Peptides were typically stored as concentrated stocks in 100% dimethyl sulfoxide at 4°C.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; ATF, N-terminal fragment of urokinase; EGF, epidermal growth factor; BSA, bovine serum albumin.

*To whom reprint requests should be addressed.

**Cloning and Expression of Cell-Surface uPAR.** A full-length human uPAR cDNA was isolated by PCR from a bacteriophage λ lysate DNA preparation of a phorbol 12-myristate 13-acetate-stimulated U937 cell cDNA library obtained from P. Olson (Chiron), using the following oligonucleotide primers, based on the published sequence (25): N terminus, 5′-CTAGAAGCTTATGGGTCACCCGCCGCTGCTG-3′; and C terminus, 5′-CGTAGTCGACTTAGGTCCAGAGGAGAGTGC-3′. PCRs were performed in 100 μl with the following components: 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.2 mM each dATP, dGTP, dTTP, dCTP/1 μM sense and antisense primer/100 ng of template cDNA preparation/2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). The reaction conditions were 94°C (1 min), 37°C (2 min), and then 72°C (3 min) for 30 cycles. The resulting PCR fragment was subcloned into *Hin*dIII/*Sal* I-digested pBR322, and its identity with the published sequence was confirmed by DNA sequencing. To transfer the full-length sequence to pAc373 (a baculovirus expression vector), the subclone was digested with *Hin*dIII/*Sal* I; the ends of the isolated fragment and the *Bam*HI linearized expression vector were filled in with Klenow fragment and deoxynucleoside triphosphates and then ligated to yield pflu-PAR, the expression vector.

Cell-surface human uPAR expression in COS-7 cells was accomplished by using the Mo3 cDNA in the vector pCDNA1 as described (26).

**Expression and Purification of Soluble Recombinant Proteins.** A truncated, soluble version of human uPAR was secreted from baculovirus-infected Sf9 insect cells. The expression vector encoding the soluble receptor (psu-PAR) was obtained by digesting the pBR322 subclone of the full-length receptor with *Sph* I/*Drd* I, yielding a fragment encoding amino acids 27–278 of uPAR. The sets of adaptors shown below were ligated to the 5′ end, introducing a *Bgl* II site and restoring the original first 26 amino acids, and to the 3′ end replacing amino acids Val and Glu (positions 278 and 279) with Leu and Val, while introducing a premature stop codon at position 280. For the 5′ end:

```
5'-GATCTATGGGTCACCCGCCGCTGCTGCCGCTGCTGCTGCT-
   3'-ATACCCAGTGGGCGGCGACGACGGCGACGACGACGA-

GCTCCACACGTGCGTCCCAGCCTCTTGGGGCCTGCGGTGCATG-3'
CGAGGTGTGCACGCAGGGTCGGAGAACCCCGGACGCCAC-5'
```

For the 3′ end:

```
5'-ATCTTGTTTAAA-3'
3'-CCTAGAACAAATTTCTAG-5'
```

The resulting fragment was cloned directly into the *Bam*HI site of pAc373, and correct clones were validated by restriction analysis and DNA sequencing. Soluble uPAR was purified from conditioned medium of baculovirus-infected Sf9 cells, by DEAE ion-exchange and Sephacryl S-100 size-exclusion chromatography, followed by preparative C-4 reversed-phase HPLC (S.R. and R. Yamamoto, unpublished data).

**Receptor Binding Assay.** Purified soluble uPAR was biotinylated with NHS-biotin (Molecular Probes) and immobilized at 0.3 μg/ml in phosphate-buffered saline (PBS)/0.1% BSA on streptavidin-coated Immulon-2 96-well Removawell plates (27). Human uPA N-terminal fragment (ATF; from M. Shuman, University of California, San Francisco) was iodinated by the Iodo-Gen method (Pierce). Unincorporated $^{125}I$ was separated from labeled protein by Sephadex G-25 chromatography. The specific activity of the labeled protein was between $5 \times 10^5$ and $1 \times 10^6$ dpm/pmol. The iodinated tracer (100–500 pM) was incubated with synthetic peptides in triplicate for 2 h at room temperature in PBS/0.1% BSA in a total vol of 200 μl. The plates were washed three times with PBS/0.1% BSA, and remaining bound radioactivity was measured on an LKB 1277 Gammamaster. Scatchard analysis was performed by using the LIGAND program (Biosoft, Milltown, NJ) (28).

**Construction of a Positive Control Bacteriophage.** A positive control bacteriophage, encoding human uPA residues 13–32 with Cys-19 converted to Ala (13-32C19A), was constructed by PCR with the M13 bacteriophage derivative pLP67 (15) as template. The sense primer for the reaction was 5′-TTTAGTGGTACCTTTCTATTCTCACTCCGCTGAATGTCTAAATGGAGGAACAGCTGTGTCCAACAAGTACTTCTCCAACATTCACTGGTGCAACCCGCCTCCACCTCCACCGACTGTTGAAAGTTGTTTAGC-3′, encoding an in-frame N-terminal *Kpn* I site in the P3 gene at position 1615 of pLP67. This *Kpn* I site is followed by a P3 sequence through the first 2 residues of the mature P3 protein (Ala-Glu), residues 13–32 of the epidermal growth factor (EGF)-like domain of human uPA with Cys-19 changed to Ala, and 6 proline residues separating the uPA sequence from P3. The antisense primer 5′-AAAGCGCAGTCTCTGAATTTACCG-3′ encodes the *Alw*nI site at position 2192 of pLP67. The PCR (38 cycles) was carried out as described above and the products were ethanol precipitated, resuspended, and digested with *Kpn* I and *Alw*nI. The major product of ≈640 bp was isolated from a 1% low melting point agarose gel by agarase digestion and was ligated with the purified large *Kpn* I/*Alw*nI-digested fragment of pLP67. After transformation, insert positive clones were identified by PCR and were confirmed by DNA sequencing.

**Peptide Library Affinity Selection.** Sf9 insect cells ($10^6$), infected 48 h previously with a baculovirus expressing the human substance P receptor, were incubated with $10^{10}$ library bacteriophage for 30 min at room temperature in 0.5 ml of Grace's insect cell medium containing 2% nonfat dry milk (GBB) to remove nonspecifically adherent bacteriophage. Cells were removed by centrifugation and the bacteriophage-containing supernatant was incubated for 30 min with gentle agitation with $10^6$ Sf9 cells displaying human uPAR. Cells were then washed five times with 10 ml of GBB, and bacteriophage were eluted with 0.5 ml of 6 M urea (pH 3) for 15 min at room temperature. The eluate was neutralized with 2 M Tris base (10 μl), and the bacteriophage yield was determined by titering on *E. coli* JM103. Bacteriophage were then amplified as plaques on large agar plates at $\approx 2 \times 10^5$ plaques per plate as described (15). Bacteriophage were eluted with Tris-buffered saline for 6 h, precipitated with polyethylene glycol 8000 (Sigma), and stored at 4°C at $\approx 10^{13}$ plaque-forming units/ml. Eluted, amplified bacteriophage were then affinity selected on transfected COS-7 cells overexpressing human uPAR. Cells, transfected 48 h previously by the DEAE-dextran method with the uPAR expression plasmid (26), were washed once with Dulbecco's modified Eagle's high glucose medium containing 2% nonfat dry milk and 20 mM Hepes (pH 7.2) (DBB). Cells were incubated with $\approx 10^{11}$ bacteriophage from the round 1 eluate for 30 min at room temperature and washed 10 times with 5 ml of DBB; bound bacteriophage were eluted as described above. Eluted bacteriophage were amplified and $10^{10}$ infectious particles were used as input for a third round of affinity selection on uPAR-expressing Sf9 cells, as described for round 1. Bacteriophage eluted from this final round of selection were plated, and individual plaques were picked and analyzed by DNA sequencing.

**Peptide Bacteriophage Competition Experiments.** *E. coli* JM103 were infected with bacteriophage and grown for 6–8 h, and bacteriophage particles were isolated by centrifugation and polyethylene glycol precipitation. Immulon-2 96-well plates were coated overnight at 4°C with 10 μg of streptavidin per well in 100 μl of 0.1 M sodium bicarbonate (pH 9.0). The wells were drained, washed once with 300 μl of PBS, and

blocked with 200 μl of 5% BSA in PBS for 1 h at room temperature with gentle agitation. The wells were rinsed three times with PBS and 1 μg of biotinylated uPAR was added and incubated for 1 h, drained, and washed three times with 300 μl of PBS. Aliquots of fresh bacteriophage stocks were mixed with either PBS or 2 μM peptide in 0.2% dimethyl sulfoxide. Two peptides were used: clone 20, AEPMPHSLNFSQYLWYT; control peptide, AEWVWPTEDSPTPSYDY (WVWP). These samples (100 μl) were incubated in uPAR-coated wells for 2 h at room temperature, the wells were washed 10 times with 300 μl of PBS, and remaining bacteriophage were eluted with 100 μl of 0.1 M glycine (pH 2.2) for 15 min at room temperature. Eluted bacteriophage were neutralized with 20 μl of 1.5 M Tris·HCl (pH 8.8) and titered in duplicate for the inputs and in triplicate for the eluates as described (15).

## RESULTS

To affinity select the bacteriophage peptide library, we first cloned and expressed human uPAR, a glycosyl phosphatidylinositol-linked integral membrane protein of 313 amino acids (25, 29). A full-length receptor cDNA was inserted into vectors for expression in mammalian cells (26) and for production of a recombinant baculovirus. Transfection of the plasmid into COS-7 cells or infection of Sf9 cells with the recombinant baculovirus yielded cells that displayed high levels of functional uPAR, as shown by immunological detection with an anti-receptor monoclonal antibody and by the binding of $^{125}$I-labeled ATF (S.R. and R. Yamamoto, unpublished data).

A peptide derived from the EGF-like domain of human urokinase, residues 12–32, with Cys-19 converted to Ala, competes with ATF for binding to uPAR with an $IC_{50}$ of 100 nM (30). To verify that bacteriophage displaying a uPAR ligand could be specifically selected by cell-surface uPAR, we constructed a positive control bacteriophage, encoding uPA 13-32C19A. As shown in Table 1, both COS-7 and baculovirus-infected Sf9 cells displaying human uPAR selectively enriched for the uPA 13-32C19A bacteriophage over a control bacteriophage by 500- and 800-fold, respectively. In addition, control cells expressing the substance P receptor did not enrich for this uPA bacteriophage. The random peptide bacteriophage display library, consisting of $10^7$ different 15-mers, was then affinity selected for three rounds alternately on Sf9 cells and COS-7 cells expressing uPAR. Enrichment for uPAR ligands was initially assessed by bacteriophage yield. After two rounds of selection the yield had increased 30-fold over the first round, and the third round showed a further increase of 130-fold to 5.4%, approximately that seen for the positive control bacteriophage. The overall yield increase was 4000-fold.

Individual plaques were picked from the third round eluate and subjected to DNA sequence analysis. From 66 plaques, 19 different DNA and peptide sequences were obtained, and these individual bacteriophage were tested for binding to cells displaying uPAR. Each of them showed 20- to 500-fold greater yields than an irrelevant bacteriophage. Peptides corresponding to the selected sequences were synthesized, purified, and tested as competitors in a uPAR binding assay with iodinated ATF as ligand (27). These results are summarized in Table 2, in comparison with known uPAR ligands.

To further map the sites of receptor interaction for these ligands, we asked whether the clone 20 synthetic peptide blocks uPAR binding of bacteriophage displaying three peptides: uPA 13-32C19A, clone 16, and the homologous clone 20. The results, shown in Fig. 1, indicate that clone 20 peptide prevents >95% of the binding of all three bacteriophage to uPAR, whereas a control peptide had little effect.

Table 1. Affinity selection of a positive control bacteriophage displaying human urokinase 13-32C19A by cell-surface uPARs

| Cells/receptor | % recovery | | Ratio positive control/M13 |
|---|---|---|---|
| | Positive control | M13 | |
| Sf9/uPAR | 6.8 | 0.009 | 756 |
| Sf9/SPR | 0.008 | 0.01 | 0.8 |
| COS-7/uPAR | 1.5 | 0.003 | 500 |
| COS-7/ETRB | 0.006 | 0.003 | 2.0 |
| COS-7/Mock | 0.003 | 0.003 | 1.0 |

Sf9 cells ($1 \times 10^6$ cells) expressing full-length human uPARs or substance P receptors (SPR) were used 48 h postinfection with the appropriate recombinant baculoviruses. COS-7 cells expressing uPAR or the human endothelin B receptor (ETRB) were tested 48 h posttransfection with a DEAE-dextran chloroquine protocol essentially as described (26) at $2 \times 10^5$ cells per well in six-well tissue culture dishes. Controls consisted of mock-transfected COS cells and polyhedron mutant baculovirus (CΔ3)-infected Sf9 cells. uPAR expression was validated by using murine monoclonal antibody 3936 (American Diagnostica) and anti-mouse horseradish peroxidase conjugate with tetramethylbenzidine as substrate (31). In each experiment, between $6 \times 10^8$ and $1.2 \times 10^9$ plaque-forming units of positive control bacteriophage and a 10-fold excess of M13 were used as input. Bacteriophage were selected as described for selection from the 15-mer library. Elutions were with 0.5 ml of 6 M urea (pH 3) for 15 min at room temperature with gentle mixing. The eluted bacteriophage were separated from the cells by centrifugation and then neutralized with 10 μl of 2 M Tris base. The inputs and eluates were titered with JM103 cells on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates containing 1 mM isopropyl β-D-thiogalactopyranoside to distinguish between M13 plaques (blue) and positive control phage plaques (clear).

## DISCUSSION

The molecular details of the interaction between uPA and uPAR have been investigated by several groups. Stoppelli *et al.* (32) showed that the N-terminal fragment of uPA (residues

Table 2. Receptor binding affinities and sequences of peptides derived from panning of 15-mer phage library on human uPAR

| Clone | Sequence | Frequency* | $IC_{50}$, μM† |
|---|---|---|---|
| 20 | AEPMPHSLNFSQYLWYT | 11 | 0.01 |
| 26 | AEHTYSSLWDTYSPLAF | 8 | 0.34 |
| 54 | AELDLWMRHYPLSFSNR | 1 | 0.38 |
| 16 | AESSLWTRYAWPSMPSY | 5 | 0.40 |
| 12 | AEWHPGLSFGSYLWSKT | 6 | 0.40 |
| 18 | AEPALLNWSFFFNPGLH | 1 | 1.0 |
| 9 | AEWSFYNLHLPEPQTIF | 2 | 1.0 |
| 11 | AEPLDLWSLYSLPPLAM | 2 | 2.0 |
| 42 | AEPTLWQLYQFPLRLSG | 1 | 2.5 |
| 48 | AEISFSELMWLRSTPAF | 1 | 5.0 |
| 75 | AELSEADLWTTWFGMGS | 1 | 7.0 |
| 17 | AESSLWRIFSPSALMMS | 1 | 8.0 |
| 13 | AESLPTLTSILWGKESV | 1 | 8.0 |
| 10 | AETLFMDLWHDKHILLT | 4 | 8.0 |
| 44 | AEILNFPLWHEPLWSTE | 2 | 9.0 |
| 14 | AESQTGTLNTLFWNTLR | 8 | 10.0 |
| 38 | AEIKTDEKMGLWDLYSM | 1 | 23.0 |
| 19 | AEMHRSLWEWYVPNQSA | 9 | >23 |
| 36 | AESHIKSLLDSSTWFLP | 1 | >47 |
| — | uPA 1–135 (ATF) | — | 0.00012 |
| — | uPA 12–32C19A | — | 0.25 |

Phage were selected as described in the text. Peptide sequences were determined by translation of DNA sequencing results from single-stranded phage templates. Receptor binding assays were done in 96-well microtiter plates as described.

*Number of separate times a given DNA and amino acid sequence was obtained from randomly picked plaques.

†Apparent inhibition constant of the synthetic peptide or uPA fragment for the uPAR–ATF interaction.

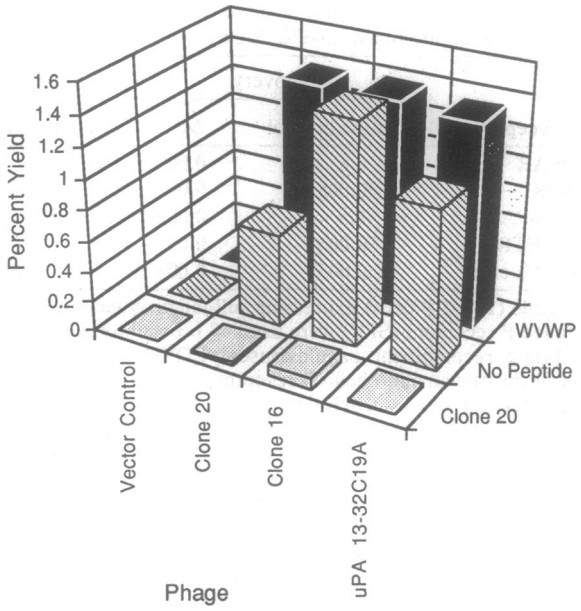


FIG. 1. Soluble peptide competition for phage binding to immobilized uPAR. Bacteriophage displaying uPA 13-32C19A (Table 1), clone 16, clone 20 (Table 2), or no peptide (LP67) ($6 \times 10^8$–$2 \times 10^9$ plaque-forming units input) were incubated in uPAR-coated wells, with and without the indicated peptides, washed, and eluted as described. The peptides used as competitors at 2 $\mu$M final concentration were clone 20 or a control peptide (WVWP). The data are shown as percentage yield of the various input phage in the presence and absence of the indicated peptides; the standard errors of these measurements are in the range of 20–50%.

1–135 including the EGF-like and kringle domains) bound to U937 cell uPAR with equal or greater affinity than uPA itself, thus separating the catalytic and receptor binding regions. Subsequent work showed that deletion of the EGF-like domain (residues 9–45) from pro-uPA reduced uPAR binding at least $10^3$-fold (33) and that recombinant human uPA EGF-like domain (residues 1–45 or 1–48) binds to uPAR with affinity comparable to that of uPA (ref. 34; S.R. and J. Stratton-Thomas, unpublished data).

Appella *et al.* (30) used limited proteolysis and synthetic peptides to further localize the key receptor binding region of uPA. They showed that a disulfide-bonded cyclic peptide encompassing residues 12–32 of uPA, with Cys-19 converted to Ala, competed with labeled ATF for binding, with an $IC_{50}$ of 40 nM. Cleavage of ATF at Lys-23 or Phe-25 drastically reduced uPAR binding, further supporting the requirement for a specific ligand secondary structure (30). The structures of molecules related to the uPA EGF-like domain, including human EGF, murine EGF, and human transforming growth factor $\alpha$, have been determined by multidimensional NMR methods (35, 36). In these cases the central loop of the molecule, corresponding approximately to residues 13–32 in the uPA EGF-like domain, is the major secondary structural element, composed of two $\beta$ strands and a turn. Preliminary NMR analysis of the uPA EGF-like domain shows a similar secondary structure (E. K. Bradley, personal communication).

uPAR consists of three domains of ≈90 amino acids, which are related to the Ly-6 superfamily (25). The murine and human receptors show little or no cross-species binding (37). A chymotryptic fragment of human uPAR (residues 1–87) can be cross-linked to ATF (38), and recent work with a closely related receptor fragment (residues 1–92) suggests that the determinants of species specificity reside primarily in the first 13 residues, as determined with chimeric receptors (39).

We have used a 15-mer random peptide library displayed on bacteriophage M13 to isolate uPAR ligands. This library consists of $10^7$ different bacteriophage displaying three to five copies of a random 15-mer peptide near the N terminus of the P3 protein in the sequence $AEX_{15}P_6$ (15). Since the total number of 15-mer peptides is $3.3 \times 10^{19}$, only 1 in $10^{12}$ sequences has been displayed. The probability of a particular sequence being expressed is not uniform due to codon bias, so that some sequences will be further underrepresented (40, 41). Despite these limitations, we have identified 15-mer peptide ligands, the most potent of which (clone 20, $IC_{50}$ = 10 nM) is only 25- to 100-fold less potent than uPA itself (32, 42). The less potent peptides likely represent suboptimal sequences due to the limited size of the library. None of the selected peptides contained cysteine residues, despite a requirement for correct disulfide bond formation in high-affinity uPA binding to uPAR, as only a single disulfide-bonded isomer of recombinant uPA EGF-like domain (residues 1–48) binds human uPAR (S.R. and J. Stratton-Thomas, unpublished data).

An alignment of the more potent uPAR binding peptides ($IC_{50} < 5$ $\mu$M), based on conserved subsequences, is shown in Fig. 2. It is difficult to align all of the bacteriophage-derived sequences with each other, and especially with the receptor binding region of uPA, since the bacteriophage peptides are linear and the uPA binding region is cyclic. There also appear to be two subsets of phage-derived peptides, with the motifs FXXYLW (clones 20 and 12) and LWXXY (clones 16 and 26). These common sequence motifs are in different registers within the variable region (Fig. 2), suggesting that subsequences of the 15-mers are likely active.

The vast majority of the peptides identified compete for ATF binding, consistent with the idea that they bind to uPAR in the uPA binding site. This suggests either that the bacteriophage peptides are capable of a relatively defined secondary structure, mimicking a disulfide-bonded loop, or that the molecular details of the binding interactions of uPAR with the bacteriophage-derived peptides and uPA are overlapping but distinct. Alternatively, these different ligands may bind to the same uPAR amino acid residues, as seen for the two unrelated binding sites on human growth hormone binding to the human growth hormone receptor (43). The likelihood of a common set of molecular interactions between uPAR and both uPA and clone 20 peptide is strengthened by the observation that the peptide is a species-specific ligand for

| Sequence Name | Alignment | IC50 (uM) |
|---|---|---|
| | Set 1 | |
| 20 | AEPMPHSLNFSQYLWYT | 0.01 |
| 12 | AEWHPGLSFGSYLWSKT | 0.40 |
| 48 | AEISFSELMWLRSTPAF | 5.0 |
| | Set 2 | |
| 26 | AEHTYSSLWDTYSPLAF | 0.34 |
| 54 | AELDLWMRHYPLSFSNR | 0.38 |
| 16 | AESSLWTRYAWPSMPSY | 0.40 |
| 18 | AEPALLNWSFFFNPGLH | 1.0 |
| 9 | AEWSFYNLHLPEPQTIF | 1.0 |
| 11 | AEPLDLWSLYSLPPLAM | 2.0 |
| 42 | AEPTLWQLYQFPLRLSG | 2.5 |

FIG. 2. Alignment of highest-affinity bacteriophage-derived peptide sequences. The highest-affinity peptide sequences derived from the bacteriophage display library selection are aligned. The sequences are divided into two subsets, which have subsequence motifs of FXXYLW and LWXXAr (Ar = Y, F, H, or W). Residues in boldface are conserved within or between sequence subsets. A speculative alignment of these sequences with each other is shown.

uPAR and does not bind to the murine uPAR (H. Y. Min and S.R., unpublished data).

The frequency of a given bacteriophage sequence in the third round pool of bacteriophage does not correlate with the inhibition constants for ATF binding (see, for example, clones 14 and 20 in Table 2). This could be due to variable protease sensitivity of the displayed peptides or to the polyvalent display method used (44). Alternatively, some of the bacteriophage may bind to sites other than the uPA ligand binding site. The possibility that other functional binding sites are present on uPAR is suggested by the three-domain structure of the molecule, in which the first domain is required for uPA binding, and the other two domains are of unknown function (38, 39).

In summary, we have identified a family of potent peptide antagonists for the human urokinase receptor from a random peptide bacteriophage library by selection on receptor-bearing cells. This work validates the bacteriophage peptide display technology using cell-surface receptors. These molecules will serve as leads for the discovery of pharmaceutical agents that inhibit cell-surface proteolysis and as unique tools for analysis of the uPA–uPAR interaction.

We thank Ralph Yamamoto and Bob Drummond for soluble recombinant uPAR, Simon Ng and Lisa McGuire for peptide synthesis, and Jean Joh for DNA sequencing. We also thank Marc A. Shuman (University of California, San Francisco) for many helpful discussions; our colleagues, especially Hye Yeong Min and Erin Bradley, for critical reading of this manuscript; and Chiron Corporation for support.

1. Saksela, O. & Rifkin, D. B. (1988) *Annu. Rev. Cell Biol.* **4**, 93–126.
2. Zini, J.-M., Murray, S. C., Graham, C. H., Lala, P. K., Karikó, K., Barnathan, E. S., Mazar, A., Henkin, J., Cines, D. B. & McCrae, K. R. (1992) *Blood* **79**, 2917–2929.
3. Folkman, J. & Shing, Y. (1992) *J. Biol. Chem.* **267**, 10931–10934.
4. Vassalli, J.-D., Wohlwend, A. & Belin, D. (1992) *Curr. Top. Microbiol. Immunol.* **181**, 65–86.
5. Mignatti, P., Robbins, E. & Rifkin, D. B. (1986) *Cell* **47**, 487–498.
6. Ossowski, L. (1992) *Cancer Res.* **52**, 6754–6760.
7. Ellis, V. F., Behrendt, N. & Dano, K. (1991) *J. Biol. Chem.* **266**, 12752–12758.
8. Emonard, H. P., Remacle, A. G., Grimaud, J. A., Stetler-Stevenson, W. G. & Foidart, J. M. (1992) *Cancer Res.* **52**, 5845–5848.
9. Pyke, C., Kristensen, P., Ralfkiaer, E., Grondahl-Hansen, J., Eriksen, J., Blasi, F. & Dano, K. (1991) *Am. J. Pathol.* **138**, 1059–1067.
10. Pyke, C., Græm, N., Ralfkiær, E., Ronne, E., Hoyer-Hansen, G., Brünner, N. & Dano, K. (1993) *Cancer Res.* **53**, 1911–1915.
11. Hollas, W., Blasi, F. & Boyd, D. (1991) *Cancer Res.* **51**, 3690–3695.
12. Ellis, V., Pyke, C., Eriksen, J., Solberg, H. & Dano, K. (1992) *Ann. N.Y. Acad. Sci.* **667**, 13–31.
13. Crowley, C. W., Cohen, R. L., Lucas, B. K., Liu, G., Shuman, M. A. & Levinson, A. D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 5021–5025.
14. Scott, J. K. & Smith, G. P. (1990) *Science* **249**, 386–390.
15. Devlin, J. J., Panganiban, L. C. & Devlin, P. E. (1990) *Science* **249**, 404–406.
16. Lowman, H. B., Bass, H. B., Simpson, N. & Wells, J. A. (1991) *Biochemistry* **30**, 10832–10838.
17. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990) *Nature (London)* **348**, 552–554.
18. Roberts, B. L., Markland, W., Siranosian, K., Saxena, M. J., Guterman, S. K. & Ladner, R. C. (1992) *Gene* **121**, 9–15.
19. Scott, J. K., Loganathan, D., Easley, R. B., Gong, X. & Goldstein, I. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5398–5402.
20. Cwirla, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6378–6382.
21. O'Neil, K. T., Hoess, R. H., Jackson, S. A., Ramachandran, N. S., Mousa, S. A. & DeGrado, W. F. (1992) *Proteins* **14**, 509–515.
22. Koivunen, E., Gay, D. A. & Ruoslahti, E. (1993) *J. Biol. Chem.* **268**, 20205–20210.
23. Marks, J. D., Ouwehand, W. H., Bye, J. M., Finnern, R., Gorick, B. D., Voak, D., Thorpe, S. J., Hughes-Jones, N. C. & Winter, G. (1993) *Biotechnology* **11**, 1145–1149.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
25. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Dano, K., Appella, E. & Blasi, F. (1990) *EMBO J.* **9**, 467–474.
26. Min, H. Y., Semnani, R., Mizukami, I. F., Watt, K., Todd, R. F., III, & Liu, D. Y. (1992) *J. Immunol.* **148**, 3636–3642.
27. Kaufman, S. E., Brown, S. & Stauber, G. B. (1993) *Anal. Biochem.* **211**, 261–266.
28. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239.
29. Ploug, M., Ronne, E., Behrendt, N., Jensen, A. L., Blasi, F. & Dano, K. (1991) *J. Biol. Chem.* **266**, 1926–1933.
30. Appella, E., Ullrich, S. J., Stoppelli, M. P., Corti, A., Cassani, G. & Blasi, F. (1987) *J. Biol. Chem.* **262**, 4437–4440.
31. Sheldon, E. L., Kellogg, D. E., Watson, R., Levenson, C. H. & Erlich, H. A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9085–9089.
32. Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F. & Assoian, R. K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4939–4943.
33. Robbiati, F., Nolli, M. L., Soffientini, A., Sarubbi, E., Stoppelli, M. P., Cassani, G., Parenti, F. & Blasi, F. (1990) *Fibrinolysis* **4**, 53–60.
34. Blasi, F. & Stoppelli, M. P. (1990) in *Growth Regulation and Carcinogenesis*, ed. Paukovits, W. R. (CRC, Boca Raton, FL), Vol. 2, pp. 149–162.
35. Hommel, U., Harvey, T. S., Driscoll, P. C. & Campbell, I. D. (1992) *J. Mol. Biol.* **227**, 271–282.
36. Harvey, T. S., Wilkinson, A. J., Tappin, M. J., Cooke, R. M. & Campbell, I. D. (1991) *Eur. J. Biochem.* **198**, 555–562.
37. Estreicher, A., Wohlwend, A., Belin, D., Schleuning, W.-D. & Vassalli, J.-D. (1989) *J. Biol. Chem.* **264**, 1180–1189.
38. Behrendt, N., Ploug, M., Patthy, L., Houen, G., Blasi, F. & Dano, K. (1991) *J. Biol. Chem.* **266**, 7842–7850.
39. Pollanen, J. J. (1993) *Blood* **82**, 2719–2729.
40. Labean, T. H. & Kauffman, S. A. (1993) *Protein Sci.* **2**, 1249–1254.
41. Arkin, A. P. & Youvan, D. C. (1992) *Biotechnology* **10**, 297–300.
42. Blasi, F., Behrendt, N., Cubellis, M. V., Ellis, V., Lund, L. R., Masucci, M. T., Moller, L. B., Olson, D. P., Pedersen, N., Ploug, M., Ronne, E. & Dano, K. (1990) *Cell Differ. Dev.* **32**, 247–254.
43. De Vos, A. M., Ultsch, M. & Kossiakoff, A. A. (1992) *Science* **255**, 306–312.
44. Barrett, R. W., Cwirla, S. E., Ackerman, M. S., Olson, A. M., Peters, E. A. & Dower, W. J. (1992) *Anal. Biochem.* **204**, 357–364.