A Monoclonal Antibody Against Human Urokinase: The Epitope Structure and Sequence Homology with a Human Tissue-Type Plasminogen Activator

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ABSTRACT. Our previous study showed that an epitope defined by a monoclonal antibody against human urokinase is located on the 33-Kdalton catalytic domain of the enzyme (Nakamura, M. et al., Cell Struct Funct., 9, 167–179, 1984). The epitope structure was further determined and characterized on one-dimensional SDS-polyacrylamide slab gel maps of CNBr-cleaved polypeptide fragments as well as on their Western blots. A single homogeneous polypeptide with an approximate molecular weight of 3.4-Kdaltons was found to be antigenic. The monoclonal antibody exhibited a stronger inhibition of the enzyme activity than the polyclonal antibodies tested, and cross-reacted with a 65-Kdalton tissue-type plasminogen activator present in Detroit 562 cells. From these results and data made up with the help of a computer comparison of known sequences of urokinase and a tissue-type plasminogen activator, we concluded that the epitope is Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys and contains a catalytically active residue, serine.

Plasminogen activators belong to a subgroup of serine-type proteases (21). The activators occur in multiple molecular weight forms (33, 35, 36) and play a prominent physiological role in the dissolution of thrombin (17). Among the activators, well studied ones are urokinase (7, 28) and a tissue-type activator (22); the molecular weights are around 54- and 65-Kdaltons, respectively. Salerno et al. (24) discovered a homogeneous mRNA of human kidney coding a single-chain pro-urokinase with an approximate molecular weight of 54-Kdaltons. Verde et al. (32) and Heynkeker et al. (9) determined the full-length of nucleotide sequence of urokinase. Other lines of studies (1, 14, 19, 29, 37) suggest that the 54-Kdalton enzyme is not a degradation product of a precursor molecule, although the de novo function of the enzyme is still not well understood.

Recent studies on amino acid (7, 28) as well as nucleotide sequence (22) have made certain the molecular configuration of urokinase and the tissue-type activator and suggested that both enzymes are closely related. Of particular interest is that both have a characteristic preserved structure, kringle, which has no catalytic domain but...
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shows affinity for fibrin as plasminogen (9, 22, 27).

Conventionally, the discrimination of both type of activators is based on the
difference in their immunological properties by the use of specific antibodies (33,
35, 36). To investigate further the functional role of enzymes which might have more
stringent and distinct immunological properties, several authors (8, 11, 19, 24, 34)
have produced monoclonal antibodies against urokinase. The usefulness of mono-
clonal antibodies for the quantification, purification, and characterization of the
antigen has been described by us (19) and others (8, 34).

The present study deals with an effort to determine the epitope structure of
urokinase that a hybridoma producing monoclonal antibody defines with a help
of a computer program searching for homologous amino acid residues between the
two proteins. This epitope might exist within a limited sequence; its homolog is
present in a similar subgroup of serine-type proteases. The cross-reactivity of our
monoclonal antibody is also discussed. Comparison of the two proteins in question
led us to conclude that both proteins are evolutionally related. A more precise
analysis with particular attention to kringles is presented in the accompanying paper
(see ref. 30).

MATERIALS AND METHODS

**Cells.** Detroit 562 cells used were a gift from Dr. Masaaki Tanifuji (Daigo Nutritive
Chemicals, Ltd., Osaka, Japan) and were grown at 37°C in MEM supplemented with 10%
fetal bovine serum, 0.1% lactalbumin hydrolysate, 1% non-essential amino acids, and 1 mM
sodium pyruvate.

**Chemicals.** Monoclonal antibodies against human urokinase were produced in our
laboratory, and a hybridoma (G62) producing IgG-isotype antibody was purified and
characterized (19). This antibody was used throughout the study. Peroxidase-conjugated
IgG of rabbit anti-mouse IgG was purchased from Miles-Yeda (Rehovot, Israel). Crude
urokinase preparations, purified urokinase, and rabbit antibodies to human urokinase were
kindly supplied by Dr. Masaaki Tanifuji. Nitrocellulose sheets (pH 79, 0.1μm) were from
Schleicher and Schuell (Keene, NH, USA). The chromogenic substrate, L-pyroglutamyl-
glycyl-L-arginine-p-nitroanilide (S-2444), was purchased from Kabi Diagnostica (Stockholm,
Sweden). Other chemicals were of reagent grade.

**Alignment of amino acid sequences.** The amino acid sequences of the A-chain (22-
Kdaltons) and B-chain (33-Kdaltons) of urokinase and the tissue-type plasminogen activator
(65-Kdaltons) were referred to Günszler et al. (7), Steffens et al. (28), and Pennica et al. (22),
respectively. We also used complete amino acid sequences of urokinase deduced from
the nucleotide sequences reported by Heyneker et al. (9) and Verde et al. (32). The alignment
of the homologous amino acids between the two proteins was established using a
computer program (PROTEIN HOMOLOG) constructed by us which determines at least three
consecutive and identical amino acid residues between the two proteins. A best alignment
of the two protein sequences with maximum match was made by the rearrangement of these
sequences essentially according to the method described by Needleman and Wunsch (20).
The method of matrix analysis of protein sequences was due to Maizel and Lenk (16).

**Affinity-purification of 55-Kdalton urokinase from crude enzyme preparations.** Urokinase
with an approximate molecular weight of 55-Kdaltons in a crude preparation was purified
on an affinity column containing immobilized-monoclonal antibodies to Sepharose 4B as
described previously (19). The purified materials were extensively dialyzed against 10 mM
ammonium bicarbonate, pH 7.5, and then lyophilized and kept at −20°C until use.

**Cleavage of urokinase by cyanogen bromide.** The method employed here was essentially due to Soderling et al. (26). Purified urokinase (1 mg) was dissolved in 100 μl of 70% formic acid. An excess amount of cyanogen bromide (more than 300 moles per methionine residue) was added and incubated at 20°C for 20 h and lyophilized. The CNBr-peptides were dissolved in distilled water and utilized for further analysis.

**SDS-polyacrylamide slab gel electrophoresis.** One-dimensional 10% or 13% SDS-polyacrylamide slab gel electrophoresis followed the method of Laemmli (13), and the procedures for the two-dimensional SDS-polyacrylamide slab gel electrophoresis were by Hirsch et al. (10). The first dimension was isoelectric focusing on a 4% polyacrylamide gel containing 2% ampholyte (pH 3.5–10) and 8 M urea. The electrophoresis was run for 750 V·h. For the second dimension, 10% SDS polyacrylamide slab gels were used. After the electrophoresis, the slab gel was stained either with Coomassie brilliant blue or silver reagent (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

**Identification of antigenic CNBr-peptide blotted on nitrocellulose from SDS-polyacrylamide slab gels (Western blots).** The CNBr-peptides separated on polyacrylamide slab gels were electrophoretically blot-transferred to nitrocellulose sheets (15, 18, 31). The detection of the antigenic peptide followed the procedures described by Glass et al. (5) except that the incubation with monoclonal antibody was performed at 4°C overnight to ensure complete binding of the antibody to the responsible antigenic polypeptide.

**Enzyme-linked immunosorbent assay (ELISA).** The essential technique was the method described by Kelsey et al. (12). The method of competitive ELISA was described previously (19).

**Enzyme assay.** The procedure was due to Hérion and Bollen (8). Enzyme samples were reconstituted in 1 ml of a reaction mixture containing 50 mM Tris-HCl, pH 8.8, 38 mM NaCl, and 0.3 mM S-2444, and then they were incubated for the desired time at 37°C. The reaction was stopped by the addition of 100 μl of 50% acetic acid. The absorbance at 405 nm was spectrophotometrically determined. The enzyme activity was expressed in International Units (IU), assuming that an increase in the optical density by 0.00125 per min gives one IU per ml under the experimental conditions according to the manufacturer’s instructions.

**Fibrin autography.** To detect plasminogen-mediated activity of the proteins separated on SDS-polyacrylamide slab gels, fibrin-agar indicator plates were prepared according to the method of Granell-Piperno and Reich (6).

**Protein determination.** The amount of proteins was determined by Bio-Rad protein assay (2), using bovine gamma globulin as a standard.

**RESULTS**

**Sequence homology between urokinase and a tissue-type plasminogen activator.** An amino acid sequence deduced from the nucleotide sequence of a full-length human tissue-type plasminogen activator (t-PA) was reported by Pennica et al. (22). We compared this sequence with those of the A- (7) and B-chain (28) of urokinase (UK). Since the activator has two kringle structures (22), each formed by a set of three disulfide bonds and connected by a linker sequence (indicated by arrowheads in Fig. 1), we tentatively termed them the K₁ and K₂ kringles, from the N-terminus. Fig. 1 shows the summarized amino acid alignment of these proteins with maximum match. Out of the 157 residues of the A-chain, 65 residues are identical (41%) to residues of the first half domain. Of the 253 residues of the B-chain, 122 residues
Fig. 1. Alignments of urokinase and tissue-type plasminogen activator. Single-letter amino acid abbreviations are used. Residues are aligned with maximum match on the base of a homology matrix (see Fig. 2). The identical residues between the two proteins are boxed in. The preserved cysteine residues that constitute kringle structures are marked by stars, and those that form intermolecular disulfide bonds are marked by closed circles. The six amino acid residues between the two kringles of the tissue-activator are indicated by arrowheads. A putative cleavage site at Arg 275-Ile 276 of the tissue-activator, the N-terminus of the B-chain, is indicated by an arrow. Asterisks mark the active sites of the enzyme. The CNBr-cleaved fragments of the B-chain are underlined and numbered beginning from the N-terminus.

are homologous (48%) to the second half domain of the t-PA sequence beginning at the Arg 275-Ile 276 cleavage site, which forms the two-chain molecular structure (22). Between the two domains of homologs, there are 76 residues which form the K2 kringle in the activator; they do not match either the A or B-chain sequence of the UK.

To visualize, a homology matrix was constructed as described in MATERIALS AND METHODS. As depicted in Fig. 2, most of the identical residues in the A-chain such as TCYE, YRG, WNS, LGLG, HNYCRNP, and PWCYV are reiterated along the sequence in the t-PA, and these duplicates belong to the K1 and K2 kringles.
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Fig. 2. Homology matrix comparison of amino acid sequences of urokinase and tissue-type plasminogen activator. Sequences of three consecutive amino acid identical to the two proteins are found by a computer program (PROTEIN HOMOLOG, unpublished). The sequences of A- and B-chain of urokinase (ordinate) were rearranged as a single protein architecture and plotted against the full-length of the tissue-type plasminogen activator (abscissa). K₁ and K₂ are kringle present in the tissue-type activator, where K₁ corresponds to a kringle present in the A-chain. The Arg 275-Ile 276 cleavage site is indicated by an arrow. The asterisk marks one of the active sites of the enzymes, serine.

Obviously, these kringles are homologous to those present in plasminogen and prothrombin (4, 27, and see ref. 30). It is of particular interest to note here that the longest homologous segment, consisting of eleven residues (CQGDSGGPLVC) contains one enzyme-active site, the serine residue (7, 22, 28, and see below), and that this segment is located in a fragment (No. 5) generated by CNBr-cleavage (Fig. 1, and see below).

**Immunological identification of a CNBr-peptide combining with the antibody.** Our previous study demonstrated that a hybridoma (G62) producing antibody could recognize 33-Kdalton heavy-chain polypeptide (B-chain), which is derived from 55-Kdalton whole mass, but did not bind to the 22-Kdalton polypeptide chain (A-chain) (19). We made similar experiments on two-dimensional SDS-polyacrylamide slab gels and on their Western blots using affinity-purified antigens (Figs. 3A and 3B). The 55- and 33-Kdalton proteins (indicated by arrows), each of which had a pI value of around 8.4, were antigenic. Thus, the results confirm that an epitope is located on the B-chain polypeptide (33-Kdaltons).

To further characterize the epitope, the 55-Kdalton antigen was utilized in CNBr-cleavage experiments. As illustrated in Fig. 1, the A and B-chain contain two and five methionine residues, respectively. Accordingly, the chemical cleavage would produce nine polypeptides. The B-chain generates six fragments with calculated molecular weight species of 8.9-, 6.1-, 3.7-, 3.0-, 3.4-, and 5.3-Kdaltons, while the A-chain gives three fragments of 8.0-, 7.2-, and 3.6-Kdaltons from each N-terminus, respectively. The binding of the monoclonal antibody to a certain polypeptide among
these fragments was examined by a competitive ELISA. The results demonstrated that the CNBr-fragments competed with the binding of the antibody to urokinase used as a standard (results not shown). This indicates that the chemical treatment does not alter the antibody binding to the epitope.

Next, these polypeptides were separated on SDS-polyacrylamide slab gels. Nine...
polypeptides were detected by silver staining of the gels (Fig. 4, track a), among
which the six polypeptide species derived from the B-chain were each numbered
beginning from the N-terminus on the basis of their molecular weights (see Fig. 1).
Similar electrophoresed gels were subsequently blot-transferred to nitrocellulose
sheets and an antigenic polypeptide fragment was detected by incubation with
the monoclonal antibody as described in MATERIALS AND METHODS. A single
homogeneous polypeptide fragment, No. 5 (3.4-Kdaltons), was revealed to be anti-
genic (Fig. 4, track b). To compare with this finding, a similar blot-transferred
nitrocellulose sheet was incubated with rabbit antibody to human urokinase (Fig. 4,
track c). In contrast, multiple polypeptide species with approximate molecular weights
of major 5.3-, and 3.7-Kdaltons were found to be antigenic. The experiments were
repeated on two-dimensional polyacrylamide slab gel maps. A single polypeptide
fragment with an approximate molecular weight of 3.4-Kdaltons and pI value of
7.2 was identified to be antigenic (data not shown), which is a similar polypeptide
(No. 5) as shown in Fig. 4 (track b).

**Enzyme inhibition by monoclonal antibody.** We then addressed the question

![Graph showing enzyme inhibition by monoclonal antibody.](image-url)
whether the monoclonal antibody could inhibit the enzymatic activity of urokinase. If so, it is possible that the active site of the enzyme might be occupied by the antibody, and thus this complex is inaccessible to a synthetic peptide-substrate. For comparison of the inhibitory effect, two samples of rabbit antibodies to human urokinase were used. Fig. 5 demonstrates that in the two cases, the polyclonal antibodies inhibited 20 and 70% of the enzyme activity, respectively, as referred to a control which did not contain antibodies. The monoclonal antibody inhibited more strongly than the polyclonal antibodies; the inhibition was as high as 80%. These results suggest that the monoclonal antibody could combine directly with an antigenic site which contains an important active site of the enzyme.

Cross-reactivity of monoclonal antibody with the tissue-type plasminogen activator. Because a CNBr-cleaved fragment, a polypeptide No. 5, contained an epitope and the enzymatic activity of urokinase was inhibited by the monoclonal antibody, it was considered that the epitope might exist within a sequence containing an important active site, the serine residue. Thus, the sequence would be CQGDSGGPLVC, which is homologous to the tissue-type activator (see Fig. 1). Therefore, our monoclonal antibody might cross-react with a certain tissue-type activator, if it contains a similar epitope sequence as well as urokinase. We examined this possibility using Detroit 562 cells, which had shown a bright fluorescence in the cytoplasm by the FITC-

Fig. 6. Identification and characterization of tissue-type plasminogen activators in Detroit 562 cells. Cellular proteins were extracted (see Text) and separated on 10% SDS-polyacrylamide slab gels (A). One of the electrophoresed gels was blot-transferred to nitocellulose sheets and the immunonochemical identification was performed as described in MATERIALS AND METHODS (B). The other gel was subjected to fibrin-agar indicator gel assay (see MATERIALS AND METHODS) (C). Numbers denote molecular weight standards in kilodaltons.
conjugated monoclonal antibody provided in the previous study (19) (data not shown). Therefore the cells were harvested and homogenized in a buffer solution containing 50 mM Tris-HCl, pH 7.5, and 0.05 % Triton X-100. The homogenate was centrifuged at 700 \( \times \) g for 10 min to remove nuclei and other cell debris. The proteins in the supernate were collected and subjected to 10 % SDS-polyacrylamide slab gel electrophoresis, and then the separated proteins were blotted on nitrocellulose sheets. Fig. 6A and 6B reveal the presence of multiple species of antigenic proteins. Out of 20 positive bands detected, one major band had a molecular weight of 65-Kdaltons. These antigens were subsequently examined by fibrin autography (see MATERIALS AND METHODS). Apparently, the antigen of interest (65-Kdaltons) had plasminogen-mediated activity (Fig. 6C). The results suggest that the epitope of the antigen is the same as or is similar to that of urokinase.

**DISCUSSION**

In the previous study, a monoclonal antibody against urokinase (UK), which has
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an approximate molecular weight of 55-Kdaltons, was developed (19). The epitope domain of the antigenic enzyme was estimated to occur in the 33-Kdalton polypeptide domain which is derived from the 55-Kdalton whole enzyme (see Fig. 3B). To further characterize this epitope structure, we prepared CNBr-cleaved fragments of the enzyme and determined the antibody binding on the Western blots. Using known sequence data, we also compared the amino acid sequence of this enzyme with that of a tissue-type plasminogen activator (t-PA) assisted by a computer program.

Amino acid alignment of the desired proteins (Fig. 1) shows that the CNBr-cleaved fragment No. 5 contains the longest sequence homolog to the t-PA, CQGDSGGGPLVC. If 6–8 amino acid residues define an epitope structure (23), this segment can be considered as a candidate for the epitope since the antibody could cross-react with a similar antigen present in Detroit 562 cells, which had been probed by FITC-conjugated antibody (unpublished observation). The presence of 50- and 60-Kdalton plasminogen activators in Detroit cells has been reported (33). The cells contained antigenic proteins, one of which had plasminogen activator activity with an approximate molecular weight of 65-Kdaltons (Fig. 6), probably similar to the t-PA cited in the present study.

CNBr-fragment No. 5 was an antigenic polypeptide (Fig. 4), indicating that the epitope sequence exists within the fragment. Because this fragment contains a segment homologous to the t-PA, as examined by the computer alignment, and it has one important active site of the enzyme, namely the serine residue (7, 22, 28), the enzymatic activity should be strongly inhibited if the monoclonal antibody binds to this site or its proximity. We examined this possibility using purified enzyme and a chromogenic substrate (S-2444) in the enzyme assay system. A substantial inhibition was occurred upon incubation with the monoclonal antibody (Fig. 5). It was feasible to calculate the molar ratio of the bound antibody to the enzyme; the molecular weights of the mouse IgG and that of the enzyme were assumed to be 165- and 55-Kdaltons, respectively. Thus, at the level of 50% inhibition, the ratio was around 1, indicating that one molecule of the antibody could bind to one molecule of the enzyme. Although the enzymatic activity was not completely inhibited under the experimental conditions, this stoichiometry is enough to suggest that the monoclonal antibody combines with the active site. The polyclonal antibody, that is, anti-rabbit IgG, however, showed only 20–70% inhibition at a molar ratio of over 40, suggesting that these antibodies mostly combine with site(s) other than the active one. Accordingly, the binding of the antibody might cause some conformational change of the enzyme, thus partially inactivating it.

From these results, the epitope defined by the monoclonal antibody is most probably a limited sequence region which has homology to the t-PA and contains a catalytically functional amino acid residue, i.e., it falls within the CQGDSGGGPLVC segment.

As the epitope sequence is found to be present in other serine-type proteases such as prothrombin, plasmin, kallikrein, and trypsin (28), it is possible that our monoclonal antibody could combine with those. The results did reveal that both the human plasminogen and bovine trypsin were definitively positive on Western blots (Fig. 7). Human thrombin, however, showed little or a little positivity (data not shown). Thus, it seems plausible that within the epitope sequence noted above, one amino acid residue, Gln, located before the active serine residue is important in the antibody binding, since the only difference in the sequence is Glu in thrombin, instead of the
Gln. This evidence might support our conclusion above. Therefore, it is important to examine the plasminogen activator activity of the antigens that antibody specifies, either by using fibrin-agar plates (Fig. 6), or by testing the specific conversion of plasminogen to plasmin (25).

Finally, in concurrence with the finding of the possible epitope sequence, our computer search for identical residues between the two proteins demonstrates that both UK and t-PA had a high degree of homology along their entire sequences (see Figs. 1 and 2). Thus, the results strongly suggest that these proteins are evolutionally related (see ref. 30).

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