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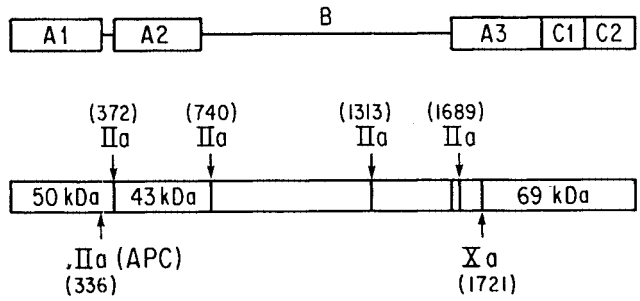
DIRECTED MUTAGENESIS IN THE STUDY OF THE REQUIREMENTS FOR FACTOR VIII ACTIVITY IN VITRO AND IN VIVO. Randal J. Kaufman, Debra D. Pittman, Louise C. Wasley, W. Barry Foster, Godfrey W. Amphlett, Alan R. Giles+. Genetics Institute, Cambridge, MA and +Departments of Pathology and Medicine, Queen's University, Kingston, Canada.

Factor VIII is a high molecular weight plasma glycoprotein that functions in the blood clotting cascade as the cofactor for factor IXa proteolytic activation of factor X. Factor VIII does not function proteolytically in this reaction but itself can be proteolytically activated by other coagulation enzymes such as factor Xa and thrombin. In the plasma, factor VIII exists as a 200 kDa amino-terminal fragment in a metal ion stabilized complex with a 76 kDa carboxy-terminal fragment. The isolation of the cDNA for human factor VIII provided the deduced primary amino acid sequence of factor VIII and revealed three distinct structural domains: 1) a triplicated A domain of 330 amino acids which has homology to ceruloplasmin, a plasma copper binding protein, 2) a duplicated C domain of 150 amino acids, and 3) a unique B domain of 980 amino acids. These domains are arranged as shown below. We have previously reported the B domain is dispensible for cofactor activity in vitro (Toole et al. 1986 Proc. Natl. Acad. Sci. 83: 5939). The in vivo efficacy of factor VIII molecules harboring the B domain deletion was tested by purification of the wildtype and modified forms and infusion into factor VIII deficient, hemophilic, dogs. The wildtype and the deleted forms of recombinant derived factor VIII exhibited very similar survival curves (T1/2 = 13 hrs) and the cuticle bleeding times suggested that both preparations appeared functionally equivalent. Sepharose 4B chromatography indicated that both factor VIII molecules were capable of binding canine plasma vWF.

Further studies have addressed what cleavages are necessary for activation of factor VIII. The position of the thrombin, factor Xa, and activated protein C (APC) cleavage sites within factor VIII are presented below. Site-directed DNA medicated mutagenesis has been performed to modify the arginine at the amino side of each cleavage site to an isoleucine. In all cases, this modification resulted in molecules that were resistant to cleavage by thrombin at the modified site. Modification of the thrombin cleavage sites at 336 and 740 and modification of the factor Xa cleavage site at 1721 resulted in no loss of cofactor activity. Modification of the thrombin cleavage site at either 372 or 1689 destroyed cofactor activity. Modification of the thrombin cleavage site at 336 resulted in a factor VIII having an increased activity, possibly due to resistance to inactivation. These results suggest the requirement of cleavage at residues 372 and 1689 for cofactor activity.



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INACTIVATION OF FACTOR VIII BY ACTIVATED PROTEIN C AND PROTEIN S. P.J. Fay (1), S.I. Chavin (1) and F.J. Walker (2) Departments of Medicine and Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, NY, U.S.A. (1) and American Red Cross, Connecticut Region, Farmington, CT, U.S.A. (2).

Human factor VIII has been isolated from factor VIII concentrates. The isolated protein is composed of a heavy chain and light chain. The heavy chain was heterogenous with respect to molecular weight ranging from 110-170 kDa. The light chain appeared as a 81/84 kDa dimer. When factor VIII was treated with activated protein C in the presence of calcium and phospholipids factor VIII procoagulant activity was rapidly lost. Analysis of the activated protein C catalyzed cleavage products of factor VIII indicated that loss of activity was correlated with cleavage of the heavy chains. The heavy chains appeared to be converted into 93 kDa and 53 kDa peptides. A separate factor VIII preparation has been prepared that contained only a 93 kDa heavy chain as well as the 81/83 kDa light chain. When this preparation was inactivated with activated protein C, a pathway in which the 93 kDa peptide was degraded into a 68 kDa peptide which was subsequently degraded into 48 and 23 kDa polypeptides. This result suggested that the 53 kDa polypeptide was not derived from the 93 kDa domain of the heavy chain, but must have been derived from the variable molecular weight portion of the heavy chain. These results suggest that activated protein C catalyzed a minimum of four cleavages in the heavy chain. Activated protein C did not appear to alter the factor VIII light chain. Protein S has been observed to be a protein cofactor both the anticoagulant and proteolytic action of activated protein C with factor Va. It is thought that protein S forms a lipid bound complex with activated protein C which then can rapidly inactivate factor Va. When factor VIII was inactivated in the presence of both activated protein C and protein S the rate of activity loss was enhanced. The effect of protein S could be observed on the cleavage of the heavy chains and on secondary cleavages of the smaller products including the 93, 68, and 53 kDa polypeptides. In an analogous reaction, the addition of factor Xa has been observed to inhibit the inactivation of factor Va by activated protein C. The addition of factor IX to the factor VIII-activated protein C reaction mixture resulted in the inhibition of factor VIII inactivation. The effect of factor IX was dose dependent. Finally, as both factor Va and factor VIII have structural similarities and are substrates for activated protein C the possibility that they might compete as substrates was tested. Factor VIII was observed to compete with factor Va for activated protein C. The concentration dependence of factor VIII inhibition of factor Va inactivation suggested that factor VIII and factor Va were equivalent substrates for activated protein C.