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ARTIFICIAL EXON SHUFFLING: CONSTRUCTION OF HYBRID cDNAs CONTAINING DOMAINS OF TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) AND UROKINASE (u-PA). C.J.M. de Vries, H. Veerman and H. Pannekoek. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Dept. of Molecular Biology, Amsterdam, The Netherlands.

The intriguing finding that functions of t-PA coincide with structural domains and that these domains occur in related proteins, has been the basis to construct hybrid proteins by artificial exon shuffling to prove the conservation of functions in the shuffled domains. The heavy chain (Hch) of t-PA mediates both binding to fibrin and stimulation of plasminogen activator activity via its Finger- and Kringle-2 domain, whereas the light chain (Lch) contains the serine protease moiety of the protein. The Hch of u-PA is very homologous to the Lch of t-PA, but exhibits a higher plasminogen activator activity. This activity of u-PA is not stimulated by fibrin. We employed the 'M13 in vitro outlooping' technique to fuse the Hch of t-PA cDNA and the Hch of u-PA cDNA, to create two different hybrid cDNAs. On one hybrid cDNA, the t-PA and the u-PA sequences are coupled precisely at the exon-intron boundaries of the corresponding genes, while the other hybrid cDNA lacks a u-PA segment at the junction, encoding 13 amino acids of u-PA. The hybrid cDNAs were transiently expressed in mouse Ltk- cells and the recombinant proteins were characterized. The plasminogen activator activity of these proteins was determined in an indirect amidolytic assay, using plasminogen and the chromogenic substrate S2251. As anticipated, the activity of both t-PA/u-PA hybrid proteins is stimulated by fibrin, however, not to the same extent as t-PA. Remarkably, we found a decreased inhibition of the hybrid proteins by the endothelial plasminogen activator inhibitor (PAI-1) as compared to t-PA and u-PA, although stable complexes between the hybrid proteins and the inhibitor are formed. We conclude that functions of structural domains are maintained during exon shuffling.

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PROPERTIES OF A HUMAN RECOMBINANT FUSION PROTEIN OF THE 'FINGER' DOMAIN OF TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) AND A TRUNCATED SINGLE CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (scu-PA). D. Gheysen (1), L. Piérard (2), P. Jacobs (2), H.R. Lijnen (3), A. Bollen (2) and D. Collen (3). Dept. Molecular Cellular Biology, SK-RIT Rixensart (1), Dept. Applied Genetics ULB, Nivelles (2) and Center for Thrombosis and Vascular Research, University of Leuven (3), Belgium.

A hybrid between human tissue-type plasminogen activator (t-PA) and human single chain urokinase-type plasminogen activator (scu-PA) was obtained by ligation of cDNA fragments encoding the NH_2-terminal amino acids 1 to 67 of t-PA and the COOH-terminal amino acids 136 to 411, of scu-PA. Both this chimaeric cDNA and cDNA encoding scu-PA were expressed in a mammalian system (HAK-cells) using bovine papilloma virus (BPV) derived vectors. Two stable cell lines were obtained which secreted the recombinant hybrid and the scu-PA at 1 µg/ml and 2 µg/ml u-PA related antigen respectively into the culture medium. Following purification by Zinc chelate Sepharose, immunoadsorption chromatography, benzamidine-Sepharose and Ultrogel AcA44 gel filtration, highly purified proteins were obtained with a yield of about 200 µg/l. SDS gel electrophoresis under reducing conditions showed single bands with M_r 43,000 and M_r 50,000 respectively. Following conversion to urokinase with plasmin, both proteins had a specific amidolytic activity comparable to that of natural scu-PA. Both proteins activated plasminogen directly with K_m 1.4 and 0.5 µM and k_2 0.0034 s^{-1} and 0.0027 s^{-1}. Neither protein bound specifically to fibrin.

Thus the fusion of the finger-like domain of t-PA to the COOH-terminal part of scu-PA does not confer fibrin affinity of t-PA to this chimaeric protein. However, peptide material can be fused to the COOH-terminal part of scu-PA without perturbing its enzymatic properties.

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MUTANT AND CHIMAERIC RECOMBINANT PLASMINOGEN ACTIVATORS. L. Piérard (1), P. Jacobs (1), D. Gheysen (2), M. Hoylaerts (1), A. Cravador (1), A. Herzog (1), D. Collen (3) and A. Bollen (1). Dept. Appl. Genetics, ULB, Nivelles (1); SK-RIT, Rixensart (2); Center Thromb. Vasc. Research, Leuven (3) Belgium.

In order to produce plasminogen activators (PA) more specific and more active than their natural counterparts, we designed recombinant genes encoding mutant forms of urokinase (u-PA) and chimaeric molecules combining fragments of tissue type plasminogen activator (t-PA) and of u-PA. The following constructs have been realized : 1°) u-PA where amino acids Arg156 and Lys158 have been replaced by Thr. The purpose of this approach was to obtain a prourokinase molecule displaying similar properties as the natural single chain urokinase (scu-PA) but resistant to the cleavage by plasmin ; 2°) u-PA where the second cleavage site, Lys135-Lys136, was also eliminated either by replacing amino acid 132 to amino acid 147 by a shorter link (Ser-Thr) as found in t-PA, or by replacing the two lysines by glutamine residues. The resulting molecules correspond thus to completely uncleavable scu-PA forms ; 3°) an hybrid composed of the finger domain of t-PA and of the B-chain of u-PA ; 4°) an hybrid made of the A-chain of t-PA and of the B-chain of u-PA ; 5°) an hybrid where the kringle 2 of t-PA has been inserted between the kringle domain and the B-chain of u-PA. The last three constructs have been made to confer the fibrin binding specificity of t-PA to the B-chain of u-PA.
All recombinant DNAs were introduced, via an expression vector, into R1610 and CosI cells. Secretion of the recombinant products was monitored by ELISA and activities were assayed in an immobilized system involving a monoclonal antibody (AAU2) raised against 33K u-PA, plasminogen and the specific chromogenic substrate S2251. In this assay, all recombinant products, except the plasmin resistant (156-158) scu-PA, showed apparent specific activities comparable to the activity of natural two-chain u-PA. Potential interest of these new plasminogen activators in therapy will be discussed and further characterization of the new molecules will be presented.

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A FUSION PROTEIN OF THE A-CHAIN OF t-PA WITH LOW M_r scu-PA COMBINES THE FIBRIN-SPECIFICITY OF BOTH MOLECULES. H.R. Lijnen, L. Nelles, G. Lemmens, D. Collen and W.E. Holmes. Center for Thrombosis and Vascular Research, University of Leuven, Belgium.

A hybrid human cDNA was constructed by ligation of a cDNA fragment of tissue-type plasminogen activator (t-PA), encoding 5'-untranslated, the pre-pro region and amino acids Ser 1 through Thr 263, with a cDNA fragment of urokinase-type plasminogen activator (u-PA), encoding amino acids Leu 144 through Leu 411. The hybrid cDNA was expressed in Chinese Hamster Ovary Cells and the translation product purified from the conditioned cell culture media in the presence of aprotinin. On SDS-gel electrophoresis under reducing conditions, the protein migrated as a single band with approximate M_r 70,000 and on immunoblotting, it reacted with rabbit antisera raised against human t-PA and against human u-PA. The urokinase-like amidolytic activity (S-2444) of the protein was 320 IU/mg but increased to 43,000 IU/mg after treatment with plasmin, which resulted in conversion of the single chain molecule (t-PA/scu-PA) to a two-chain molecule (t-PA/tcu-PA).

Both proteins activated plasminogen directly with Michaelis constant (K_m) 1.5 µM and catalytic rate constant (k_2) 0.0058 s^{-1} for t-PA/scu-PA and with K_m = 80 µM and k_2 = 5.6 s^{-1} for t-PA/tcu-PA. CBNr-digested fibrinogen stimulated the activation rate of plasminogen with t-PA/tcu-PA (increase of k_2/K_m of 88-fold).

Both t-PA/scu-PA and t-PA/tcu-PA bound specifically to fibrin albeit more weakly than t-PA. In an in vitro system composed of a human ^{125}I-fibrin labeled plasma clot immersed in human plasma, the t-PA/tcu-PA hybrid has a higher fibrin-selectivity of clot lysis than tcu-PA, but this difference was not evident between t-PA/scu-PA and scu-PA. The stability of the t-PA/scu-PA hybrid in plasma was much higher than that of the t-PA/tcu-PA hybrid, a difference comparable to that between scu-PA and tcu-PA.

It is concluded that these t-PA/u-PA hybrid proteins combine fibrin-affinity of t-PA with the enzymatic properties of u-PA (either scu-PA or tcu-PA), resulting in improved fibrin-mediated plasminogen activation.

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