Ligand Binding Regions in the Receptor for Urokinase-type Plasminogen Activator

STRUCTURAL REQUIREMENTS FOR A MULTIDOMAIN BINDING REGION AND RECEPTOR-RECEPTOR INTERACTION

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The interaction between urokinase plasminogen activator (uPA) and its cellular receptor (uPAR) is a key event in cell surface-associated plasminogen activation, relevant for cell migration and invasion. In order to define receptor recognition sites for uPA, we have expressed uPAR fragments as fusion products with the minor coat protein on the surface of M13 bacteriophages. Sequence analysis of cDNA fragments encoding uPA-binding peptides indicated the existence of a composite uPA-binding structure including all three uPAR domains. This finding was confirmed by experiments using an overlapping 15-mer peptide array covering the entire uPAR molecule. Four regions within the uPAR sequence were found to directly bind to uPA: two distinct regions containing amino acids 13–20 and amino acids 74–84 of the uPAR domain I, and regions in the putative loop 3 of the domains II and III. All the uPA-binding fragments from the three domains were shown to have an agonistic effect on uPA binding to immobilized uPAR. Furthermore, uPAR-(154–176) increased uPAR-transfected BAF3-cell adhesion on vitronectin in the presence of uPA, whereas uPAR-(247–276) stimulated the cell adhesion both in the absence or presence of uPA. The latter fragment was also able to augment the binding of vitronectin to uPAR in a purified system, thereby mimicking the effect of uPA on this interaction. These results indicate that uPA binding can take place to particular part(s) on several uPAR molecules and that direct uPAR-uPAR contacts may contribute to receptor activation and ligand binding.

Cell migration and invasion are important processes in many pathological situations, such as inflammation, angiogenesis, and tumor metastasis. The proteolytic cascade system of plasminogen activation, directed by the urokinase plasminogen activator (uPA) and its receptor (uPAR), has long been recognized as performing a central role in these processes (1). Experimental evidence of their involvement has come from studies in a variety of model systems, in which the ability of tumor cells to invade and metastasize could be down-regulated by uPA inhibitors (2), anti-uPA antibodies (3), and uPAR antagonists (4). Several reviews have provided details on the molecular and functional properties of the uPA-uPAR system and its role in extracellular matrix degradation (5–7). Recent investigations have indicated that uPAR also takes part in other protein interactions, relevant not only to proteolysis but also to cell adhesion and signal transduction, where uPAR serves as a pleiotropic interactive cell surface molecule (8–10).

Human uPAR is a glycoprotein encoded as a 335-residue non-processed polypeptide (11). The entire sequence of the fully processed and completely extracellular uPAR (amino acids 1–283) is divided into three homologous domains primarily defined by a conserved pattern of cysteine residues and linked to a glycosylphosphatidylinositol moiety (12–15). Although the N-terminal domain I of uPAR has the primary role in uPA binding (16), the integrity of the multidomain structure of uPAR is required for the maintenance of high affinity binding (17, 18). The specific, high affinity interaction between uPA and uPAR is governed by the growth factor-like region of uPA, which also contains a kringle domain and a serine protease domain (19). Besides uPA, uPAR is also able to interact with Vn, and uPAR promotes uPAR-dependent cell adhesion on Vn. Multimeric Vn, rather than monomers, serves as predominant high affinity ligand for uPAR. In addition, uPAR has been reported to interact with several other soluble and membrane proteins, such as high molecular weight kinogen and integrins (10).

In an approach to uncover the uPA recognition regions on the uPAR molecule and to identify epitopes responsible for promotion of uPAR-dependent cell adhesion on Vn, we have employed two techniques in the current investigation, i.e. phage display and peptide array. The phage display technique was first reported in 1985 by Smith and co-workers (20, 21) and relied on the ability to display a peptide of interest on the surface of a bacteriophage capsid. Goodson et al. (22) reported the identification and characterization of peptide antagonists with nanomolar affinity for the human uPAR by using a 15-mer phage display library. Utilizing the second method (23–25), the uPAR peptides were directly synthesized as spots on the cellulose membrane and used in solid phase binding tests. Together, four uPA-binding regions with distinct sequences on the uPAR molecule were identified and partially characterized, two of which could promote uPAR-mediated cell adhesion on Vn reminiscent of uPA itself. These results indicate that ligation with uPA can...
induce conformational changes to uPAR allowing direct uPAR-uPAR contacts and, thereby, may influence its functional activity in cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Materials—**Vn was purified from human plasma and converted to the multimeric form as described previously (26, 27). The plasmid pBSW87 containing the full-length uPAR cDNA was obtained from Dr. N. B. R. Coquet. The monoclonal antibodies (mAbs) R3 and R4 to uPAR (28) were a gift from Dr. G. Hayer-Hansen (both from the Finsen Laboratory, Copenhagen, Denmark). The phagemid vector pComb3B (29) was obtained from Dr. H. Pannekoek (University of Amsterdam, Amsterdam, The Netherlands). The expression vector pGEX-6P-1 and pBSW87 containing the full-length uPAR cDNA was obtained from Dr. W. H. Oostra (University of Amsterdam, Amsterdam, The Netherlands). The expression vector pGEX-6P-1 and pBSW87 containing the full-length uPAR cDNA was obtained from Dr. W. H. Oostra.

**Production of uPA-Fc Fusion Protein—**In previous studies, the uPAR contacts and, thereby, may influence its functional activity in cell adhesion.

**Affinity Selection of a uPA-Binding Phagemid Library**—Dynambeads M-280 (tosyl-activated) (Dynal, Hamburg, Germany) have been designed as a solid phase for biomagnetic separation, which have been applied among others also for screening of phage display libraries (32, 33). High molecular weight human uPA was coated onto tosyl-activated Dynambeads M-280 according to the manufacturer's instructions, and the uPA-coated beads (5 μg of uPA/10^6 beads in 1 ml) were stored in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) and 0.02% sodium azide at 4 °C. After washing three times in PBS, 100 μl of uPA-coated magnetic beads were blocked with 1.5 ml of PBS containing 3% BSA and 0.05% Tween 20 (PBST-3% BSA) for 1.5 h at room temperature. The beads were then resuspended in 50 μl of PBST-3% BSA and mixed with 50 μl of the non-diluted or in PBST-3% BSA 1:10. 1:100 or 1:1000 diluted phage display library, containing 1.6 × 10^11, 1.6 × 10^10, 1.6 × 10^9, or 1.6 × 10^8 phagemid particles, respectively, at room temperature for 2 h with gentle agitation. After binding, the bead suspension was transferred into a new 1.5-ml Eppendorf tube to avoid plastic-bound phagemid particles. With the help of a magnetic particle concentrator (DynaMPC E-1), the beads carrying uPA-binding phages were separated from the supernatant containing uPA-non-binding phages, which, after proper dilution, were used to infect cells of E. coli XL-1 Blue MRF' were purchased from Stratagene (Amsterdam, The Netherlands). The plasmid pCDNA3 and BstXI linker were from Invitrogen (Groningen, The Netherlands). Human high molecular weight uPA, antibody 3936 to uPAR, and polyclonal rabbit antibodies against uPAR were from American Diagnostica (Greenwich, CT). Recombinant human soluble uPAR (suPAR) from insect cells was a gift from Dr. D. Cines (University of Pennsylvania, Philadelphia, PA). Recombinant bacterial uPA (Saruplase) was from Grünenthal (Stolberg, Germany). All other chemicals and reagents were of analytical grade.

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Expression and Purification of uPA-binding Fragments as GST Fusion Proteins—The uPAR fragments selected from the phage display library were cloned into expression vector pGEX-6P-1 and expressed as GST fusion proteins. The fusion proteins as well as GST were purified through a GSTrap prepacked column. Cloning, expression, and purification were performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The fusion proteins were recognized by polyclonal rabbit antibodies against uPAR (1.6 μg/ml) as well as antibodies against GST (1 μg/ml) in Western blots.

Microtiter Plate Binding Assay—The microtiter plate binding assay was performed as described previously elsewhere (34). Briefly, high molecular weight human uPA at a concentration of 5 μg/ml in PBS was coated onto Maxisorb 96-well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4 °C. After the wells were blocked with 3% BSA-PBST for 1.5 h at 37 °C, various concentrations of GST/GST-uPAR fragments (0–2 μM) and suPAR (90 nM) in blocking buffer were added and incubated for another 1 h. Bound suPAR was detected by the antibody R4 (10 μg/ml), which was further detected by 1:1000 diluted peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Hamburg, Germany). Bound peroxidase was quantified using 2,2’-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) as substrate (Roche Applied Science, Mannheim, Germany). Similarly, suPAR was coated at a concentration of 2.5 μg/ml in PBS overnight at 4 °C. After BSA blocking, various concentrations of GST/GST-uPAR fragments (0–2 μM) and uPA-Fc (8 μg/ml) or Vn (2 μg/ml) in the absence or presence of uPA (50 nM), in blocking buffer were added and incubated for another 1 h. Bound uPA-Fc was detected by 1:5000 diluted peroxidase-conjugated rabbit anti-mouse immunoglobulins, and bound Vn was detected by antibody Vn-7 (125 ng/ml) against Vn (35). Bound peroxidase was quantified as above. Nonspecific binding to BSA-coated wells was used as a blank and was subtracted to calculate specific binding. Experiments were performed in duplicate and repeated at least three times.

Cell Culture and Cell Adhesion Assay—BAF-3 (interleukin-3-dependent mouse B-cell line) cells were from the American Type Culture Collection (ATCC, Rockville, MD), and cultured in RPMI 1640 medium containing 10% fetal calf serum and 2 mg/ml interleukin-3. BAF-3 cells were transfected by electroporation with uPAR cDNA in the sense and antisense orientation using the expression vector pCDNA3. Cells were selected in the presence of G418 (1.2 mg/ml) (Calbiochem, San Diego, CA) and determined to express uPAR by fluorescence-activated cell sorter analysis, Northern blotting, and uPAR-enzyme linked immunosorbent assay. Cell adhesion of uPAR sense-transfected BAF-3 cells to Vn-coated plates was performed according to a previously described protocol (34, 36). Briefly, multiwell plates were coated with 2 μg/ml Vn and blocked with 3% BSA. Cells were washed in serum-free RPMI and plated onto the precoated wells for 60 min at 37 °C in the absence or presence of competitors in serum-free RPMI. After the incubation period, the wells were washed and the number of adherent cells was quantified by crystal violet staining at 590 nm.

RESULTS

Construction and Characterization of a uPAR Random Epitope Phage Library—The phagemid library contained a total of 1.67 × 10^14 clones with a phagemid titer of 3.2 × 10^12 phagemids/ml. Eighteen randomly picked phagemid clones from this library contained a uPAR cDNA fragment with a size of 30–120 bp from either strand, which were distributed over the entire uPAR cDNA sequence (data not shown).

Affinity Selection, Analysis, and Alignment of uPA-binding uPAR Fragments—Affinity selection of uPA-binding phagemid particles was performed as described, and the results are summarized in Table I. From the eluted phagemid particles, 30
FIG. 3. Sequences and alignment of uPA-binding uPAR fragments obtained from phage display and peptide array experiments. Overlapping regions from these two independent selections are framed.

A

Urokinase-binding fragments in uPAR domain I:

| Sequence | Alignment |
|----------|-----------|
| uPAR (5-20) | □□□□□□□□□□ □□□□□□□□□□ |
| uPAR (13-33) | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-11 | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-12 | □□□□□□□□□□ □□□□□□□□□□ |
| uPAR (24-38) | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-17 | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-18 | □□□□□□□□□□ □□□□□□□□□□ |

B

Urokinase-binding fragments in uPAR domains II and III:

| Sequence | Alignment |
|----------|-----------|
| uPAR (64-84) | □□□□□□□□□□ □□□□□□□□□□ |
| uPAR (74-122) | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-23* | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-24* | □□□□□□□□□□ □□□□□□□□□□ |
| uPAR (154-176) | □□□□□□□□□□ □□□□□□□□□□ |
| SPOT-34 | □□□□□□□□□□ □□□□□□□□□□ |

* Contains amino acids characterized by Gledhill et al (Arg[5], Leu[7] and Tyr[7])

FIG. 4. Interference of the uPAR fragments with binding of uPA-Fc to immobilized suPAR. Top, effect of the uPA-binding fragments from domain I on the binding of uPA to immobilized uPAR. Bottom, effect of the uPA-binding fragments from domains II and III on the binding of uPA to immobilized uPAR. As a control, both GST and uPA-non-binder uPAR (198–213) were used in the assay.
clones from the pH 5.2 elution, 30 clones from the pH 2.2 elution, and 120 clones from the E. coli adsorption were subjected to plasmid extraction and DNA insert sequencing. All clones were found to contain a DNA insert. Only one of the clones eluting at pH 5.2, uPAR-(26–38), had a partial uPAR peptide, whereas 9 of the 120 clones eluted by E. coli adsorption, designated as, e.g., uPAR-(5–20), had a correct insert orientation and a correct open reading frame expressing a uPAR peptide. Results of the analysis and alignment of uPA-binding uPAR fragments is presented in Fig. 1, with de-
the two independent screening procedures for uPA-binding uPAR peptides have produced largely congruent results.

Interference of the GST Fusion uPAR Fragments with the uPA-uPAR Interaction—When uPA binding to immobilized uPAR was performed, all the uPA-binding uPAR fragments from its three domains, and especially the two fragments from the domains II and III were shown to have an agonistic effect, whereas the uPA-non-binding uPAR fragment uPAR-(198–213) and GST had no effect (Fig. 4). The influence of uPAR fragments was also studied in a reverse system utilizing binding of suPAR to immobilized uPA. Interestingly, whereas the fragments uPAR-(5–20) and uPAR-(13–33) as well as uPAR-(64–84) and uPAR-(74–122) from the domain I could partially inhibit suPAR binding to immobilized uPA, the fragments uPAR-(154–176) and uPAR-(247–276) from the domains II and III again increased the association between suPAR and uPA (data not shown). Additionally, in the converse experiment, the fragment uPAR-(198–213) and GST had no effect on suPAR binding to immobilized uPA.

Role of the GST Fusion uPAR Fragments in uPAR-dependent Cell Adhesion to Vn—As established previously, the adhesion of leukocytes to immobilized Vn is predominantly mediated by uPAR, and uPA augments this adhesion by increasing the affinity of the Vn-uPAR interaction (38). Only if BAF-3 cells were transfected with full-length uPAR-cDNA did they adhere to Vn and display uPA-augmented adhesion (36). Consequently, the influence of various uPAR fragments at different concentrations on uPAR-transfected BAF-3 cell adhesion to Vn in the absence or presence of uPA (50 nM) was tested. Fragment uPAR-(247–276) from the domain III increased Vn adhesion both in the absence or presence of uPA, whereas uPAR-(154–176) from the domain II stimulated adhesion only in the presence of uPA (Fig. 5, A and B). Neither peptide stimulated adhesion of non-transfected BAF-3 cells to Vn. The uPAR-(247–276)-induced cell adhesion to Vn was blocked by anti-uPAR mAb R3 and mAb 3936, respectively, which are known to inhibit uPAR-mediated adhesion to Vn (Fig. 5C). A similar reactivity of both peptides was observed in a purified system when the uPAR fragments (1 μM) were tested in the binding of Vn to immobilized uPA. Although uPAR-(247–276) enhanced the Vn-uPAR interaction in a dose-dependent manner in the absence or presence of uPA (50 nM), uPAR-(154–176) stimulated only the uPA-induced Vn-binding to uPAR (Fig. 6), whereas the other uPA-binding uPAR peptides had no effect on the Vn-uPAR interaction.

DISCUSSION

The urokinase plasminogen activator has been implicated in angiogenesis, fibrinolysis, wound healing, and tumor metastasis. The ability of uPA to participate in cell invasion and the remodeling of connective tissue through its enzymatic activity and to promote cell adhesion through non-enzymatic processes is modulated by interaction with its cellular receptor uPAR (10). The unique topography of uPAR appears to be essential for multiple binding contacts with uPA and other uPAR ligands, since isolated domains or proteolytic fragments of the receptor show by far less efficiency and affinity in ligand binding. The requirement of uPAR domain I as well as the contribution of domains II and III in ligand recognition has been reported, particularly in high affinity binding to uPA (16–18, 39–41). Gårdvoll et al. (37) have performed an alanine-scanning analysis of the putative loop 3 of uPAR domain I and have identified four positions (Arg53, Leu55, Tyr57, and Leu66) exhibiting significant changes in the contribution to the free energy of uPA binding upon single-site substitutions to alanine. Furthermore, a recent report suggested that a region in domain II of uPAR (Arg137–Arg145) plays a role in uPA binding (42).

Utilizing two different analytical approaches, the binding of uPA to several distinct regions in uPAR, including the above described uPA-binding determinants was observed. The regions in the putative loop 3 of domains II and III, uPAR-(154–176) and uPAR-(247–276), were shown to have an agonistic influence on the uPA-uPAR interaction independent of the experimental setting, whereas the fragments from domain I could either augment the binding of uPA to immobilized suPAR or partially inhibit suPAR binding to immobilized uPA. However, the physiological relevance of the observed inhibition caused by the fragments from the domain I in the latter experimental setting remains to be further explored. Additional contact sites between uPA and its receptor cannot be excluded, since the constraints imposed within the receptor may well be different from those displayed by phage or immobilized on nitrocellulose membrane. Shliom et al. (43) reported novel interactions between uPA and its receptor and postulated that suPAR in solution is present in
equilibrium between oligomer/dimer/monomer forms, and that the mixed population of uPAR might alter the affinity as well as the kinetics with regard to binding of uPA. Our results are the first direct experimental evidence for the fact that direct uPAR-uPAR contacts may contribute to the functional activities of the receptor. This contention is based on the observation that the fragment uPAR-(154–176) in the domain II and the fragment uPAR-(247–276) in domain III could promote uPAR-dependent cell adhesion on Vn either in the presence of uPA or independently of uPA stimulation in the latter case.

The display of proteins or their functional domains provided a system for the analysis of structure-function relationships, and the potential to generate proteins with altered binding characteristics or novel catalytic properties (44–46). The phagemid pComb3B was employed here to construct a uPAR phage display random epitope library, which required a library of only limited size in contrast to random peptide epitope mapping (29). Through affinity selection, phagemid particles displaying a functional epitope, e.g., uPA binding, could be selected from the library and by sequence analysis of the corresponding DNA fragment, the primary structure could be determined. The reasons that, in our study, the uPAR phage library was panned for only one round against immobilized uPA were as follows: first, to cover all possible uPA-binding regions; second, to minimize identical clones; and third, to avoid the risk of losing uPA-binding uPAR fragments. Although the peptide array screening (see Fig. 2) was performed under physiological conditions, the uPA-binding phage-displayed uPAR fragments were obtained after pH 5.2 and 2.2 treatment, which suggested considerable affinity for the interactions. The two independent screening procedures for uPA-binding uPAR-derived peptides have generated largely congruent results. Both the SPOT membrane uPAR peptide array as well as the uPAR phage display library have proved useful in determination of binding motifs, whereas the latter has the advantage over immobilized linear peptides that the fused uPAR fragments have a greater potential to adapt to a structural conformation. It is worth noting that both experimental approaches in this study share technical limitations, and that not all possible linear sequences were taken into account, nor were the non-attainable discontinuous sequences. In addition, some of these sequences may not be expressed within the intact receptor, whereas other undetected discontinuous sequences may dominate binding. Antibody and peptide inhibition studies with intact cellular receptor are currently being carried out in order to achieve a complete understanding of the physiological relevance of specific parts of the uPAR molecule.

Our results confirm the existence of a composite uPA-binding structure including all three uPAR domains and that an interdomain cooperation involving all three domains appears to be required to achieve an appropriate functional conformation of uPAR (Fig. 7). Furthermore, our data also support the notion that, when uPA binds to uPAR, both components of the complex undergo conformational changes required for cell adhesion, signal transduction, or the induction of enzymatic activity (47–50). The former aspect was analyzed in more detail using uPAR-transfected BAF3 cells. The results from adhesion experiments to Vn indicated that uPAR-(247–276) could stimulate uPAR-dependent adhesion by increasing both uPAR-uPA and uPAR-Vn interactions, whereas uPAR-(154–176) could only affect uPA binding to uPAR and thereby augmented cell adhesion. The fragment uPAR-(247–276) could possibly by itself induce a conformational change and expose Vn-binding site(s) of the receptor, acting in a manner similar to that of uPA. In the presence of uPA, the fragment of domain III would in a non-competitive manner enhance the uPA effect synergistically. The fragment uPAR-(154–176), on the other hand, could either, in a non-competitive manner, directly enhance the effect of uPA in terms of exposing Vn-binding site(s) of the receptor or, in the presence of uPA, unfold uPAR molecule exposing domain III and augment the effect induced by the fragment uPAR-(247–276).

In agreement with our findings, Trigwell et al. (51) observed that incubation of macrophages with either full-length or two-domain (domains II + III) soluble uPAR could increase the adhesion of THP-1 cells to vitronectin and fibronectin, and this effect was independent of the epitope SRSRYLE. Similarly, a synthetic peptide comprising the central heparin binding region of Vn (residues 348–361) not only could bind to plasma Vn but also induced multimerization of Vn (27). Moreover, Sitrin et al. (52) demonstrated that clustering of uPAR induces proinflammatory signaling in human polymorphonuclear neutrophils. Taken together, these results indicate that uPAR activation can occur (a) by direct contact between several receptor molecules and (b) when multiple binding sites on different uPAR molecules serve to form a composite ligand-receptor complex.

These aspects can well be related to recent observations on cluster formation of uPAR within lipid rafts or caveolae (48) and the cross-talk of uPAR with other receptors such as integrins (50). Based on the present data, clustering of uPAR could occur through contacts involving uPAR-(154–176) and uPAR-(247–276) regions, followed by interactions of these clusters with integrins, both in cis or trans interactions. Additionally, such uPAR-uPAR contacts might play a role in cell surface-associated plasminogen activation and this hypothesis is currently under investigation. Whether fragments of uPAR derived from shedding and cleavage of the receptor in vivo (53) have any potential biological activities similar to those observed in our study remains to be analyzed.

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REFERENCES
1. Blasi, F. (1997) ImmunoL. Today 18, 415–417
2. Rabbani, S. A., Harakidas, P., Davidson, D. J., Henkin, J., and Mazar, A. P. (1995) Int. J. Cancer 63, 840–845
3. Oosowski, L., and Reich, E. (1988) Cell 55, 611–619
4. Cremona, C. W., Cohen, E., Liu, G., Shuman, M. A., and Levinson, A. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5021–5025
5. Andreassen, P. A., Kjølner, L., Christensen, L., and Duffy, M. J. (1997) Int. J. Cancer 72, 1–22
6. Blasi, F. (1999) Thromb. Haemostasis 82, 298–304
7. Andreassen, P. A., Egelund, R., and Petersen, H. H. (2000) Cell Mol. Life Sci. 57, 25–40
8. Chapman, H. A., Wei, Y., Simon, D. I., and Waltz, D. A. (1999) Thromb. Haemostasis 82, 291–297
9. Koshelnick, Y., Ehrati, M., Stockinger, H., and Binder, B. R. (1999) Thromb. Haemostasis 82, 305–311
10. Preisnner, K. T., Kanse, S. M., and May, A. E. (2000) Curr. Opin. Cell Biol. 12, 621–628
11. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Dano, K., Appella, E., and Blasi, F. (1990) EMBO J. 9, 467–474
12. Ploog, M., Ronne, H., Behrendt, N., Jensen, A. L., Blasi, F., and Dano, K. (1991) J. Biol. Chem. 266, 1926–1933
13. Behrendt, N., Ploog, M., Ronne, K., Hoyer-Hansen, G., and Dano, K. (1993) Methods Enzymol. 223, 207–223
14. Ploog, M., and Ellis, V. (1994) FEBS Lett. 349, 163–168
15. Behrendt, N., and Stephens, E. W. (1998) Fibrinolysis Protocols 12, 191–204
16. Behrendt, N., Ploog, M., Pathly, H., Houen, G., Blasi, F., and Dano, K. (1991) J. Biol. Chem. 266, 7842–7847
17. Ploug, M., Ellis, V., and Dano, K. (1994) Biochemistry 33, 8991–8997
18. Behrendt, N., Ronne, K., and Dano, K. (1996) J. Biol. Chem. 271, 22885–22894
19. Appella, E., Robinson, E. A., Ulrich, S. J., Stoppelli, M. P., Corti, A., Cassani, G., and Blasi, F. (1987) J. Biol. Chem. 262, 4437–4440
20. Smith, G. P. (1985) Science 228, 1315–1317
21. Scott, J. K., and Smith, G. P. (1990) Science 240, 386–390
22. Goodman, R. J., Doyle, M. V., Kaufman, S. E., and Rosenberg, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7129–7133
23. Frank, R. (1995) *J. Biotechnol.* **41**, 259–272
24. Frank, R., and Overwin, H. (1996) *Methods Mol. Biol.* **66**, 149–169
25. Tegge, W. J., and Frank, R. (1998) *Methods Mol. Biol.* **87**, 99–106
26. Preissner, K. T., Wasmuth, R., and Müller-Berghaus, G. (1985) *Biochem. J.* **231**, 349–355
27. Stockmann, A., Hess, S., DeClerck, P., Timpl, R., and Preissner, K. T. (1993) *J. Biol. Chem.* **268**, 22874–22882
28. List, K., Hoyera-Hansen, G., Ronne, E., Dano, K., and Behrendt, N. (1999) *J. Immunol. Methods* **222**, 125–133
29. van Zonneveld, A. J., van den Berg, B. M., van Meijer, M., and Pannekoek, H. (1995) *Gene (Amst.)* **167**, 349–355
30. Tressler, R. J., Pitot, P. A., Stratton, J. R., Forrest, L. D., Zhuo, S., Drummond, R. J., Fong, S., Doyle, M. V., Doyle, L. V., Min, H. Y., and Rosenberg, S. (1999) *APMIS* **107**, 168–173
31. Nelles, L., Lijnen, H. R., Collen, D., and Holmes, W. E. (1987) *J. Biol. Chem.* **262**, 5682–5689
32. Hawkins, R. E., Russell, S. J., and Winter, G. (1992) *J. Mol. Biol.* **226**, 889–896
33. Russell, S. J., Hawkins, R. E., and Winter, G. (1993) *Nucleic Acids Res.* **21**, 1081–1085
34. Chavakis, T., Kans, S. M., Lupu, F., Hammes, H. P., Muller-Esterl, W., Pixley, R. A., Colman, R. W., and Preissner, K. T. (2000) *Blood* **96**, 514–522
35. Kost, C., Stuber, W., Ehrlich, H., Pannekoek, H., and Preissner, K. T. (1992) *J. Biol. Chem.* **267**, 12098–12105
36. Chavakis, T., May, A. E., Preissner, K. T., and Kanse, S. M. (1999) *Blood* **93**, 2976–2983
37. Gårdenfors, H., Dano, K., and Ploug, M. (1999) *J. Biol. Chem.* **274**, 37985–38003
38. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. (1994) *J. Biol. Chem.* **269**, 32380–32388
39. Ronne, E., Behrendt, N., Ellis, V., Ploug, M., Dano, K., and Hoyera-Hansen, G. (1991) *FEBS Lett.* **288**, 233–236
40. Pollanen, J. J. (1993) *Blood* **82**, 2719–2729
41. Ploug, M. (1998) *Biochemistry* **37**, 16494–16505
42. Bdeir, K., Kuo, A., Mazar, A., Sachs, B. S., Xiao, W., Gawlak, S., Harris, S., Higazi, A. A., and Cines, D. B. (2000) *J. Biol. Chem.* **275**, 28532–28538
43. Shliom, O., Huang, M., Sachs, B., Kuo, A., Weisel, J. W., Nagaswami, C., Nassar, T., Bdeir, K., Hess, E., Gawlak, S., Harris, S., Mazar, A., and Higazi, A. A. (2000) *J. Biol. Chem.* **275**, 24304–24312
44. O'Neil, K. T., DeGrado, W. F., Mosu, S. A., Ramachandran, N., and Hoess, R. H. (1994) *Methods Enzymol.* **245**, 570–586
45. Hill, H. R., and Stockley, P. G. (1996) *Mol. Microbiol.* **20**, 685–692
46. Hoogenboom, H. R., and Chames, P. (2000) *Immunol. Today* **21**, 371–378
47. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) *Science* **273**, 1551–1555
48. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) *J. Cell Biol.* **144**, 1285–1294
49. Dumler, I., Stepanova, V., Jerke, U., Mayboroda, O. A., Vogel, F., Bouvet, P., Tkachuk, V., Haller, H., and Galha, C. (1999) *Curr. Biol.* **9**, 1468–1476
50. Tarui, T., Mazar, A. P., Cines, D. D., and Takada, Y. (2001) *J. Biol. Chem.* **276**, 3983–3990
51. Trigwell, S., Wood, L., and Jones, P. (2000) *Biochem. Biophys. Res. Commun.* **278**, 440–446
52. Sitrin, R. G., Pan, P. M., Harper, H. A., Todd, R. F., Harsh, D. M., and Blackwood, R. A. (2000) *J. Immunol.* **165**, 3341–3349
53. Sidenius, N., Sier, C. P., and Blazi, P. (2000) *FEBS Lett.* **475**, 52–56

**Ligand Binding Regions in uPAR**
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