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# Involvement of Furin and Proprotein Convertase-5/6 in Proteolytic Processing of Factor VIII Albumin Fusion Protein

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## Abstract

Proprotein Convertases (PCs) cleave protein precursors at specific basic amino acid residue to form the mature bioactive protein. The processing event leads to activation or sometimes inactivation of these proteins while trafficking through secretory pathway. Coagulation Factor VIII (FVIII) essential in blood hemostasis also undertakes proteolytic cleavages during biosynthesis, and its processing sites consist of Arg-X-X-Arg motif. We have screened nine mammalian PC family members for FVIII cleavage activity, by transfecting those into CHO cells that expressed a B-domain deleted FVIII-albumin fusion protein. Human Furin and PC5/6 proteins were found to process the FVIII-albumin fusion protein, suggesting their potential involvements in FVIII cleavages *in vivo*. We also found that there is no activity difference between Furin-treated or PC5/6-treated FVIII-Albumin and a processing-site-variant R1648I.

**Keywords:** Coagulation factor VIII; Proteolytic cleavage; Proprotein convertases

## Abbreviations

| | | |
|---|---|---|
| aPTT | : | Thrombo-plastin assay |
| BMP | : | Bone morphogenetic protein |
| ER | : | Endoplasmic reticulum |
| FVIII | : | Factor VIII |
| HAS | : | Human serum albumin |
| MTX | : | Methotrexate |
| PC | : | Proprotein convertase |
| SDS-PAGE: electrophoresis | | Sodium dodecyl sulfate polyacrylamide gel |
| TGN | : | trans Golgi network |

## Introduction

Many extracellularly-destined proteins and peptides are synthesized as protein precursors and undergo proteolytic cleavage before or upon reaching their final locations. A family of proteases, termed Proprotein Convertases (PCs) [1-5], which share sequence homology with bacterial subtilases and yeast kexin, has been found to process many of these precursors. Most of these specific cleavages occur at relatively conserved sites often with single or pairs of basic amino acids (aa) at the general motif (R/K)-2nX-R↓ (n=0-3 aa) [3,4]. The cleavages are likely carried out by one or more of seven basic-residue-specific mammalian PC family members known as PC1/3, PC2, Furin, PC4, PC5/6, PACE4, and PC7 [1-5]. The PCs family also comprises two non-basic-residue-specific family members, SKI-1 [6,7] and PCSK9 [8,9]. SKI-1

cleaves substrates like sterol regulatory element binding protein SREBP 1/2 [6] at the motif (R/K)-X (hydrophobic)-X↓ [9], whereas PCSK9 was found to cleave auto-catalytically its own propeptide at VFAQ152↓ [8].

The PCs family members themselves are produced as inactive zymogens [1,2,4,5]. Auto-cleavage in Endoplasmic Reticulum (ER) or in immature secretory granules (PC2 only) results in an inactive heterodimer of the mature protein associated with inhibitory propeptide. A second cleavage event takes place on the prosegment at specific subcellular compartments to free the active enzyme from the inhibitory propeptide. This might ensure that these PCs are only active at specific intracellular sites. Furin, PACE4, PC5/6, PC7, and SKI-1, have been shown to be active at the Golgi complex, whereas PC1/3 and PC2 are maximally active in secretory granules [1,2,5]. PCSK9 is secreted as catalytically inactive heterodimer [8].

When overexpressed in cell lines, basic-residue-specific PC members have been found to display some functional redundancy, yet inactivation studies in mice or human indicate that each of them seems to have unique processing functions and participate different biological events *in vivo* [4]. PC1/3 [10] and PC2 [11] disruption in mice reveals their dominant neuroendocrine functions, whereas PC4 deficiency in rodents leads to severe impairment of fertility in homozygous mutant males [12]. Furin, PC5/6, PACE4, and SKI-1 are essential genes. Furin Knock-out (KO) in mice causes numerous embryonic malfunctions such as absence of axial rotation and heart loop, implying its critical role in cardiac development [13]. PC5/6 KO results in death at birth with an altered antero-posterior pattern, kidney agenesis, hemorrhages, and retarded ossification [14,15]. PACE4 KO develops incompletely penetrant left-right patterning defects and cardiac malformations in some embryos [16]. Tissue-specific KO of SKI-1 confirms its regulatory roles in cholesterol and fatty acid synthesis [17]. Though not being essential genes, PC7 and PCSK9's KO mice exhibit interesting phenotypes, with anxiolytic and novelty seeking phenotype for PC7 KO mice [18] and significantly less circulating total cholesterol/ LDLc for PCSK9 KO mice [19,20]. Different phenotypic consequences of PC family member inactivation are likely due to the inefficient processing of their targeted substrates. Identifying these individual protein substrates should provide a further understanding of the physiological roles of these proteases.

Factor VIII (FVIII) is a nonenzymatic cofactor in the intrinsic pathway of blood coagulation for the activated factor IX-mediated activation of Factor X [21]. FVIII gene defects may result in the bleeding disorder hemophilia A. In plasma, FVIII contains a heavy chain sized heterogeneously up to 200kDa in a metal ion complex with an 80kDa light chain. FVIII has a domain organization of A1-A2-B-A3-C1-C2 [22,23], with A domains similar to the A domains of the copper-binding protein ceruloplasmin [24], C domains homogenous to the phospholipid binding domains present in discoidins and milk fat globule protein [25], and B domain diverged among species 26-28.

FVIII is a complex plasma protein, undergoing extensive post-translational modifications [29], such as disulfide bond formation, N-linked and O-linked glycosylation, tyrosine sulfation, and phosphorylation. It is also subjected to proteolytic processing. A 19-amino-acid signal sequence is removed from its single chain precursor of 2351 amino acids by signal peptidase upon protein translocation across endoplasmic reticulum membranes. The mature polypeptide is further processed at R1313 and R1648 of the B domain within the secretory pathway in cells to yield a heterodimer. Additional minor cleavage sites in B-domain (R740, S817, K1115, S1657) have also been detected in purified FVIII preparations [30]. The predominant heavy chain (residue 1-1313) is composed of domains A1-A2-B* while the major light chain (residue 1649-2332) has domains A3-C1-C2. Both R1313 and R1648 major processing sites contain an Arg-X-X-Arg motif which can be cleaved by some PC family members [31-34], but it remains unknown whether or not these cleavages are enzyme-specific and whether or not other family members can also process FVIII. In this study, nine known members of the PC family were examined for their activities on the processing of a B-domain deleted FVIII-albumin fusion protein stably expressed in Chinese Hamster Ovary (CHO) cells. Furin and Proprotein convertase-5/6 (PC5/6) were found to process FVIII. The data also shows that this cleavage event has no substantial effect on FVIII functional activity *in* vitro [31].

## Materials and Methods

### DNA Constructs

A PCR fragment, encoding a human B-domain-deleted form of FVIII (LAFVIII) [28,35] fused to N-terminus of Human Serum Albumin (HSA) with a linker of GGSGGSGG-EDENQSPR (thrombin cleavage site)-GSGSGG in between (Figure 1B), was cloned into a murine cytomegalovirus promoter containing vector pSMED2 (pSMED2-LAFVIII-HSA). A similar PCR fragment encoding LAFVIII-HSA with Arg-to-Ile variant of R1648I was cloned into pSMED2 (pSMED2-LAFVIII-HSA-R1648I based on full length FVIII). To generate a catalytically inactive variant of human PC5/6 [36], a PCR fragment encoding a full-length PC5/6 with Ser 386 changed to Ala was cloned into pSMED2 (PC5/6-S386A). To generate a catalytically inactive mutant of human Furin [37], a PCR fragment encoding a full-length Furin with Ser 368 changed to Ala was cloned into pSMED2 (Furin-S368A). All constructs were confirmed by DNA sequencing.

### Cell lines and Cell Culture

Mammalian cell lines were grown and maintained in a humidified incubator with 5% $CO_2$ at 37°C. CHO cells lacking

dihydrofolate reductase (CHO-DUKX) were grown in alpha medium supplemented with adenosine (10mg/L), deoxyadenosine (10mg/L), thymidine (10mg/L), and 10% fetal bovine serum (FBS). The CHO-DUKX cells were stably transfected with pSMED2-LAFVIII-HSA or pSMED2-LAFVIII-HSA-R1648I, linearized with PvuI and subjected to selection with 10nM, or 20nM, or 50nM methotrexate (MTX). The stable colony formation was allowed to undergo selection for 3 weeks. The colonies were picked and expanded in media with MTX. Following selection, condition medium was harvested and analyzed by SDS-PAGE and Immunoblot. One clone designated for each construct was chosen for further analysis. The stable CHO-DUKX cells were maintained in alpha medium supplemented with 10% FBS and 20nM MTX. Conditioned media was harvested at the end of 3-day culturing, cleared by centrifugation prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Immunoblot.

### Immunoblot Analysis

Conditioned media were separated by SDS-PAGE, and analyzed by anti-human serum albumin antibody HRP conjugated (Abcam, Cambridge, MA, 1.4µg/ml).

### Assay for FVIII Activity

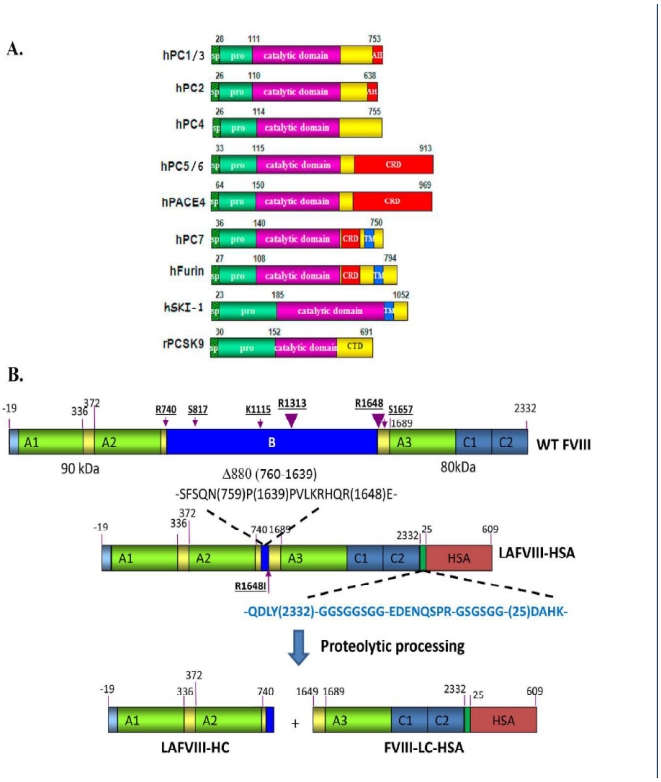
FVIII activity was measured as described [31]. Conditioned medium was diluted, added to the Activated Partial Thromboplastin Assay (aPTT) reagent containing Actin® FSL (Siemens) and FVIII deficient plasma (George King Bio-Medical, Inc., Overland Park, KS). The time to clot was measured using a StarT4 coagulation instrument (Diagnostica Stago, Parsippany, NJ). Standard curves were prepared by dilution of pooled normal plasma (FACT, George King Bio-Medical, Inc.). One International Unit (IU) of FVIII activity was defined as that amount measured in 1 mL of normal human pooled plasma.

## Results

- Nine members of proprotein convertase family in mammalian expression vector and stable CHO expression of a FVIII B-domain deletion mutant with C-terminal human serum albumin fusion.

To study FVIII processing, human PC1/3, PC2, PC4, PC5/6, PC7, PACE4, Furin, SKI-1, and rat PCSK9 (rPCSK9), were cloned into a mammalian expression vector (Figure1A). They all contain a signal peptide, a pro-domain, and a catalytic domain.

To simplify processing study, we have made use of a B-domain-deleted form of FVIII (LA-FVIII) [28,35]. This FVIII molecule has a deletion from residue 760 through 1639 and exhibits activity similar to that of wild type recombinant FVIII, as B-domain is dispensable for procoagulant activity. LA-FVIII is expressed at 10- to 20- fold greater level compared with wild type FVIII, likely due to increased levels of mRNA and secretion efficiency [28,35]. To extend half-life of FVIII for potential *in vivo* studies, human serum albumin (HSA, aa25-609) [38,39] was fused to the C-terminus of the protein with a linker in between (LAFVIII-HSA, 2060aa, Figure1B). One major processing site at R1313 is consequentially eliminated along with the 880aa B-domain deletion, whereas another major processing site at R1648 remains in this construct [31]. The resulting heterodimer after cleavage contains LAFVIII-HC (759aa, A1-A2-B*) and FVIII-LC-HSA (1291aa, A3-C1-C2-HSA).




**Figures 1(A,B):** Nine members of proprotein convertase family and a FVIII B-domain deletion mutant with C-terminal human serum albumin fusion. **A**. Diagram of nine members of proprotein convertase family. SP, signal sequence; Pro, prodomain; AH, amphipathic helix; CRD, cysteine rich domain; TM, transmembrane domain; CTD, C-terminal domain. **B**. Diagram for wild type FVIII and a B-domain deletion mutant FVIII (LAFVIII) with a C-terminal human serum albumin fusion. Major processing sites as well as some reported minor clipping sites in FVIII are annotated.

To generate a CHO-DUKX stable clone constitutively expressing LAFVIII-HSA, the DNA construct was transfected into CHO-DUKX cells for methotrexate selection. A positive clone was isolated by screening the conditioned medium from a number of colonies with anti-HSA SDS-PAGE and Immunoblot. This stable clone of FVIII-HSA (Figure 2. lane 1) produced one 220kDa-band and one 120kDa-band which were absent in the

negative CHO clone (lane 2). The 220kDa-band corresponds to the full length unprocessed based on detection with an HSA antibody that recognizes the C-terminal albumin fusion. The 120kDa-band corresponds to FVIII-LC-HSA (A3-C1-C2-HSA). The processing of LAFVIII-HSA was not completed, as the full-length LAFVIII-HSA was still detected. This observation is consistent with reported findings [27,30,31,35]. Wild type FVIII contains both R1313 and R1648 sites, which might ensure efficient processing, as no full-length unprocessed polypeptide is measurable [30].

The detection of unprocessed LAFVIII-HSA is consistent with the notion that processing of FVIII is not required for its extracellular secretion. As shown in Figure 2, cleavage site variant of LAFVIII-HSA-R1648I was also stably transfected into CHO-DUKX cells. Positive clones were isolated with anti-HSA immunoblotting. As shown in Figure 2 Lanes 3 &4, the 220kDa-unprocessed LAFVIII-HSA band was prominent while the 120kDa-band was also present. The data suggested that R1648 was the major processing site for LAFVIII-HSA.

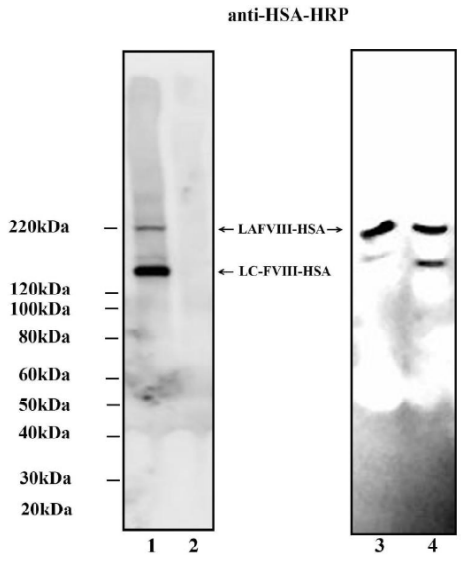
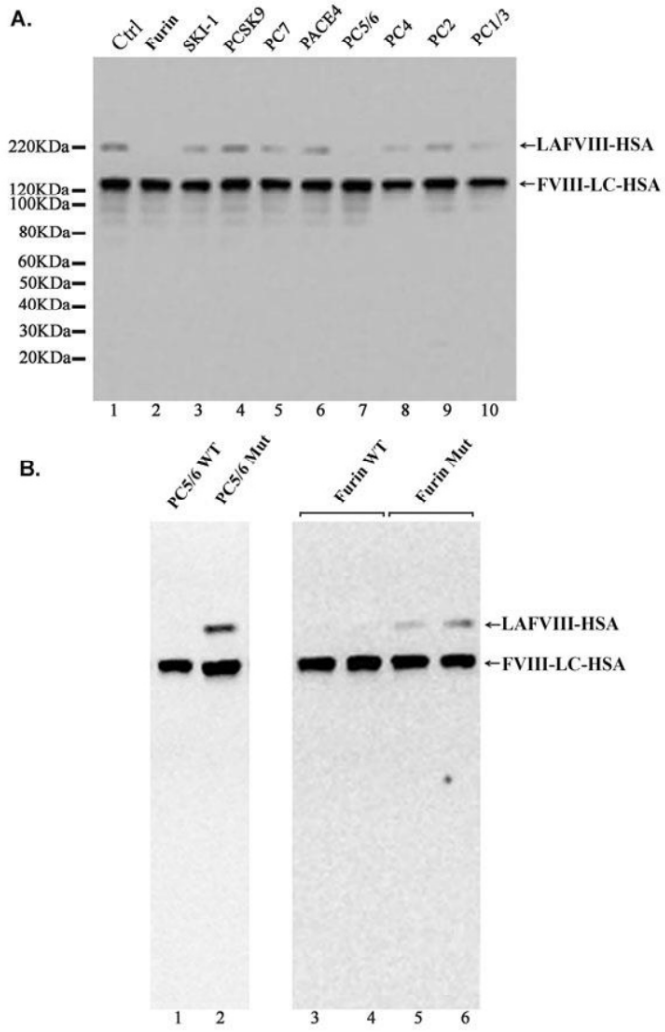



**Figure 2:** Stable CHO expression of LAFVIII-HSA and R1648I LAFVIII-HSA. with a C-terminal human serum albumin fusion. Conditioned media from stable CHO-DUKX cell clones established for LAFVIII-HSA were analyzed by SDS-PAGE and immunoblotted with anti-HSA-HRP, as described in Materials & Methods. **Lanes 1** is for LAFVIII-HSA CHO clone and **lane 2** is negative CHO clone. **Lane 3 & 4** are individual clones for R1648I LAFVIII-HSA.

- Furin and PC5/6 can specifically process LAFVIII-HSA. To address which PC family members may cleave FVIII, DNAs of the PC constructs were transiently transfected into the CHO-DUKX cell line stably expressing LAFVIII-HSA. Conditioned media was harvested after 3 days and analyzed by SDS-PAGE and detected by anti-HSA Immunoblot. As shown in Figure 3A, when human PC1/3, PC2, PC4, PC7, PACE4, SKI-1, or rat PCSK9, were transfected into stable CHO cells, the 220kDa band of LAFVIII-HSA was still detectable (Lanes 3-6, & 8-10). In contrast, the 220kDa-band disappeared completely when the cells were transfected with Furin (Lane 2) or PC5/6 (Lane 7). To confirm that the disappearance of the 220kDa band is associated with the enzymatic activity of Furin or PC5/6, catalytically-inactive mutation S368A in Furin [37] or S386A in PC5/6 [36] were generated. These mutations completely inactivated the processing activity of Furin or PC5/6 by destroying the key Ser residue within the catalytic pockets [36,37]. When the DNA constructs were transfected into CHO cells, processing of LAFVIII-HSA was no longer detectable (Figure3B, Lane 1 vs 2, lanes 3, 4 vs 5, 6). This data indicates that LAFVIII-HSA processing was associated with the activities of Furin and PC5/6.
- There is no activity difference between Furin-treated or PC5/6-treated LAFVIII-HSA and the R1648I LAFVIII-HSA variant.

CHO-DUKX cells stably expressing R1648I variant produced predominantly full-length LAFVIII-HSA (Figure 2. lanes 3 &4), whereas Furin-transfected (Figure 3B, lane 3) or PC5/6-transfected (Figure 3B, lane 1) CHO-DUKX cells stably expressing wild type LAFVIII-HSA produced no detectable full-length protein. These samples could allow us to do a direct comparison to ascertain if there is a functional activity difference among them. As shown in Table I, R1648I and PC5/6-treated or Furin-treated LAFVIII-HSA exhibited a similar activity in a one stage clotting assay.




**Figures 3(A,B):** Furin and PC5/6 process FVIII-HSA. **A.** Furin and PC5/6, but not the other seven PC family members, can process LAFVIII-HSA. DNAs of nine PC constructs (Figure 1) were transiently transfected into the CHO-DUKX cell line stably expressed LAFVIII-HSA. Conditioned medium (20μL) after three days was analyzed by SDS-PAGE and anti-HSA Immunoblot. **B.** Processing of FVIII-HSA by Furin and PC5/6 is associated with their enzymatic activities. Wild Type (WT) and catalytically-inactive mutant (Mut) constructs of Furin and PC5/6 were transiently transfected into the CHO-DUKX cell line stably expressing LAFVIII-HSA, and analyzed as described in Materials & Methods. Catalytically-inactive mutants of PC5/6 (S386A) and Furin (S368A) did not effectively process LAFVIII-HSA precursor.

| Constructs | Activity (IU/mL) |
|---|---|
| CHO Cell Control | 0.00 |
| LAFVIII-HSA | 0.68 |
| LAFVIII-HSA-R1648I | 1.12 |
| PC5/6+LAFVIII-HSA | 1.10 |
| Furin+LAFVIII-HSA | 1.09 |

**Table I:** FVIII activity of conditioned medium of LAFVIII-HSA. CHO-DUKX cells stably expressing wild type LAFVIII-HSA, LAFVIII-HSA R1648I variant or those transfected with Furin or PC5/6, were collected after three days' culturing. The conditioned media were analyzed for its FVIII activity as described in Materials & Methods.

## Discussion

In this study, we have shown that Furin and PC5/6 of the PC family members could process a B-domain-deleted form of FVIII with a C-terminal HSA fusion, while seven other PC family members appear not to possess such activity. The cleavage is associated with the enzymatic activity of Furin or PC5/6 because the corresponding catalytically-inactive mutants lost this capability. Native mature FVIII is a processed heterodimeric cofactor, but under recombinant conditions both processed and unprocessed molecules have been found secreted out of producing cells. The finding that PC5/6 or Furin can process full-length LAFVIII-HSA fusion efficiently has allowed us to compare the activity between fully processed molecules and predominantly unprocessed R1648I variant in an *in vitro* assay. Both forms of FVIII molecules seem similarly active, consistent with the previous finding [27,31].

The involvements of Furin and PC5/6 in FVIII processing are aligned with the notion that each PC family member has unique substrates *in vivo*, even though they exhibit a certain degree of functional redundancy during overexpression. Among these nine PC members, PCSK9 and SKI-1 are least likely involved in FVIII processing, as PCSK9 is secreted as catalytically inactive enzyme and SKI has a unique cleavage site sequence. The not-involvement of PC1/3 and PC2 seems possible, because they are dominant in the regulated secretory pathway of endocrine and neural cells [1,4]. For the processing in the constitutive secretory pathway, Furin, PC5/6, PACE4, and PC7 are the most common [2,4]. PC7 is unique in its ability to cleave transferrin receptor 1 [40], whereas PACE4 shares some substrates with Furin such as TGFβ-related Nodal precursor [41]. Some protein substrates like angiopoietin-like 3 were found to be processed by Furin, PC5/6, and PACE4 *in vitro* [42]. Our data might provide another substrate that is shared by PC5/6 and Furin.

The data suggesting that FVIII can be processed by PC5/6 and Furin might imply possible FVIII's processing location in cells, because these two PC enzymes are only active at specific intracellular sites. Furin activation occurs in *trans* Golgi Network (TGN), cell surface, as well as recycling endosome [43]. PC5/6 is activated predominantly at the cell surface, and is localized by binding to heparin sulfate proteoglycans [2]. Since both PC5/6 and

Furin could process FVIII, the cleavage event might take place at TGN or cell surface where these two enzymes are both active.

Identifying the involvement of PC5/6 and Furin in FVIII cleavage adds to the list of a number of therapeutically important proteins or peptides processed by the PCs. Proinsulin is processed by PC1/3 and PC2 [1]. Bone morphogenetic protein (BMP)-2 precursor [44] is processed by PC5/6 [45], whereas BMP10 is mainly cleaved by Furin [46]. For hemostasis pathway, presegments of Pro-factor VII [1] and Pro-factor IX (FIX) [47] are processed by Furin. Different from that of FVIII, processing of Pro-FIX to the zymogen FIX is critical for activated FIX to formaphospholipid interaction [48]. These findings together emphasize the critical roles of the PC family members in therapeutic protein biogenesis. Further investigation on the potential roles of PC5/6 and Furin in FVIII processing *in vivo* should shed new light on their molecular mechanisms.

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