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CHARACTERIZATION OF RECOMBINANT HUMAN TISSUE-TYPE PLASMINOGEN ACTIVATOR MISSING THE FINGER DOMAIN. Deborah L. Higgins and William E. Holmes. Dept. of Cardiovascular Research, Genentech, Inc., South San Francisco, CA 94080, USA.

Site-specific mutagenesis was used to produce a mutant form of tissue-type plasminogen activator (t-PA) which was missing the first 44 amino acids. This domain has sequence homology with the type 1 regions in proteins such as fibronectin and is commonly called the finger domain. The mutant protein (des 1-44 t-PA) was expressed in Chinese Hampster Ovary cells, and was purified using chromatography on Zn-chelate sepharose and lysine-sepharose. Sequence analysis indicated that the resulting protein was homogeneous and started at amino acid 45 in the sequence of the normal protein. The two-chain forms of both des 1-44 t-PA and normal sequence t-PA exhibited similar kinetic constants with a small synthetic substrate (H-D-Isoleucyl-L- prolyl-L-arginyl-p-nitroanilide). The ability of des 1-44 t-PA to activate plasminogen was decreased to 70% of the rate of normal t-PA. The rate of plasminogen activation by normal t-PA was stimulated 51-fold in the presence of fibrin, whereas with des 1-44 t-PA it was stimulated only 40-fold. Although des 1-44 t-PA bound to lysine-agarose, little (if any) binding was observed to either intact or degraded fibrin indicating that fibrin stimulation is due in part to the ability of t-PA to recognize plasminogen bound to fibrin as a preferable substrate. The mutant t-PA was capable of forming complexes in vitro with all of the inhibitors in blood which react with normal sequence t-PA. The rate of reaction with α2-macroglobulin, however, was slower with des 1-44 t-PA than with normal sequence t-PA. The similar resistance of des 1-44 t-PA and normal sequence t-PA to proteolysis and the ability to react with a battery of monoclonal antibodies suggests that the deletion did not cause perturbed folding, but rather that alterations in function of des 1-44 t-PA were due to the lack of the finger domain.

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ROLE OF PROTEOLYSIS AT ARGININE-275 OF TISSUE PLASMINOGEN ACTIVATOR (t-PA) AS ASSESSED BY SITE-DIRECTED MUTAGENESIS. G.A. Vehar, K.M. Tate, D.L. Higgins, W.E. Holmes, H.L. Heyneker. Genentech, Inc., South San Francisco, CA 94080 USA

The significance of the cleavage at arginine-275 of human t-PA has been the subject of debate. It has been reported, as expected for a member of the serine protease family, that the single chain form is a zymogen and that generation of catalytic activity is dependent upon cleavage at arginine-275. Other groups, in contrast, have found considerable enzyme activity associated with the one-chain form of t-PA. To clarify the functional significance of this proteolysis and circumvent cleavage of one-chain t-PA by itself or plasmin, site-directed mutagenesis was employed to change the codon of arginine-275 to specify a glutamic acid. The resulting plasmid was used to transfect CHO cells. The single chain mutant [Glu-275 t-PA] was expressed in CHO cells and the protein purified by conventional techniques. The mutant enzyme could be converted to the two-chain form by V8 protease, but not by plasmin. Glu-275 t-PA was 8 times less active in the cleavage of a tripeptide substrate and 20-50 times less active in the activation of plasminogen in the absence of firbrin(ogen) than its two-chain form. In the presence of fibrin(ogen), in contrast, the one and two-chain forms of Glu-275 t-PA were equal in their ability to activate plasminogen in the presence of fibrin(ogen). The activity in these assays was equal to the activity of wild type t-PA. In addition, it was observed that fibrin bound considerably more of the one-chain form of t-PA than the two chain forms of t-PA and the Glu-275 mutant. The one and two-chain forms of the wild type and mutated t-PA were found to slowly form complexes with plasma protease inhibitors in vitro, although the one-chain forms were less reactive with alpha-2-macroglobulin. It can be concluded that the one-chain form of t-PA appears to be fully functional under physiologic conditions and has an increased affinity for fibrin compared to two-chain t-PA.

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ENHANCEMENT AND INHIBITION OF THE ACTIVITY OF RECOMBINANT ANALOGS OF TISSUE PLASMINOGEN ACTIVATOR. L.A. Erickson, P.W. Bergum, E.V. Hubert, N.Y. Theriault, E.F. Rehberg, D.P. Palermo, G.A.W. deMunk*, J.H. Verheijen*, and K.R. Marotti. The Upjohn Company, Kalamazoo, MI USA and *The Gaubius Institute, Leiden, The Netherlands.

Both the affinity of tissue plasminogen activator (tPA) for fibrin, which is associated with enhancement of its activity, and its susceptibility to inhibition contribute to the ability of this molecule to initiate vascular fibrinolysis. Full-length tPA is thought to consist of five structural regions designated the finger (F), growth factor-like (G), kringle 1 (K_1), kringle 2 (K_2), and protease (P) domains. Previous studies have suggested that the interaction of tPA with fibrin is primarily dependent upon the presence of the F and K_2 domains, with its inhibition minimally requiring an intact active site. We have generated analogs of tPA with which to study further the relationship between the structure and the function of these domains. Synthetic DNA molecules, encoding specific tPA analogs and containing unique restriction sites in the interdomain regions, were expressed in Chinese hamster ovary cells. Serum-free media conditioned by transfected cells were then analyzed for both tPA antigen and activity. Analogs studied thus far include the full-length molecule FGK_1K_2P, GK_1K_2P, FK_1K_2P, FGK_2P, FGK_1P, FGK_2K_1P, and FK_2P. In each case, samples of conditioned medium contained from 100 to 200 ng of tPA-like material per ml. Based on active-site titration of immunopurified analogs with ^{3}H-DFP and assessment of tPA activity in the presence of CNBr-digested fibrinogen, the specific activities of analogs containing the K_2 domain adjacent to the P domain were similar to that of native full-length tPA. In contrast, the specific activities of analogs with the K_2 domain deleted (FGK_1P) or transposed (FGK_2K_1P) were significantly reduced, indicative of the decreased ability of the fibrinogen fragments to enhance their respective activities. The extent of neutralization of the activity of each analog by platelet-derived PA inhibitor appeared to be similar to that of full-length tPA, confirming that the P domain contains the primary site of interaction between tPA and inhibitor. These data suggest that not only the presence of but also the position of the K_2 domain are important for proper expression of tPA's activity.

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CATALYTIC ACTIVITIES OF ONE-CHAIN tPA AS REVEALED BY AN ANALOGUE RESISTANT TO PLASMIN CLEAVAGE. L.C. Petersen (1), M. Johannessen (1), D. Foster (2), A. Kumar (2), and E. Mulvihill (2). Novo Research Institute, Bagsvaerd, Denmark, (1) and Zymogenetics Institute, Seattle, U.S.A. (2).

Substitution of Arg_{275} with Gly in the activation site of tPA provides a one-chain recombinant analogue, tPA-Gly_{275}, which is very resistant to cleavage by plasmin. The amidolytic activity of tPA-Gly_{275} with simple synthetic substrates was investigated and compared to the kinetics obtained with authentic one- and two-chain tPA. Both one-chain (zymogen) forms possess enzymatic activity, however, in the absence of fibrin it is much lower than that of two-chain tPA. Fibrin enhances the activity of the one-chain tPA forms, but not of two-chain tPA.

A chromogenic assay was developed for measurement of plasminogen activation. Due to the presence of a high affinity plasmin substrate (D-Val-Phe-Lys-pNA) and aprotinin in the reaction mixture, the assay ensures a low steady-state concentration of free plasmin during the measurement. With this assay and with tPA-Gly_{275} it is possible to measure plasminogen activation kinetics of one-chain tPA without any significant two-chain tPA formation even in the presence of fibrin. The intactness of tPA-Gly_{275} was confirmed by direct measurement of the one-/two-chain tPA content by means of reduced SDS-PAGE combined with Western blotting after exposure to plasmin digestion in the presence and absence of fibrin. The results suggest that one-chain tPA possesses enzymatic activity also with plasminogen as the substrate, however, the activity is much lower than that of two-chain tPA. Addition of fibrin profoundly enhances the plasminogen activation rate of both tPA-Gly_{275}, one-chain, and two-chain authentic tPA to approx. the same maximal level. Taken together these observations indicate that fibrin binding can induce an activated state of the intact tPA 'zymogen'.

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