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MOLECULAR DEFECT IN PROTHROMBIN MADRID: SUBSTITUTION OF ARGININE 273 BY CYSTEINE PRECLUDES ACTIVATION.

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Prothrombin Madrid, a mutant prothrombin, was detected in a patient with a excessive bleeding history. The defect was characterized by a low coagulant activity contrasting with a normal level of prothrombin antigen in plasma. Activation of the purified protein was impaired by the absence of one of the two factor Xa catalyzed cleavages, generating meizothrombin which expressed a thrombin-like activity but was inactive on fibrinogen (Guillin et al., Ann. N.Y. Acad. Sci. 370:414, 1981). Prothrombin and prothrombin Madrid were isolated directly from plasma, with high yield, by immunoaffinity chromatography using conformation specific antibodies immobilized on Sepharose. After reduction and alkylation, purified proteins were hydrolyzed by trypsin. Resulting peptides were separated by reverse phase HPLC. Comparison of the two peptide maps showed that the prothrombin Madrid digest contained an additional peptide, identified by automated Edman degradation as residues 269 to 287 in prothrombin with the substitution of cysteine for arginine at position 273. Peptide 274-287, present in the prothrombin digest, was missing in the prothrombin Madrid digest. The mutation, precluding cleavage by factor Xa and normal generation of thrombin, is identical to the one described for prothrombin Barcelona. The two patients families are not related, raising the possibility that the gene coding for the cysteine 273 mutation in prothrombin is more common than anticipated. Of the seven mutants of vitamin K-dependant blood clotting proteins structurally characterized to date, three are functionally defective due to the presence of the propeptide on the mature amino-terminus (factor IX Cambridge, Oxford 3 and San Dimas) and three are due to an alteration that precludes zymogen activation (factor IX Chapel Hill, prothrombin Barcelona and Madrid). This sample remains too small to anticipate the different classes of point mutations seen in the human population but functional abnormalities of protein processing, metal and lipid binding, zymogen activation, substrate recognition and enzyme catalysis will likely be important phenotypes. However genetic defects may be limited to a discrete group of point mutations that have significant functional implication for the proteins.

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A SHARED EPITOPE ON HUMAN PROTEIN C, FACTOR X, FACTOR VII, AND PROTHROMBIN DEFINED BY A MONOCLONAL ANTIBODY. W. Church (1), T. Messier (1), P. Howard (1), J. Amiral (2), D. Meyer (3), K. Mann (1). Department of Biochemistry, University of Vermont, Burlington, VT, USA (1); Diagnostica Stago, Francoville, FRANCE (2); and Hospital Bicetre, Paris, FRANCE (3).

A monoclonal antibody prepared against human protein C (HPC) was found to react with several other vitamin K-dependent blood proteins. Using a competitive inhibition solid-phase radioimmunoassay with HPC, binding of ^{125}I-HPC to the antibody was inhibited by purified prothrombin, Factor X, and Factor VII in addition to protein C. Other vitamin K-dependent proteins including Factor IX, protein S, and bone-GLA protein did not compete for binding of ^{125}I-HPC to the antibody. The effect of calcium ion on the binding of antibody to ^{125}I-HPC was examined in a solid-phase immunoassay system with the antibody bound to rabbit anti-mouse immunoglobulin adsorbed to microtiter plates. In the presence of 5 mM calcium ion, radiolabeled protein C did not bind to the antibody; radiolabeled protein C did bind, however, in the presence of 5 mM EDTA suggesting that the epitope is expressed only after removal of calcium ion. The antibody bound to prothrombin and to decarboxylated prothrombin after adsorption of the antigens onto nitrocellulose indicating that the presence of GLA was not required for antibody binding. Immunoblotting of proteins which were reduced, the peptides separated by SDS-PAGE, and transferred to nitrocellulose showed that the antibody reacts with a determinant found on the light chains of protein C and Factor X and with prothrombin Fragment 1. Comparison of the protein sequences of protein C light chain, Factor X light chain, Factor VII, and prothrombin Fragment 1 identified a segment of amino acid sequence that is highly conserved in all four proteins and might contain the antigenic site. The monoclonal antibody thus defines an antigenic determinant which is masked by calcium ion and is found on the surface of several related, yet different coagulation proteins. This antibody should prove useful in understanding the evolutionary relationships amongst the vitamin K-dependent proteins and also in understanding the effect of calcium ion on the structure of protein C, Factor X, prothrombin, Factor VII and possibly other related proteins. (Supported by NIH grant NHLBI HL35058)

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COVALENT Mr ~92,000 HYBRID PLASMINOGEN ACTIVATOR DERIVED FROM THE PLASMIN FIBRIN-BINDING DOMAIN AND THE TISSUE PLASMINOGEN ACTIVATOR CATALYTIC DOMAIN. K.C. Robbins and I. Boreisha. Joint Section of Hematology/Oncology, Michael Reese Hospital and Medical Center, Chicago, IL., U.S.A.

A covalent hybrid plasminogen activator was prepared from the sulfhydryl forms of the NH_2-terminal A chain of human plasmin (Pln_A) containing the fibrin-binding domain, and the COOH-terminal B chain of tissue plasminogen activator ($t\text{-}PA_B$) containing the catalytic domain. The $Pln_A(SH)_2$ and $t\text{-}PA_B(SH)$ chains were mixed in a 1:1 molar ratio, and hybridization was allowed to proceed at 4°C for 6 days. The covalent $Pln_A\text{-}t\text{-}PA_B$ hybrid activator was isolated from the mixture by a two-step affinity chromatography method, with L-lysine-substituted Sepharose and Zn-chelated agarose. The protein yield of purified hybrid was 10% with a major component (77%) of Mr ~92,000. The covalent $Pln_A\text{-}t\text{-}PA_B$ hybrid activator, contained 1 mol of each chain; after reduction, it gave the two parent chains, Pln_A and $t\text{-}PA_B$, also shown to be present by double immunodiffusion. The specific plasminogen activator activity, with soluble fibrin, and the specific amidolytic activity, of the purified covalent hybrid activator was determined to be 200,000 IU/mg of protein, about 40% of the specific activity of the parent t-PA. In a fibrin clot lysis assay, the covalent hybrid activator and t-PA have similar specific fibrinolytic activities, 500,000 IU/mg of protein; however, the clot lysis time curves were not parallel. The binding of the covalent $Pln_A\text{-}t\text{-}PA_B$ hybrid activator and t-PA to forming fibrin were found to be similar; at physiological fibrinogen concentrations, binding of both activators to forming fibrin was about 90%.

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CONSTRUCTION AND EXPRESSION OF HYBRID PLASMINOGEN ACTIVATORS PREPARED FROM TISSUE-PLASMINOGEN ACTIVATOR (t-PA) AND UROKINASE (u-PA) GENES. S.G. Lee, N.K. Kalyan, J. Wilhelm, W-T. Hum, R. Rappaport, S.M. Cheng, S. Dheer, C. Urbano, M. Levner and P.P. Hung. Microbiology Division, Wyeth Laboratories Inc., P.O. Box 8299, Philadelphia, PA 19101, USA.

Recent data from several studies have suggested that the non-protease domains in t-PA and u-PA determine their biological specificity including binding to fibrin clots and survival in the circulatory system. Structural manipulations (e.g. deletions, additions or substitutions) in these domains can thus be utilized to maximize the desired biological effects. Using recombinant DNA technology, we constructed a number of hybrid molecules from the t-PA and u-PA genes. In hybrid A, the EGF and finger domains of t-PA (a.a.1-91) were replaced by the EGF and kringle of u-PA (a.a.1-131). In hybrids B and C, the u-PA kringle (a.a.50-131) was inserted either before (a.a.92) or after (a.a.261) the double kringle region of t-PA. All these hybrid PAs containing three kringles were expressed in mouse fibroblast cells (C-127). The hybrid proteins were synthesized in predominantly a single-chain form with molecular weights of 70-80 K by SDS-PAGE and were enzymatically active as assayed by the fibrin-agar plate method. In vitro studies on the binding of hybrid PAs to fibrin showed that while hybrid B, like t-PA, possessed high affinity toward fibrin, hybrid A showed lower binding. This suggests that the finger domain, which is not present in hybrid A, plays a role in conferring fibrin affinity to the hybrid PAs. The enzymatic activities of hybrid B were compared with that of recombinant t-PA expressed in the same vector/host system. Hybrid B and t-PA were found to be very similar in activity toward a chromogenic peptide substrate. In addition, their activities in fibrin-dependent plasminogen activation were almost equal.