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The Receptor-binding Sequence of Urokinase

A BIOLOGICAL FUNCTION FOR THE GROWTH-FACTOR MODULE OF PROTEASES*

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Previous studies have shown that the region of human urokinase-type plasminogen activator (uPA) responsible for receptor binding resides in the amino-terminal fragment (ATF, residues 1–135) (Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 4939–4943). The area within ATF responsible for specific receptor binding has now been identified by the ability of different synthetic peptides corresponding to different regions of the amino terminus of uPA to inhibit receptor binding of ^{125}I-labeled ATF. A peptide corresponding to human [Ala^{19}]uPA-(12–32) resulted in 50% inhibition of ATF binding at 100 nM. Peptides uPA-(18–32) and [Ala^{13}]uPA-(9–20) inhibit at 100 and 2000 μM, respectively. The human peptide uPA-(1–14) and the mouse peptide [Ala^{20}]uPA-(13–33) have no effect on ATF receptor binding. This region of uPA is referred to as the growth factor module since it shares partial amino acid sequence homology (residues 14–33) to epidermal growth factor (EGF). Furthermore, this region of EGF is responsible for binding of EGF to its receptor (Komoriya, A. Hortsch, M., Meyers, C., Smith, M., Kanety, H., and Schlessinger, J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1351–1355). However, EGF does not inhibit ATF receptor binding. Comparison of the sequences responsible for receptor binding of uPA and EGF indicate that the region of highest homology is between residues 13–19 and 14–20 of human uPA and EGF, respectively. In addition, there is a conservation of the spacings of four cysteines in this module whereas there is no homology between residues 20–30 and 21–33 of uPA and EGF. Thus, residues 20–30 of uPA apparently confer receptor binding specificity, and residues 13–19 provide the proper conformation to the adjacent binding region.

Plasminogen activators catalyze the proteolytic activation of plasminogen to the broad-specificity protease plasmin (1) which can activate or degrade extracellular proteins (zymogens, fibrinogen, fibronectin, etc.). Thus, plasminogen activators can regulate extracellular proteolysis, fibrin clot lysis, tissue remodeling, developmental cell migration, and such pathological processes as inflammation and metastasis (2). Two types of plasminogen activators are known, tissue-type (tPA)[1] and urokinase-type (uPA). tPA has been primarily associated with fibrinolytic function, while uPA is believed to also have a regulatory role in other forms of extracellular proteolysis (3). uPA is synthesized and secreted as a single-chain pro-uPA (4). The expression of the uPA gene (5) is controlled by growth factor stimulation in normal quiescent cells (6), in a manner similar to that shown for the oncogenes *myc* and *fos* (7, 8). Synthesis of uPA by normal fibroblasts is maximal during logarithmic growth (when uPA is membrane-associated) and is decreased as the cells reach confluence (9). Normal and neoplastic cells possess a specific uPA receptor (10–12) which may serve to focus the proteolytic activity of uPA at the cell membrane.

In several types of malignant cells it has been shown that membrane uPA receptors are fully occupied by biosynthetic uPA, via an autocrine mechanism (12), since they overexpress the uPA gene and are insensitive to growth regulation of uPA gene expression (13). uPA binds to its receptor with high affinity through its amino-terminal portion (11). Part of this region has sequence homology with the murine epidermal growth factor (EGF) and is referred to as the growth factor (GF) module (14). EGF is a 53-residue, three-disulfide bridged, single-chain peptide which binds to a specific surface receptor having cytoplasmic tyrosine-kinase activity which mediates its mitogenic and pleiotropic activities (15, 16). The receptor-binding region of EGF has been identified through the use of biologically active synthetic peptides (17, 18). This region is located within residues 19–31 (see Fig. 5) which is within one of the three disulfide loops of EGF. In this paper we show that the receptor binding domain of uPA is located within the GF module, in a position equivalent to that of EGF.

MATERIALS AND METHODS

Human urinary urokinase-type plasminogen activator (uPA) was obtained from Lepetit S. P. A. Laboratories (Milan, Italy). Degradation products of the enzyme produce by incubation at pH 8.0 were separated by gel filtration on Sephadex G-100 as described (11). Fractions containing peptides with a molecular weight of 17,000 were pooled and subjected to cation-exchange chromatography on a Mono S HR 5/5 column (11). Mono S fractions were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Amino-terminal sequencing of peptides was performed on an Applied Biosystems 470A Protein Sequencer. Phenylthiohydantoins were analyzed by HPLC on a Hewlett-Packard 1090A instrument. Solid-phase synthesis (19) of peptides was performed with an Applied Biosystems 430A Peptide Synthesizer; peptides were purified by reverse-phase HPLC. Oxidization of peptides was carried out as described (20).

ATF was radioiodinated and binding assay was performed as described (10, 12). Briefly, 1×10^6 U937 cells were washed in binding buffer (serum-free RPMI 1640 medium containing 20 mM HEPES (pH 7.5) and 1 mg/ml of bovine serum albumin), then incubated with ^{125}I-labeled ATF in binding buffer for 150 min at 4 °C. For binding

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[1] The abbreviations used are: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; ATF, amino-terminal fragment; EGF, epidermal growth factor; GF, growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.

inhibition studies, peptides were dissolved in binding buffer and examined at various concentrations for their ability to inhibit ^{125}I-labeled ATF binding.

RESULTS AND DISCUSSION

Incubation of uPA at pH 8.0, at room temperature, results in the degradation of the enzyme; different fragments from that degradation have been isolated and used to identify the receptor-binding region. Initially, uPA is degraded into two fragments which can be separated by gel filtration. We have previously shown that the carboxyl-terminal (catalytically active) half-molecule has no binding activity, while the amino-terminal fragment (ATF, residues 1–125) contains all of the binding determinants (11). When the fraction containing ATF was chromatographed on a Mono S column, three fractions were obtained (11). Fraction I, eluting at the highest salt concentration, has been previously shown to be the intact receptor-binding ATF (11). The two other fractions (II and III) eluted at lower salt concentrations. Nevertheless, they had the same amino acid composition as ATF (data not shown). Fig. 1 shows the electrophoretic behavior of fractions I, II, and III on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. Although the electrophoretic mobility of the ATF (fraction I) was not changed by reduction of disulfide bonds, the apparent molecular weights of fractions II and III were decreased after reduction from 17,000 to about 14,000. These results indicate that fractions II and III consist of ATF molecules having additional internal proteolytic cleavages apparent only upon reduction. We have tested whether fractions II and III can bind to the urokinase receptor in human U937 cells by competition with ^{125}I-labeled ATF. As shown in Fig. 2, neither fraction II nor III competed with ATF at concentrations up to 10^{-7} M, indicating that the additional proteolytic cleavage of ATF has reduced the binding activity by at least several hundredfold. We therefore located the extra cleavage sites of fractions II and III by amino-terminal sequencing. As shown in Fig. 3, two amino-terminal sequences were presented in fraction II in approximately a 1:1 ratio. The knowledge of the amino acid sequence of uPA (21) allowed an unequivocal identification of both sequences: one was the amino-terminal sequence of ATF (Ser^{1}) while the other started at residue 24 of ATF (Tyr^{24}). A third amino-terminal sequence was also present which represented only about 21% of the total and this sequence started at Ser^{26} of the ATF (data not shown). Similarly, analysis of fraction III also revealed two major sequences and a third, minor, sequence. However, the major sequences, again in a 1:1 ratio, commenced at Ser^{1} and Ser^{26} while the minor sequence (13% of the total) started at Tyr^{24}, *i.e.* the major cleavage site in fraction III was the minor cleavage site in fraction II and vice versa. From these experiments, we conclude that fraction II primarily contains an ATF molecule cleaved at Lys^{23}-Tyr^{24} and that fraction III is an ATF molecule cleaved primarily at Phe^{25}-Ser^{26}. The binding data show that cleavage of the peptide chain, at either position 23 or 25, strongly decreases the ability of ATF to bind the uPA receptor. These results indicate a critical involvement of this region for receptor binding.

Four synthetic peptides of human uPA have been tested for

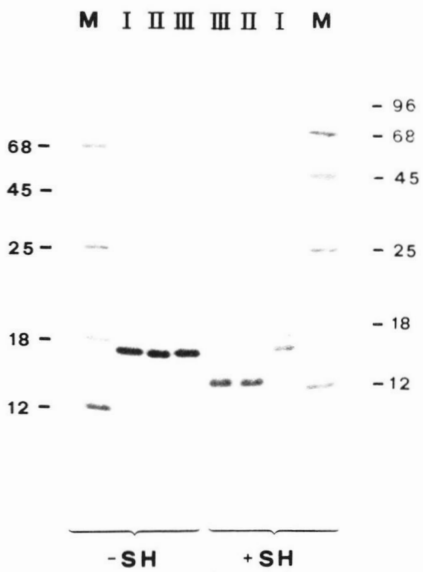

FIG. 1. **Electrophoretic analysis of amino-terminal fragments of human uPA.** Human urinary uPA (30 mg/ml) was incubated for 8 h at room temperature in 50 mM sodium phosphate buffer, pH 8.0, 0.2 M NaCl (10) and purified as described under "Materials and Methods." Analysis of Mono S fractions (I–III) by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing (+*SH*) or nonreducing (−*SH*) conditions. *M*, molecular size markers (values are given in kilodaltons).

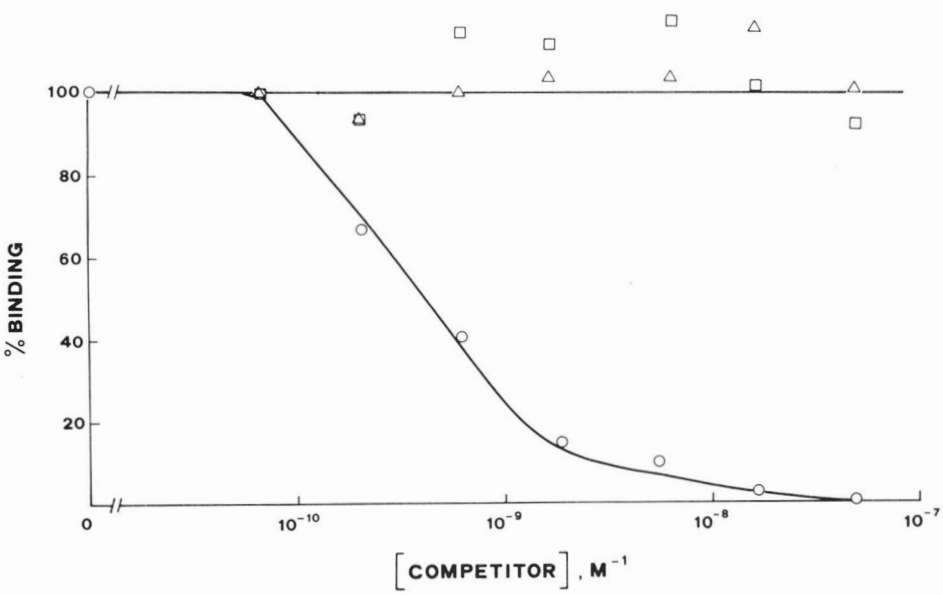

FIG. 2. **Binding inhibition analysis of fractions II and III with ^{125}I-labeled ATF.** Binding assay and radioiodination conditions have been described (10). The concentration of the unlabeled fractions II and III was established by amino acid analysis. Each assay contained 1×10^{6} U937 cells; data are the average of duplicate experiments corrected for nonspecific binding (amounting to 5–6% of maximal binding). Binding was carried out at 4 °C for 2 h with about 5×10^{4} cpm of ^{125}I-labeled ATF (corresponding to about 10 ng). ○—○, unlabeled ATF: △, unlabeled fraction II; □, unlabeled fraction III.

FIG. 3. **Amino-terminal sequences of ATF fractions II (300 pmol) and III (316 pmol).** The *numbers* below the one-letter amino acid symbols are the yields (pmol) at each cycle. *Dashes* in the sequences indicate that no amino acid was detected. ↓, over the ATF sequence (20) indicate the bonds cleaved in II and III.

receptor binding. As shown in Fig. 4, peptide uPA-(1–14),[2] covering the amino-terminal 14 residues of ATF, had no effect on binding of ^{125}I-labeled ATF to the uPA receptor. On the contrary, peptides uPA-(18–32), [Ala^{13}]uPA-(9–20), and [Ala^{19}]uPA-(12–32) effectively competed with ATF for binding to the receptor. The two former displace 50% of ^{125}I-labeled ATF at 100 or 2000 μM, respectively, and the latter at 100 nM.

Peptide [Ala^{19}]uPA-(12–32) contains two cysteines (the third, Cys^{19}, was replaced by Ala to avoid undesired disulfide bond formations). We tested the binding of the oxidized form of this peptide having an intrachain disulfide bond and the results show only a slightly higher affinity (40 nM) (Fig. 4). These data suggest that the introduction of a disulfide bond stabilizes the module conformation without affecting its folding.

Our results can be summarized as follows. 1) The receptor binding sequence of human uPA is located within the GF module. 2) The amino acid residues which appear critical for binding are located within the sequence 12–32. In fact, in fractions II and III, where the ATF molecule is cleaved at positions 23 and 25, respectively, their binding affinity is reduced by at least 500-fold. Furthermore, the oxidized synthetic peptide [Ala^{19}]uPA-(12–32) has an affinity for the uPA receptor of about 40 nM. *i.e.* about 100-fold less than ATF itself. The affinity of this peptide is surprisingly high for a synthetic ligand lacking most of the secondary structure features of the protein. In many examples, the affinity of synthetic peptides is much lower than that of the natural protein. In the case of epidermal growth factor, synthetic peptide EGF (15–53) has at least 10^4-fold lower affinity than natural EGF or its identical synthetic analog EGF-(1–53), yet it can be shown to contain all the binding specificity (18). 3) Separation of the binding sequence (12–32) in two different peptides, [Ala^{13}]uPA-(9–20) and uPA-(18–32), results in a dramatic loss of binding capacity, compared to the peptide [Ala^{18}]uPA-(12–32). However, peptide uPA-(18–32) is still able to compete with an affinity of about 100 μM. This value is similar to that obtained with the epidermal growth factor synthetic peptide EGF-(20–31) (17). This result indicates that both the first and middle disulfide loops of the GF module are necessary for binding or, alternatively, that only the middle loop (*i.e.* residues 19–30) confers binding specificity while the first (residues 13–20) may be required to attain the proper conformation. The following observations make us favor this second interpretation. (i) The sequence of the first loop is highly homologous in EGF and uPA (Fig. 5), yet unlabeled EGF does not compete with ATF for receptor binding (10, 11) at a

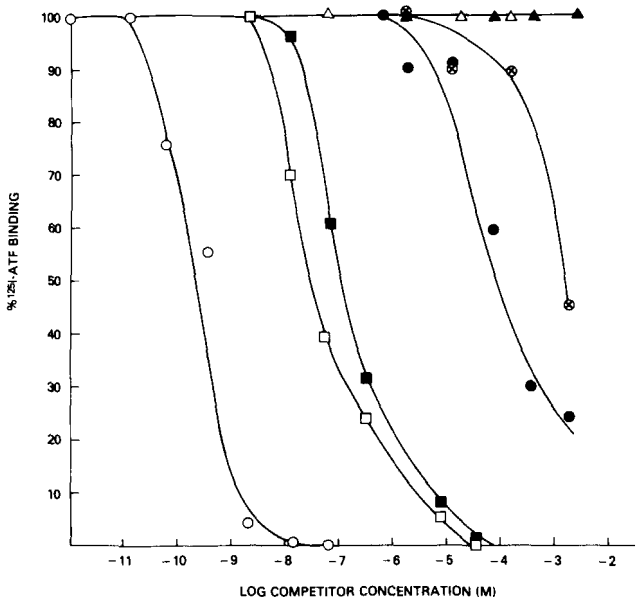

FIG. 4. **Inhibition of ^{125}I-labeled ATF binding by synthetic peptides.** Binding conditions are described in Fig. 2. Peptides were purified by HPLC reverse-phase column: ▲——▲, human uPA (1–14); ●——●, human uPA (18–32); ■——■, human [Ala^{19}] uPA (12–32); □——□, oxidized human [Ala^{19}] uPA (12–32); △——△, mouse [Ala^{20}] uPA (13–33); ⊗——⊗, human [Ala^{13}] uPA (9–20). ○——○, competition with unlabeled ATF.

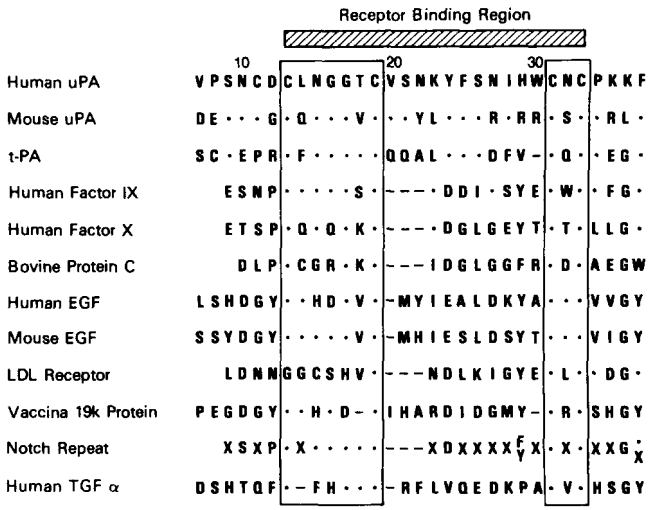

FIG. 5. **Comparison of the amino acid sequence of uPA receptor binding region with other proteins (14).** The amino acid residues corresponding to the aligned sequences are indicated in parenthesis: mouse uPA (8–38; human tPA (50–79); human factor IX (48–73); human factor X (51–76); bovine protein C (60–84); human epidermal growth factor (8–37); mouse epidermal growth factor (8–37); low density lipoprotein receptor (319–344); vaccina virus 19-kDa protein (47–75); Notch repeat sequence where X = variable amino acid residues; and human transforming growth factor α (10–38). The regions of highest homology to the uPA receptor binding region are enclosed within the *boxes*. — indicates gaps introduced to align sequences. *Hatched box* corresponds to receptor binding regions determined for human and mouse uPA and mouse EGF.

[2] The numbers in parenthesis following the urokinase synthetic peptides correspond to the amino acid residues of native urokinase present in the peptide, where the NH_2 terminus of urokinase corresponds to residue 1. The amino acid and residue number in square brackets indicate those positions where a cysteine residue of native urokinase was replaced by another amino acid in the synthetic peptide.

concentration of 10 μM.[3] (ii) Available data show that the middle loop of EGF contains the structural information for receptor binding specificity and biological activity (17), although adjacent sequences confer higher affinity (18). No amino acid sequence homology between uPA and EGF is present within the middle loop (Fig. 5). (iii) Binding to the uPA receptor is quite specific. Closely related molecules, tPA (10,11) or mouse uPA (Fig. 5), fail to bind to the human uPA receptor. The mouse synthetic peptide [Ala^{20}]uPA-(13–33) does not compete for human uPA receptor binding at concentrations up to 1 mM (Fig. 4) while it competes with mouse uPA for binding to murine receptors (50% inhibition at a concentration of 1–3 $\times$ 10^{-7} M peptide).[4] Analysis of sequence homology (Fig. 5) between mouse and human uPA indicates that there are five amino acid differences in the middle loop (Asn^{22}, Lys^{23}, Asn^{27}, His^{29}, and Trp^{30}) as opposed to only two in the first loop (Leu^{14} and Thr^{18}). These five divergent residues of the middle loop of uPA are also different in the tPA sequence while one of the two positions of the first loop is conserved.

Hence we propose that the binding specificity for the uPA receptor resides in the middle loop of the GF module of uPA (residues 19–32). On the basis of the results obtained with the epidermal growth factor in which the specificity appears to reside in the homologous position (17) with the flanking first and third loop conferring the proper conformation (18), we would therefore expect that all three disulfide loops be required for optimum binding of uPA to the uPA receptor.

Proteases of the fibrinolytic and blood coagulation system share large homologous segments with digestive proteases. Noncatalytic modules, however, are present at the amino-terminal region of these regulatory proteases (14). One such module, which shows some homology with EGF, links together one group of proteins with regulatory protease activity (uPA and tPA, protein C, and coagulation factors IX and X) with another group having growth factor and growth factor receptor properties (EGF precursor, transforming growth factor-α (TGF-α), vaccinia virus 19-kDa protein, and low density lipoprotein receptor), and the two newly identified differentiation controlling genes, Notch in *Drosophila melanogaster* and lin-12 in *Caenorrabditis elegans* (22, 23) (Fig. 5). The presence of the GF module in receptor binding proteins and in proteins known to be receptors (*e.g.* low density lipoprotein receptor) or suggested to function as receptors (*e.g.* Notch (22) and lin-12 (23)) is highly relevant and reinforces the concept of the evolutionary exchangeability of ligand and receptor structure (24).

Two very different molecules, EGF and uPA, share sequence homology within the GF module. Although the two proteins have very different physiological roles, they both have a specific, high-affinity membrane receptor; moreover, in both molecules a heptapeptide sequence (residues 13–19) and a conserved cysteine spacing in the GF module increases the binding affinity of the adjacent sequence involved in specific receptor binding, thus stabilizing the basic tertiary structure of these molecules. Therefore, conservation of this homologous structure must be important to achieve the proper protein folding necessary to expose the stretch of amino acids responsible for receptor binding specificity. The species specificity of receptor binding between mouse and human uPA suggests that certain amino acid residues, which may confer receptor binding specificity, are at positions 22, 23, 27, 29, and 30 of human uPA, since they are not conserved in mouse uPA. In contrast, the EGF receptor does not appear to exhibit species specificity in receptor binding since the human receptor apparently binds mouse EGF (25) and human transforming growth factor-α (26) and perhaps vaccinia virus growth factor (19-kDa protein) (27) (see Fig. 5). Examination of the amino acid sequence of the above proteins in the region of EGF responsible for receptor binding (residues 21–30) (17) indicates little homology in this region except for the conservation of an aspartic acid residue in all these proteins at the position equivalent to residue 27 of EGF. The present study suggests that the existence of the GF module in tPA, coagulation factors IX and X, protein C, low density lipoprotein receptor, and the Notch repeat and lin-12 gene products may be related to a specific property of recognition and binding to other proteins. For example, this module may allow coagulation factors to interact with other proteins during the process of blood coagulation. This hypothesis can be tested by the use of various synthetic peptides corresponding to the respective GF module sequence.

In conclusion, many proteins have been found to have the heptapeptide sequence homology and the conserved cysteine spacing in their GF modules (Fig. 5). The presence of this highly conserved module in these proteins may mean that it is part of a specific receptor/ligand system responsible for diverse biological functions.

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[3] M. P. Stoppelli, unpublished data.

[4] J. D. Vassalli and D. Belin, personal communication.

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