The N-Terminal Domain of Human Urokinase Receptor Contains Two Distinct Regions Critical for Ligand Recognition

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The high-affinity receptor that binds human urokinase-type plasminogen activator (hu-PAR) is a glycosyl-phosphatidylinositol (GPI)-anchored cell-surface glycoprotein that belongs to the Ly-6 superfamily of T-cell-activating receptors. Binding of urokinase (u-PA) to u-PAR is species-specific, since neither murine (mu-PAR) nor hu-PAR binds u-PA from the other species. I designed and analyzed a series of exchanges between hu-PAR and mu-PAR in the N-terminal first domain to which ligand-binding function had been independently mapped. Introduction of as few as 13 murine residues (six of 13 variables) into the N-terminal region of hu-PAR abrogated binding to recombinant human pro-u-PA, whereas the opposite chimera, a mu-PAR carrying six of 13 human residues, was positive for binding.

Within this region, the mu-PAR domain 1 could be minimally humanized to bind human pro-u-PA by a substitution of as few as four of the six nonconserved residues, thereby identifying the residues arginine-2, lysine-7, threonine-8, and glycine-10 as important in determining binding specificity. By alanine-scanning mutagenesis, a second recognition site within domain 1 was discovered between residues 47 and 53, a segment that is fully conserved between the human and the mouse receptors. Taken together, these results provide identification of two confined subregions within the N-terminal domain of hu-PAR critically involved in pro-u-PA recognition.

The prediction that u-PAR is structurally related to members of the Ly-6 superfamily/T-cell-activating proteins enabled structural alignment of the u-PAR molecule with domains of known Ly-6 structures. As a result of such alignments, u-PAR was predicted to have three internally homologous domains, characterized by a unique pattern of cysteine residues. Analyzing chymotryptic fragments of purified hu-PAR protein, ligand-binding activity was mapped to the first N-terminal domain. The first N-terminal domain of the mu-PAR and hu-PAR share an overall 60% identity at the amino acid level, however, mu-PAR fails to bind human u-PA. Using an in vitro transcription-translation system, I confirm that the first 92 residues of u-PAR are sufficient for maintaining ligand-binding specificity. I have further examined this region of the molecule in detail by construction of a series of human/mouse u-PAR receptor domain chimeras. Analysis of these truncated molecules and several smaller substitutions allowed the identification and the biochemical characterization of the purified u-PAR protein as a highly glycosylated 55- to 60-kD protein.

The complete cDNA of the hu-PAR was cloned and found to encode a 313-amino acid residue polypeptide, preceded by a single peptide. Following carboxyterminal processing, u-PAR becomes attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. A mu-PAR cDNA was also cloned, and shown to have high sequence homology to the human counterpart.

The prediction that u-PAR is structurally related to members of the Ly-6 superfamily/T-cell-activating proteins enabled structural alignment of the u-PAR molecule with domains of known Ly-6 structures. As a result of such alignments, u-PAR was predicted to have three internally homologous domains, characterized by a unique pattern of cysteine residues.

The N-terminal domain of hu-PAR is critical for ligand recognition.
cation of two regions in the N-terminal domain of u-PAR that are important for ligand binding, as well as individual amino acid residues that may be involved in direct contact with the ligand.

MATERIALS AND METHODS

Plasmids. For mu-PAR DNA, the p-mu-PAR-1 plasmid subcloned into pBluescriptKS(+) (Stratagene, La Jolla, CA) was used. For hu-PAR, the p-u-PAR-1 DNA was used: it encodes the complete hu-PAR sequence and can be used for expression in eukaryotic cells of an active u-PAR protein. A 1,221-bp NruI-Sac1 fragment containing the entire coding region of hu-PAR was subcloned into EcoRV-Sac1 sites in pBluescriptSK(+)(Stratagene), and is here designated pJP2. Two in-frame deletion mutants were subsequently prepared by first cleaving pJP2 at unique NruI and EcoRV restriction sites. The overhangs were then treated with T4 nuclease, and in one case a XbaI stop-codon linker (Pharmacia, Uppsala, Sweden) was inserted, before self-ligation of the purified DNA fragments with T4 ligase. These plasmid constructs were designated pJP3 and pJP4 (the latter containing the stop codon). They encode for residues 1-92 (containing the entire N-terminal domain) and in the case of pJP3, also the residues 278-313, a region involved in GPI membrane attachment. These constructs and the other mutants listed in the text all used the T7 promoter.

Oligonucleotide primer design and polymerase chain reaction. The polymerase chain reaction (PCR) mixture included 0.2 mmol/L of each primer, 100 ng of double-strand U-PAR cDNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 100 μL. After varying with 50 μL of mineral oil, the reactions were performed in a Hybaid DNA thermal cycler (Omnigene, Middlesex, UK) at three different temperatures (94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes) for 30 cycles. The PCR-amplified DNA fragments were examined by agarose gel electrophoresis. The appropriately sized fragments were cut from the gel and purified by QIAEX (Qiagen, Chatsworth, CA).

Mutant u-PAR cDNAs were generated by a two-step recombinant PCR method as described elsewhere. In this way a wide variety of changes can be made into one piece of DNA, and two DNA sequences can be joined at almost any position. Briefly, in a primary PCR amplification step, two separate DNA fragments were generated using four primers: left, right, and a pair of overlapping inside primers, containing mismatched bases for making a mutation. These two gel-purified PCR products were then combined into one longer product in a secondary PCR amplification, performed in the presence of the left and right primers only under the above conditions for 12 cycles. Secondary PCR products were also gel-purified by QIAEX. Taking the chimera JP15 as an example, two primers were prepared to make this construct that encodes for hu-PAR residues 1-46 and murine ones 47-92. The first inside primer, a 29-mer oligonucleotide 5'-TGGAGCTGGTGGAGA-AAAAGCTGACCCAC-3' which carried the hu-PAR sequence coding for amino acids 38-47, was allowed to anneal to the mu-PAR cDNA template, together with a constant murine left primer used, 5'-TGGAGGCCCCTAGGC GCCTGAGG-3'. The second inside primer was a 29-mer oligonucleotide complementary to the first one, and was used in the other primary PCR amplification on the human template with a constant right primer, a 24-mer oligonucleotide 5'-GTAAAACGAGCGCCAGTGAGCGCG-3' (M13-20 sequence). These overlapping primary products were then combined in secondary PCR, and the final products all contained T7 promoter sequence at their 5'-end. Oligonucleotides were all synthesized on an Applied Biosystem 381 DNA synthesizer (Perkin Elmer Corp, USA).

Mutagenesis was confirmed by direct PCR sequencing: products of the secondary PCR were used for asymmetric reamplification using 50 pmol of one primer and 5 pmol of the other or reverse, using the same cyclic profile as above, but for 40 cycles. The single-stranded products of the asymmetric PCR were purified from residual primers and dNTPs by selective isopropanol precipitation. All of the templates were sequenced by Sequenase Version 2.0 Kit (United States Biochemical, Cleveland, OH), using either the primer present in lower amount in the preceding asymmetric PCR or an internal primer.

Transcription and translation. The capped u-PAR cRNAs were synthesized by in vitro transcriptions of linearized human/mouse cDNAs or the purified PCR-generated mutant DNA fragments with 70 U/μL T7 RNA polymerase according to instructions from the manufacturer (Promega, Madison, WI). The cRNAs were purified from the transcription mixtures by treatments with DNase-free DNase (Promega), 0.5% sodium dodecyl sulfate (SDS) and 5 μg proteinase K (Boehringer-Mannheim, Mannheim, Germany), after which they were extracted with phenol and chloroform-isooamyl alcohol, and then ethanol-precipitated. The cRNAs were resuspended in 20 μL of nuclease-free water containing 1 U RNasin (Promega). The quality and quantity of the newly synthesized cRNAs were routinely estimated by running aliquots of them on reducing 1.2% agarose-formaldehyde gels, before starting the translation reactions.

The u-PAR polypeptides were synthesized by an in vitro transcription of the cRNA in nuclease-treated rabbit reticulocyte lysates (Promega) in the presence of 1/10 vol of 35S-cysteine (34.8 TBq/mmol, 408.5 MBq/mL; NEN-DuPont, Boston, MA). In some experiments, canine pancreatic microsomal membranes (Promega) were included in the translation reactions. Translations were performed for 1 hour at 30°C, and stopped by chilling on ice. The reticulocyte lysates (50 μL) were then solubilized by adding 200 μL of a solution of 1.25% Triton X-100, 190 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 6 mmol/L EDTA, and 1 μg/mL aprotinin, and used immediately in each experiment.

Pro-u-PAR binding assay and immunoprecipitation. For measuring pro-u-PAR binding activity of the mutant u-PAR polypeptides, 20- to 40-μL aliquots of the translation mixture were further diluted in 200 μL phosphate-buffered saline (PBS) and then mixed with 30 μL (50% wt/vol) of human recombinant pro-u-PAR-Sepharose and incubated for 16 hours on a rotator at 4°C. The affinity beads were prepared by coupling 20 mg of recombinant human prourokinase (Leptin SPA, Milan, Italy) with 20 mL of activated CNBr-Sepharose (Pharmacia) according to the instructions from the manufacturer, and afterwards blocking unoccupied binding sites with 1 mol/L ethanolamine (pH 8.0) and 1 mg/mL bovine serum albumin overnight on a rotator at 4°C. In some experiments, murine u-PA (American Diagnostica, Greenwich, CT) and gelatine (Pharmacia) coupled to Sepharose in the same way was used. For immunoprecipitation, diluted reticulocyte lysates were first treated with 3 μg/mL 3R monoclonal antibody directed against the domain 1 of hu-PAR overnight on a rotator at 4°C, and then immune complexes were collected with 30 μL (50% wt/vol) of protein G-Sepharose (Pharmacia). The competition with 200 mmol/L soluble two-chain u-PA (Leptin SPA) or a soluble hu-PAR, here designated INXS, was included as positive specificity controls. The precipitates were washed four times with 500 μL of a solution containing 0.1% Triton-X-100, 190 mmol/L NaCl, 5 mmol/L Tris-HCl, pH 7.5, 6.5 mmol/L EDTA, and 1 μg/mL aprotinin. The bound material was then resuspended in 30 μL of reduced Laemmli sample buffer, and resolved on a 15% SDS-polyacrylamide gel electrophoresis.
Fixed gels were treated with Amplify (Amersham) and dried and exposed to X-Omat AR film (Kodak, Rochester, NY) for 12 to 48 hours.

Expression of u-PAR protein chimera in LB6 cells. The human-murine chimera, JP14 and JP16, were selected for transfection experiments, and were reproduced using the M13-20 reverse primer as the left primer in the PCR, to make them include the entire murine C-terminal sequence (ie, domains 2 and 3). They were subsequently subcloned as HinClI cassettes into the p-u-PAR-1 expression vector.23 The murine wild-type u-PAR was also subcloned into the same vector as a control, but using the SacII-Apal insert. Cell culture and stable transfections of murine LB6 cells by the calcium phosphate coprecipitation method were performed, essentially as described.23 For human wild-type u-PAR-expressing cells, a stably transfected clone also under the control of SV-40 promoter, LB6-Cl19, was used.23 LB6 transfected with the RSV-neo gene43 were used as a control. Pools of transfected cells were analyzed for surface expression of the constructs by immunofluorescence, using the 3R monoclonal antibody as described.28,40 For ligand-binding assays, cells were seeded onto 24-well microtiter plates (Nunc, Roskilde, Denmark) at 1.5 × 10⁵ cells per well. Radiiodinated ATF (a kind gift from Dr Jack Henkin, Abbott Laboratories, North Chicago, IL) was used as the ligand in the cell-binding experiments.15,33 Each experiment was performed in triplicate. Specific binding was determined by subtracting cell-associated radioactivity in the presence of 200 nmol/L cold u-PA (Lepetit) from the values in the absence of it.23 Nonspecific binding to the cell layer was less than 5%. Dissociation constants were graphically estimated from Scatchard plots, the SE being less than 6% for the binding values.

RESULTS

Carboxy-terminal boundary of the ligand-binding region. In this study, I have used an in vitro binding assay involving Sepharose-coupled recombinant human prourokinase to determine the regions of the u-PAR polypeptide that are critical for stable interaction with its ligand. For these experiments, the open-reading frame of the hu-PAR cDNA was cloned downstream of the bacteriophage T7 promoter, allowing u-PAR cRNA to be synthesized directly from the cDNA by in vitro transcription. The cRNA was then used to program an in vitro translation reaction in rabbit reticulocyte lysates, in the presence and absence of canine pancreatic microsomal membranes. Mutant or wild-type ³⁵S-labeled u-PAR products were then assayed for pro-u-PA binding activity. Both the full length and two truncated forms of human u-PAR were used. They were constructed as follows: (1) JP-3 is an in-frame deletion of the cDNA at two unique restriction sites NsiI and EcoRV,41 ie, deletion of residues 93-278, generating a u-PAR-containing domain 1 plus the C-terminal GPI-anchor signal sequence; (2) JP-4 was made by introduction of a stop-codon linker at the NsiI site, generating a truncated form (residues 1-92) of u-PAR lacking the GPI-tail signal (Fig 1C).

When human full-length u-PAR polypeptides synthesized in vitro in the presence of membranes were assayed for pro-u-PA binding, a major band of Mr, 46 kD, compatible with the full-length u-PAR protein, was produced. In addition, some smaller fragments, presumably arising from premature terminations of the in vitro translation reactions, were seen (Fig 1A). The truncated u-PAR polypeptides (JP3 and JP4) were translated into products of the expected sizes, and they all bound to pro-u-PA (Fig 1A). When translations were performed without membranes, the products still showed good binding activity, despite a lower molecular weight, compatible with them being nonglycosylated. As these u-PAR polypeptides also contained the signal peptide, its presence did not appear to alter u-PAR-binding avidity, which was also found to be the case with subsequent mutants.

The carboxy-terminal GPI-anchor signal sequence (residues 281-313 in u-PAR) does not appear to be processed in this system, as the migration of the mutant JP3 is slower than that of JP4 in SDS-PAGE (Fig 1A). However, it seems that the lack of the GPI signal in the absence of microsomal membranes significantly increases the binding activity of the truncated u-PAR polypeptides. These results demonstrated that the sequences to the carboxy-terminal side of the amino acid 92 were not required for ligand binding. These residues form exactly the first of three repeats according to structural alignments with other Ly-6 family/T-cell-activating antigens.46 and thus this domain is likely to function independently. These results also confirmed earlier findings obtained by analyzing chymotryptic fragments of purified hu-PAR protein by chemical cross-linking.36

The specificity of the binding to the immobilized pro-u-PA was confirmed by competitions with excess soluble u-PA and a purified recombinant soluble u-PA receptor termed INXS,41 as shown for the deletion mutant JP4 in Fig 1B. This truncated u-PAR could also be specifically immunoprecipitated with a blocking monoclonal antibody 3R40 directed against the N-terminal domain (Fig 1B, lanes 4 and 5). Most (up to 60% for JP4) of the translated polypeptides added to the assay were bound to the human pro-u-PA-Sepharose.

I next analyzed the corresponding region of the mu-PAR (residues 1-92, JP5) produced by in vitro translation and tested for pro-u-PA binding activity. As shown in Fig 2, no detectable binding could be observed to human pro-u-PA, whereas JP5 was functional with the murine ligand. Two mutant constructs, JP6 and JP7, were then produced by taking advantage of a unique BclI restriction site that allowed swapping within the N-terminal first domain of the u-PAR sequence. By PCR, residues 1-62 (JP6) and 63-92 (JP7) of hu-PAR sequence were subcloned into appropriate sites in the murine cDNA vector.35 The binding properties of these two opposite receptor chimeras to human and mouse u-PA are shown in Fig 2 in parallel with their reactivities to polyclonal u-PA antibodies. These results strongly imply that the sequence within residues 1-62 of the human and mouse u-PA proteins contain all of the structural determinants needed for the species-specific binding to pro-u-PA.

Generation of a series of human/mouse u-PAR chimeras by recombinant PCR. To define further the sites within the domain 1 of hu-PAR that are critical for binding to pro-u-PA, a series of truncated u-PAR mutants that contained murine substitutions in the u-PAR coding region were produced, using a variation on the PCR technology termed recombinant PCR.38 Using this strategy, a series of human/mouse chimeras (JP14-19; Fig 3B) were designed by...
Fig 1. (A) The carboxy-terminal boundary of pro-u-PA binding sequence determined after linearizing the indicated hu-PAR cDNA constructs with the ScaI restriction enzyme (JP2, 3, 4). 35S-cysteine–labeled u-PAR polypeptides were synthesized by in vitro transcription/translation from the T7 promoter-driven u-PAR cDNA constructs in the presence and absence of microsomal membranes. The synthesized u-PAR polypeptides were precipitated with recombinant human pro-u-PA-Sepharose or immunoprecipitated with anti-hu-PAR amino terminal antibody, 3R. The generally stronger bands in the absence of membranes represent higher translation efficiencies, rather than changes in binding affinity. Equal volumes of the reticulocyte lysate (20 μL diluted in 200 μL) were used in this experiment. (B) Human pro-u-PA binding and immunoprecipitation of JP4 polypeptides (translated without membranes) in the presence of competitors: u-PA at 200 nmol/L, INXS (recombinant soluble hu-PAR) at 0.1 mmol/L. Protein complexes were separated by electrophoresis on a 15% SDS-PAGE and detected by fluorography (A: nonreduced; B: reduced). (C) Structural diagram of the three u-PAR cDNA constructs; JP2: human wild-type u-PAR; JP3: deletion mutant hu-PAR lacking amino acid residues 93-278; JP4: human u-PAR domain 1 (residues 1-92).
render this mutant u-PAR less functional for hu-PA. The region implicated as important for binding (see top lines in Fig 5) contains six nonconserved amino acids between human and mouse u-PAR sequences, and was therefore selected as a target for subsequent mutagenesis.

The structures of the mutants I made in this region are presented diagrammatically in Fig 5, together with a summary of their binding properties. By changing individually the six nonconserved residues (human for murine) into hu-PAR sequence—mutants JP20-25—virtually no effects on binding affinity could be observed (Fig 4A). This result implied that these single substitutions were not capable of weakening the interaction enough for it to be readily detectable by the relatively high-capacity pro-u-PA binding assay. A group of three mutations involving combinations of human for murine exchanges was tested next. As shown in Fig 4C and E, the triple mutant JP26, featuring murine substitutions of residues Arg-2, Lys-7, and Thr-8, and the mutant JP27, having residues 7, 8, 10, and 11 substituted, both expressed a weaker binding activity, as compared with the mutant JP28 (substitutions at positions 10, 11, and 13) that bound better to prourokinase.

I tested next the reverse approach, by introducing human residues in to the nonbinding mu-PAR. This kind of critical humanization of mu-PAR would thus allow positive identification of residues involved in ligand-recognition specificity. Initially, I produced three such mutants, JP30-32 (Fig 5), and the results are shown in Fig 4B and D. The mutant JP31, featuring four human residues at positions 2, 7, 8, and 10, had the highest binding activity in four independent assays. By contrast, mutant JP32, carrying another combination of four human residues (8, 10, 11, and 13), displayed only very weak binding to pro-u-PA. By selecting the four charged human residues for substitution (2, 7, 11, and 13), as in mutant JP30, an intermediate level of binding activity in relation to the two other mutants and wild-type u-PAR could be seen. In addition to these, I produced another mutant designated JP31B as a control, by a substitution of the human residues only at positions 2 and 7. This mutation was only partially sufficient in conferring adequate binding activity to hu-PA: the average pro-u-PA binding score for JP31B was 33% versus 85% for JP31 in four independent experiments (Fig 4D). Taken together, these data imply that the combination of human residues 2, 7, 8, and 10 is required for the high-affinity binding to hu-PA.

Furthermore, a group of four alanine substitutions to hu-PAR domain 1 within this critical region were also produced and tested for pro-u-PA-binding activity (JPs 33-36). Of these, the greatest loss of binding activity was achieved in mutant JP36 that had alanine residues at positions 2, 7, 8,
Fig 3. Human pro-u-PA binding of a series of truncated, human/mouse u-PAR ligand-binding domain chimeras generated by recombinant PCR mutagenesis (A). 35S-cysteine–labeled polypeptides, containing the constructions indicated in the diagram (B: •, human sequence; ●, murine sequence; ◊, overlapping conserved area), were synthesized in vitro translation reactions and mixed with human pro-u-PA-Sepharose. Protein complexes were separated by SDS-PAGE and labeled proteins detected by fluorography.

and 10 (Figs 4 and 5). This complements the previous data from the humanization trials and suggests a primary role for these four amino acids in determining ligand specificity to the human receptor.

Expression of two human/murine u-PAR chimera in LB6 cells. To ensure that the binding specificities observed in vitro were of functional and physiological significance, I subcloned and expressed two positive chimera, JP14 and JP16 (see diagram in Fig 3B), with the exception that they now contained the full murine C-terminal sequence, as well as wild-type hu-PAR and mu-PAR cDNAs in LB6 cells. The LB6 cells are unique in that they do not express endogenously U-PA nor U-PA inhibitors, and produce a mu-PAR that does not bind human u-PA, making them well suited for transfection experiments. Overexpression was attained under the control of the SV-40 promoter. Cell surface expression of these chimera was demonstrated by immunofluorescence labeling of pools of transfected cells with the 3R monoclonal antibody (not shown). I tested the ability of these transfected cells to bind human 125I-ATF in the presence and absence of cold ligand, and estimated relative binding affinities by Scatchard analysis (Table 1 and Fig 6).

Ligand binding to each of the two chimera was comparable to wild-type hu-PAR (Table 1), whereas cells expressing mouse u-PAR showed no specific binding above the level observed in neo-transfected cells. This result confirms that the region conferring binding specificity lies within the first 13 amino acids. The kd for LB6-C19 cells was estimated from the Scatchard plot to be 1.5 nmol/L (Fig 6), and the one for LB6-JP14 cells 2.0 nmol/L. This, combined with the fact that both receptor chimeras also reacted with the blocking 3R monoclonal antibody in immunofluorescence, supports the conclusion that I have mapped the primary region that controls the ligand specificity to the hu-PAR.

Alanine scanning of hu-PAR domain I identifies a second site critical for ligand binding. The rest of the hu-PAR domain 1 was then screened by alanine scanning mutagenesis. This strategy consists of mutagenizing clusters of charged amino acids (E, D, H, R, K) to alanine with the aim of disrupting interactive sites. As charged residues are likely to be exposed to the solvent, and at least part of the binding domain would be composed of hydrophilic amino acid residues, such clusters would include probable candidates for critical residues. Because alanine does not impose steric or electrostatic effects on neighboring side chains, an alanine substitution for a charged amino acid is not likely to introduce global effects on the polypeptide structure.

Four cluster mutations (Fig 7B) outside residues 1-13 were generated by the recombinant PCR protocol, and each was confirmed by direct PCR sequencing. The PCR-DNAs encoding the mutant polypeptides were directly transcribed in vitro, and the cRNAs translated in rabbit reticulocytes. These mutant polypeptides, JP37-40, were then tested in group for binding to the immobilized human pro-u-PA (Fig 7A).

Starting from the N-terminal side of hu-PAR, alanine substitutions at a quadruple glutamic acid cluster (residues 33-37; JP37), and a triple mutation located between residues 39 and 43 (E39A E42A K43A; JP38) did not significantly affect binding to pro-u-PA (Fig 7). On the contrary, the mutation between amino acids 47 and 53 (H47A E49A K50A R53A; JP39) was found to have a markedly lower
HUMAN u-PAR LIGAND BINDING SITE

**Fig 4.** Human pro-u-PA binding of u-PAR–binding domain; human/murine, murine/human, and human/alanine substitution mutants targeted at the N-terminal region between amino acid residues 1-13 (A–C). 35S-cysteine–labeled polypeptides, containing the substitutions detailed in Fig 5, were synthesized in in vitro translation reactions and mixed with human pro-u-PA-Sepharose. On these gels, equal amounts of TCA-precipitable u-PAR polypeptides were used in each binding reaction, except for JP4 in (A) of the amount was used. Protein complexes were separated by SDS-PAGE and labeled proteins detected by fluorography. One representative experiment of four is shown. (D–E) Competition controls of pro-u-PA binding by certain key constructs scoring weak or intermediate signals in the assay, with 0.1 mmol/L of unlabeled soluble human u-PAR (INXS), demonstrating ligand-binding specificity and accuracy. Note the difference in specific binding avidity between JP31 and JP31B, the two “minimally humanized” mu-PAR constructs, the former carrying four human residues (see Fig 5) and the latter with human residues at positions 2 and 7 only.

binding activity. Interestingly, this region is completely conserved between the human and the mouse receptors. A double-mutation JP40 (R58A K62A) displayed only a slightly reduced binding activity to pro-u-PA (Fig 7). In conclusion, the highly charged region between amino acids 47 and 53 was found to be important for binding activity. Although the exact structural reason for this result is not known, the fact that this sequence is conserved suggests that
Fig 5. Summary showing the structure of all substitution mutants targeted at the region between amino acids 2726-2731. The left column represents the nomenclature and specification of all u-PAR binding domain mutants made in this region. The right column shows the individual binding score of each mutant (bound radioactivity): ++, 80% to 100% binding; +, 30% to 80%; (+), <30%; --, very weak or no detectable binding activity. This semiquantitative estimate of the binding avidity, densitometrical scanning of autoradiographs was performed from experiments, in which constant amounts of [125I]-ATF bound were graphically estimated from these plots. Table 1. Binding of [125I]-ATF to LB6 Cells Expressing u-PAR Chimeras

<table>
<thead>
<tr>
<th>u-PAR Construct</th>
<th>Bound (cpm)</th>
<th>% Binding</th>
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<tbody>
<tr>
<td>H-PAR (C119)</td>
<td>18,387</td>
<td>100</td>
</tr>
<tr>
<td>hu/mu JP14</td>
<td>17,326</td>
<td>94.2</td>
</tr>
<tr>
<td>hu/mu JP16</td>
<td>16,485</td>
<td>89.7</td>
</tr>
<tr>
<td>Mouse u-PAR</td>
<td>2,104</td>
<td>11.4</td>
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<tr>
<td>LB6-neo</td>
<td>2,001</td>
<td>10.9</td>
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LB6 cells were stably transfected with cDNA constructs and analyzed for ligand-binding activity, using [125I]-ATF (200,000 cpm/well). Surface expression was confirmed with the anti-hu-PAR monoclonal antibody 3R6, by immunofluorescence analysis. Semiconfluent layers were used (1.5 x 10^5 cells/2 cm^2), and ATF binding is expressed as total counts bound per well, as well as the percentage of specific binding, determined by using 200 nmol/L cold ligand as a competitor. The concentration of ATF bound was 0.22 nmol/L for 100% binding. SE values for the ligand binding data were less than 4%.

must be the case of the amino terminal domain of u-PAR, since cleavage by chymotrypsin released an 87-residue fragment with conserved ligand-binding activity.36 u-PAR is extremely rich in cysteine (28 of 282 residues of the mature membrane-anchored protein), the spacing of which is uniquely conserved. u-PAR appears to be a highly compact molecule in structure; in fact, it is quite resistant to proteolytic cleavage.36 However, chymotrypsin and AspN are able to efficiently cleave u-PAR at specific positions.36 It is not yet known whether or how the cysteines are arranged in disulfide bonds, but their existence is implied by the compact nature of the molecule and by its localization on the extracellular side of the plasma membrane.

This study has confirmed that the N-terminal domain of it could serve an overall structural function that is needed to allow the correct folding of the ligand-binding pocket, or it could be participating in a direct contact with the ligand. As this segment is the most hydrophilic one of the entire binding domain, the two possibilities need not to be mutually exclusive. Future x-ray crystallographic data are likely to resolve this issue.

DISCUSSION
The primary structure of u-PAR suggests that the molecule is composed of three domains similar to an original 92-residue polypeptide, and that these repeats constitute functionally independent domains.33,36,37 In particular, this...
hu-PAR is independently responsible for the ligand-binding function, and I have further identified two essential subregions by homologue and alanine-scanning mutagenesis. One distinct region between hu-PAR residues 2 and 10, RCMQCKTNG, appears to be critical for the ligand-binding specificity; a second region between amino acids 47 and 53, HSEKTNR, was likewise found to be important for ligand binding.

One surprising aspect of these results was that the mutant u-PAR products of in vitro translation appeared to bind pro-u-PA with or without the presence of a signal peptide. This feature is of particular interest, as the most N-terminal part of each receptor contains the sequences that discriminate between human and murine ligands (Fig 2). The major contribution of the microsomal membranes to this system was found to be a constant decrease in expression levels, rather than any significant variations in binding affinity. Unprocessed, yet functionally active intracellular receptor forms—with signal peptides attached—can also be detected in several types of transfected and wild-type cell cultures by chemical cross-linking (Pöllänen, unpublished observation). Therefore, in this respect, the conclusions drawn from the present in vitro measurements lacking the membranes may be considered relevant to the case in vivo.

The region of the first 13 residues identified as important for binding specificity is short, and probably localizes key residues rather than defines the entire contact surface. Within this segment, the recently published sequence of bovine u-PAR differs from the human sequence at positions 4, 7-11, and 13; and from the murine sequence at residues 2, 4, 8-10, and 13. Whether these new pieces of information also explain why the hu-PAR seems to act in a more specific way than the bovine receptor, which is able to bind both human and murine ligands, requires further confirmation from studies using a three-dimensional structural approach. In general, it appears that interspecies conservation is a little more pronounced for the ligands than the so-far cloned u-PARs. The second region, between residues 47 and 53, is fully conserved between the mouse and human receptors and shares three important charged amino acids with bovine u-PAR. This region also contains the only glycosylation site (Asn52) of the human u-PAR-binding domain. Interestingly, a single point mutation at this particular site is able to induce a significant decrease in the affinity for the ligand, when tested in transfected LB6 cells. The present results indicate that domains 2 and 3 are not critically required for the ligand-binding function. Therefore, I propose that the cysteine-rich domain 1 might provide a framework on which shorter segments, like those two now identified, are optimally arranged to allow direct interaction. Such a spatial structure may prove hard to mimic using synthetic peptides (Pöllänen, unpublished observations). Clearly, additional work, including crystallization of the ligand-receptor complex, remains to be done to define further the specific interaction between u-PAR and pro-u-PA. At present, no such information is available for any of the Ly-6 receptor family. However, these data on the ligand-binding site of u-PAR may also have important implications for future modeling of these interactions in other structurally similar members of the Ly-6 family.

The functional significance of the u-PAR:pro-u-PA interaction is linked to the context of cell surface plasminogen activation, as it provides a mechanism for proteolytic events to escape the dominating effect of soluble plasmin inhibitors, normally presented in great abundance in extracellular fluids. An additional mechanism highlighting the role of
the receptor, u-PA interaction seems to be the augmentation of the enzymatic activity of u-PA upon binding to u-PAR. In particular, studies of cellular invasiveness have demonstrated the need for u-PA to act receptor-bound to become biologically efficient. Not only can u-PA-dependent tumor cell dissemination occur when the cells express both u-PA and its receptor, it was recently observed that a paracrine interaction for u-PA and u-PAR could operate in an in vivo model of invasion, by genetic complementation analysis. Against this biological background, it is evident that the careful definition of the binding regions in u-PAR will allow more rational strategies for interfering with the process of cell surface plasminogen activation, e.g., via the development of potential new antagonists for the urokinase receptor.

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