Cell Surface Heparan Sulfate Proteoglycans Participate in Factor VIII Catabolism Mediated by Low Density Lipoprotein Receptor-related Protein*

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We have demonstrated previously that catabolism of a coagulation factor VIII (fVIII) from its complex with von Willebrand factor (vWF) is mediated by low density lipoprotein receptor-related protein (LRP) (Saenko, E. L., Yakhyaev, A. V., Mikhailenko, L. Strickland, D. R., and Sarafanov, A. G. (1999) J. Biol. Chem. 274, 37685–37692). In the present study, we found that this process is facilitated by cell surface heparan sulfate proteoglycans (HSPGs). This was demonstrated by simultaneous blocking of LRP and HSPGs in model cells, which completely prevented fVIII internalization and degradation from its complex with vWF. In contrast, the selective blocking of either receptor had a lesser effect. In vivo studies of clearance of 125I-fVIII-vWF complex in mice also demonstrated that the simultaneous blocking of HSPGs and LRP led to a more significant prolongation of fVIII half-life (5.5-fold) than blocking of LRP alone (3.5-fold). The cell culture and in vivo experiments revealed that HSPGs are also involved in another, LRP-independent pathway of fVIII catabolism. In both pathways, HSPGs act as receptors providing the initial binding of fVIII-vWF complex to cells. We demonstrated that this binding occurs via the A2 domain of fVIII, since A2, but not other portions of fVIII or isolated vWF, strongly inhibited cell surface binding of fVIII-vWF complex, and the affinities of A2 and fVIII-vWF complex for the cells were similar. The A2 site involved in binding to heparin was localized to the region 558–565, based on the ability of the corresponding synthetic peptide to inhibit A2 binding to heparin, used as a model for HSPG.

Factor VIII (fVIII) is an essential component of the intrinsic pathway of blood coagulation, since genetic deficiency in fVIII results in a coagulation disorder known as hemophilia A and occurs in 1 per 5000 males. In the intrinsic pathway, activated fVIII (fVIIIa) functions as a cofactor for the serine protease factor Xa, and their membrane-bound complex (Xase complex) activates factor X to factor Xa (1). Factor Xa subsequently participates in activation of prothrombin into thrombin, the key enzyme of the coagulation cascade.

fVIII is a glycoprotein (~300 kDa, 2332 amino acid residues) consisting of three homologous A domains, two homologous C domains, and the unique B domain, which are arranged in the order of A1-A2-B-A3-C1-C2 (2). Prior to its secretion to plasma, fVIII is processed intracellularly to a series of Me2+–linked heterodimers produced by cleavage at the B-A3 junction (3) and by a number of additional cleavages within the B domain (2). These cleavages generate a heavy chain (HCh) consisting of the A1 (residues 1–336), A2 (residues 373–740), and B domains (residues 741–1648), and a light chain (LCh) composed of the domains A3 (residues 1690–2019), C1 (residues 2020–2172), and C2 (residues 2173–2332).

In circulation, most of fVIII is bound to vWF, which confers from physiological concentrations of the proteins, which are ~1 (4) and ~50 nM (5), respectively, and a high affinity (0.2–0.5 nM) of their interaction (6, 7). Binding to vWF prevents fVIII from premature interaction with components of Xase complex and is also required for maintenance of the normal fVIII level in plasma (8), since vWF deficiency in both humans (8, 9) and animals (10, 11) leads to a secondary deficiency of fVIII.

We have recently shown that fVIII catabolism from its complex with vWF in vitro and in vivo is mediated by low density lipoprotein receptor-related protein (LRP) (12). LRP, a member of the low density lipoprotein receptor family (13), is responsible for plasma clearance of lipoprotein remnants, serine proteinases, and their complexes with inhibitors (serpins) (13, 14). LRP is most prominent in liver on hepatocytes, and in vasculature it is presented on the surface of smooth muscle cells, fibroblasts, and macrophages (15). Besides fVIII, LRP mediates the clearance of a number of other proteins involved in blood coagulation and fibrinolysis, such as factors IXa (16) and Xa (17, 18), plasminogen activators, and their complexes with plasminogen activator inhibitor (19–21). A unique place among LRP ligands belongs to 39-kDa receptor-associated protein (RAP), which binds to LRP with a high affinity (Kd = 4 nM) and efficiently inhibits binding and endocytosis of all known LRP ligands (22).

The sites of fVIII involved in interaction with LRP were localized within the A2 domain residues 484–509 (12) and becbo’s modified Eagle’s medium; Xase, membrane-bound complex of fVIIIa and factor IXa.

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within the C-terminal portion of the C2 domain (23). Since the latter region of fVIII is likely to be blocked by vWF (23, 24), the C2 site could contribute to the clearance of fVIII only in the absence of vWF. This is consistent with the reported faster clearance of fVIII in vWF-deficient patients and animals (8, 25, 26), which was shown to be mediated by LRP (11).

The LRP-mediated endocytosis of many ligands is facilitated by cell surface heparan sulfate proteoglycans (HSPGs), one of the components constituting extracellular matrix. Among the LRP ligands, lipoprotein lipase (27), apoE-containing lipoproteins (28, 29), thrombomodulin (30), and thrombin-protease nexin 1 complex (31), and tissue factor pathway inhibitor (19, 32) are HSPGs-binding proteins. HSPGs serve either as coreceptors of LRP providing the initial binding of the ligands to the cell surface and their subsequent presentation to LRP (14, 29), or function as catabolic receptors themselves, acting independently of LRP (33). All LRPs ligands interacting with HSPGs are also able to bind to heparin (34), which is structurally similar to carbohydrate portions of HSPG molecules, and represent a useful model for studying these interactions in a purified system.

Noteworthy, the recently reported $K_d$ of 116 nM for fVIII interaction with LRP (12) is much higher than the normal (~1 nM) concentration of fVIII in plasma (4). This implies that the direct binding of plasma fVIII-vWF complex to LRP is negligible and suggests possible involvement of other receptor(s) in this process. In the present study, we examined participation of cell surface HSPGs in the binding and catabolism of fVIII-vWF complex, based on the ability of fVIII to interact with heparin (35). We demonstrated that HSPGs are indeed responsible for the initial binding of fVIII-vWF complex to the surface of various LRPI-expressing cells and subsequent facilitation of fVIII catabolism both in cell culture and in vivo. We found that the binding occurs via the fVIII moiety of fVIII-vWF complex and localized the major heparin-binding site of fVIII within its A2 domain.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Chondroitin sulfate A, heparin (average molecular weight 17–19 kDa), and biotinylated heparin were purchased from Sigma and Celsus Laboratories Inc., respectively. Chondroitin sulfate A was biotinylated using EZ-Link Biotin-LC-Hydrazide (Pierce) as described (36). Human coagulation factors IXa, X, and Xa were purchased from Enzyme Research Laboratories, and heparinase I was purchased from Sigma. Active site fluorescently labeled factor IXa (FI-FFR-IXa) was a generous gift of Dr. Philip Fay. Monoclonal antibody 8860 recognizing the A1 domain of fVIII was kindly provided by Baxter/Hyland Healthcare Inc. The rabbit polyclonal anti-LRP antibody RAB2629, RAP, and 125I-labeled activated $\alpha_{2}$-macroglobulin were kindly provided by Dr. Dudley Strickland. Phosphatidylinerine (PS) and phosphatidylcholine (PC) were purchased from Sigma. Phospholipid vesicles containing 25% PS and 75% PC were prepared as described previously (37). The fVIII peptides 432–456, 484–509, and 558–565 were synthesized using a 9050 Milligen synthesizer (Millipore) by the Fmoc ((9-fluorenylmethoxycarbonyl) method and pentafluoro-ester activation chemistry and were purified by reverse phase high-pressure liquid chromatography using a C18 column (Waters) in a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The 2.2–3.5 mM solutions of peptides were dialyzed versus 20 mM HEPES, pH 7.4, 0.15 mM NaCl (HBS) using membrane with 1-kDa cut-off (Pierce).

**Proteins**—fVIII was purified from therapeutic concentrates prepared by Method A, American Red Cross (38). HCh and LCh of fVIII were prepared as described previously (6). The A1 and A2 subunits were obtained from thrombin-activated fVIII using ion exchange chromatography on a Mono S column (Amersham Pharmacia Biotech) (12). 125I-labeled fVIII (fVIII and Its A2 Subunit—Prior to labeling, fVIII and A2 were dialyzed into 0.2 mM sodium acetate, pH 6.8, containing 5 mM calcium nitrate. Five $\mu$g of fVIII or A2 in 30 $\mu$l of the above buffer were added to lactoperoxidase beads (Worthington) containing 5 $\mu$g of Na125I (100 mCi/ml, Amersham Pharmacia Biotech) and 5 $\mu$l of 0.03% H2O2 (Mallinkrodt) and incubated for 4 min at room temperature. Unreacted Na125I was removed by chromatography on a PD10 column (Amersham Pharmacia Biotech). The specific radioactivities of 125I-labeled fVIII and A2 were 3–6 $\mu$Ci/$\mu$g of protein. The activity of 125I-fVIII (3650 units/mg) determined in the one-stage clotting assay (39) was similar to that of unlabeled fVIII (3840 units/mg).

**Assays for Cell-mediated Surface Binding, Internalization, and Degradation of Ligands**—LRP-expressing mouse embryonic fibroblast cells (MEF) and mouse embryonic fibroblast cells genetically deficient in LRP biosynthesis (PEA 13) were obtained from Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX) and maintained as described (40). Cells were grown to a density of $2 \times 10^6$ cells/well as we described previously (12). Human smooth muscle cells (SMC) and human alveolar epithelial cells (T2) were obtained from American Tissue Culture Collection. SMC and T2 cells were grown to a density of $10^6$ cells/well in DMEM and Leibovitz’s L-15 medium, respectively, containing 10% fetal bovine serum (Life Technologies, Inc.). The complex of 125I-fVIII or unlabeled fVIII with vWF was prepared by incubating the proteins at a 1:50 molar ratio in HBS, 5 mM CaCl2, for 30 min at 25°C. The complex formation was verified by gel filtration as described previously (12). To assess the contribution of HSPGs in fVIII uptake, the cells were preincubated in the medium containing heparinase-I (Sigma) at a concentration of 0.005 IU/ml for 30 min at 37°C followed by three washes with HBS containing 0.1% bovine serum albumin. Surface binding, internalization, and degradation assays were conducted as described previously (41). In some experiments, surface binding and degradation after incubation at 4°C to prevent endocytosis (42). Surface binding of radiolabeled ligands was defined as the amount of radioactivity released by treatment with mixture of trypsin (50 $\mu$g/ml) and proteinase K (50 $\mu$g/ml) (Sigma) as described (43). The radioactivity, which remained associated with the cells, was considered as internalized (41). Degradation was defined as radioactivity in the medium that was soluble in 10% trichloroacetic acid. The value of degradation was corrected for noncellular degradation by subtracting the acid-soluble radioactivity in parallel wells lacking cells.

**Factor Xa Generation Assay**—The rate of conversion of factor X to factor Xa was measured in a purified system (39), in which fVIIIa was substituted by its A2 subunit as described (44, 45). The A2 subunit (200 nM) was preincubated with varying concentrations of heparin (0–100 $\mu$g/ml) in HBS, 5 mM CaCl2, 0.01% Tween 20, and 200 $\mu$g/ml bovine serum albumin for 30 min at room temperature. This was followed by the addition of factor IXa (5 nM) and PSPC vesicles (10 $\mu$m) and incubation for 10 min, prior to addition of factor X (300 nM). To determine the initial rates of factor Xa generation, the aliquots were taken at 10, 20, 30, and 45 min, and the reaction was stopped with 0.05 mM EDTA. Factor Xa generation was determined from conversion of synthetic chromogenic substrate S-2765 (Chromogenix, Sweden).

**Fluorescence Anisotropy Measurements**—The measurements of interaction of the A2 subunit with FI-FFR-IXa were performed as described (45). A2 was preincubated with varying concentrations of heparin for 15 min at 25°C in HBS, 5 mM CaCl2. The anisotropy was measured in a 0.2-mI cell upon addition of PSPC vesicles (50 $\mu$m) and FI-FFR-IXa (30 nM) in the presence or absence of factor X (400 nM). The measurements were conducted using a Raman spectrofluorometer (Jobin Yvon) at the excitation wavelength of 495 nm and emission wavelength of 524 nm. The data were recorded 5 times for each reaction and averaged.

**Kinetin Measurements Using Surface Plasmon Resonance (SPR)**—The kinetics of interaction of fVIII-vWF complex, fVIII, its fragments, and vWF with heparin or chondroitin sulfate was measured by SPR technique using Biacore 3000 (Biacore, Sweden). Biotinylated heparin (100 $\mu$g/ml) or chondroitin sulfate (100 $\mu$g/ml) was immobilized at the level of 300 resonance units on the surface of a biosensor SA chip in HBS, 5 mM CaCl2, 0.05% Tween 20. The binding of the above ligands was measured in the same buffer at a flow rate of 10 $\mu$l/min. Dissociation was measured upon replacement of the ligand solution for the buffer without ligand. The chip surface was regenerated by washing with 1 mM NaCl, 0.05% Tween 20. The kinetic parameters were derived from the kinetic curves using Biacore BIA evaluation 3.1 software.

**Immunofluorescence Microscopy**—Human hepatocellular carcinoma cells HEP G2 (American Tissue Culture Collection) were grown on coverslips to 80% confluence in DMEM containing 10% fetal bovine serum at 37°C, 6% CO2. In some cells or cells treated with heparinase as described previously (12) were incubated with 10 mM of fVIII-vWF complex with 0.1% bovine serum albumin of DMEM, 1% bovine serum albumin for 2 h at 4°C in the absence or presence of RAP (1 $\mu$m). The cells were washed twice with phosphate-buffered saline, fixed in 2% formaldehyde in phosphate-buffered saline, and stained for fVIII, LRP, and HSPGs by triple label immunofluorescence staining. Staining for fVIII was performed by subsequent incubation of cells with mouse anti-fVIII monoclonal antibody 8860, biotin-
RESULTS

HSPGs Are the Primary Receptors Responsible for the Initial Binding of fVIII-vWf Complex to LRP-expressing Cells—We demonstrated previously that RAP inhibited endocytosis and degradation of fVIII from its complex with vWF by LRP-expressing cells, indicating that LRP is involved in catabolism of fVIII (12). Therefore, we examined the role of LRP in the initial binding of 125I-fVIII-vWf complex to the cell surface. As seen in Fig. 1A, the binding levels of 125I-fVIII-vWf complex were similar for both LRP-expressing MEF cells and LRP-deficient PEA13 cells. Moreover, RAP did not have a significant inhibitory effect on the binding to MEF cells. These findings suggest that receptor(s) other than LRP is responsible for the surface binding of 125I-fVIII-vWf complex. We next examined whether these receptors could be HSPGs by testing the effect of heparin on the surface binding. As seen in Fig. 1A, heparin significantly reduced the cell surface binding for both LRP-expressing and LRP-deficient cells, supporting our assumption that HSPGs are the major surface receptors responsible for the initial binding of fVIII-vWf complex. Consistent with this assumption, heparin also inhibited degradation of fVIII by MEF cells (Fig. 1B). To exclude the possibility that heparin inhibited fVIII degradation by interfering with fVIII binding to LRP, we tested the effect of heparin on 125I-fVIII binding to immobilized LRP in the assay described previously (12). We found that heparin did not inhibit the binding (data not shown), suggesting the following: (i) inhibition of fVIII degradation by heparin was not related to heparin binding to fVIII and (ii) heparin’s effect on the binding to MEF cells. These findings suggest that receptor(s) other than LRP is responsible for the surface binding of 125I-fVIII-vWf complex to LRP-deficient PEA13 cells.

The Major fVIII Site Involved in Binding to HSPGs Is Located within Its A2 Domain—Since the LRP binding site of fVIII is significantly lower in comparison with MEF cells, the ability of LRP-deficient cells to degrade fVIII implies the existence of an alternative, LRP-independent pathway of fVIII catabolism. Since it was inhibited by heparin (Fig. 1B), HSPGs appear to be also required for this LRP-independent pathway.

Fig. 1. Effect of RAP and heparin on the surface binding and degradation of 125I-fVIII-vWf by MEF and PEA13 cells. 125I-fVIII-vWf complex (1 nM) was added to wells containing 2 × 106 LRP-expressing MEF cells (solid bars) or LRP-deficient PEA13 cells (gray bars) in the absence or presence of heparin (100 μg/ml) or RAP (1 μM) and incubated for 6 h at 37 °C. The surface binding of 125I-fVIII (A) and its degradation (B) were subsequently determined as described under “Experimental Procedures.” Each data point represents the mean value and S.D. of duplicate determinations.
As seen in Fig. 2, this treatment reduced the surface binding and degradation of \(^{125}\text{I}-\text{A2}\) and \(^{125}\text{I}-\text{fVIII}\) to the same level, indicating that interaction of \(\text{fVIII-vWf}\) complex with HSPGs is likely to occur primarily via the A2 domain. Notably, the addition of RAP to heparinase-treated cells did not inhibit the surface binding of the ligands (data not shown); however, it further reduced their degradation (Fig. 2B). In the control experiment, we confirmed that the functional activity of LRP was not impaired by heparinase treatment, since this treatment did not have any effect on internalization of a direct LRP ligand \(^{125}\text{I}-\text{a2-macroglobulin}\) (40), data not shown. Altogether, the above experiments demonstrated that the A2 subunit behaves equivalently to \(\text{fVIII-vWf}\) complex in the catabolic process.

To examine whether involvement of HSPGs in LRP-mediated catabolism of A2 is a common feature of LRP-expressing cells, we tested the binding of A2 to human smooth muscle cells (SMC) and human alveolar epithelial cells (T2) expressing LRP and HSPGs (15). In both cell types heparin and heparinase significantly inhibited the surface binding, internalization, and degradation of \(^{125}\text{I}-\text{A2}\) (Fig. 3). Addition of RAP to heparinase-treated cells had no effect on the \(^{125}\text{I}-\text{A2}\) binding but led to a further decrease of its internalization and degradation. Thus, the effects of heparinase and RAP on A2 catabolism in MEF (Fig. 2), SMC, and T2 (Fig. 3) are similar, indicating that LRP and HSPGs are both involved in the A2 catabolism by different LRP-expressing cells.

To confirm that the A2 domain is fully responsible for the binding of \(\text{fVIII-vWf}\) complex to cell surface HSPGs, we studied the effects of increasing concentrations of \(\text{fVIII fragments}\) and \(\text{vWf}\) on the surface binding of \(^{125}\text{I}-\text{fVIII-vWf}\) complex to MEF cells. As seen in Fig. 4, at a concentration of 200 nM, \(\text{A2}\) inhibited this binding by 84%. Notably, pretreatment of MEF cells with heparinase resulted in a similar (87%) reduction of \(^{125}\text{I}-\text{fVIII-vWf}\) binding as shown in Fig. 2A. In contrast, neither \(\text{A1/A3-C1-C2 heterodimer}\) nor \(\text{vWf}\) were able to inhibit the binding of \(^{125}\text{I}-\text{fVIII-vWf}\) complex. This indicates that \(\text{fVIII but not vWf}\) is responsible for the binding of \(\text{fVIII-vWf}\) complex to cell surface HSPGs, and the major HSPGs-binding site of \(\text{fVIII}\) is located within the A2 domain.

The A2 Domain and \(\text{fVIII-vWf}\) Complex Bind to the Cells with Similar Affinities—The presence of the major HSPGs-binding site of A2 implies that affinities of A2 and \(\text{fVIII-vWf}\) complex for the cell surface should be similar. To verify this, we first determined the affinity of \(^{125}\text{I}-\text{A2}\) to MEF cells in a saturation binding experiment similar to that presented in Fig. 2 but performed at 4 °C to exclude internalization (data not shown). The nonspecific binding measured in the presence of 100-fold excess of unlabeled A2 constituted 18% of the total \(^{125}\text{I}-\text{A2}\) binding. The specific binding was adequately described by a model implying existence of a single class of binding sites.
Participation of Proteoglycans in Factor VIII Catabolism

Interaction of fVIII fragments and vWF with heparin in SPR-based assay. Heparin was immobilized to a biosensor chip at a level of 300 resonance units (RU) as described under “Experimental Procedures.” The binding of 500 nM of either fVIII (curve 1a), its HCh (curve 2), LCh (curve 3), A2 (curve 4), or A1 (curve 5) was measured for 5 min at a flow rate of 10 μl/min. In the control experiment (dotted curve 1b), VIII binding to chondroitin sulfate, immobilized on the chip at the same level as heparin, was tested. Dissociation kinetics was measured upon replacement of the ligand solution for the buffer without a ligand. Inset shows interaction of 1000 nM vWF (curve 6) and 500 nM fVIII-vWF complex formed from 500 nM fVIII and 1000 nM vWF (curves 7) with heparin-coated chip.

![Image](image_url)

**FIG. 5.** Kinetic parameters of the binding of fVIII-vWF complex, fVIII, its fragments, and vWF to heparin.

Interaction of fVIII, fVIII-vWF complex, fVIII, and its fragments with immobilized heparin was assessed in an SPR-based experiment shown in Fig. 6. The kinetic data for fVIII-vWF complex and fVIII were optimally fitted to a model implying existence of two independent heparin-binding sites within the fVIII molecule, referred to as 1 and 2. The kinetics of HCh, A2, and LCh interaction with heparin were optimally fitted to a model assuming existence of one heparin-binding site within each fragment. The association rate constants (k_on), dissociation rate constants (k_off), and affinities (K_d) were derived from the SPR data by using Biacore software BIAevaluation 3.1.

### Table I

| Ligand    | k_on | k_off | K_d (nM) |
|-----------|------|-------|----------|
| fVIII     | 1.14 | 3.91  | 27.9     |
|           | 0.034 | 0.4 | 652.9    |
| HCh       | 0.16 | 5.38 | 2.5     |
| A2        | 0.035 | 0.7 | 10.3    |
| LCh       | 0.16 | 0.7 | 571.0   |
| vWF       | 0.16 | 0.06 | 571.3   |
| fVIII-vWF | 0.053 | 0.5 | 31.3    |
|           | 0.053 | 0.05 | 357.25   |

(9.6 × 10^5 sites per cell) with K_d of 15 ± 2.8 nM. To verify that A2 and fVIII-vWF complex bind to the same sites, we performed displacement of [125I]-A2 (1 nM) by unlabeled A2 or fVIII-vWF complex. In this assay, A2 and fVIII-vWF complex were found to be equal as competitors (Fig. 5) with K_d values of 18.8 ± 2.2 and 21.4 ± 1.9 nM, respectively. The similarity of the K_d values further supports that the binding of fVIII-vWF complex to HSPGs is mediated by the A2 domain of fVIII.

The Major Site within A2 and the Minor Site within LCh Are Involved in fVIII Binding to Heparin—To examine whether A2 is the only site responsible for interaction of isolated fVIII with HSPGs, we tested the direct binding of VIII, A2, and other fVIII fragments to heparin in SPR-based assay (Fig. 6). The kinetic parameters for fVIII and its fragments derived from Fig. 6 are shown in Table I. We found that fVIII, its A2 domain, and HCh (containing A2), but not A1, were able to bind to heparin, consistent with the presence of the heparin-binding site within the A2 subunit. The determined affinity of the A2 subunit for heparin is 25.8 nM. Unexpectedly, LCh was also able to bind heparin with a low affinity (K_d = 571 nM), indicating that it contains another heparin-binding site. Consistent with this observation, the fVIII binding kinetics was optimally fitted to a model implying existence of two heparin-binding sites, which affinities (K_d values of 28 and 652 nM) are similar to the values determined for A2 and LCh, respectively. The 23-fold lower affinity of the LCh site for heparin implies that its involvement in heparin binding is limited.
contribution to fVIII binding to HSPGs is not significant. In control experiments, the specificity of fVIII and A2 interaction with heparin was demonstrated using Biacore SA chip coated with biotinylated chondroitin sulfate which, similarly to heparin, is composed of negatively charged sulfated polysaccharides. The binding of fVIII (Fig. 6, curve 1b) and its A2 subunit (data not shown) constituted less than 7.3% of the corresponding binding of the ligands to heparin.

We next compared the parameters of fVIII-vWF complex and vWF binding to heparin (derived from Fig. 6, inset). As seen in Table I, although the parameters of fVIII-vWF interaction with heparin are similar to those of isolated fVIII, isolated vWF bound to heparin with a low affinity ($K_d = 8.4 \mu M$). This confirms that its contribution to interaction of fVIII-vWF complex with heparin is negligible. Remarkably, the affinities of fVIII-vWF complex and A2 for heparin determined in a purified system (Table I) are close to the affinities of fVIII-vWF complex and A2 (18.8 and 21.4 nM, respectively) for the surface of MEF cells. Altogether, these data further support our hypothesis that the major fVIII site responsible for the binding to HSPGs is located within the A2 domain.

The A2 Domain Heparin-binding Site Includes the Residues 558–565—Localization of the heparin-binding site within the A2 domain was initiated by the previous findings that heparin inhibits Xase activity (35, 49), and fVIIIa can be substituted by its A2 subunit in the Xase assay (45). As seen in Fig. 7A, heparin was inhibitory in the A2-dependent Xase assay; the effect was dose-dependent; and 90% inhibition was observed at 10 mg/ml (600 nM).

Since it was previously shown that heparin does not inhibit interaction of the Xase complex with its substrate factor X (49), we proposed that heparin inhibits Xase assembly by preventing the A2 binding to factor IXa. To examine this possibility, we tested the effect of heparin on the A2 binding to factor IXa by fluorescent anisotropy technique. The experiment was based on the previous observation that anisotropy of Fl-FFR-fIXa increases moderately upon the binding of A2 (44, 45). We found that heparin inhibited the increase of anisotropy in a dose-dependent fashion, both in the absence or presence of factor X (Fig. 7B). The maximal effect of heparin was observed at its concentration of 30 mg/ml, which is similar to the concentration completely suppressing the factor Xase assay (Fig. 7A).
heparin did not affect the anisotropy of Fl-FFR-fIXa in either absence or presence of factor X.

The above findings suggest that heparin blocks interaction between the A2 subunit and factor Xa, which might be due to the overlapping of the A2 domain binding sites for heparin and factor Xa. Since the A2 domain regions comprising residues 484–509 and 558–565 are directly involved in the interaction with factor Xa (45, 50), we tested the effects of the corresponding synthetic peptides on the A2 binding to heparin. In the SPR-based experiment, the peptide 558–565 inhibited the binding by 78% at a concentration of 800 μM (Fig. 8). In contrast, at the same concentration, the peptide 484–509 inhibited the binding by ~25%, and the peptide 417–428 was not inhibitory at all. This suggests that the A2 domain region 558–565 is involved in the fVIII binding to hepatic and, possibly, to cell surface HSPGs.

Cell Surface Proteoglycans Participate in fVIII Catabolism in Vivo—To examine whether HSPGs contribute to fVIII clearance in vivo, we performed clearance studies in mice in the presence of protamine, which prevents HSPGs from interaction with their ligands (32, 51). The data shown in Fig. 9 were fitted to the previously used double exponential model (12), implying existence of the fast and slow phases of fVIII clearance. This model is described by the following Equation 1:

\[
C = C_1e^{-k_1t} + C_2e^{-k_2t} \quad \text{(Eq. 1)}
\]

where C is the percentage of 125I-fVIII remaining in plasma at a given time; \(k_1\) and \(k_2\) are the kinetic rate constants corresponding to the fast and slow phases of fVIII clearance; and \(C_1\) and \(C_2\) are percentages of radioactivity removed during the fast and slow phases of clearance. The values of \(k_1\), \(k_2\), \(C_1\), and \(C_2\) constants were derived for each clearance curve by fitting C versus t to the above Equation 1. At the saturating concentration of RAP, the rate of the fast phase of clearance was dramatically reduced (Table II), resulting in prolongation of the half-life of fVIII by 3.5-fold, similar to that reported previously (12). Administration of protamine prolonged the fVIII half-life by 1.6-fold and reduced the rates of both phases of clearance (Table II). This indicates that HSPGs contribute to both RAP-sensitive and RAP-independent pathways of fVIII clearance. Notably, coinjection of RAP and protamine resulted in a greater increase of the fVIII half-life (5.5-fold) than injection of RAP alone (3.5-fold), suggesting that LRP (12) and HSPGs are simultaneously involved in fVIII clearance. To confirm that the effect of protamine on fVIII clearance in vivo was specific, we performed a control experiment in which protamine was administered together with 100 units (1 mg) of heparin. This amount of heparin is sufficient to neutralize 1 mg of protamine by irreversible binding, thus preventing interaction of protamine with HSPGs (51, 52). Administration of heparin abolished the effect of protamine both in the presence of RAP (Fig. 9) and in its absence (data not shown), supporting our assumption that the effect of protamine is due to prevention of fVIII-vWf binding to HSPGs. Thus, the above data suggest that HSPGs participate in fVIII clearance in vivo and are involved in LRP-mediated and LRP-independent catabolic pathways.

FVIII Is Colocalized with HSPGs on the Surface of LRP-Expressing Hepatic Cells—We found previously that injection of 125I-fVIII-vWf complex into mice led to accumulation of most of the radioactivity in liver (12), where LRP is present in high abundance (15). To verify whether HSPGs are involved in the initial fVIII binding to the liver cells, we performed direct visualization of fVIII, HSPGs, and LRP in human hepatic cells HEP G2, expressing both LRP and HSPGs (53). The cells were incubated with fVIII-vWf complex at 4°C followed by triple label immunofluorescent staining for fVIII, LRP, and HSPGs and microscopy (Fig. 10). For each preparation, the distribution of fVIII, HSPGs, or LRP is shown in red, blue and green images, respectively. For control cells, the individual stainings for fVIII, HSPGs, and LRP are represented by the images a, b, and c, respectively. FVIII was distributed on the cell surface in a grainy pattern, typical for cell surface but not for cytoplasmic staining. The merged image (image d) demonstrates that fVIII colocalized predominantly with HSPGs as the purple areas, resulting from the superimposing of red and blue staining for fVIII and HSPGs, respectively. Colocalization of surface-bound fVIII with LRP was negligible, since large areas in the merged image remained green but not yellow, as would be expected for superimposed red and green images. Consistent with this observation, treatment of the cells with heparinase removing glycosaminic residues from HSPGs (image f) led to a dramatic reduction of bound fVIII (image e) and to disappearance of purple areas on the merged image (image h). In contrast, blocking of LRP by RAP (images i, j, k, and l) did not appreciably alter the level of fVIII binding (image i) when compared with the control cells (image a). In the merged image (image l) fVIII remained colocalized with HSPGs, consistent with a negligible role of LRP in the initial surface binding of fVIII-vWf complex. Thus, the microscopy study confirmed that HSPGs are the major receptors responsible for the initial binding of fVIII-vWf complex to the surface of LRP-expressing hepatic cells.

DISCUSSION

In the present study we found that cell surface HSPGs facilitate LRP-mediated catabolism of fVIII from its complex with vWf in cell culture and in vivo. In LRP-expressing cells, the bulk of the initial binding of fVIII-vWf complex occurs via HSPGs, which cooperate with LRP receptor in the consequent internalization of the fVIII molecule. In mice, the simultaneous blocking of HSPGs and LRP led to a significant prolongation of the fVIII half-life compared with fVIII half-lives when HSPGs...
and LRP were blocked independently.

The interaction of fVIII-vWF complex with HSPGs occurs via the A2 domain of fVIII, which is based on the following findings: (i) the cell surface binding of A2 and fVIII-vWF complex displayed a similar dose dependence and was inhibited by heparinase treatment to the same extent; (ii) the A2 subunit, but not other portions of fVIII or isolated vWF, strongly inhibited the cell surface binding; (iii) the A2 subunit and fVIII-vWF complex bound to the cell surface with similar affinities; (iv) the A2 subunit and HSPGs had similar affinities and LRP were blocked independently.

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TABLE II

Effect of RAP and protamine on the parameters of fVIII clearance from plasma of mice

| Added agent          | $C_1$          | $C_2$          | $k_1$          | $k_2$          |
|----------------------|----------------|----------------|----------------|----------------|
| None                 | 58 ± 3.6       | 42 ± 3.7       | 0.0208 ± 0.0026| 0.00345 ± 0.0009|
| RAP (150 μM)         | 7.4 ± 3.8      | 92.6 ± 6.2     | 0.00107 ± 0.0008| 0.00367 ± 0.0012|
| Protamine (0.2 mM)   | 63 ± 6.5       | 37 ± 7.6       | 0.0118 ± 0.0007| 0.00225 ± 0.0004|
| RAP + protamine      | 12 ± 3.4       | 88 ± 6.5       | 0.0007 ± 0.0006| 0.00252 ± 0.0025|

The values of the kinetic rate constants $k_1$ and $k_2$, corresponding to the fast and slow phases of fVIII clearance, and the percentages of total radioactivity ($C_1$ and $C_2$, respectively) removed during these phases were determined by fitting the clearance data shown in Fig. 9 Equation 1 described under “Results.”

Fig. 10. Microscopy studies of surface binding of fVIII from its complex with vWF by HEP G2 cells. Control untreated HEP G2 cells (upper panel, images a–d) and the cells treated with heparinase (middle panel, images e–h) or RAP (lower panel, images i–l) were incubated with 10 nM of fVIII-vWF complex for 2 h at 4°C. This was followed by fixing the cells and triple label staining for fVIII using Texas Red (red images a, e, and i), for HSPGs using AMCA (blue images b, f, and g) and for LRP using FITC (green images c, g, and k) as fluorophores, as described under “Experimental Procedures.” Each type of staining was visualized using a selective fluorescent filter block. The merged images (d, h, and l) were obtained by superimposing single-stained images as described under “Experimental Procedures.”
which confirms the involvement of LRP in fVIII catabolism previously reported by us (12). In LRP-deficient PEA13 cells the level of fVIII degradation was similar to that determined in the previous study (12) and significantly lower (22%) in comparison with LRP-expressing MEF cells. We found that this less effective, LRP-independent degradation by PEA13 cells was strongly inhibited by heparinase treatment, suggesting existence of a different pathway of fVIII catabolism, which involves HSPGs. These findings are consistent with the biphasic character of fVIII clearance in vivo, reflecting the existence of two distinct pathways of fVIII catabolism. The inhibitory effect of protamine on the fast and slow phases of clearance points to involvement of HSPGs in both pathways of fVIII catabolism in vivo. Since only the fast phase of fVIII clearance was RAP-sensitive, we propose that in this phase fVIII bound to cell surface HSPGs undergoes LRP-mediated endocytosis, whereas in the slow phase, also facilitated by HSPGs, fVIII follows LRP-independent pathway. Unlike in cell culture, the simultaneous blocking of HSPGs and LRP by protamine and RAP, respectively, did not completely block the fVIII clearance in mice. This can be explained by either incomplete inhibition of these receptors due to clearance of RAP and protamine or by the existence of another mechanism, which does not involve HSPGs and LRP. It cannot be completely excluded, however, that the inhibitory effect of protamine on fVIII clearance could be due to prevention of fVIII interactions with negatively charged cell surface-associated molecules other than HSPGs.

The proposed role of HSPGs is depicted in Fig. 11, which indicates that catabolism of fVIII from its complex with vWF occurs via initial binding of the complex to HSPGs, followed by both LRP-mediated and LRP-independent endocytosis and degradation of fVIII. The model implies that vWF dissociates prior to fVIII internalization, based on our previous finding that vWF does not follow fVIII in the endocytic pathway (12). Our demonstration that catabolism of the A2 subunit is equivalent to that of fVIII from its complex with vWF suggests that dissociation of the complex is not a rate-limiting step of the catabolic process. The previous finding that the isolated fVIII is catabolized more efficiently than from its complex with vWF (12) can be explained by the presence of the second LRP-binding site within the C2 domain of LCh (23), which is likely to be blocked by interaction with vWF. The overlapping of the C2 domain LRP- and vWF-binding sites (24) was suggested from the inhibitory effect of monoclonal anti-C2 domain antibody ESH4 on fVIII binding to both LRP (23) and vWF. The proposed contribution of the LRP-binding site located within the C2 domain to catabolism of fVIII is consistent with a significantly faster clearance of fVIII in vWF-deficient patients (8).

Our finding that the isolated A2 domain of fVIII can also be catabolized by HSPGs and LRP-mediated mechanisms may reflect the existence of a specific pathway for clearance of activated fVIII. Heterotrimeric fVIIIa (A1/A2/A3-C1-C2) is an unstable molecule due to its rapid but reversible dissociation to A2 and A1/A3-C1-C2 (59, 60). Since the A2 subunit retains a weak fVIIIa-like ability to support Xase and may also reassemble with A1/A3-A3-C1, it is

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tempting to speculate that clearance of the isolated A2 subunit may have evolved as a mechanism preventing formation of the Xase complex at inappropriate coagulation sites.

In summary, we demonstrated that FVIII catabolism from its complex with vWF involves the initial binding of the complex to cell surface HS PGs due to interaction between polysaccharide portions of HS PGs and the major heparin-binding site of FVIII localized within the A2 domain. The FVIII molecule is subsequently catabolized via LRP-mediated and LRP-independent pathways. Our finding that the simultaneous blocking of LRP and HS PGs receptors dramatically prolonged the lifetime of FVIII in a mouse model supports the physiological role of LRP and HS PGs in FVIII catabolism.

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