1611

REC. t-PA GENETICALLY MODIFIED AT THE CLEAVAGE SITE OF ONE-CHAIN TO TWO-CHAIN CONVERSION: ENZYMOLOGY AND DIAGNOSTIC APPLICATIONS.

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Native one-chain t-PA is cleaved by plasmin or by trypsin after the Arg in the sequence -Gln-Phe-Arg-Ile-Lys-. Variants of one-chain t-PA where the -Arg- was replaced by a His (Arg to His) or by a Lys (Arg to Lys) or by a Thr (Arg to Thr) were made through genetic modification. The three mutants and the wild type were expressed in animal cells and purified in the one-chain form by affinity chromatography as was t-PA from Bowes melanoma cells.
In contrast to wild type and melanoma t-PA the mutants reacted poorly with polyclonal antibodies raised against the peptide -Gln-Pro-Gln-Phe-Arg-Ile-Lys--Gly-Gly- indicating mutation in the sequence. Of these proteins only the Arg to Thr mutant was resistant to plasmin cleavage as evidenced by SDS-PAGE. t-PA antigen values (ELISA) and fibrinolytic activity values (fibrin clot lysis assay) yielded the following specific activities expressed in IU/μg: 810 (Arg to His), 640 (Arg to Lys), 290 (Arg to Thr), 810 (wild type) and 660 (melanoma t-PA). The amidolytic activities for the one-chain proteins against D-Ile-Pro-Arg-pNA at pH 9.0 and 37°C, expressed in mOD per minute at 1 μg/mL of enzyme were: 15.8 (Arg to His), 13.6 (Arg to Lys), 8.3 (Arg to Thr), 10.0 (wild type), 9.6 (melanoma t-PA) as compared to 55.2 for two-chain melanoma t-PA.
All mutants including the uncleavable Arg to Thr mutant could be used in determination of PAI activity in plasma sampies. Only one-chain t-PA reacts selectively with PAI 1. Thus, use of the Arg to Thr mutant represents a theoretical advantage in PAI 1 activity determination since preparations of this mutant most likely is free of contaminating two-chain t-PA.
The plasminogen activation rate as measured in a coupled assay in the presence and absence of fibrin at 0.5 μM plasminogen and 37°C was measured and the stimulation factor calculated. This was about 950 fold for the Arg to Thr mutant wich was considerably higher than that of melanoma one chain t-PA and the other mutants wich all were about 550 fold. The stimulation factor for melanoma two-chain t-PA was in the same experiment about 120 fold. The extra fibrin sensitivity of the Arg to Thr mutant resulted in improved soluble fibrin assay according to Wiman and Rånby Thromb. Haemostas. (1986) 55: 189-193.
In conclusion: the use of a plasmin insensitive protein-engineered mutant of t-PA gives advantages in assays for PAI 1 and soluble fibrin.

1612

MUTANTS OF TPA AND THEIR CATALYTIC PROPERTIES. B. Chaudhuri, J. Heim, B. Meyhack, and J. van Oostrum. Ciba-Geigy Ltd. Department of Biotechnology, Basle, Switzerland.

Mutants of tPA were obtained by in vitro mutagenesis to study the effects of N-linked glycosylation, and to gain insight into the properties of pure single-chain tPA. The glycosylation mutants were obtained by modification of the last residue of the sequence Asn.X.Ser/Thr, known to be specific for N-linked glycosylation, whereas the plasmin sensitive sequence Arg275 Ile276 was modified to Asp 275 Ile276 to obtain a plasmin insensitive tPA. Mutant proteins were expressed in yeast under the control of the repressible acid-phosphatase promoter, and in CHO-cells. The novel proteins were isolated mainly by affinity chromatography using the selective protease inhibitor DE-3. A tPA mutant containing a modification in the sequence Asn184 Gly185 Ser 186 showed similar results as compared to recombinant yeast tPA in the clot lysis test, a direct fluoresence assay (FU test), and the indirect double rate assay according to Verheyen (Verheyen test). A mutant with modifications in the sequences Asn117 Ser 118 Ser119, Asn184 Gly185 Ser186, and Asn448 Arg449 Thr450, showed an increased clot lysis activity with no increase in activity in the FU test and Verheyen test. However, the stimulation by fibrinogen fragments in the Verheyen assay was found to be doubled as compared to yeast tPA. An even further increase in stimulation by fibrinogen fragments, upto 500% over yeast tPA, was observed with the plasmin insensitive tPA, although this mutant only showed 20% of the yeast tPA activity in the FU and Verheyen tests

1613

CONSTRUCTION AND EXPRESSION OF HYBRID PLASMINOGEN ACTIVATOR GENES, B. Rajput (1,3), D. Alaimo (2), F. A.M. Asselbergs (2) and E. Reich (1,3). Friedrich Miescher Institute, P.O. Box 2543 (1) and Dept. of Biotechnology, CIBA-GEIGY Ltd. (2), CH-4002 Basel, Switzerland. (3) present address: Dept. Pharmacol. Sciences, School of Medicine, State University of New York, Stonybrook NY 11794-8651.

Hybrid plasminogen activator (PA) genes containing fragments of cDNA encoding the non-catalytic part of tissue-PA and the catalytic domain of urokinase and vice versa were constructed and expressed in Chinese Hamster ovary (CHO) cells. The hybrid nature of the products in stably transformed cells was analyzed at the level of DNA and RNA using probes derived from different regions of the urokinase and tissue-PA cDNAs and at the protein level by means of polyclonal antibodies raised against tissue-PA and urokinase. The hybrid genes made hybrid RNAs and proteins of the expected sizes. The proteins were enzymatically active as determined by zymography and chromogenic enzyme assays and this activity was blocked by the appropriate antibodies. The effect on hybrid PAs of cyanogen bromide cleaved fibrinogen fragments, poly-D-lysine and heparin which are known to affect the activity of tissue-PA and urokinase differently was also studied

1614

EFFECT OF THROMBIN-CLEAVAGE OF PRO-UROKINASE ON ITS AFFINITY TO FIBRIN. Y. Eguchi (1), Y. Sakata (1), M. Matsuda (1) and K. Kondo (2). Institute of Hematology, Jichi Medical School, Tochigi-Ken (1), Sagami Chemical Research Center, Kanagawa-Ken (2), Japan

Pro-urokinase (PUK) has been reported to bind to fibrin-Sepharose specifically but not to fibrin clot. As thrombin (TH) clotted fibrinogen and cleaved the Arg 156-Phe 157 peptide bond of PUK simultaneously, we tested effect of this cleavage on the binding of PUK to fibrin by using high molecular weight urckinase (HMUK), PUK purified from culture media of human kidney cell (nPUK) and two kinds of recombinant PUK (rPUK) expressed in genetically transformed E. Coli. One rPUK designated as PO lacks the carbohydrate side chain present in nPUK but has the same amino acids sequence as nPUK does. The other rPUK designated as P3 is a mutant whose Phe 157 was replaced by Asp so as to be resistant against the proteolytic cleavage by TH. The rate of conversion of single-chain $^{125}I$ labeled PUK ($^{125}I$ PUK) to two-chain form by TH or plasmin (PM) was determined by both amidolytic activity toward S-2444 and autoradiography of polyacrylamide gel under reducing conditions. Although 100 U/ml TH did not cleave $^{125}I$ P3 in 2h at 37°C, 10 U/ml TH completely cleaved 400 U/ml $^{125}I$ nPUK and PO instantaneously. $^{125}I$ PUK, which had been totally or partially cleaved by either TH or PM, was mixed with fibrinogen in the presence of aprotinin. Then the mixture was clotted with 2.5 mM $Ca^{2+}$ and 0.5 U/ml TH which cleaved PUK only a little for 10 min. Clots were obtained by clot-syneresis and washed with Tris-buffer containing albumin, aprotinin and hirudin. The bound PUK was calculated by measuring the radioactivity of the clot and the supernatant. Although the binding of nPUK and PO to fibrin decreased from 12% to 1% as cleavage by TH or PM proceeded, that of P3 to fibrin decreased only when two-chain conversion was processed by PM. HMUK did not bind to fibrin. These results strongly suggest that single-chain PUK binds to fibrin and imply that P3, which is TH-resistant, may be useful as an activator in the presence of TH surrounding the clot.