A lack of factor VIII:C, manifested as a bleeding disorder due to the absence of clot formation, is known as hemophilia A, an X chromosome-linked inherited disease affecting 1–2 males/10,000. To determine the minimum functional domain(s) essential for factor VIII:C activity, we have expressed the amino-terminal (92-kDa) and carboxyl-terminal (80-kDa) proteolytic cleavage products as individual, secreted polypeptides in monkey cells without the 909-residue central region. We have found that neither terminal domain alone is able to promote coagulation in factor VIII:C-deficient plasma. However, when the 92- and 80-kDa peptides are co-expressed, clotting activity is readily detected. Thus, these two chains alone constitute an active or activatable complex. The central domain is required neither for activity nor for the assembly of an active complex from two chains expressed in trans. These results suggest that a truncated derivative of factor VIII:C may be useful in coagulation therapy.

Factor VIII:C functions at a midpoint in the multistep coagulation cascade as a cofactor in the activation of factor X by activated factor IX. The end point of the cascade is the rapid conversion of fibrinogen into insoluble fibrin to form a clot around a platelet plug. Factor VIII:C is a very large glycoprotein (native Mr = 330,000–360,000) (1–3) present in plasma at extremely low concentrations (4) and very susceptible to cleavage by thrombin, plasmin, activated protein C, and other serine proteases (5). As a result, it is isolated from plasma as a series of related polypeptides ranging from 210–40 kDa with predominant species of 92 kDa and 80 kDa (1, 2, 6–9, 12, 13). This complex pattern has made the analysis of the structure of active factor VIII:C difficult, although species of 92 kDa and 80 kDa seem to co-purify with material of high specific activity (10, 13).

The primary sequence, consisting of 2332 amino acids for the mature protein, has recently been deduced from the DNA sequence (7, 8, 11), and the cleavage sites responsible for the generation of the 92- and the 80-kDa proteins (Fig. 1A) have been determined by analysis of the amino acid sequences of the respective isolated fragments (6, 8, 9, 13). First, the 80-kDa protein is generated from the amino terminus by a thrombin cleavage at amino acid 740 (6, 12, 13). Both of these polypeptides are susceptible to further thrombin processing; the 92-kDa species is cleaved at amino acid 372 into 52- and 40-kDa proteins, and the 80-kDa molecule is cleaved at amino acid 1689 to a slightly smaller 73-kDa species (6, 9, 12, 13).

In addition to the proteolytic processing, an examination of the sequence of factor VIII:C suggests the existence of three domains. The amino and carboxyl termini contain the majority of the cysteine residues, but a paucity of potential N-linked glycosylation sites, whereas the interior linker region has the opposite distribution. The homology of factor VIII:C to another protein in the coagulation cascade, factor V, also suggests a multidomain structure. Activated bovine factor V has been shown to consist of two polypeptide chains, the amino- and carboxyl-terminal species, linked by a calcium bridge (14–17). Both chains seem to be required for factor V activity. The presence of a similar calcium bridge linking the amino-terminal domain to the carboxyl-terminal domain of factor VIII:C has been demonstrated by us and others (8, 13, 18).

By engineering the factor VIII:C cDNA, we have expressed the amino-terminal 92-kDa chain and the carboxyl-terminal 80-kDa chain as separate proteins. Co-expression of these individual 92- and 80-kDa regions results in a level of factor VIII:C activity comparable to that obtained from the expression of the whole factor VIII:C coding region. These proteins assemble together to form a thrombin-activatable complex. The presence of the intracellular linker region, containing 909 amino acids or about 40% of the total for the intact protein, is not required for factor VIII:C activity assayed in vitro. Moreover, assembly of the complex does not require the presence of the central region and occurs efficiently for the two chains expressed in trans.

**MATERIALS AND METHODS**

**DNA Transfections**

COS 7 cells (19) obtained from R. Tjian University of California, Berkeley, were cultured in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% fetal calf serum. To assay transient expression of factor VIII:C activity in mammalian cells, COS 7 cells were transfected with various plasmid DNAs using the calcium phosphate co-precipitation method (20) coupled with chloroquine dihydrophosphate treatment (21) essentially as described by Gorman (22). Transfections utilized 50 µg of the indicated plasmid DNA per 5 × 10⁵ cells plated on a 6-cm plate. Conditioned medium samples were obtained from a 48-h collection of media at 37 °C post-transfection.

**Factor VIII:C Assays**

Coagulation—Aliquots of 75 µl of medium conditioned by the growth of COS cells transfected with the indicated plasmids or mock-transfected were assayed for their ability to decrease the prolonged partial thromboplastin time of factor VIII:C-deficient plasma in the one-stage assay (23). Briefly, 75 µl of Platelin (General Diagnostics) was incubated for 3 min at 37 °C followed by the addition of 75 µl of factor VIII:C-deficient plasma plus 75 µl of the test sample for an additional 5-min incubation at 37 °C. A 75-µl aliquot of prewarmed 0.025 M CaCl₂ was added, and the clotting time was measured with a Becton Dickinson fibrometer. Normal human plasma diluted in COS...
Functional Domains of Factor VIII:C

12575

cell medium was used as a standard and is assumed to contain 1 milliunit of factor VIII:C activity per μl, corresponding to approximately 100 pg of factor VIII:C protein (4). For the coagulation inhibition assay, 160 μl of the indicated conditioned medium was incubated with 20 μl of a 100-fold dilution of a human factor VIII:C inhibitory serum (24) (Bethesda titer 1500 units/ml) or a 10-fold dilution of pooled human serum (50 mM imidazole, 0.1 M NaCl, 100 μg/ml bovine serum albumin, pH 7.3) for 2 min at 37°C. These samples were then assayed for residual coagulation activity.

Chromagen — The Coatest assay (obtained from Kabi) measures the generation of activated factor X (Xa) as a linear function of the concentration of factor VIII:C (25). The concentration of factor Xa is measured by the proteolytic cleavage of p-nitroaniline from a synthetic peptide substrate for Xa. Normal human plasma diluted in COS cell medium was used as the standard. Aliquots of 25 μl of conditioned medium were measured in the end point assay as outlined by the manufacturer except that the assay volumes were uniformly reduced 5-fold.

Radioimmunoassay (RIA) Specific for the 92-kDa Polypeptide— Purified dog factor VIII:C inhibitory IgG was coated onto the wells of a 96-well polystyrene microtiter plate at a concentration of 3.5 μg/ml in 0.1% sodium carbonate buffer, pH 9.8, by overnight incubation at 37°C. The plates were washed 3 times with 0.1 M NaCl, 0.05% Tween 20 followed by an incubation with a mixture of test medium samples and iodinated VIII:C 92-kDa protein, both diluted in 0.05 M imidazole, 0.1 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 7.3. The VIII:C 92-kDa protein was isolated from plasma as described (8) and was greater than 50 percent homogenous as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining. After incubation for 16 h at room temperature, the plates were washed, and the amount of 125I in the individual wells was measured in a γ counter. An intermediate purified commercial factor VIII:C preparation (factor VIII, NORDISK) with a specific activity of 0.5 unit of coagulation activity per mg was used as the standard. This standard was calibrated against the World Health Organization Third International factor VIII:C standard. We defined our intermediate purified standard to contain a 92-kDa RIA activity/factor VIII:C coagulation activity ratio of 1.

Enzyme-linked Immunosorbent Assay (ELISA) Specific for the 80-kDa Polypeptide — The ELISA assay was performed as described (24). Normal human plasma was used as a standard.

Partial Purification of a Coagulation Active Complex

One hundred μg of an anti-80-kDa monoclonal antibody (56 IgG) (26) specific for the 80-kDa protein in the medium was incubated overnight at 20°C with 1.4 ml of medium obtained from COS cells co-transfected with plasmids pSVF8-92 and pSVF8-80 containing a total of 6.2 milliunits of activity (measured by the Coatest assay). After incubation, the slurry was loaded into a column, and the unretarded fraction was collected. The column was washed with 300 μl of 50 mM imidazole, 0.1 M NaCl, 0.1% sodium insulin, 0.2% NaN3, pH 7.3, and then eluted with 300 μl of 2.5 M NaCl, 50% ethylene glycol, 0.5 M imidazole, 0.1 M CaCl2, 0.1% sodium insulin, 0.2% NaN3, pH 7.3.

DNA Manipulations

Standard DNA manipulations followed protocols included in Maniatis et al. (27).

RESULTS AND DISCUSSION

To determine the functional significance of the apparent domain structure of factor VIII:C, a series of three plasmids was constructed to express and secrete the complete factor VIII:C protein, as well as both terminal regions as separate proteins. The amino-terminal 92-kDa domain is encoded by the expression vector pSVF8-92 (Fig. 1C), a plasmid which specifies the first 759 amino acids of factor VIII:C (Fig. 1A) including the 19-residue signal sequence and which terminates at a thrombin site (Arg574-Ser575) present in the intact protein. The carboxy-terminal 80-kDa domain was expressed as a 684-residue polypeptide by plasmid pSVF8-80 (Fig. 1D). A heterologous signal sequence was provided at the amino terminus of this protein by a precise fusion to the first 35 amino acids of tissue type plasminogen activator (28, 29). This fusion generates an Arg-Ser cleavage site at the tissue plasminogen activator-factor VIII:C junction to substitute for the Arg454-Glu455 proteolytic site used in the release of the 80-kDa domain from intact factor VIII:C. For purposes of comparison, the entire factor VIII:C coding sequence was placed into the vector pSV7d to generate the plasmid pSVF8-200 (Fig. 1E) (8).

Plasmids pSVF8-92, pSVF8-80, and pSVF8-200 were transfected into COS 7 cells both singly and together as noted in Table I to achieve transient expression of the cloned genes. At specific intervals post-transfection, conditioned medium was removed from the cells and tested for the ability to decrease the prolonged partial thromboplastin time of factor VIII:C-deficient plasma in a standard coagulation assay (23). The more specific Coatest assay (25), which measures the generation of activated factor X (Xa) as a linear function of the concentration of exogenously supplied factor VIII:C, was used to verify the results of the coagulation assay. The concentration of immunologically reactive factor VIII:C protein in the medium was determined by the use of a radioimmunoassay developed to detect the 92-kDa polypeptide and by an ELISA specific for the 80-kDa polypeptide (24).

As shown in Table I, expression of the 92-kDa polypeptide or of the 80-kDa polypeptide alone produced no detectable activity even though high levels of each of the individual proteins were present in the conditioned medium. However, the medium obtained from cells co-transfected with both plasmids contained about 20 milliunits/ml of coagulation activity. The same relative level of coagulation activity was secreted by cells transfected with the plasmid pSVF8-200 encoding the complete factor VIII:C protein. These results indicate that a complex of the amino- and carboxyl-terminal domains of factor VIII:C retains intrinsic coagulation activity and that the interior domain is not essential for activity nor for the assembly of an active complex from separate chains.

The results from Table I would suggest that the level of coagulation activity of the two-chain complex is limited by the expression of the 92-kDa chain since the ratio of factor VIII:C chromogenic activity/92-kDa RIA activity is 0.4, whereas the ratio of factor VIII:C chromogenic activity/80-kDa ELISA activity is 0.018. Also, it appears that the ratio of Coatest activity/92-kDa RIA activity is approximately the same for the entire protein, 0.4 and 0.38, respectively, suggesting that assembly of the complex is a relatively efficient process. However, we note that the absolute amounts of the 92-kDa protein, the 80-kDa protein, and the 330-kDa protein are not strictly comparable, as the immunosassays and the coagulation assays may be dependent on the exact molecular form of the molecule(s). Furthermore, different standards are used in the assays. It is, however, obvious that there is a large excess of coagulation-inactive 80-kDa protein in the medium from the co-transfectants.

When conditioned media from the pSVF8-92 and the pSVF8-80 single transfectants were mixed together (using several different conditions as outlined in Table I), no activity was measurable. The failure to reconstitute the activity of the complex in vitro could result from the instability of one (or both) polypeptides when present individually. Alternatively, the concentration of one (or both) of the individual peptides in the media may be too low to support complex formation. These results contrast with those obtained with factor V where the analogous polypeptides can be reassembled into an active complex from the isolated chains in vitro (16, 17). The
**Functional Domains of Factor VIII:C**

**A**, diagram of human factor VIII:C cDNA and protein coding sequences. **B**, the mammalian cell expression vector pSV7d (2423 bp) (8) consists of a 1845-bp fragment of pML (30) (nucleotides 4209 to 972 bp of pBR322 (31) with a deletion of 2490 to 1098 bp). The NruI site is fused to the 323-bp puuII to StuI segment of SV40 (nucleotides 270-5190) encompassing the early promoter and origin of replication (32). A synthetic 47-bp oligomer is fused to the StuI site followed by a 214-bp fragment of SV40 containing a polyadenylation site from the BglII site at nucleotide 2770 to nucleotide 2556. Tick marks indicate 1000-bp segments. Selected restriction sites shown for the plasmids are: B, BamHI; Bc, BclI; Bg, BglII; E, EcoRI; H, HindIII; Hp, HpaI; K, KpnI; N, NdeI; Nr, NruI; P, PstI; Pu, PvuII; S, SalI; Sc, SacI; Sm, SmaI; St, StuI; X, XbaI. Not all of these sites are shown for each plasmid. Those sites enclosed within parentheses were used in the vector construction, but were not regenerated. C, the 92-kDa polypeptide expression vector. Starting from the BamHI site in the polylinker of pSV7d, pSVF8-92 consists of a 49-bp synthetic linker-adapter molecule from BarnHI to SacI encoding nucleotides -30 to +14 of the factor VIII:C protein (numbering from the first nucleotide of the translational start site) (8), a 2267-bp SacI to HindIII fragment from the factor VIII:C cDNA, and pSV7d from HindIII to BamHI. D, the 80-kDa polypeptide expression vector. Starting from the SalI site in the polylinker of pSV7d, pSVF8-80 consists of a 201-bp fragment of a tissue plasminogen activator cDNA from nucleotides -98 to +103 terminating at a BglII site (28, 29), a 29-bp synthetic BglII to BclI linker-adapter encoding nucleotides +5002 to +5031 of factor VIII:C (8) ligated to a 2464-bp BclI fragment of factor VIII:C spanning from a BclI site created at nucleotide 5028 of the factor VIII:C cDNA through in vitro mutagenesis (33), to a BclI site in the 3' untranslated region, at nucleotide 7492, and a 400-bp fragment of tissue plasminogen activator 3' untranslated sequence spanning from a BglII site at nucleotide 2076 to a synthetic PstI site generated from cDNA cloning, followed by the polylinker from the vector M13mp9 (34) and then pSV7d. E, the complete factor VIII:C protein expression vector pSVF8-200. This plasmid contains the entire factor VIII:C cDNA coding and 3' untranslated sequences (8). The 5' untranslated sequences are the same as described above for pSVF8-92.

Sensitivity limits of the assays correspond to about 0.9 milliunit/ml for the coagulation test and 1.0 milliunit/ml for the Coatest assay. Thus, if active individually, the separate polypeptide chains must be at least 1000-fold less active than the complex under the conditions tested.

To verify that the observed coagulation activity was due to factor VIII:C, the sensitivity of the coagulation to inhibition by an antibody specific for factor VIII:C was determined. Prior to assay, aliquots of conditioned medium was preincubated with normal human serum or serum from a hemophiliac who had developed a high titer of inhibitory antibodies to factor VIII:C (24). The coagulation activity of the complete molecule and that of the 92-80-kDa complex were both reduced specifically by the inhibitory serum (Table II). The same results were obtained using three different inhibitory mouse monoclonal antibodies which bind to the 80-kDa species (data not shown).

To demonstrate more clearly the existence of a two-chain complex, we have partially purified the active species from the COS cell medium by passage over a monoclonal antibody column directed against the 80-kDa portion. Approximately 65% of the applied activity was retained, and 50% of this bound material was eluted in an active and 5-fold concentrated form (Table III). Thus, an active complex can be
plasmids pSVF8-92 plus pSVF8-80, 1.4 ml in total, containing 6.2 milliunits of Coatest activity, was absorbed batchwise to an 80-kDa titer 1500 units (92 kDa).

Earlier experiments had collected. The column was washed with 50 mM imidazole, 0.1 M NaCl, 0.1% sodium insulin, 0.2% NaN₃, pH 7.3, and then eluted with the activity is readily eluted by a buffer containing 0.5 M N-acetylglucosamine. However, when the recombinant 92-kDa complex was bound. This result suggests that the presence of the internal domain, which contains the vast majority of potential N-linked glycosylation sites, is necessary for binding to wheat germ lectin.

We have shown here by expression of specific domains of the factor VIIIC protein that the amino- and carboxyl-terminal two-chain complex is sufficient to generate activity in an in vitro coagulation assay. This conclusion has also been reached by other workers in the field based on very different experimental approaches. For example, monoclonal antibodies which inhibit the coagulation activity of factor VIIIC in vitro have been found to bind to epitopes within the 80-kDa domain, as well as within the 92-kDa domain, whereas monoclonal antibodies directed against epitopes within the central region showed no inhibition (2, 3, 25). It has recently been shown that a M₄ = 170,000 complex consisting of two polypeptide chains of M₄ = 90,000 and M₄ = 80,000 can be purified from human plasma (10). Although this material is a minor fraction of the factor VIII:C activity, it has the same high specific activity as the major peak (13).

A 160-µl aliquot of conditioned medium from COS7 cells transfected with the indicated plasmids was preincubated with 20 µl of a 100-fold dilution of a human factor VIII:C inhibitory serum (Bethesda titer 1500 units) or a similar dilution of pooled normal human serum or diluent alone for 2 h at 37 °C. These samples were then assayed using the one-stage coagulation assay (29).

### TABLE I

**Assay of recombinant factor VIII:C activity in conditioned media**

| Plasmid expressed | Coagulation activity | Chromogenic activity |
|-------------------|----------------------|----------------------|
|                   | Coagulation time     | Chromogenic activity |
|                   | s milliunits/ml      | (92 kDa)             |
|                   |                      | ELISA (80 kDa)       |
| pSVF8-92          | 95.7                 | <0.5                 |
|                   |                      | <1.0                 |
|                   |                      | 150                  |
|                   |                      | <10                  |
| pSVF8-80          | 97.2                 | <0.9                 |
|                   |                      | <1.0                 |
|                   |                      | <10                  |
|                   |                      | 1,360                |
| pSVF8-92 + pSVF8-80 | 56.1               | 22.5                 |
|                   |                      | 20.4                 |
|                   |                      | 50                   |
|                   |                      | 1,130                |
| pSVF8-200         | 47.7                 | 70.0                 |
|                   |                      | 43.2                 |
|                   |                      | 120                  |
|                   |                      | 280                  |
| None              | 94.6                 | <0.9                 |
|                   |                      | <1.0                 |
|                   |                      | <10                  |
|                   |                      | <10                  |
| pSVF8-92: mixed in vitro<sup>a</sup> | 95.7 | <0.9 | <1.0 | <10 | <10 |
| pSVF8-80          |                      |                      | nd<sup>a</sup> | nd |

<sup>a</sup> A variety of mixing conditions were tested including preincubation for various times up to 2 h at 37 °C, 20 °C, or 4 °C in the presence or absence of 10 mM CaCl₂. The value reported in this table is representative of the data obtained. Some transfections have produced as much as 950 milliunits/ml of 92-kDa RIA activity and up to 4350 milliunits/ml of 80-kDa ELISA activity. Even at these much higher expression levels, neither individual subunit has coagulation activity nor is an active complex produced by in vitro mixing.

### TABLE II

**Coagulation inhibition assay**

A 160-µl aliquot of conditioned medium from COS7 cells transfected with the indicated plasmids was preincubated with 20 µl of a 100-fold dilution of a human factor VIII:C inhibitory serum (Bethesda titer 1500 units) or a similar dilution of pooled normal human serum or diluent alone for 2 h at 37 °C. These samples were then assayed using the one-stage coagulation assay (29).

| Plasmid                | Serum<sup>a</sup> | Coagulation activity |
|------------------------|-------------------|----------------------|
|                        | s milliunits/ml   | (92 kDa)             |
|                        |                   | ELISA (80 kDa)       |
| pSVF8-80 + pSVF8-92    | N                 | 51.9                 |
|                        | I                 | 74.5                 |
|                        | D                 | 54.4                 |
| pSVF8-200              | N                 | 46.4                 |
|                        | I                 | 69.4                 |
|                        | D                 | 46.8                 |

<sup>a</sup> N, normal serum; I, inhibitory serum; D, diluent.

### TABLE III

**Partial purification of 92-80-kDa coagulation active complex**

Conditioned medium from COS7 cells co-transfected with the plasmids pSVF8-92 plus pSVF8-80, 1.4 ml in total, containing 6.2 milliunits of Coatest activity, was absorbed batchwise to an 80-kDa chain-specific monoclonal antibody coupled to Sepharose CL-4B. The slurry was loaded into a column, and the unbound fraction was collected. The column was washed with 50 mM imidazole, 0.1 M NaCl, 0.1% sodium insulin, 0.2% NaN₃, pH 7.3, and then eluted with the same buffer supplemented with 2.5 M NaCl, 50% ethylene glycol, and 0.1 M CaCl₂. The chromogenic and 80-kDa ELISA activity of each fraction was measured as noted under "Materials and Methods."

| Fraction | Chromogenic assay | 80-kDa ELISA |
|----------|------------------|--------------|
|          | milliunits/ml    |              |
| Media    | 4.4              | 175          |
| Unbound  | 1.7              | 13           |
| Eluate   | 20.0             | 760          |

isolated by affinity chromatography using an antibody specific for only the 80-kDa species.

We have also tried to purify the complex by chromatography on wheat germ lectin-Sepharose. Earlier experiments had shown that human factor VIII:C purified from cryoprecipitate binds quantitatively to this matrix, and that 50% of the activity is readily eluted by a buffer containing 0.5 M N-acetylglycosamine.<sup>7</sup> However, when the recombinant 92-kDa–80-kDa complex was applied to the same column, no activity was bound. This result suggests that the presence of the internal domain, which contains the vast majority of potential

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<sup>7</sup> R. L. Burke and M. Truett, unpublished results.

<sup>8</sup> R. L. Burke, unpublished results.
not clear. Are the intact 92- and 80-kDa chains partially active? Is it cleavage of the 92-kDa chain or of the 80-kDa chain or both which is required for activation? These types of questions may be answered by altering the molecules by in vitro mutagenesis so as to eliminate the possibility of thrombin cleavage at particular sites. Our results shown here, that the interior domain is not essential for the assembly of an active complex, and, moreover, that each terminal chain may be made as a separate protein, make a functional dissection of the protein simpler.

These results also point to the question of what the central domain of factor VIII:C does in vivo. The work of Brinkhous et al. (10) showed that a 170-kDa complex of the two terminal chains could combine with von Willebrand's factor and restore hemostasis in hemophilic dogs lacking factor VIII:C and that this preparation had a slightly longer half-life than larger complexes with active components of M_i = 185,000–280,000. Thus, it is clear that a 92–80-kDa complex is sufficient to restore hemostasis, and that a definition of the role of the linker region requires further analysis.

Acknowledgements—We would like to thank Pablo Valenzuela, Bruno Hansen, and Dino Dina for their continued support and encouragement. I. Anderson, A. Danielson, and R. Jergensen for expert technical assistance, our co-workers at Chiron for helpful discussions, and Dana Topping and Peter Anderson for preparation of this manuscript.

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