Factor VIII circulates as a noncovalent heterodimer consisting of a heavy chain (HC, contiguous A1-A2-B domains) and light chain (LC). Cleavage of HC at the A1-A2 and A2-B junctions generates the A1 and A2 subunits of factor VIIIa. Although the isolated A2 subunit stimulates factor IXa-catalyzed generation of factor Xa by ~100-fold, the isolated HC, free from the LC, showed no effect in this assay. However, extended reaction of HC with factors IXa and X resulted in an increase in factor IXa activity because of conversion of the HC to A1 and A2 subunits by factor Xa. HC cleavage by thrombin or factor Xa yielded similar products, although factor Xa cleaved at a rate of ~1% observed for thrombin. HC showed little inhibition of the A2 subunit-dependent stimulation of factor IXa activity, suggesting that factor IXa-interactive sites are masked in the A2 domain of HC. Furthermore, HC showed no effect on the fluorescence anisotropy of fluorescein-Phe-Phe-Arg-factor IXa in the presence of factor X, whereas thrombin-cleaved HC yielded a marked increase in this parameter. These results indicate that HC cleavage by either thrombin or factor Xa is essential to expose the factor IXa-interactive site(s) in the A2 subunit required to modulate protease activity.

Factor VIII, the plasma protein deficient or defective in individuals with hemophilia A, is synthesized as a 300-kDa precursor (1, 2) with the domain structure A1-A2-B-A3-C1-C2 (3). It is processed to a series of divalent metal ion-dependent heterodimers after cleavage at the B-A3 junction, generating a HC1 (A1-A2-B domains) and a LC (A3-C1-C2 domains). Additional cleavage sites within the B domain result in variably sized HCs minimally represented by contiguous A1-A2 domains. The two chains can be separated by chelating reagents (4, 5) and isolated after ion exchange and/or immunoaffinity chromatography. Factor VIII activity can be reconstituted from the separated chains by combining them in the presence of a divalent metal ion (6).

Factor VIII functions in the intrinsic factor Xase complex as a cofactor for the serine protease, factor IXa in the surface-dependent conversion of factor X to Xa. This activity is dependent upon conversion of factor VIII to the active cofactor form, factor VIIIa, by thrombin or factor Xa. These enzymes cleave factor VIII HC at Arg-740, removing the B domain (or fragments) and at Arg-372, bisecting the HC into the A1 and the A2 subunits (7). The proteases also cleave factor VIII LC at Arg-1689 (7), liberating an acid-rich region and creating a new NH$_2$ terminus. Thus, factor VIIIa is a heterotrimer of subunits designated as A1, A2, and A3-C1-C2 (8, 9). The A1 and A3-C1-C2 subunits retain the divalent metal ion-dependent linkage, whereas the A2 subunit is weakly associated with the A1-A3-C1-C2 dimer by primarily electrostatic interactions (9, 10).

Two regions of factor VIII have been identified as interactive sites for factor IXa. A high affinity site ($K_\text{d}$ ~15 nM (11)) was localized to the A3 domain of the LC in and around residues 1811–1818 (12). A second, lower affinity site ($K_\text{d}$ ~300 nM (13)) was localized within the (isolated) A2 domain and comprises residues 558–565 (14). Recently, isolated A2 subunit was shown to stimulate the $k_\text{cat}$ for factor IXa-catalyzed conversion of factor X by ~100-fold (13). This property appeared unique to A2 and was not observed for either the isolated A1 or A3-C1-C2 subunit. However, the A1 subunit synergistically increased the cofactor activity of the isolated A2 subunit by ~10-fold (15).

Proteolysis of the LC during activation is responsible for the dissociation of factor VIIIa from its carrier protein, von Willebrand factor (16). This cleavage also appears to increase the cofactor activity of factor VIIIa (17, 18). The function of HC cleavage (at Arg-372) is not well understood. However, this step is essential for generating cofactor activity based upon mutations at this which result in severe hemophilia (19). The isolated A2 subunit of factor VIIIa shows a several hundredfold weaker affinity for factor IXa and ~1% of the cofactor activity compared with intact factor VIIIa (13). It is unknown whether these low level activities are intrinsic to the HC or whether cleavage of the HC is necessary to manifest them. In this report we examine the capacity of isolated HC and derived subunits to modulate the catalytic activity of factor IXa in a purified system. Studies were performed in the absence of LC to preclude any interactions of this chain with factor IXa or factor X. Results show that intact HC possesses no detectable cofactor-like activity and fails to compete with isolated A2 subunit for interaction with factor IXa. However, its resultant cleavage by thrombin or (less efficiently) by factor Xa generates active subunits that show cofactor activity and modulation of factor IXa activity similar to that observed previously using the purified subunits. These results indicate that a primary role for HC cleavage during cofactor activation is the exposure of a functional factor IXa-interactive site.
MATERIALS AND METHODS

Reagents—The reagents α-thrombin, factor IXaβ, factor X, factor Xa (Enzyme Research Laboratories), and Fl-FFR-factor IXaβ (Molecular Innovations) were purchased from the indicated vendors. Phospholipid vesicles composed of 20% PS, 40% PC, and 40% PE (Sigma) were prepared using octyl glucoside as described previously (20). Tick anticoagulant peptide was a gift from Dr. S. Krishnaswamy. The anti-factor VIII monoclonal antibody, RSB12, which recognizes the COOH-terminal portion of the A2 domain (21), was prepared as described (9). Antibody 10104, an inhibitory monoclonal that binds the NH2-terminal region of factor VIII light chain (5), was obtained from QED BioScience. Monoclonal antibody 413 binds an epitope defined by residues 484–508 (22) and was a generous gift from Dr. Leon Hoyer.

Factor VIII Subunits—Recombinant factor VIII preparations were gifts from the Bayer Corporation and the Genetics Institute. Factor VIII HC was prepared as described previously (23) and is illustrated in Fig. 1. Potential trace levels of factor VIII LC present in the HC preparation were removed following chromatography using antibody 10104 coupled to Affi-Gel 10 as described previously (6). The A2 subunit was prepared following fractionation of factor VIIa using Mono S as described previously (9).

Factor Xa Generation Assays—The rate of conversion of factor X to factor Xa was monitored in a purified system (24). HC forms were reacted with factor IXa in 20 mM Hepes pH 7.2, 50 mM NaCl, 5 mM CaCl2, and 0.01% Tween 20 (buffer A) in the presence of 100 μM bovine serum albumin and 10 μM phospholipid vesicles. Reactions were initiated with the addition of factor X (for reactant concentrations, see figure legends). Aliquots were removed at appropriate times to assess the initial rates of product formation and added to tubes containing EDTA (80 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined by the addition of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were read at 405 nm using a Vmax microtiter plate reader (Molecular Devices).

Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli (25) with a Bio-Rad minigel system. Electrophoresis was performed at 200V for 1 h. Proteins were stained using Gel-Code Blue (Pierce). Alternatively, the proteins were transferred to a polyvinylidene difluoride membrane using a Bio-Rad mini-transblot apparatus at 0.5 amps for 30 min in a buffer containing 10 mM CAPS, pH 11, and 10% (v/v) methanol. Western blotting used the R8B12 monoclonal followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody. The secondary antibody signal was detected using the ECL system (Amersham PharmaSciences) with luminol as substrate, and the blots were exposed to film for various times. Films were scanned, and band densities (obtained from a linear exposure range) were quantitated by using ImageQuant software (Molecular Devices).

Rates of HC cleavage were calculated from the linear portion (initial time points) of the plotted data. Initial rates were estimated using best fit lines through points where ≤50% of the substrate had been converted to product. The concentration of HC remaining was calculated from band densities using the formula HC = (density of HC/density of HC + density of A2 subunit) × initial HC concentration. The concentration of A2 subunit formed following HC cleavage was calculated using the formula A2 subunit = (1 – density of HC/density of HC + density of A2 subunit) × initial HC concentration.

Fluorescence Spectroscopy—Fluorescence anisotropy measurements were conducted using an Amico-Bowman series 2 spectrometer equipped with automatic polarizers arranged in a 1-format. Reactions (0.2 ml) were run at room temperature in buffer A containing 90 nM Fl-FFR-Ixa, 460 nM HC form, and 50 μM PSPCPE vesicles in the absence or presence of 500 nM factor X. Samples were excited at 495 nm, and the emission intensity was monitored at 520 nm (band pass = 4 nm) for 2 s at each polarizer position. Anisotropy values were calculated automatically after subtraction of blank readings. Data were averaged for at least five anisotropy measurements.

RESULTS

Cleavage of Isolated HC Is Required for Cofactor Activity—Earlier we showed that the isolated A2 subunit of factor VIII stimulated the kcat for factor IXa-catalyzed conversion of factor X by ~1% the level observed for factor VIIIa (13). The effect was enhanced further by 1 order of magnitude in the presence of saturating A1 subunit (15). Isolated factor VIII HC was assessed similarly for cofactor activity using a factor Xa generation assay and employing purified components. HC was obtained from EDTA-treated factor VIII as described under “Materials and Methods” and was essentially free from LC.

Titration of intact HC in the factor Xa generation assay yielded virtually no activity increase under the reaction conditions described in Fig. 2. However, prior cleavage of HC by thrombin to yield the HCIIa (free A1 and A2 subunits) resulted in a marked increase in the rate of factor Xa formed which was dose-dependent and saturable with respect to the cleaved HC. The concentration of substrate factor X used in these reactions (500 nM) represented near Vmax levels (data not shown). The extent of factor IXa stimulation observed at near saturating levels of HCIIa (1 μM; kcat ~ 9 nM factor Xa generated/min/nM factor IXa) was similar to that observed for factor IXa in the presence of an equivalent concentration of purified A2 plus A1 subunits (kcat ~ 14 factor Xa generated/min/nM factor IXa) (15). These results indicate that intact HC possesses no detectable factor IXa-stimulating activity and that this property is expressed only after cleavage of the HC to component subunits.

To help exclude any contribution of trace factor VIII to these results, a series of experiments was performed in which HC was reacted first with one of three anti-factor VIII monoclonal antibodies prior to treatment with thrombin (Table I). Antibody 10104, a potent inhibitor that binds factor VIII LC (5), showed no effect in inhibiting HCIIIa activity, consistent with the absence of functional factor VIII in the assay. RSB12, an anti-A2 domainal monoclonal with little inhibitory activity, also showed no effect. However, antibody 413, which binds an epitope defined by A2 domain residues 484–508 (22) and blocks...
### Table I

| Antibody | Residual activity | Epitope (LC/HC, residues) |
|----------|------------------|--------------------------|
| None     | 100              |                          |
| 19104    | 98               | LC (1649–1689)           |
| R8B12    | 102              | HC (563–740)             |
| 413      | <1               | HC (484–508)             |

* Purified IgG (1 μM) was reacted with HC (300 nM) for 1 h prior to cleavage by thrombin and addition to the factor Xa generation assay containing 5 nM factor IXa, 10 μM PSpCPE vesicles, and 500 nM factor X in buffer A.

## Discussion

To gain insights into the contribution made by HC cleavage to cofactor activation, a series of experiments was performed using purified HC in the absence of LC to preclude any interaction of the latter with components of the assay. In this report we show that cleavage of isolated factor VIII HC is essential for subsequent interaction with factor IXa and stimulation of its catalytic activity. The isolated A2 subunit of factor VIII is capable of binding factor IXa and stimulating the activation of factor Xa.

The rate of A2 formation catalyzed by thrombin was significantly greater than that observed for factor Xa. Examination of initial time points (≤50% substrate utilized) using a linear curve fit indicated rates of ~33.3 min⁻¹ and 0.4 min⁻¹ for thrombin and factor Xa, respectively. Although these values obtained at a single HC concentration allow for a limited comparison of rate for substrate cleavage by the two enzymes under these reaction conditions, cleavage by factor Xa appeared to occur at ~1–2% the rate observed for thrombin. One likely reason for this markedly slower rate by factor Xa is the absence of LC, which is required for association of factor VIII with the phospholipid surface, a requisite step for efficient cleavage of factor VIII by this protease (27).

### Effects of HC and HClIa on the Fluorescence Anisotropy of Fl-FFR-Factor IXa

The above results using a functional assay demonstrate essentially no effect of isolated HC on the catalytic activity of factor IXa. To determine whether this chain influences the active site of the protease, fluorescence anisotropy using a Fl-FFR-labeled factor IXa was performed. Earlier results demonstrated that this parameter was affected little if any by isolated A2 subunit, whereas a significant increase in anisotropy was observed in the presence of substrate factor X (13). Inclusion of isolated HC showed no effect on the anisotropy of Fl-FFR-factor IXa, either in the absence or presence of factor X (Table II). However, prior cleavage of HC with thrombin resulted in a marked increase in anisotropy observed in the presence of factor X, consistent with the generated A2 subunit altering the factor IXa active site. These results indicate the uncleaved HC does not affect the active site of factor IXa, consistent with its lack of effect in enhancing catalytic activity.

### Competition Analysis between HC and A2 Subunit for Binding Factor IXa

The isolated A2 subunit binds factor IXa with a $K_d \sim 300$ nM, whereas the A1 subunit does not appear to bind the protease directly (13) nor affect the affinity of A2 subunit for factor IXa (15). Thus any potential factor IXa-interactive site in the HC is likely contained within the A2 domain. To assess the relative affinity of HC and the derived A2 subunit for factor IXa, a competition assay was performed using stimulation of factor IXa activity by A2 subunit as an indicator of the interaction. The effect of increasing amounts of HC on this stimulation was subsequently determined. Results in Fig. 5 show stimulation of factor IXa-catalyzed generation of factor Xa in the presence of 100 nM A2 subunit. Addition of HC resulted in little change in the A2-dependent stimulation of activity. At 1 μM HC, a 10-fold excess relative to A2 subunit, the A2-dependent activity was reduced by ~20%. This result suggests that the affinity of factor IXa for HC is at least 1 order of magnitude weaker than that for the A2 subunit.

### Discussion

To gain insights into the contribution made by HC cleavage to cofactor activation, a series of experiments was performed using purified HC in the absence of LC to preclude any interaction of the latter with components of the assay. In this report we show that cleavage of isolated factor VIII HC is essential for subsequent interaction with factor IXa and stimulation of its catalytic activity. The isolated A2 subunit of factor VIII is capable of binding factor IXa and stimulating the activation of factor Xa.
factor X (13); however, the isolated HC (i) showed no stimulation of factor IXa in the generation of factor Xa, (ii) did not affect the conformation in and around the active site of the enzyme in the absence or presence of substrate, and (iii) failed to compete effectively with the A2 subunit for binding factor IXa as judged by a functional assay. Thus, these results indicate that (isolated) HC is devoid of the basal cofactor-like activities associated with its component subunits. These results are consistent with the observed lack of cofactor activity in the unactivated factor VIII heterodimer (27).

It is of interest to note that both thrombin and factor Xa cleave the isolated HC, although with markedly disparate efficiency. The slow cleavage of HC by low levels of factor Xa in the Xa generation assay resulted in the time-dependent stimulation of the reaction rate. This can be attributed to production of the A2 subunit, which could then serve as cofactor to factor IXa. In a more controlled experiment, we show that HC cleavage by factor Xa occurred at 1–2% the rate observed for thrombin. These reactions contained a phospholipid surface, which is requisite for optimal factor Xa activity. However, the absence of factor VIII LC precluded association of HC with surface and this condition severely retarded cleavage by the protease. Consistent with this observation was the recent identification of a factor Xa-interactive site within the C2 domain of factor VIII (28).

### Table II

**Effects of HC cleavage and substrate on the fluorescence anisotropy of Fl-FFR-factor IXa**

| Condition* | Anisotropy§ | Δr  
|------------|-------------|-------|
| Fl-FFR-factor IXa | 0.228 ± 0.002 (5) | 0.011 |
| + HC | 0.226 ± 0.002 (6) | 0.016 |
| + HCIIa | 0.230 ± 0.002 (6) | 0.041 |

* Reactions contained 90 nM Fl-FFR-factor IXa and 50 μM PSPCPE vesicles in the absence and presence of 460 nM each intact HC or thrombin-cleaved HC (HCIIa). Factor X, when present, was at 500 nM.

§ Anisotropy values represent the mean ± S.D. for the number of determination indicated in parentheses.

Ar reflects the change in anisotropy observed in the presence versus the absence of factor X.
factor X site localized to HC residues 337–372 (29) may serve as an interactive site for (solution phase) factor Xa binding and cleavage of the subunit. Support for this speculation comes from studies employing a zero-length cross-linker that indicated that this site was bound by a region of the protease-forming domain of factor X distinct from the activation peptide sequence (30).

Using intact porcine factor VIII as substrate, Lollar et al. (27) showed that the catalytic efficiency ($k_{cat}/K_m$) for cofactor cleavage was ~5-fold greater for thrombin compared with factor Xa. Results of that study also indicated that factor VIIIa activity generated by thrombin was ~3-fold greater than that generated by factor Xa. Our data do not suggest that the isolated (human) HC cleaved by factor Xa possesses significantly less activity than thrombin-cleaved HC. Results obtained in Fig. 2 showed that the complete conversion of 300 nM HC by factor Xa yielded activity similar to that observed earlier with an equivalent concentration of isolated A2 plus A1 subunits prepared following cleavage of factor VIII by thrombin (15). Thus, the activator-dependent disparity in cofactor activity may reflect a differential effect and/or contribution of the factor VIII LC cleavage, or it may indicate a species difference.

Recently, Lollar and co-workers (31) characterized more fully factor Xa-activated (porcine) factor VIIIa and noted cleavage sites in the HC at Arg-219 (A1 domain) and Arg-490 (A2 domain) in addition to those HC sites cleaved by thrombin (Arg-372 and Arg-740). Although the Arg-490 site is present in the human protein, residue 219 is Gln and thus would not be attacked efficiently by the protease. However, Arg-490 in the isolated human HC did not appear to be cleaved at any appreciable level because the fragment generated (residues 491–740) would retain reactivity with the R8B12 antibody, and this fragment was not detected in our blots. We did note, however, that the spacing between the A2 doublet bands was somewhat greater for the Xa-cleaved material, reflecting a slightly smaller, lower $M_\text{r}$ band in the doublet. The reason for this is unclear at the present time.

The results presented in this study are compatible with, at best, a weak affinity interaction between HC and factor IXa. In an earlier report, Lenting et al. (11) used a solid phase binding assay in the absence of phospholipid to show that isolated factor VIII LC bound factor IXa with high affinity ($K_d \sim 15 \text{nM}$). However, no detectable factor IXa binding was observed using HC up to ~300 nM. A factor IXa site has been localized to residues 558–565 in the A2 subunit (14). Recently, we showed that isolated A2 subunit bound factor IXa in a phospholipid-containing system with a functional $K_d \sim 300 \text{ nM}$ (13). In the absence of a surface, saturation of factor IXa with A2 subunit is not readily achieved reflecting a $K_d > 5 \mu M$. Thus the phospholipid surface likely orients factor IXa such that collisions with A2 subunit are more productive. In this report, we show that a 10-fold molar excess of HC relative to A2 subunit marginally inhibited the A2-dependent stimulation of factor IXa activity, suggesting that the affinity of isolated HC for the enzyme is at least 10-fold weaker ($>3 \mu M$) than that of the derived A2 subunit.

One model consistent with these observations is that cleavage of HC is required to expose the factor IXa-interactive site(s). Several lines of evidence demonstrate a conformational change in factor VIII HC following the conversion of factor VIII to factor VIIIa. For example, reaction of factor VIIIa with the zero-length cross-linker, EDC, resulted in formation of a covalent linkage between A1 and A2 subunits (32), indicating the presence of a salt bridge at the site of cross-linking. However, treatment of factor VIII with EDC prior to cleavage by thrombin showed no linkage between the subunits, suggesting that the interdomain salt bridge was not present in the unactivated form. In addition, examination of binding of the apolar probe bisnaphthalenesulfonic acid to isolated factor VIII and factor VIIIa subunits revealed two exposed hydrophobic sites on the isolated HC (33) possessing affinities ($K_d$ values) of 0.21 and 1.4 $\mu M$. However, these sites contrast the single sites localized to the isolated A1 subunit (0.77 $\mu M$) and A2 subunit (0.11 $\mu M$), suggesting a change in conformation in and around these regions following thrombin cleavage. Finally, CD studies suggest an increase in $\beta$-sheet structure in factor VIIIa formed from factor VIII (34).

Little evidence exists for a specific conformational change in and around the factor IXa-interactive site in A2 after cofactor activation. Suggestive evidence comes from observations of the inactivation of factor VIII and factor IXa by activated protein C. In collaboration with Walker, we showed that the bovine protease binds the cofactor near the COOH-terminal region of the A3 domain in the light chain (35) and preferentially attacks Arg-562 (21) within the factor IXa-interactive site. Interestingly, the rate of cleavage at this site in factor VIIIa is ~5-fold faster than its cleavage in factor VIII (21). In factor VIIIa, this site is protected from cleavage by factor IXa (36). However, no factor IXa-dependent protection was observed using factor VIII (37). These results are compatible with differential exposure of the scissile bond at Arg-562 in the two substrates, with this region partially masked in the unactivated form. Taken together with the results of this study, these observations suggest that the factor IXa site localized to residues 558–565 in the A2 subunit is not fully formed in the contiguous A1-A2 domains of uncleaved HC. This lack of a functional factor IXa site in HC likely represents a primary requirement for cofactor activation at this domain junction and provides an explanation for the molecular basis of severe hemophilia attributed to cleavage-resistant mutations at Arg-372.

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