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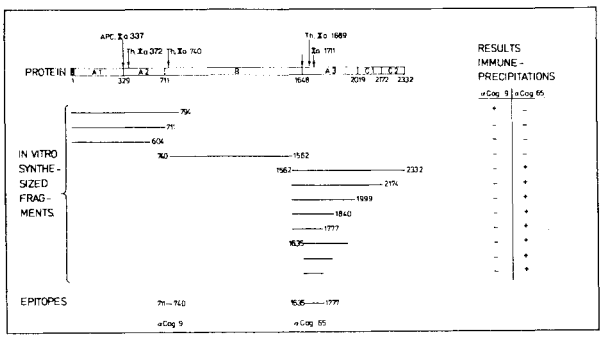
EPITOPE MAPPING OF HUMAN COAGULATION FACTOR VIII WITH IN VITRO SYNTHESIZED FRAGMENTS OF THE PROTEIN. A. Leyte, R.F. Evers and M. Ph. Verbeet. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Dept. of Molecular Biology, Amsterdam, The Netherlands.

We have used recombinant DNA techniques to map the epitopes of the coagulation Factor VIII for several monoclonal antibodies, raised against this protein. For this purpose, we cloned full-length- and partial Factor VIII cDNA sequences in the vector pSP64. Corresponding RNA fragments were synthesized in vitro with SP6 RNA polymerase and translated in a rabbit reticulocyte lysate system. The resulting Factor VIII polypeptide fragments were immunoprecipitated. We have located the binding sites of a panel of monoclonal anti-Factor VIII antibodies. Two examples are shown in the figure below. The epitopes for anti-Factor VIII CLB-9 and CLB-65 have been confined to areas of 29 (711-740) and 142 (1635-1777) amino acids, respectively. The results of these studies will be useful in determining structure-function relationships of Factor VIII.



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ANALYSIS OF STRUCTURAL REQUIREMENTS FOR FACTOR VIII FUNCTION USING SITE-DIRECTED MUTAGENESIS. Debra D. Pittman, Louise C. Wasley, Beth L. Murray, Jack H. Wang, Randal J. Kaufman, Genetics Institute, Cambridge, MA

Factor VIII (fVIII) functions in the intrinsic pathway of coagulation as the cofactor for Factor IXa proteolytic activation of Factor X. fVIII contains multiple sites which are susceptible to cleavage by thrombin, Factor Xa, and activated protein C. Proteolytic cleavage is required for cofactor activity and may be responsible for inactivation of cofactor activity. In order to identify the role of the individual cleavages of fVIII in its activation and inactivation, site-directed DNA mediated mutagenesis of fVIII was performed and the altered forms of fVIII produced and characterized. Conversion of Arg residues to Ile residues at amino acid positions 740, 1648, and 1721 resulted in resistance to thrombin cleavage at those sites with no alteration of in vitro procoagulant activity. Modification of the thrombin cleavage sites at either positions 372 or 1689 resulted in loss of cofactor activity suggesting that these sites are important for activation. Modification of the postulated activated protein C cleavage site at position 336 resulted in fVIII with a higher specific activity than wild type, possibly due to resistance to proteolytic inactivation.

DNA mediated mutagenesis was also used to study the role of post-translational biosynthetic modifications of fVIII. Structural characterization of recombinant fVIII suggested the presence of sulfated tyrosine residues within two acidic regions located between amino acid residues 336-372 and 1648-1689. Individual modification of these Tyr residues to Phe had negligible effect on synthesis and in vitro cofactor activity. The effect of combinations of these mutations on secretion, cofactor activity, and vWF interaction will be presented.

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ACTIVATION AND INACTIVATION OF PURIFIED HUMAN FACTOR VIII BY THROMBIN. K. Sewerin, A. Lundin, E. Hellström, K. Larsen and H. Sandberg. Department of Research & Development, KabiVitrum AB, Stockholm.

Highly purified human factor VIII was activated by human thrombin. The factor VIII material used showed variation in molecular weight and was separated into one heterogeneous form with a molecular weight ranging from 280 to 185 kDa and into one homogeneous 170 kDa form. Incubation of the (280-185) kDa form of factor VIII with thrombin resulted in proteolytic degradation of the heavy chain and formation of a 90 kDa peptide chain. This chain was further split into one 52 kDa and one 43 kDa peptide chain, as was the 90 kDa chain present in the 170 kDa form of factor VIII. The light chain of 80 kDa was split into a 70 kDa peptide chain. The change in peptide composition was followed by scanning and integration of SDS-PAGE gels on samples from the activation of both forms of factor VIII with thrombin. No strict relationship between factor VIII activity and degradation or formation of a new peptide chain was seen.

When factor VIII bound to von Willebrand factor matrix was incubated with thrombin, activated factor VIII was released. The released material consisted mainly of a 90 kDa and a 70 kDa peptide chain, but the presence of the 52 and 43 kDa peptide chains increased with the passage of time. The concentration of Ca^{2+} ions was of great importance for the degree and rate of activation. A high concentration of $CaCl_2$ resulted in a lower degree of activation but also slower inactivation of the factor VIIIa formed. However, no permanent stabilization of factor VIIIa was noted at either low or high concentrations of $CaCl_2$.

Inhibition or removal of thrombin from the factor VIII system, by the use of protease inhibitors or matrix bound thrombin, did not prevent the loss of factor VIII activity following the activation. The peptide composition was stabilized, however, when thrombin was removed. This indicates that the inactivation of factor VIIIa is due to an intramolecular change in the factor VIII molecule.

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THE INTERACTION OF THE FACTOR VIII/vWF COMPLEX WITH IMMOBILISED METAL AFFINITY CHROMATOGRAPHY (IMAC) MATRICES R.H. Saundry, P. Kopp and G.F. Savidge. Division of Haematology, United Medical and Dental School, Guy's and St. Thomas' Hospital, Lambeth Palace Road, London, SE1. ENGLAND.

IMAC principles were used to investigate the binding of Factor VIII/vWF from either $Al(OH)_3$ - absorbed citrated PPP or cryoprecipitate using citrate-saline buffers. Factor VIII/vWF showed high affinity for both Zn^{2+} - or Cu^{2+} - immobilised on biscarboxymethylamino Sepharose 4B at ambient room temperature, but no interaction with Ca^{2+}, Mg^{2+}, Mn^{2+} or Cd^{2+} - immobilised matrices.

Factor VIII/vWF could be re-eluted as a single component from Zn^{2+} - columns using either decreasing pH, or increasing competitor ligand concentrations. In pH gradients comprising 14mM citrate, 2.14mM $CaCl_2$, 0.15m NaCl Factor VIII eluted at pH 6.50, whereas in pH gradients comprising 0.1m Tris, 2.5mM $CaCl_2$ pH 7.5 and 0.1M succinate, 2.5mM $CaCl_2$ pH 5.5 Factor VIII eluted at pH 6.06. Citrate behaves as a competitor ligand; at 4°C in citrate buffer Factor VIII did not bind.

In citrate-saline buffers pH 7.2 Factor VIII could be re-eluted in high yield (100% Factor VIII:Ag; 70% VIII:C; 90% vWF:Ag) through application of linear competitor ligand gradients (at 11.5mM imidazole, 15.4mM dl-Histidine, 45mM L-Lysine, or 1.7% BSA), whereas 1.0M NaCl or 1.0M NH_4Cl were ineffective. The Factor VIII activities co-eluted ahead of, but incompletely resolved from the re-eluted Fg and Fn. If 5 mg/ml. BSA was incorporated into the buffers Factor VIII was quantitatively recovered in the column wash - through fractions leaving all the Fg and Fn bound to the matrix.