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TWO INDEPENDENT DOMAINS OF FACTOR VIII CO-EXPRESSED USING RECOMBINANT VACCINIA VIRUSES HAVE PROCOAGULANT ACTIVITY

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Using recombinant DNA technology, the NH_2 and COOH terminal domains of the human Factor VIII molecule were co-expressed in baby hamster kidney 21 (BHK21) cells using the vaccinia virus system. Procoagulant activity was detectable in cell supernatants, thus suggesting that the central portion present in the FVIII protein (domain B) is not required for FVIII function.

Factor VIII (FVIII) is an essential protein cofactor participating in the intrinsic pathway of coagulation at the level of Factor IXa-catalysed activation of Factor X (FX) to FXa (1). The chromosome X-linked bleeding disorder haemophilia A is due to a deficiency in FVIII procoagulant activity (FVIII:C). Purification of the natural protein (2-7) and gene cloning (8) have provided insight into the structure and function of FVIII and have enabled the mapping of processing sites responsible for the activation and subsequent inactivation of this molecule. An initial cleavage by an unknown protease at amino acid (aa) 1649 leads to proteolytic degradation of the central portion (domain B), and following thrombin cleavage of the NH_2 portion at aa position 740, polypeptides of approximately 90 Kd and 80 Kd are produced which are held together by a calcium bridge (see Fig. 1B). It has been shown (2-7) that the presence of these heavy and light chains is

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necessary and sufficient for FVIII:C, the thrombin activation sites being present at aa position 372 on the heavy chain and aa 1689 on the light chain. This observation suggests that the B region may not be required for the biological function of this molecule. Further cleavage of both chains by thrombin and additional factors (activated protein C or FXa) is thought to be responsible for the inactivation of FVIII (5).

Recently the entire human FVIII cDNA has been expressed in mammalian cell systems resulting in a biologically functional molecule (9-12). We have now tested the hypothesis that the heavy and light chains may be sufficient for procoagulant activity by co-expressing different portions of the FVIII cDNA using the vaccinia virus (VV) as vector (13-17). Our results confirm that the 90 Kd and 80 Kd moieties co-expressed in the same cell can function in a coagulation assay replacing FVIII and therefore the function of the B domain remains unassigned.

MATERIALS AND METHODS

Numbering of nucleotides (ntd) and aa is according to Wood, W.I. et al. (9).

Construction of recombinant VVs. pTG1016 (12) which carries the entire FVIII coding sequence was modified by deleting DNA between ntd 3936 and 6585 cleaving with SphI and subsequent religation (Fig. 1A). This heavy chain construct (pTG1021) contains sequences encoding aa 1-1293 of the FVIII molecule followed by 46 aa plus stop codon resulting from a frameshift due to the position of the 3' SphI site (dotted portion in Fig. 1A). A similar light chain construct (pTG1025) was made by deleting the DNA between the two BclI sites at ntd 403 and ntd 4419 (Fig. 1A). This construction encodes the FVIII signal peptide, aa 1 to aa 115 (heavy chain), aa 1455 to 2232 and the natural FVIII stop codon. Both pTG1021 and pTG1025 were recombined in vivo with wild type (WT) VV (Copenhagen ts 26) thus creating VV.TG.FVIII HC-1 and VV.TG.FVIII LC-1, respectively (13-17).

Cell infection and sample collection. $2x10^6$ BHK21 cells were infected with recombinant VVs or WT VV at 1 pfu/cell for one hour. After extensive washing, medium containing 4 % BSA, 1 mM $CaCl_2$, 0.5 units ml^{-1} of von Willebrand factor (vWf), but lacking fetal calf serum (FCS), was applied. At 24 and 48 hours after infection, supernatants were collected and quick frozen in liquid N_2 for subsequent testing.

FVIII : C levels were measured by a classical activated partial thromboplastin time (APTT) test (18).

Immunoradiometric assay (IRMA). Determination of FVIII antigen (FVIII:Ag) levels was performed using a "sandwich" assay (19). Tubes were coated with immunoglobulin G isolated from the serum of a haemophilic inhibitor-patient. Subsequently a monoclonal anti FVIII antibody (specific for the light chain domain) was used as the labelled antibody.

RESULTS AND DISCUSSION

FVIII heavy and light chains were studied by expressing the corresponding portions of the cDNA sequence in BHK21 cells using VV as vector. Both chains contain the FVIII signal peptide and include the thrombin cleavage sites necessary for proteolytic activation of the molecule (Fig. 1). These DNA constructions were integrated in the VV genome so that expression was initiated at the VV 7.5 K promoter (13, 14). The recombinant viruses were designated VV.TG.FVIII HC-1 and VV.TG.FVIII LC-1 containing the heavy and light chains, respectively.

After co-infection of BHK21 cells with these two recombinant VVs, supernatants were tested for the presence of FVIII:C (Table 1). Significant

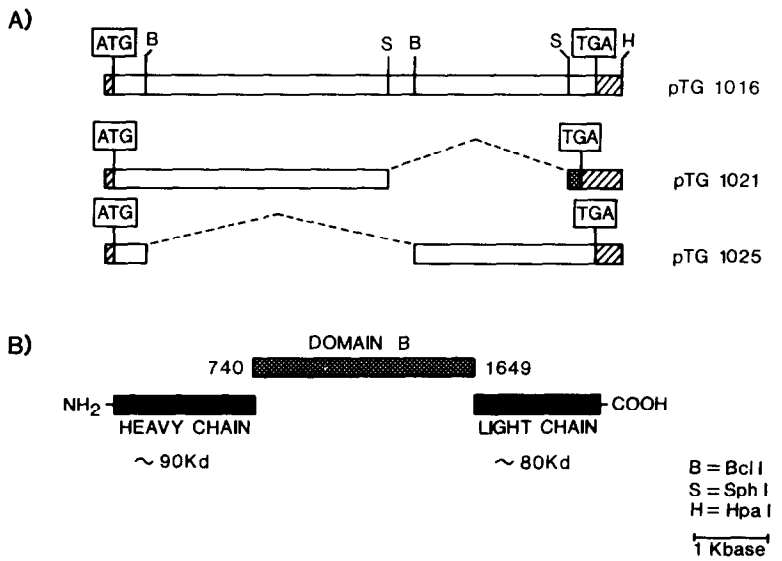

Fig. 1.

A) Schematic representation of the construction of two separate plasmids containing either the heavy chain (pTG1021) or the light chain (pTG1025) FVIII cDNA sequence. At the top the FVIII cDNA sequence in pTG1016 is shown. 5' and 3' untranslated regions are hatched. The start (ATG) and stop (TGA) codons are indicated. The restriction endonuclease sites used for the assembly of the two sequences are shown (see MATERIALS AND METHODS for their ntd positions). The dotted portion indicates the cDNA sequence encoding the novel (non-FVIII) amino acids resulting from the frameshift at the 3' SphI site.

B) Primary (pre-activation) proteolytic products of the FVIII protein are shown. The size of the peptides are in scale with that of the cDNA. Note that the excision of the central domain B is achieved by progressive proteolytical degradation starting from its own COOH terminus. Numbers indicate the aa at which cleavage occurs.

TABLE 1 . FVIII:C and FVIII:Ag levels (mU ml^{-1}) in the supernatants of BHK21 cells infected with either recombinant VVs or WT VV

	24 hours		48 hours	
	FVIII:C	FVIII:Ag	FVIII:C	FVIII:Ag
VV.TG.FVIII 10.2-1	140	412	235	412
VV.TG.FVIII HC-1 + VV.TG.FVIII LC-1	10	195	-	95
VV.TG.FVIII HC-1	-	*	-	*
VV.TG.FVIII LC-1	-	407	-	247
WT VV	-	-	-	-

$2x10^6$ BHK21 cells were infected at 1 pfu/cell for one hour (see MATERIALS AND METHODS). Time 0 represents the time when medium lacking FCS and containing 4 % BSA + 1 mM $CaCl_2$ + 0.5 units ml^{-1} of vWf was applied. The APTT and IRMA assays were performed as described in MATERIALS AND METHODS. - : below detection limits (detection limit = 1 mU ml^{-1}). * : FVIII:Ag was detected using an anti FVIII heavy chain monoclonal antibody (CAG-1 72A3) but, due to the different assay system (liquid phase) used, these values are not comparable with those presented in Table 1.

procoagulant activity was detected after 24 hours, albeit lower than that found when the entire molecule (VV.TG.FVIII 10.2-1) is expressed. However a decrease in activity was observed at the 48 hour time point. This procoagulant activity was inhibited by either an anti-FVIII monoclonal antibody (CAG-1 175A7) (20) or serum from a haemophilic-inhibitor patient (data not shown). No FVIII:C was detected with WT VV (negative control) or with VV carrying either the heavy or the light chain portions. Moreover, FVIII:C was not demonstrated when supernatants from cells infected with VV containing either the heavy or the light chain domains were mixed in vitro.

In Table 1 FVIII:Ag data, obtained using a monoclonal antibody against the light chain, are shown. It is difficult to correlate the antigen values with those of FVIII:C since FVIII:Ag levels are higher compared to the functional activity. However, as was shown for FVIII:C, FVIII:Ag decreased significantly between 24 and 48 hours. A two-fold increase in FVIII:Ag is

observed in the supernatants of cells infected with VV.TG.FVIII LC-1 alone, when compared to the supernatant of cells which co-expressed the two chains. This is to be expected as in the latter case two viruses are competing for the available host cell machinery.

From these data it seems that only the co-expression of the two domains can restore the procoagulant activity of the FVIII molecule, since each single chain expressed independently does not give rise to detectable FVIII:C. However this novel FVIII molecule is less stable than the entire protein as is evidenced by the loss of activity and low antigen levels observed at the 48 hour time point. The addition of vWf in the cell supernatant had no stabilising effect on the procoagulant activity observed in the co-expression experiments, whereas the expression of the entire molecule was, as we already have observed (12), stabilized in the presence of vWf. At present we do not have the explanation for such an observation.

It was evident that the FVIII:C detected after co-expression of the heavy and light chains was lower than in the case of the complete molecule. Some other constraint, either lower expression levels of the heavy chain or inefficient association of the two chains, could be responsible for the observed lower FVIII:C. The failure to reconstitute FVIII:C by mixing supernatants of cells expressing either the light or heavy chains would indicate that either the concentration is prohibitively low for the formation of the two chain complex or that this association demands specific conditions yet to be discovered.

Similar results have been reported using a transient expression system in COS-1 cells (21). Furthermore Toole J.J. *et al.* (22) and Eaton D.L. *et al.* (23) have reported the expression of a functional FVIII molecule deleted in the central region. In the former case ten times more procoagulant activity was detected in cell supernatants when compared to the entire molecule. This truncated molecule is expressed ten times more efficiently possibly due to its smaller size. When compared to the co-expression of the two separate chains a more effective association of both domains after

thrombin cleavage is assured owing to the covalent linkage of the heavy and light chains. In fact the association kinetics of the separate heavy and light chains may well limit the formation of the active complex thus reflecting the observed lower FVIII:C found in the co-expression experiments.

In this paper we have demonstrated that two functional domains are responsible for FVIII:C, the central B portion of the molecule not being implicated.

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