Molecular determinants of FVIII immunogenicity in hemophilia A
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Pharmacological modulation of the uptake of blood coagulation FVIII by antigen presenting cells

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Abstract

Endocytosis of blood coagulation factor VIII (FVIII) by antigen-presenting cells (APCs) comprises the initial step leading to activation of FVIII-specific T and B cell responses in patients with hemophilia A. Although multiple receptors have been implicated in FVIII uptake, the detailed (intra)cellular mechanism of this process has not been investigated. In this study we characterized the pharmacological profile of FVIII endocytosis by dendritic cells. We employed number of reference compounds such as transferrin, reported to be internalized via clathrin-mediated endocytosis, FITC-labeled high molecular dextran, a commonly used marker for macropinocytosis and Lucifer Yellow (LY), a small compound taken up via fluid-phase internalization. Endocytosis of both transferrin and dextran was strongly inhibited following disruption of the actin cytoskeleton by cytochalasin D. Endocytosis of FVIII was blocked only partially, even when high concentrations of the inhibitor were used. Uptake of LY was only marginally affected. Similar effects were observed for dimethyl amiloride (DMA), an established inhibitor of macropinocytosis – a strong decrease in uptake was observed for transferrin and FITC-dextran, none for LY and only a partial effect for FVIII. The phosphoinositide 3-kinase (PI3K) inhibitor – wortmannin, was able to partially block endocytosis of FITC-dextran, FVIII and transferrin, but not LY. Blockage with dextran sulfate suggested that heparan sulfate proteoglycans (HPSGs) could play a role in FVIII endocytosis by DCs. However, down-modulation of one of the components of the HS-copolymerase did not affect FVIII uptake. Studies using blocking antibodies emphasized the importance of the C1 domain but also suggested a potential modulating role for the C2 domain of FVIII. Taken together, our data suggest that C1 domain-mediated internalization proceeds via an unusual endocytic pathway that is distinct from classical macropinocytosis or clathrin-mediated endocytosis.
Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that play a fundamental role in the initiation of both innate and adaptive immune responses. The main functions of DCs include efficient endocytosis of antigens, migration from peripheral tissues to the lymphoid organs, presentation of antigen-derived peptides on MHC class I and II molecules, production and release of cytokines as well as expression of co-stimulatory molecules that allow them to efficiently prime naïve T cells. 1-4 Antigens can be taken up by DCs via various mechanisms, such as clathrin-mediated endocytosis, macropinocytosis or caveolin-dependent endocytosis. 5 Clathrin- and caveolin-independent pathways of endocytosis used for internalization of fluid phase markers and bacterial toxins have also been described. 5 Different endocytic mechanisms have been shown to transport cargo to distinct endosome populations thereby modulating antigen presentation. 6-9 Recently, it has been suggested that release by dendritic cells of untouched, macropinocytosed antigens in the draining lymph nodes plays an important role in the activation of B-cell responses. 10 Replacement therapy comprising frequent, intravenous administration of either recombinant or plasma-derived FVIII is a common treatment introduced in hemophilia A patients. Unfortunately, in 25% of severe patients therapy leads to the development of anti-FVIII antibodies, mainly IgG1 and IgG4, 11,12 interfering with the cofactor function of FVIII and therefore often referred to as “inhibitors”. Formation of high affinity IgG antibodies is a CD4+ T cell-dependent process. 13,14 Studies with human DCs have indicated that in vitro administration of FVIII does not result in the activation and maturation of DCs. 15 We have previously shown that endocytosis of FVIII by dendritic cells is mediated by its C1 domain. 16 Modification of a critical, surface-exposed loop within the C1 domain leads to diminished endocytosis of FVIII and, subsequently, greatly reduced B- and T-cell responses upon in vivo administration. 17 However, it still remains unclear which endocytic receptor directs internalization of FVIII. Although the mannose receptor has been suggested to play a role in this process, 18 we have shown that siRNA-mediated knock-down of this receptor does not influence FVIII uptake by DCs. 16 Moreover, roles for other commonly present on DCs endocytic receptors, such as LDL receptor related protein (LRP) and DC-SIGN, have been ruled out. 16,19 However, detailed (intra)cellular mechanism of FVIII uptake by DCs has not been investigated. Here, we employed several inhibitors to verify a role for actin polymerization, membrane ruffling, regulation of intracellular pH, as well as signaling molecules such as phosphatidylinositol-3 kinase (PI3K) in the endocytosis of FVIII by monocyte-derived dendritic cells. We show that this process is dependent on cytoskeleton reorganization, involvement of PI3 kinase and ion channels. Moreover, endocytosis of FVIII could be efficiently blocked by the sulfated polyanion – dextran sulfate, suggesting that this process could be mediated by charged polysaccharides or other sulfated molecules present on the cell surface. We also investigated potential involvement of the C2 domain in FVIII uptake. However, we show that its role is only marginal. Altogether, our
data provides novel insight into the cellular mechanism of FVIII endocytosis by human dendritic cells.

## Methods

### Materials

Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), CD14 microbeads (Miltenyi Biotech, Auburn, CA, USA), human recombinant GM-CSF and IL-4 and serum-free CellGro DC medium (both Cellgenix Technology Transfer, Freiburg, Germany) were used for the generation of human monocyte-derived dendritic cells. Penicillin/streptomycin was from Lonza (Walkersville, MD, USA). Ultrapure methanol-free paraformaldehyde was from Polysciences (Eppelheim, Germany). Antibodies used were: mouse IgG isotype control antibodies conjugated with FITC and PE (Dako, Glostrup, Denmark); mouse IgG isotype control IgG conjugated with APC, anti-human CD80-FITC, anti-human CD83-APC, anti-human CD86-APC, anti-human CD206-APC (BD Biosciences, San Jose, CA, USA); anti-human CD209 (R&D Systems, Minneapolis, MN, USA). Heparan sulfate-specific monoclonal antibody 10E4 (IgM) was from United States Biological (Salem, MA, USA). Monoclonal anti-FVIII antibodies CLB-CAg117 (targeting C2 domain of FVIII), KM33 (targeting C1 domain), EL-14 (C2 domain) and VK34 (A2 domain) have been described previously. For flow cytometry, antibody CLB-CAg117 was labeled with FITC using the FluoReporter® FITC Protein Labeling Kit (Invitrogen, Breda, The Netherlands), and antibody EL-14 was labeled using DyLight™ 549 Labeling kit (Thermo Scientific, Rockford, IL, USA).

### FVIII endocytosis, blocking and siRNA experiments

Human monocyte-derived dendritic cells (moDCs) were prepared as described before. In uptake experiments, 0.2×10^6 moDCs were incubated with various concentrations of recombinant FVIII at 37°C for 30 minutes in serum-free CellGro (CellGenix). Uptake was analyzed by flow cytometry (LSRII flow cytometer; BD Biosciences). Histograms were processed using FlowJo Version 7.6.5 software (TreeStar). To block the endocytosis of FVIII, 100 μM wortmannin (covalent inhibitor of phosphoinositide 3-kinases (PI3K) and thereby receptor-mediated endocytosis), 1-25 μM cytochalasin D (actin polymerization inhibitor), 50-1000 μM dimethyl amiloride (DMA; inhibitor of macropinocytosis by blockage of Na^+/H^+ ion-exchange and membrane ruffling) and 0.1-1 mg/ml dextran sulfate were first preincubated with cells at 37°C for 60 minutes. Subsequently, 20 nM of FVIII was added for 30 minutes at 37°C. Cells were fixed and internalized FVIII was detected with monoclonal antibody CLB-CAg117-FITC in the presence of saponin. Transferrin-488 (Invitrogen), 400 kDa dextran-FITC (Merck) and Lucifer Yellow were used as control antigens. In the experiments were anti-FVIII antibodies used, increasing concentrations of CLB-CAg117, KM33 and VK34 were first incubated with 20 nM FVIII for 30 minutes at 37°C and then subsequently added to the cells. Internalized FVIII was detected with recombinant human monoclonal antibody EL14-DyLight 549 in the presence of saponin. For siRNA experiments,
4x10⁶ immature moDCs were pulsed with 250 V, 150 mF and ∞Ω by using 4-mm cuvettes in a Biorad GenePulser adding 6 mL of either non-targeting control (scramble) or EXT-1-specific siRNA pools (Dharmacon, Thermo Fisher Scientific) in serum-free medium on day 3. After 72 hours, the cells were analyzed for heparan sulfate proteoglycans (HSPGs), mannose receptor (MR), DC-SIGN, CD80, CD83 and CD86 expression. To study uptake, increasing concentrations (0-160 nM) of FVIII were incubated for 30 minutes at 37°C with siRNA treated moDCs. Mean fluorescence intensities were determined using flow cytometry.

**Statistical analysis**

Unless stated otherwise, data were analyzed by Student’s t test and differences were considered significant at P < 0.05: * P < 0.05, ** P < 0.01, *** P < 0.001, ns – not significant.

**Results**

**Endocytosis of FVIII by human monocyte-derived dendritic cells is actin- and PI3 kinase-dependent and amiloride-sensitive**

We explored the mechanism of FVIII endocytosis using human monocyte-derived dendritic cells (moDCs). Cells were collected after 5 days of differentiation in the presence of GM-CSF and IL-4. Endocytosis of FVIII was compared to a panel of

![Figure 1. Endocytosis of FVIII depends on actin polymerization.](image)

A, B. Prior to addition of the antigen, moDCs were incubated with 5 μM cytochalasin D or medium control for 1 hour at 37°C. Next, 20 nM FVIII, 50 μg/ml dextran-FITC, 5 μg/ml transferrin-488, 100 μg/ml Lucifer Yellow (LY) or medium control were added to the cells and incubated for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAG117-FITC in the presence of saponin. Representative histograms are shown in A, where filled grey histograms show untreated cells and open histograms – cells treated with FVIII, FITC-dextran, transferrin or LY. C. Experiments using 20 nM FVIII were carried out as in A and B, but this time various concentrations of cytochalasin D (1-25 μM) were incubated with moDCs. Results in B and C are expressed as % MFI, where 100% corresponds to mean fluorescent intensity obtained in the absence of inhibitor. Data show mean ± SD from at least 3 independent experiments.
control antigens: Alexa Fluor 488-coupled transferrin (80 kDa), reported to be taken up via receptor-mediated mechanism; FITC-labeled large dextran (400 kDa), a common marker for both macropinocytosis as well as mannose-receptor related endocytosis; and Lucifer Yellow (457 Da), a small compound taken up via fluid-phase internalization. In order to better understand mechanism of endocytic routing, immature moDCs were pre-treated with several drugs. We used cytochalasin D, an actin polymerization blocker, and dimethyl amiloride (DMA), which blocks sodium channels important for regulation of intracellular pH and actin polymerization, and is commonly referred to as the most specific inhibitor of macropinocytosis. We also evaluated the effect of wortmannin, an inhibitor of PI3K. Endocytosis of both transferrin as well as dextran was strongly inhibited by cytochalasin D (Figure 1A and 1B), indicating involvement of actin polymerization in the internalization of these antigens. Endocytosis of FVIII was blocked only partially, even when high concentrations (up to 25 μM) of cytochalasin D were used (Figure 1C). Uptake of Lucifer Yellow (LY) was decreased only by a small margin, indicating that LY might enter DCs in fluid-phase. The effect of DMA was comparable to inhibition with cytochalasin D – a strong blockage was observed for transferrin and dextran, none for LY and only a partial effect was seen in case of FVIII (Figure 2A and 2B), even at high doses of the inhibitor (Figure 2C). Wortmannin, a strong covalent inhibitor of PI3-K, was able to partially block endocytosis of dextran, transferrin and FVIII, but

Figure 2. Inhibition of FVIII uptake by DMA. A, B. Prior to addition of the antigen, moDCs were incubated with 500 μM DMA or medium control for 1 hour at 37°C. Next, 20 nM FVIII, 50 μg/ml dextran-FITC, 5 μg/ml transferrin-488, 100 μg/ml Lucifer Yellow or medium control were added to the cells and incubated for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAg117-FITC in the presence of saponin. Representative histograms are shown in A, where filled grey histograms show untreated cells and open histograms – cells treated with FVIII, FITC-dextran, transferrin or LY. C. Experiments using 20 nM FVIII were carried out as in A and B, but this time various concentrations of DMA (50-1000 μM) were incubated with moDCs. Results in B and C are expressed as % MFI, were 100% corresponds to mean fluorescent intensity obtained in the absence of inhibitor. Data show mean ± SD from at least 3 independent experiments.
not LY (Figure 3A and 3B). When all three inhibitors were used simultaneously, an additive effect was observed for dextran and transferrin (Figure 4A and 4B), but the extent of FVIII endocytosis blockage was similar to that observed in experiments where single inhibitors were used. Taken together, these data

Figure 4. Simultaneous blockage of actin polymerization, macropinocytosis and PI3K leads to partial blockage of FVIII uptake. A, B. Prior to addition of the antigen, moDCs were incubated with either 100 μM wortmannin or medium control for 1 hour at 37°C. Next, 20 nM FVIII, 50 μg/ml dextran-FITC, 5 μg/ml transferrin-488, 100 μg/ml Lucifer Yellow or medium control were added to the cells and incubated for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAg117-FITC in the presence of saponin. Representative histograms are shown in A, where filled grey histograms show untreated cells and open histograms – cells treated with FVIII, FITC-dextran, transferrin or LY. Results are expressed as % MFI, were 100% corresponds to mean fluorescent intensity obtained in the absence of inhibitor. Data show mean ± SD from 4 independent experiments.

Figure 3. Role of phosphoinositide 3-kinase in FVIII endocytosis by moDCs. A, B. Prior to addition of the antigen, moDCs were incubated with either 100 μM wortmannin or medium control for 1 hour at 37°C. Next, 20 nM FVIII, 50 μg/ml dextran-FITC, 5 μg/ml transferrin-488, 100 μg/ml Lucifer Yellow or medium control were added to the cells and incubated for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAg117-FITC in the presence of saponin. Representative histograms are shown in A, where filled grey histograms show untreated cells and open histograms – cells treated with FVIII, FITC-dextran, transferrin or LY. Results are expressed as % MFI, were 100% corresponds to mean fluorescent intensity obtained in the absence of inhibitor. Data show mean ± SD from 4 independent experiments.
suggest that FVIII is endocytosed in an actin-dependent, DMA-sensitive and PI3K-dependent manner. These results indicate that endocytic routing of FVIII proceeds via a different mechanism when compared to that of commonly used model antigens such as transferrin or high molecular weight dextran.

**Role of heparan sulfate proteoglycans (HSPGs) in FVIII endocytosis by moDCs**

To further explore the mechanism of FVIII uptake by moDCs, we decided to use sulfated polyanions, such as dextran sulfate, known to interfere with endocytosis via scavenger receptors as well as competing for binding to negatively charged polysaccharides present on the cell surface. Dextran sulfate inhibited FVIII endocytosis in a dose-dependent manner; 36-39% residual FVIII uptake remained at a concentration of 0.5-1.0 mg/ml of the inhibitor (Figure 5). These results raise the possibility that binding of FVIII to sulfated polysaccharides present on the cell surface plays a role in its endocytosis by moDCs. HSPGs have been previously proposed as a co-receptor for FVIII endocytosis. HSPGs are widely expressed and composed of a protein core to which a variable number of heparin sulphate chains, each composed of 40-300 sugar residues, are attached. Heparan sulphate (HS) biosynthesis involves the consecutive action of glycosultransferases and sulfotransferases, which finally result in elongated, polydisperse heparin sulphates. Chain-elongation is catalyzed by the heparin sulphate co-polymerase (N-acetylglucosaminyl-glucuronyltransferase) EXT-1/2. shRNA-mediated down modulation of EXT-1 has been shown to prevent chain extension of HSPGs. Surface expression of HSPGs can be quantified.

![Figure 5. Role of HSPGs in FVIII endocytosis by moDCs. A. Prior to addition of the antigen, moDCs were incubated with increasing concentrations of dextran sulfate (0.1-1 mg/ml) for 30 minutes at 37°C. Next, 20 nM of FVIII or medium control were added to the cells and incubated for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAg117-FITC in the presence of saponin. B. Expression of HSPGs was measured 72 hours after transfection with either non-targeting control (scramble) or EXT-1-specific siRNA. Grey filled histograms represent isotype control, open histograms indicate staining with heparin sulfate-specific mAb 10E4. C. Internalized FVIII (0-160 nM) was quantified by flow cytometry. Results are expressed as % MFI, were 100% corresponds to mean fluorescent intensity obtained in the absence of inhibitor (A) or for 160 nM FVIII (C). Data show mean ± SD from 3 independent experiments.](image-url)
using antibody 10E4, directed to an epitope expressed on native, sulfated HS. In accordance with previous findings siRNA-mediated knockdown of EXT-1 reduced the amount of HSPGs on moDCs (Figure 5B). Interestingly, down-modulation of HSPG expression did not reduce the uptake of FVIII suggesting that other cellular determinants contribute to FVIII endocytosis by moDCs (Figure 5C).

Role of C2 domain in endocytosis of FVIII by moDCs
We have previously shown that the C1 domain plays important role in FVIII endocytosis by human and murine DCs, as well as human macrophages. As yet, a role for the highly homologous C2 domain in FVIII internalization has not been excluded. We compared the effect of monoclonal anti-FVIII antibody CLB-CAg117, recognizing the C2 domain; KM33, directed towards the C1 domain, and VK34, the A2 domain-specific antibody on FVIII endocytosis. While VK34 did not have an effect on FVIII uptake by moDCs, CLB-CAg117 blocked it partially (up to 50%) when 4- or 8-fold molar excess over FVIII was used (Figure 6). In agreement with previous data, KM33 decreased the uptake by approximately 80% (Figure 6). When KM33 and CLB-CAg117 were used simultaneously, no additive effect for CLB-CAg117 was observed (Figure 6), suggesting only a marginal role for the C2 domain in FVIII endocytosis. Taken together, these data emphasize the importance of C1 domain but also suggest a potential modulatory role for the C2 domain in directing FVIII endocytosis by DCs.

Discussion
Inhibitor development is the most serious complication in hemophilia A therapy. Somatic hypermutations and isotype switching, both of which require involvement of CD4+ T helper cells, are crucial steps for the generation of high affinity anti-FVIII antibodies. Endocytosis of FVIII by antigen presenting cells provides the initial step orchestrating CD4+ T-cell responses to FVIII. Here we investigated in more detail the cellular requirements of FVIII endocytosis by human monocyte-derived DCs. We show that FVIII endocytosis is partly reduced in the presence of high concentrations of DMA, cytochalasin D and the PI3K inhibitor wortmannin. All three compounds are establishes inhibitors of macropinocytosis. Macropinosomes originate from membrane extrusions that fold back and fuse with the plasma membrane resulting in the
generation of large vesicles that are internalized and eventually fuse with other endocytic compartments. 38 High molecular weight FITC-dextran is a well-known marker for macropinocytosis-mediated endocytosis. 10 The observed reduction in uptake of FITC-dextran upon incubation with cytochalasin D, wortmannin and DMA is in agreement with these findings. In current study, we included fluorescently labeled transferrin as a marker for clathrin-mediated, receptor-dependent endocytosis. Wortmannin has been shown to mediate pleiotropic effects on transferrin endocytosis, which may explain the reduction in transferrin uptake in the presence of this compound. 39 Actin is indispensable for clathrin-mediated endocytosis in yeast 40; in mammalian cells, however, it is not always necessary. 41 In epithelial cells, a role for actin in clathrin-mediated endocytosis has been shown 42, whereas in other studies actin has not been implicated in such process. 38 The pronounced effect of cytochalasin D on the internalization of transferrin observed in this study suggests that the actin cytoskeleton may participate in clathrin-mediated endocytosis in moDCs. PI-3K has been implicated in closure of macropinosomes. 29 The decline in FITC-dextran endocytosis observed in the presence of the PI-3K inhibitor wortmannin is consistent with that earlier report. Wortmannin also reduced clathrin-mediated endocytosis of transferrin, which may be explained by the reported inhibition of wortmannin on the late endocytic pathway. 43 It should be noted that endocytosis of LY was not affected by wortmannin, DMA and cytochalasin D suggesting that small compounds like LY are continuously internalized in moDCs, presumably through micropinocytosis. Our results clearly show that pre-incubation of moDCs with wortmannin, DMA and cytochalasin D reduces the internalization of FVIII. The observed effects however are relatively small when compared to those observed for FITC-dextran and transferrin. FVIII internalization was evaluated by fluorescence activated cell sorting. Hence, our experimental setup did not discriminate between initial binding and internalization. Binding of FVIII to the surface of moDCs is unlikely to be affected by the pharmacological inhibitors used in this study; therefore we cannot fully exclude that our current set-up underestimates the effects of DMA, wortmannin and cytochalasin D on FVIII internalization.

A two-step mechanism for FVIII endocytosis composed of an initial binding step to HSPGs and subsequent internalization via receptor-mediated pathways has been proposed. 32,44 In this study we show that the sulfated polyanion – dextran sulfate, inhibits FVIII internalization in a dose-dependent manner. These findings potentially implicate HSPGs in FVIII internalization. Thus far, HSPGs have been implicated in endocytosis of ligands and/or viruses. 33 For instance, syndecan-3 has been proposed as a receptor for HIV on dendritic cells. 45 Here, we show that knockdown of EXT-1, one of the components of heparin sulphate co-polymerase complex, does not affect internalization of FVIII. The extent of knockdown of EXT-1 was evaluated using monoclonal antibody 10E4, targeting an epitope present on heparin sulphate side-chains. 35 These findings suggest that HSPGs do not direct FVIII endocytosis. The observed effect of dextran sulfate can also results from competitive binding to surface exposed areas on FVIII required for its internalization by non-HSPGs directed mechanisms. We have previously
shown that an exposed loop in the C1 domain provides a crucial determinant for FVIII endocytosis. This exposed loop also modulates the binding of FVIII to phospholipid bilayers containing low levels of phosphatidylserine. In this study we show that the C2 domain specific antibody CLB-CAg117 also blocks FVIII endocytosis although less efficiently than the anti-C1 domain antibody KM33. This raises the possibility that a common structural determinant in the C1 and C2 domain can potentially interact with an unidentified cellular component that, in view of the observed competition with dextran sulfate, binds FVIII by electrostatic interactions. The pharmacological inhibition profile suggests that endocytosis of FVIII proceeds via a mechanism that is distinct from the classical macropinocytosis or clathrin-dependent endocytosis pathways as previously described for FITC-dextran and transferrin, respectively.
Modulation of FVIII endocytosis by APCs

References


