The Von Willebrand Factor Propeptide (VWFpp) Traffics an Unrelated Protein to Storage

Sandra L. Haberichter, Mary A. Jozwiak, Jonathan B. Rosenberg, Pamela A. Christopherson, Robert R. Montgomery

Abstract—The von Willebrand factor (VWF) propeptide (VWFpp) is critical for the targeting of VWF multimers to storage granules. VWFpp alone efficiently navigates the storage pathway in AtT-20 and endothelial cells and chaperones mature VWF multimers to storage granules when the two proteins are expressed in cis or in trans. To further define the role of VWFpp in granular sorting, we examined its ability to sort an unrelated protein, C3α, into the regulated secretory pathway. Chimeric constructs of VWFpp and the α-chain of C3 were developed. The C3α protein expressed alone did not sort to granules in AtT-20 cells. The trans expression of C3α and VWFpp resulted in granular storage of VWFpp but no corresponding storage of C3α. When C3α is expressed as a single chain molecule with VWFpp that was rendered uncleavable by furin, C3α is re-routed to storage and is colocalized with VWFpp. The uncleavable protein was expressed in bovine aortic endothelial cells where it sorted to Weibel-Palade bodies, colocalized with bovine VWF, and was released when agonist stimulated. We now demonstrate that VWFpp re-routes a constitutively secreted protein to the regulated storage pathway. Furthermore, our studies suggest that the VWFpp storage signal is contained within amino acids 201 to 741. (Arterioscler Thromb Vasc Biol. 2002;22:921-926.)

Key Words: von Willebrand factor | von Willebrand factor propeptide | protein trafficking | complement C3, secretory granules | Weibel-Palade bodies

An adhesive glycoprotein, von Willebrand factor (VWF), mediates the attachment of platelets to subendothelial tissue at the site of vascular injury and serves as the carrier protein for coagulation factor VIII. VWF is synthesized and stored exclusively in endothelial cells and megakaryocytes as prepro-VWF consisting of a 22–amino acid signal peptide, a 741–amino acid propeptide, and a 2050–amino acid mature subunit. The precursor prepro-VWF is post-translationally modified through signal peptide cleavage, C-terminal dimerization, carbohydrate processing, sulfation, N-terminal multimerization, and proteolytic processing to yield free VWFpp and mature VWF multimers.

The propeptides of many proteins serve functional roles in the processing and folding of their mature proteins and are referred to as “intramolecular chaperones”. The propeptide domain of some proteins is involved in targeting of the mature protein to the regulated secretory pathway; included in this group are the propeptides of prosomatostatin and myeloperoxidase.

VWFpp also serves a functional role in the proper processing and storage of the mature protein. VWFpp is required both for N-terminal multimerization and for granular sorting of VWF. We recently demonstrated that VWFpp functions as an intracellular chaperone, trafficking mature VWF to storage granules in AtT-20 cells. When expressed alone, VWFpp is sorted to storage granules, whereas mature VWF expressed in the absence of VWFpp is not stored. Expression of VWFpp with mature VWF either in cis (single polypeptide chain) or in trans (two separate polypeptides) results in colocalized granular storage of VWFpp and VWF. Thus, VWF is trafficked to storage only through its interaction with VWFpp.

To further define the function of VWFpp in granular targeting and to further confirm that propeptide processing occurs before granule formation, we now examine the ability of VWFpp to sort a nonsecretory granule protein of the complement system to granular storage. The advantage of using VWFpp is the potential for targeting proteins to endothelial cells where release of Weibel-Palade body contents could be stimulated with an exogenous pharmacological agent such as 1-desamino-8-D-arginine-vasopressin (DDAVP). The third component of complement, C3, is the most abundant complement protein and has been extensively characterized. The precursor C3 protein is synthesized as a 188-kDa prepro-C3 protein that contains a 22–amino acid signal peptide, a 73-kDa β-chain, and a 115-kDa α-chain. C3 was chosen for this study because its α-chain is cleaved from the β-chain by PACE/furin, the same enzyme responsible for proteolytic processing of VWF, and because C3 is not sorted to the regulated storage pathway. We constructed VWFpp/C3α chimeras containing either a functional or a disrupted furin cleavage site and expressed these chimeras in...
AtT-20 cells that possess a regulated secretory pathway and that correctly synthesize and store VWF.13 We demonstrate the role of VWFpp in the targeting of C3α to regulated storage granules with the potential for exogenous pharmacological control. We also show that the signal for sorting VWFpp to storage granules is contained within the last 540 amino acids of VWFpp.

**Methods**

See online supplementary material for details (which can be accessed at http://atvb.ahajournals.org).

**Plasmid Construction**

The full-length cDNA for human complement C3 was a gift from John Lambris (University of Pennsylvania, Philadelphia, PA).14 The VWF propeptide expression vector, pVWFpp, contains human sequence and was constructed as previously described.12 The amino-terminal truncated VWF propeptide expression vector pVWFpp-201 to 741 was constructed by using a strategy based on the type IIS restriction enzyme BsmBI, as described previously.13 The pVWFpp-201-741 vector consists of VWF signal peptide sequence followed by sequence encoding amino acids 201 to 741 of VWFpp; amino acids 23 to 200 are deleted. pVWFpp-C3α, a vector expressing a chimeric protein with an intact furin cleavage site between VWFpp and C3α was constructed by using a similar strategy, pVWFpp*C3α, a vector expressing the chimeric protein containing a known mutation that disrupts the furin cleavage site was also constructed. This vector expresses the chimeric protein containing a known mutation within the VWF propeptide coding region. This deletion mutation has been identified and characterized in our laboratory from bovine aortic endothelial cells (BAECs). A vector, pC3α, expresses the α-chain of C3 with the native C3 signal peptide. For transfections, plasmids were purified by using Qiagen Plasmid Maxi kit (Qiagen).

**Cell Culture**

Two cell lines were used in this study: mouse pituitary tumor cells (AtT-20/D16v-F2, CRL 1795, American Type Culture Collection) and BAECs harvested in our laboratory from bovine aortas. AtT-20 cells were grown as previously described.12 BAECs were cultured in RPMI-1640 (Sigma) supplemented with 15% FBS, 9 mmol/L HEPES buffer, 1 mmol/L sodium pyruvate, 2 mmol/L l-glutamine, 50 μg/mL Endothe-
lial Cell Growth Supplement (Sigma), and 6.45 U/mL heparin and grown on tissue culture flasks coated with 2% gelatin.

**Mammalian Cell Transfections**

AtT-20 cells were transiently transfected as previously described.12 BAECs, 1.8×10^6 (first passage), were plated in 100-mm dishes. After 48 hours cells were incubated with 8 μg of DNA and 22 μL of lipofectin (Gibco) in a final volume of 4 mL of OptiMEM for 5 hours at 37°C. Then, 6 mL of complete RPMI was added. After 72 hours, transfected AtT-20 cells and BAECs were fixed in buffer formalin before immunofluorescent staining.17 For agonist stimulation experiments, transfected BAECs were selected by using G418 and incubated 30 minutes with 250 μL of 2 μmol/L phorbol-12-myristate-13-acetate (PMMA) or OptiMEM (control) (supplementary material). Transfected cells were analyzed for the intracellular localization of VWFpp and C3α after staining with immunofluorescent antibodies as previously described.15

**Antibodies**

Monoclonal antibodies to VWFpp (MBC 33.4, 33.5, and 239.1, 239.7, 239.8, 239.11), rabbit polyclonal antibodies raised against VWFpp or VWF, and mouse monoclonal antibody 9E10, raised against the 10 amino acid cmyc epitope were produced in our laboratory. C3 antibodies included C3a clone H3, a mouse monoclonal antibody (Research Diagnostics, Inc), rabbit anti-C3 (Cortex Biochem, Inc), and goat anti-C3 (Sigma). Secondary antibodies used for immunofluorescence detection included donkey anti-rabbit, anti-goat, and anti-mouse IgG (H+L) [F(Ab')2] fragments (Jackson Immunoresearch) conjugated with Texas Red (TXR), Red-X, fluorescein isothiocyanate (FITC), or AlexaFluor 488 or 594 goat anti-mouse and anti-rabbit IgG (H+L) [F(Ab')2] fragments (Molecular Probes).

**Results**

Previous work in our laboratory established that VWFpp functions as an intracellular chaperone in the trafficking of VWF to regulated storage.15 To further investigate the role of VWFpp in granular targeting, we examined its ability to alter the sorting of a constitutively secreted protein, C3α, into regulated storage. In addition to exploring the sorting mechanism, we sought to define when propeptide cleavage occurs in relation to granular sorting. The prepro-VWF and prepro-C3 structures are illustrated in Figure 1A with the furin cleavage sites depicted with arrows. cDNA constructs were developed to define the sorting of human VWFpp and C3α alone, in trans, or as a full-length construct either with or without normal furin cleavage (Figure 1B). In VWFpp*C3α, the furin cleavage site has been eliminated by introducing a VWF patient mutation within the VWF propeptide coding region. This deletion mutation has been identified and characterized in our laboratory and prevents cleavage of the VWF propeptide, but it does not affect the sorting of VWF to storage granules.16,17 To determine if the entire propeptide sequence was necessary for granular sorting, we also developed the amino-terminal truncated VWFpp expressing constructs pVWFpp-201-741 and pVWFpp-201-741*C3α.

Expression plasmids were transfected into AtT-20 cells to examine the synthesis and intracellular localization of VWFpp and C3α. The conditioned media and lysates from cells expressing the proteins VWFpp-C3α or VWFpp*C3α were immunoprecipitated with either an anti-VWFpp monoclonal antibody or anti-C3 monoclonal antibody. The immunoprecipitated proteins were reduced, analyzed by SDSPAGE, and immunoblotted with either an anti-C3 polyclonal antibody (Figure 2A) or anti-VWFpp polyclonal antibody.
Analysis of conditioned medium from cells expressing VWFpp-C3/H9251 demonstrated effective cleavage of VWFpp from C3/H9251. Immunoprecipitation with an anti-VWFpp antibody and Western blot analysis (Figure 2B, lane 1) revealed a band that corresponds to the position of expressed VWFpp (Figure 2B, lane 9), while C3/H9251 was not detected (Figure 2A, lanes 1 and 9). Conversely, immunoprecipitation with an anti-C3 antibody revealed a band that migrated at a position identical to C3/H9251 expressed alone (Figure 2A, lanes 5 and 10). Taken together, these results confirm effective furin cleavage of the VWFpp-C3/H9251 protein.

The cell lysate contained both proteolytically processed protein and uncleaved pro-VWFpp-C3/H9251 that was detected by immunoprecipitation with either anti-VWFpp or anti-C3 and blotting with either antibody (Figure 2A and 2B, lanes 3 and 7). Cells expressing VWFpp*C3/H9251, which contains a disrupted furin cleavage site, produced pro-VWFpp*C3/H9251 that was found intracellularly and efficiently secreted into the medium (Figure 2A and 2B, lanes 2, 4, 6, and 8). Cells expressing VWFpp-201-741*C3/H9251 showed no evidence of furin processing (data not shown).

We expressed C3/H9251, full-length C3, and VWFpp individually in AtT-20 cells to further examine the intracellular localization of these expressed proteins. Transfected cells were immunostained with monoclonal antibodies to VWFpp and a polyclonal antibody to C3 that recognizes the C3/H9251 domain detected with labeled secondary antibodies. The dual-stained cells were examined by using confocal microscopy. In cells expressing C3/H9251 alone (B and D), no C3/H9251 was detected (A). Expression of C3α alone (B and D) showed only diffuse staining of C3α (B) with no detection of VWFpp (D). VWFpp contains the necessary signal for sorting to storage, while C3α alone does not traffic to granules.

Figure 3. Immunofluorescent staining of VWFpp and C3α. AtT-20 cells were transfected with VWFpp alone or C3α alone. Fixed cells were dual-stained and visualized by using confocal microscopy. A and B, Cells stained for C3α; C and D, cells stained for VWFpp. Expression of VWFpp alone (A and C) resulted in granular storage of VWFpp (C); no C3α was detected (A). Expression of C3α alone (B and D) showed only diffuse staining of C3α (B) with no detection of VWFpp (D). VWFpp contains the necessary signal for sorting to storage, while C3α alone does not traffic to granules.
storage granules when expressed alone, while no staining for C3α is observed (Figure 3A and 3C).

When VWFpp is co-expressed in trans or in cis with mature VWF, both proteins traffic to storage granules through a noncovalent association as shown previously. To determine whether this type of interaction could occur with VWFpp and C3α, both proteins were co-expressed in trans and examined by using confocal microscopy (Figure 4A and 4C). We observed granular sorting of VWFpp but no corresponding granular storage of C3α; only diffuse staining was detected. This demonstrates that C3α neither associates noncovalently with VWFpp nor co-trafics to storage. To examine the effect of furin cleavage on C3α storage, the furin-cleavable protein VWFpp-C3α was expressed, immunostained, and examined by using confocal microscopy (Figure 4B and 4D). We observed normal granular storage of VWFpp, but only diffuse staining for C3α; no punctate C3α-containing granules were detected. This demonstrates that effective furin cleavage occurs before maturation of the granule with clear segregation of VWFpp from C3α.

We investigated whether VWFpp could traffic C3α, a non-stored protein, to storage granules when expressed in AtT-20 cells as a single polypeptide. VWFpp·C3α, which is resistant to furin cleavage, was expressed and immunostained. Representative confocal images obtained are shown in Figure 5A, 5D, and 5G. We observed granular storage when cells were stained for either C3α (Figure 5A) or VWFpp (Figure 5D). Merging the separate images obtained for C3α and VWFpp staining revealed co-localization in granules (shown in yellow, Figure 5G). Thus, when C3α is expressed with VWFpp as a single polypeptide that is rendered uncleavable by furin, C3α is directed to storage granules with VWFpp.

To determine whether the entire VWFpp protein is necessary for targeting to storage granules, we expressed the N-terminal truncated VWFpp-201-741 and VWFpp-201-741·C3α in AtT-20 cells and examined the intracellular localization of proteins (Figure 5B, 5C, 5E, 5F, 5H, and 5I). Cells expressing VWFpp-201-741 exhibited a punctate granular staining pattern (Figure 5E and 5H). This truncated VWFpp traffics C3α to granules when expressed as a single protein. We observed colocalized granular storage when cells expressing VWFpp-201-741·C3α were immunostained for VWFpp and C3α (Figure 5C, 5F, and 5I). This demonstrates that the “sorting signal” for VWFpp resides in the C-terminal region from amino acid 201-741.

We next expressed the uncleavable full-length protein in BAEC. These cells express bovine VWF that can be detected

**Figure 4.** Intracellular distribution of proteins expressed in trans and the furin-cleavable protein VWFpp·C3α. AtT-20 cells were cotransfected with VWFpp and C3α (trans) or VWFpp·C3α. Cells were dual-stained and examined by using confocal microscopy. A and B, Cells stained for C3α; C and D, cells stained for VWFpp. Cells expressing the two proteins VWFpp and C3α (trans) (A and C) showed punctate VWFpp-containing granules (C) while C3α showed diffuse staining (A). Similarly, expression of VWFpp·C3α that contains an intact furin cleavage site also showed a granular staining pattern for VWFpp (D) but diffuse staining pattern for C3α (B), indicating that these two proteins do not cotrafic to storage through a noncovalent interaction.

**Figure 5.** Intracellular distribution of the uncleavable proteins VWFpp·C3α and VWFpp-201-741·C3α. VWFpp·C3α, VWFpp-201-741, and VWFpp-201-741·C3α were expressed in AtT-20 cells. Cells were dual-stained and examined by using confocal microscopy. A, B, and C, Cells stained for C3α; D, E, and F, cells stained for VWFpp; G, H, and I, merges of VWFpp staining and C3α staining. Colocalization is shown in yellow. Cells expressing VWFpp·C3α (A, D, and G) showed a punctate staining pattern for C3α (A) and VWFpp (D) and are colocalized in storage granules (G). Cells expressing the amino-terminal truncated VWFpp-201-741 (B, E, and H) showed a punctate granular staining pattern. Cells transfected with VWFpp-201-741·C3α also demonstrated granular staining for C3α (C) and VWFpp (F) and were colocalized in granules (I). VWFpp can direct C3α into storage granules and this storage signal is contained within amino acids 201-741 of the propeptide.
by using a polyclonal antibody to human VWF. Although there have been reports of C3 synthesis in some types of endothelial cells,15 no C3 synthesis was detected in mock-transfected cells. To circumvent any possibility of detecting endogenous C3, a cmyc epitope was added to the C-terminus of VWFpp*C3α to generate the construct VWFpp*C3α-cmyc. The monoclonal antibody MAB 33.4 does not cross-react with bovine VWFpp, but does detect human VWFpp when it was transfected alone into BAECs (online Figure IA, please see http://atvb.ahajournals.org). The human VWFpp detected was colocalized in Weibel-Palade bodies with endogenous bovine VWF (online Figure IE and II). Nontransfected cells within the same field showed no staining for VWFpp. The uncleavable protein, VWFpp*C3α-cmyc was then expressed in BAECs and immunostained by using a monoclonal antibody to either cmyc or VWFpp followed by the VWF polyclonal antibody. Transfected cells immunostained for cmyc (online Figure IB) exhibited granular storage of VWFpp*C3α-cmyc that colocalized with bovine VWF in Weibel-Palade bodies (online Figure IF and II). Similarly, immunostaining with an anti-VWFpp antibody exhibited colocalized granular storage of VWFpp*C3α-cmyc and bovine VWF (data not shown). Within the field shown, we also observed several non-transfected cells that contain bovine VWF in Weibel-Palade bodies that do not stain with anti-cmyc, providing an internal negative control.

In addition to demonstrating colocalization of VWFpp*C3α-cmyc and endogenous bovine VWF in Weibel-Palade bodies we have also demonstrated agonist-induced release. Enrichment of the population of cells expressing VWFpp*C3α-cmyc was accomplished by using the selection agent G418. This population was incubated with either an agonist (PMA) or control (OptiMEM), and the results are shown in online Figure I. We observed a clear difference between cells exposed to PMA and the control stimulation. The agonist-stimulated cells efficiently released VWFpp*C3α-cmyc and endogenous bovine VWF (online Figure IC, IG, and IK) resulting in a loss of granular staining, whereas control-treated cells did not appear to have diminished staining for either VWFpp*C3α-cmyc or VWF (online Figure ID, IH, and IL). Thus, the propeptide VWFpp can direct the constitutive secretory protein C3α to the regulated secretory pathway in endothelial cells where it can be released by agonist stimulation.

**Discussion**

The VWF propeptide, VWFpp, functions as an intracellular chaperone to transport mature VWF multimers into storage in AtT-20 cells.12 The propeptides of several proteins have been implicated in the trafficking of their mature proteins to storage granules.7–9,19–22 Stoller and Shields23 demonstrated expression of a chimera consisting of the signal sequence and propeptide of anglerfish preprosomatostatin-I fused to ape α-globin resulted in targeting of α-globin to the regulated secretory pathway. We investigated the possibility that VWFpp might function to reroute an unrelated, nonsecretory granule protein to storage granules in AtT-20 cells. In the absence of VWFpp, C3α or full-length C3 does not sort to storage granules; only diffuse staining was detected. Neither full-length C3 nor the α-chain alone possesses the structural interactions necessary for sorting to granules.

VWFpp does not traffic C3α to storage granules when expressed in trans, unlike that observed in the trans expression of VWFpp and mature VWF in which the two proteins continue to associate through a specific noncovalent interaction after furin cleavage. This implies, not unexpectedly, that VWFpp and C3α cannot form this noncovalent association. Although VWFpp is correctly sorted to storage granules, it seems that any foreign protein, not covalently bound to VWFpp, lacks the necessary sorting capability and is not routed to storage. This was also observed when the furin-cleavable construct VWFpp-C3α was expressed. This protein was correctly processed by furin to yield free VWFpp and C3α as shown by immunoprecipitation and SDS-PAGE. The two proteins neither associated noncovalently nor co-trafficked to storage, although normal VWFpp storage was preserved. Proteolytic cleavage of VWFpp from VWF has been demonstrated to occur in the trans-Golgi network, before the formation of storage granules.5 The probable VWF processing enzyme has been identified as furin/PACE, which has also been found to process C3,4,24,25 Furin has been localized to the trans-Golgi network.4,16 The processing and storage of furin-cleavable VWFpp-C3α further demonstrates that propeptide cleavage occurs before the formation of mature storage granules as only VWFpp is detected in granules. Proteolytic processing appears to occur efficiently with complete separation of C3α from VWFpp.

Selection of proteins destined for storage granules and exclusion of other proteins presumably occur in the trans-Golgi network. There are several possible variables in the sorting equation, including a transport signal (sequence or conformation), self-interaction, and selective aggregation that is pH-, calcium-, or protein-concentration dependent.26–28 Two models of protein sorting that have been recently reviewed are sorting for entry and sorting by retention.26,28 In the sorting for entry model, granule cargo and membrane protein selection and nonsecretory granule protein exclusion occur in the trans-Golgi network before granule formation. The sorting by retention model entails that exclusion or selection of proteins occur after the formation of immature granules. During the process of granule maturation some proteins are retained while others are removed from the maturing granule. The two models are not mutually exclusive and selective aggregation of proteins plays a role in both models. In the sorting by retention model, aggregation and condensation of secretory granule proteins are the driving forces in retention and limit the ability of proteins to escape from maturing granules. Our results are more consistent with a sorting for entry model with VWFpp containing the signal for granule entry, although the sorting by retention model cannot be completely excluded. Although other studies have shown multimerization of VWF to be essential for sorting,29,30 our results demonstrate that VWF multimerization is not a prerequisite for vesicular storage. Our laboratory has characterized a naturally occurring mutation in the propeptide (Y87S) that results in synthesis of dimeric VWF that is stored in granules (unpublished data).31 The propeptide independently traffics to storage granules in BAECs (present study) and in AtT-20 cells,12 where its release can be induced by an agonist (8-Br-cAMP or PMA, unpublished data). Our model of VWF storage requires intact propeptide and structurally competent VWF that can
associate with VWFpp noncovalently. Deletion of one or more domains of mature VWF may result in conformationally defective VWF that cannot maintain noncovalent association with VWFpp, resulting in loss of storage.

The apparent complete removal or exclusion of C3α after furin cleavage of VWFpp-C3α implies that removal of excluded proteins from maturing granules is a very efficient process. Furthermore, this process is fully accomplished before the granule has completely matured. Although the mechanisms for routing proteins to the regulated secretory pathway are not well defined, the overall process must be relatively conserved because human VWFpp can efficiently navigate the storage pathway in both murine AT-20 cells and BAECs.

Our results show that the VWF propeptide not only possesses the structural elements necessary for sorting to storage granules but also it is capable of rerouting an unrelated, constitutively secreted protein to the regulated secretory pathway when expressed on a single polypeptide such as VWFpp-C3α. Furthermore, the first 179 amino acids of mature VWFpp are not required for targeting to storage granules. Because the VWFpp sequence can induce the storage of a nonsecretory granule protein in a physiologically relevant cell line such as the BAEC, such sorting suggests a possible pharmacological usefulness. While it is impractical to use a 741-amino acid protein to alter targeting of a protein into a secretory granule, our studies illustrate the potential for a region of the VWFpp to be synthesized on a normally nonsecretory granule protein and to redirect its storage into the regulated pathway. Therapeutic proteins could therefore be targeted to Weibel-Palade bodies in endothelial cells and subsequently released by administration of an exogenous agonist such as DDAVP to provide clinical pharmacological control of the newly stored protein.

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References
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