Real-Time Imaging of the Dynamics and Secretory Behavior of Weibel-Palade Bodies

Thalia Romani de Wit, Mariska G. Rondaij, Peter L. Hordijk, Jan Voorberg, Jan A. van Mourik

Objective—Weibel-Palade bodies (WPBs) are specialized secretory granules found in endothelial cells. These vesicles store hormones, enzymes, and receptors and exhibit regulated exocytosis on cellular stimulation. Here we have directly visualized intracellular trafficking and the secretory behavior of WPBs in living cells by using a hybrid protein consisting of von Willebrand factor (vWF), a prominent WPB constituent, and green fluorescent protein (GFP).

Methods and Results—Immunofluorescence microscopy demonstrated that this chimera was targeted into WPBs. In resting cells, some WPBs seemed motionless, whereas others moved at low speed in a stochastic manner. On stimulation of cells with [Ca2+]i- or cAMP-raising secretagogues, membrane-apposed patches were formed, suggesting fusion of WPBs with the plasma membrane. Patches remained visible for >20 minutes. This sustained, membrane-associated retention of vWF might play a role in focal adhesion of blood constituents to the endothelium after vascular injury. In addition, stimulation with cAMP-raising agonists resulted in clustering of a subset of WPBs in the perinuclear region of the cell. Apparently, these WPBs escaped secretion. This feature might provide a mechanism to control regulated exocytosis.

Conclusions—In conclusion, the fusion protein vWF-GFP provides a powerful tool to study, in real time, signal-mediated trafficking of WPBs. (Arterioscler Thromb Vasc Biol. 2003;23:755-761.)

Key Words: von Willebrand factor  ■  Weibel-Palade bodies  ■  endothelial cells  ■  real-time imaging  ■  green fluorescent protein

Vascular endothelial cells are equipped with a machinery that, on perturbation, allows prompt delivery of a number of bioactive substances, including hormones, receptors, and adhesive molecules, to the surface of the cell. A distinct subset of proteins destined to be released on stimulation of the endothelium stems from Weibel-Palade bodies (WPBs), typical and morphologically highly organized storage vesicles that release their contents by regulated exocytosis. WPBs are endothelial cell–specific elongated organelles, enclosed by a limiting membrane, which are ~0.1 µm wide and up to 4 µm long and originate from the trans-Golgi network.2,3 They serve as storage vesicles for a variety of proteins with different biologic functions, such as the leukocyte adhesion receptor P-selectin4,5 and the chemokine interleukin-8 (IL-8).6,7 Effective translocation of P-selectin from WPBs to the cell surface is critical for the binding and rolling of leukocytes on the endothelium at sites of inflammation.8,9 Similarly, regulated exocytosis of IL-8 provides an effective means for controlling local leukocyte extravasation.10 One of the most prominent WPB residents is von Willebrand factor (vWF), an adhesive multimeric glycoprotein that contributes to platelet adhesion and hemostatic plug formation at sites of vascular injury (reviewed in Sadler11 and Ruggeri12). Regulated secretion of vWF provides an adequate means for endothelial cells to actively participate in controlling the arrest of bleeding after vascular damage. Thus, it seems likely that regulated exocytosis of WPBs serves several physiological functions, including inflammatory and hemostatic responses.

See cover

Regulated exocytosis of vWF and other WPB residents involves the translocation of WPBs from the cytoplasm toward the cell surface and the fusion of these vesicles with the plasma membrane. Increased concentrations of cytosolic Ca2+ have been implicated in the mechanism of exocytosis of a number of agonists, including thrombin and histamine.13,14 Release of WPBs can also be induced by secretagogues, such as epinephrine or forskolin, agents known for their ability to activate cAMP-dependent signaling.15,16 The cellular responses to increased cytosolic Ca2+ are most likely mediated by calmodulin and small GTP-binding proteins.17-19 The molecular mechanisms associated with cAMP-dependent exocytosis of WPBs remain to be identified. It has been
shown that cAMP-mediated responses differ from regulated secretion elicited by a rise in cytosolic Ca\(^{2+}\) in that secretion induced by Ca\(^{2+}\)-raising agents involves the release of both peripheral and central granules, whereas cAMP-mediated secretion primarily involves vesicles located in the periphery of the cell.18

Although studies performed so far have clearly provided the basis for understanding the molecular machinery responsible for exocytic trafficking of WPBs, little is known about the dynamics of this secretory process. To date, exocytosis of WPBs has been studied only morphologically, by monitoring defined stages of this process in fixed cells. These conditions do not necessarily reflect the dynamics found in intact, living cells. The aim of this study was to investigate the intracellular trafficking of WPBs in living, wild-type endothelial cells in real time. For this purpose, we introduced, by retroviral transduction, a green fluorescent protein (GFP)-tagged vWF into primary human umbilical vein endothelial cells (HUVECs). The vWF-GFP chimera was correctly targeted to WPBs, together with endogenous vWF and P-selectin. This approach, exploiting the intrinsic fluorescence of vWF-GFP, allows direct visualization of the routing and fate of WPBs on stimulation of the cell. Our data reveal novel features of the dynamics and secretory behavior of WPBs, including perinuclear redistribution and membrane-apposed accumulation of GFP-containing granules.

Methods
Please see http://atvb.ahajournals.org for details of the methods used for this study.

Results
vWF-GFP Expression and Distribution in HUVECs
HUVECs were retrovirally transfected with either the vWF-GFP hybrid protein alone or together with hTERT-NGFR (hTERT, human telomerase reverse transcriptase; NGFR, neural growth factor). The transduction efficiency by either method was \(\approx 20\%\), as determined by measuring GFP expression by flow cytometry. GFP-positive cells revealed a vesicular distribution of the green fluorescence (Figure IBI available at http://atvb.ahajournals.org). Three-dimensional analysis of the cells showed rod-shaped vesicles that were distributed throughout the cytoplasm. Closer examination of the position of the GFP-containing organelles within the cell, visualized with a color scale (Figure IBI), revealed that some vesicles were located at the bottom of the cell, whereas the majority was positioned in the middle, and some others were located at the top of the cell. Thus, transfection of HUVECs with vWF-GFP results in the formation of GFP-labeled vesicles, typical of WPBs, which were heterogeneously distributed throughout the cell.

We next confirmed whether vWF-GFP was indeed targeted to genuine WPBs. Staining of vWF-GFP–infected HUVECs with anti-vWF (CLB-RAG 35, directed against the A1 domain) revealed rod-shaped granules that were always colocalized with the GFP signal, consistent with targeting of vWF-GFP to WPBs (Figure IIA through IIC, available at http://atvb.ahajournals.org). Similarly, vWF-GFP was colocalized with P-selectin, a typical marker of WPBs (Figure IIg through IIm). GFP-positive vesicles were distinct from lysosomes, because GFP-associated vesicles were not colocalized with granules that stained with an antibody to cathepsin D, a lysosomal marker (Figure IIj through IIII). These data demonstrate that vWF-GFP is targeted to WPBs, together with endogenous, wild-type vWF. To discriminate between endogenous vWF and vWF-GFP, cells were stained with an anti-vWF antibody (CLB-RAG 50) directed against the A2 domain of vWF. All GFP-positive vesicles were stained with this antibody (Figure IId through IIII). This indicates that WPBs of vWF-GFP–infected HUVECs contained both endogenous, wild-type vWF and the vWF-GFP hybrid protein.

vWF-GFP Multimerization and Secretion
Polymerization of vWF is one of the most characteristic events that occurs during its posttranslational maturation. Only vWF in its multimeric form is stored in WPBs.11 To verify whether vWF-GFP was also able to multimerize, wild-type HUVECs and vWF–GFP–transfected HUVECs were stimulated with thrombin, and the respective media were subjected to multimer analysis. vWF–GFP–transfected cells produced vWF multimers that were similar to wild-type vWF multimers (Figure III, available at http://atvb.ahajournals.org). As expected, vWF multimers produced by wild-type HUVECs did not stain with an antibody against GFP. However, the distribution of multimers secreted by vWF–GFP HUVECs, revealed by staining with anti-GFP, was identical to the pattern observed after staining with polyclonal antibodies to vWF. Thus, replacement of the A2 domain by GFP did not affect the ability of vWF to multimerize.

We also quantified the secreted vWF-GFP. Stimulation with phorbol myristate acetate (PMA) for 60 minutes resulted in a 1.5-fold increase of vWF (\(P<0.05\)) and GFP antigen (\(P<0.01\)) secreted into the medium compared with the amounts secreted by unstimulated cells (Figure IV, available at http://atvb.ahajournals.org). Similarly, after incubation with thrombin, PMA, or forskolin for 60 minutes, we observed a significant decrease in the number of WPBs compared with the number secreted by untreated cells (Table I, available at http://atvb.ahajournals.org). Collectively, these data indicate that WPBs containing vWF–GFP retained their ability to secrete their “cargo” in a regulated manner.

Dynamics of WPBs in Resting vWF-GFP–Transfected HUVECs
Having established that vWF-GFP properly accumulated in WPBs, was releasable, and had retained its ability to multimerize, we next examined the dynamics of WPBs in living, resting cells. To follow individual vesicles, we selected cells with relatively few WPBs. Preliminary observations revealed that the secretory behavior of WPBs was not affected by the WPB density of the cell. These vesicles were monitored for 60 minutes at intervals of 2 minutes. Figure I shows individual frames of the first 40 minutes of a real-time movie of resting vWF-GFP–transfected cells (Video I, available at http://atvb.ahajournals.org). We observed vesicle traffic in an apparently random and uncoordinated fashion throughout the cell body. Some granules seemed motionless during the entire recording period, as if they were “tethered” (Figure I; WPB
1, 3), whereas others were continuously moving in a stochastic manner (Figure 1; WPB 2 and 4 through 7). Some vesicles seemed to travel longer distances, notably toward the periphery (Figure 1; WPB 4, 7), whereas others returned to their starting point (Figure 1; WPB 2, 5). We also observed WPBs that rotated along their longitudinal axes and sometimes appeared as round vesicles (Figure 1; WPB 6).

**Dynamics of WPBs in PMA-Stimulated vWF-GFP-Infected HUVECs**

We next investigated WPB trafficking induced by different agonists. Cells were monitored during 60 minutes of stimulation at intervals of 1 minute. Figure 2 shows a typical time-lapse sequence of vWF-GFP-infected HUVECs stimulated with PMA (Video II, available at http://atvb.ahajournals.org). The apparent random movement of WPBs changed radically on stimulation. In each real-time stimulation experiment performed (n=8), we observed the same succession of events. Cells contracted slightly, an event which coincided with slight movement of the WPBs toward the center of the cell. Vesicles were not seen to clearly move to the periphery of the cell. However, ~15 minutes after stimulation of the cells, the rod-shaped WPBs transformed into bright, stationary patches (Figure 2A and 2B; WPB 1 through 3). The lag phase probably reflects diffusion of the agonist after addition to the medium. Typically, individual patches remained visible for ~20 minutes. Finally, these patches completely disappeared, and the cells became depleted of fluorescent material. Interestingly, disappearance of a patch often coincided with the appearance of a diffuse “cloud” (Figure 2; WPB 2, 30 minutes and WPB 3, 40 minutes) at the surface of the cell. This feature most likely reflects gradual dispersion of vWF-GFP into the extracellular environment. The formation of patches appeared within a single cell was not synchronized. The first patches appeared, on average, 15 minutes after stimulation; others appeared only after 60 minutes. Similarly, patches disappeared at apparently different rates. Cells were totally depleted of WPBs between 60 and 120 minutes after stimulation.

Figure 3 shows a 3-dimensional analysis of the transformation of a single WPB into a patch (arrow). To facilitate this analysis, we selected a cell with few vesicles. The x-y view of this cell (Figure 3A) shows that during the first 20 minutes of stimulation with PMA, the WPB appeared as a thin, rod-shaped vesicle. Its depth-coding staining signal suggested that the vesicle was located in the middle of the cell (green). A y-z view of this WPB (Figure 3B) confirmed its tubular morphology and central localization. About 20 minutes after stimulation, this vesicle transformed into a bright, round patch located at the apical side (blue, Figure 3A; 20 minutes). Examination of the cell on the y-z plane indicated that the patch represented the same vesicle (Figure 3B, arrow). However, it had rotated ~45° and was positioned perpendicularly to the focal plane. The vesicle was apposed to the cell membrane at the apical part of the cell (Figure 3B). The patch remained visible in the x-y view for ~20 minutes (Figure 3A, 50 minutes). The y-z view of the vesicle showed that its size diminished in time (Figure 3B, 22 to 40 minutes). Finally, 50 minutes after stimulation, the vesicle had completely disappeared. On stimulation, WPBs moved at a similar speed as in untreated cells (<10 nm/s). The entire process of patch formation, disappearance of vesicles, and appearance of the vWF-GFP clouds (see Figure 2) most likely reflects fusion of WPBs with the plasma membrane and secretion of vWF-GFP at the fusion site into the extracellular milieu. No preferential movement of WPBs to either the basal or the apical side of the cell was observed. However, vesicles that were primarily located at the bottom of the cell tended to form patches at the
basal side of the cell, whereas vesicles residing at the upper part of the cell tended to dock at the apical side.

Dynamics of WPBs in Thrombin-Stimulated vWF-GFP–Infected HUVECs
When vWF-GFP–infected HUVECs were stimulated with thrombin, we often observed an immediate contraction of the cells. This somewhat obscured visualization of the dynamics of WPB secretion. This process started a few minutes after addition of the stimulus. Thrombin also induced more rapid formation of patches than did PMA. Most patches were formed within the first 5 minutes of stimulation (Figure V, available at http://atvb.ahajournals.org) and disappeared faster (after \( \approx 10 \) minutes) than did PMA-induced patches. In some cases, concomitant formation of clouds was observed. Fewer patches were formed with thrombin than with PMA. As during PMA-induced stimulation, patch formation and fading were observed at the apical as well as the basal part of the cells.

WPB Dynamics Induced by cAMP Agonists
Unlike PMA and thrombin, neither forskolin (not shown) nor epinephrine induced contraction of the cell. However, we observed that these secretagogues induced a vectorial migration of WPBs toward the nucleus (Figure 4). Perinuclear redistribution was much more pronounced than that induced by either PMA or thrombin. After 30 minutes, most of the vesicles clustered around the nucleus in a starlike structure (Figure 4A). Also, \( \gamma-z \) analysis of the cell demonstrated that the initial random distribution of WPBs changed completely on stimulation with epinephrine (Figure 4B). We clearly observed that on stimulation, vesicles moved to the center of the cell, in particular, to the luminal side. Furthermore, vesicles moved up to 10 times faster (>100 nm/s) than in resting cells.

During migration, secretion was suggested to occur by means of the formation of patches that started to appear within \( \approx 20 \) minutes of stimulation. However, they disappeared faster than after PMA stimulation. Indeed, patches lasted for \( \approx 5 \) minutes (Video III, available at http://atvb.ahajournals.org). Patches were seen both at the periphery of the cell and around the nucleus. Furthermore, patch formation

Figure 2. Dynamics of WPBs on stimulation of vWF-GFP–infected HUVECs with PMA. Living vWF-GFP/hTERT-NGFR–transduced HUVECs were monitored in real time at intervals of 1 minute during 54 minutes of incubation with 50 ng/mL PMA (Video II). A, WPB trafficking of 1 cell is shown at intervals of 10 minutes. During the first 10 minutes, WPBs showed the same behavior as in resting cells (Figure 1). After 10 minutes, WPBs started to transform into highly GFP-condensed patches. Patches were not formed simultaneously (WPB 1, 10 minutes; WPBs 2 and 3, 20 minutes). However, most of the patches were observed 20 minutes after stimulation with PMA. Patches lasted, on average, 20 minutes (WPB 1 through 3). Some patches formed a cloud before completely disappearing (WPBs 1 and 3, 40 minutes). Sixty minutes after stimulation, the cell was devoid of WPBs. Scale bar-5 \( \mu m \). B, Total fluorescence intensity produced by WPB 2 during the whole recording period. Note the sharp increase in the fluorescence signal that is produced on formation of the patch. The gradual fading of the patch was correlated with the long trailing edge of the fluorescence peak.
was observed at the basal (Figure 4, WPB 1) as well as the apical (WPB 2) part of the cell. Thus, cAMP agonists induced distinct migration of WPBs to a specific site directly above the nucleus and concomitant formation of a starlike clustering of vesicles. Tentative fusion events started within the same time interval as observed on stimulation with PMA, but the event itself was as fast as after stimulation with thrombin.
Discussion

In the past, biogenesis and regulated exocytosis of WPBs have been extensively studied by biochemical and morphological analyses of endothelial cells. So far, the dynamics of this unique endothelial cell–specific storage device could not be addressed. In this study, the first evidence is presented for the complexity of the dynamics of WPBs. We constructed a vWF-GFP chimera that was able to multimerize and was properly targeted to WPBs and secreted on stimulation. This allowed direct visualization of WPB trafficking in living, primary HUVECs.

We observed that in resting cells, these vesicles are not static but consist of pools with different motilities (Figure 1). Some vesicles barely moved, as if they were tethered; others seemed to travel in a stochastic manner and frequently reversed their direction. All WPBs moved with remarkably low speed, not exceeding 10 nm/s. Previous studies on other cell types have shown that secretory vesicles may travel in different directions and move with different speeds. Newly formed, microtubule-associated vesicles travel at high speed (≈1 μm/s) from the Golgi to the plasma membrane.20–23 On arrival at the plasma membrane, these vesicles are trapped and mature in the dense meshwork of the actin cortex, which restricts their motility (≈50 nm/s).23,24 On the basis of these data and on the similar dynamics observed in WPBs, we speculate that the tethered WPBs seen in our study (eg, Figure 1; WPB 1, 3) are “docked” at the plasma membrane or trapped in the actin cortex of endothelial cells. WPBs that moved in all directions are reminiscent of microtubule-associated granules.20 Whether the WPBs observed here are immature or mature granules cannot be concluded from this study.

Stimulation with secretagogues dramatically changes the dynamics of WPBs. We observed 2 characteristic features of this traffic: (1) formation of patches observed on stimulation with both Ca2+- and cAMP agonists and (2) perinuclear clustering of WPBs only induced by cAMP secretagogues.

Formation of Membrane-Associated Patches

Previously, in studies with fixed endothelial cells, the presence of large, extracellular patches of vWF was noticed on stimulation of the cells.25,26 Our study clearly documents the generation of this typical feature. This is most apparent in the real-time movie of PMA-stimulated cells (Video I). These observations suggest that patch formation reflects fusion of a generation of this typical feature. This is most apparent in the clustering of WPBs only induced by cAMP secretagogues.26

Observations suggest that patch formation reflects fusion of a single WPB with the plasma membrane rather than the clustering of vesicles and subsequent bulk extrusion.26 Changes in fluorescence signal could be due to changes in the pH of the microenvironment and/or changes in fluorescence quenching because of dispersion of vWF-GFP. Striking was the rather long life span of WPB patches.22 The rapid (seconds) increase of the fluorescence signal due to fusion with the plasma membrane was followed by a slow (minutes) decay of the fluorescence (Figure 2B) as vWF-GFP diffused into the extracellular space. The apparent slow release of vWF-GFP from the cell could be due to the compact, crystalloid structure of vWF, which may hamper its dissolution. The possible interaction of vWF with other WPB constituents or with proteins localized at the plasma membrane might also affect the rate of vWF dispersion.

These long-lasting patches could have physiological significance. Patches at the cell surface might not only reflect diffusion of vWF-GFP but might also provide focal sites with a high concentration of vWF. These sites could play a role in adequately recruiting and binding plasma proteins, blood cells, or matrix components to sites of vascular injury. Pertinent to this point is the observation that platelets adhered rapidly, though transiently, to vWF secreted at the luminal face of endothelial cells on triggering of the cell with Ca2+-agonists.27,28 The observation that adherence of platelets to the endothelium was transient (minutes) corresponds with the time course of the fading of patches. Although this has not been demonstrated, it is possible that vWF-containing patches also present IL-8, P-selectin, or other WPB residents at high concentrations at the cell surface. Focal sites expressing these proteins at high levels could contribute to the rapid recruitment of leukocytes to endothelial cells after stimulation. Indeed, the time frame of P-selectin–mediated rolling of leukocytes on the endothelium is on the same order of magnitude as patch fading.29

The vectorial movement of WPBs induced by stimulation with either Ca2+- or cAMP-raising agents was less prominent than expected on the basis of previous observations. Basolateral as well apical secretion of vWF after stimulation of endothelial cells has been observed.30,31 In this study, we observed that WPBs that are recruited toward the nucleus tended to secrete their contents at the apical side of the cell, whereas vesicles that resided at the periphery of the cell secreted at the basal side. The location of the microtubule-organizing center (MTOC, see next paragraph) at the apical side of the cell might contribute to preferential luminal secretion associated with exocytosis of vesicles located in the vicinity of the nucleus. However, we did not observe any preferential accumulation of WPBs or a distinct vectorial movement. Quantitative analysis of the dynamics of WPBs in real time of multiple cells might provide more insight into a possible vectorial behavior of vesicle trafficking.

Perinuclear Clustering of WPBs

Another prominent feature of the dynamics of WPBs was the perinuclear clustering of WPBs. Only when cells were exposed to cAMP-raising agonists, such as forskolin or epinephrine, were WPBs docked at a distinct site proximal to the nucleus and spatially organized in a starlike structure (Figure 4). WPBs migrated to that site at velocities ≈10 times higher than in resting cells. On the basis of the observed localization and the morphology of these clusters, we assume that the site toward which WPBs migrated is associated with the MTOC. This observation suggests that microtubules play a role in the cAMP-induced migration of WPBs. Although not studied here, the cAMP-dependent mechanism responsible for the perinuclear recruitment of WPBs is most likely caused by protein kinase A–dependent modulation of the activity of microtubule-associated motor proteins.32,33 In addition to perinuclear clustering, patch formation was also observed (Figure 4; WPB 1, 2). Notably, tethered WPBs escaped clustering and fused directly with the plasma mem-
brane. On stimulation with thrombin or PMA, WPBs did not accumulate around the nucleus (Figure 2 and Figure V). Apparently, WPBs are directly translocated to the plasma membrane under these conditions. Both actin filaments and microtubules are most likely involved in the Ca\(^{2+}\)-dependent trafficking of WPBs.\(^{18,34,35}\)

Taken together, although both cAMP- and Ca\(^{2+}\)-raising agents induce patch formation, WPB dynamics triggered by these agonists are clearly different. cAMP-dependent perinuclear recruitment of WPBs might provide a means to limit excessive release of prothrombotic and proinflammatory mediators stored in WPBs under physiological conditions that raise intracellular levels of cAMP, such as physical exercise or other stress situations.\(^{18}\) On the other hand, Ca\(^{2+}\)-mediated secretion, eg, in response to vascular damage, most likely reflects mobilization of the entire WPB population to accomplish adequate release of bioactive molecules at sites of vascular injury.

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ONLINE MATERIALS AND METHODS

Construction of vectors and production of helper-free recombinant virus
VWF-GFP was constructed by replacing the VWF A2 domain by the sequence encoding the GFP (online Fig.IA). The GFP fragment was amplified from the pEGFP-N3 vector by PCR using primers containing the XmaI and BsPI restriction sites flanking the A2 domain and the ends of the GFP coding sequence \(^1\). A recombinant retroviral vector for delivery of the VWF-GFP gene was constructed using the dicistronic retroviral LZRS vector as described previously \(^2\).

The LZRS-hTERT-NGFR construct (a generous gift from Dr. H. Spits, Dutch Cancer Institute, Amsterdam, The Netherlands), encoding the human telomerase reverse transcriptase (hTERT) gene, was made by replacing GFP present in LZRS-hTERT-GFP \(^3\) by a truncated form of the neural growth factor receptor (NGFR). NGFR is expressed downstream hTERT and was used as a marker for hTERT transfection.

Helper-free recombinant retrovirus was produced after transfection of the LZRS-VWF-GFP or LZRS-hTERT-NGFR DNA into Phoenix-A cells (a 293T-based amphotropic retroviral packaging cell line) \(^3\) by calcium phosphate transfection (Gibco). The cells were cultured in medium containing IMDM, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin (all from Gibco) and 2 \(\mu\)g/ml puromycin (Sigma). For the production of virus, transfected Phoenix-A cells were maintained for at least 6 h in medium lacking puromycin.

Cell culture and retroviral transduction
HUVEC were isolated and cultured as described \(^4\). For transduction, HUVEC were grown on 35 mm-diameter fibronectin-coated wells until they reached a cell density of 20-40%. Cells were subsequently transduced with the harvested virus supernatant of the Phoenix cells \(^5\). HUVEC were either transduced with LZRS-VWF-GFP virus alone or, to extend the life span \(^6\), co-transduced with LZRS-VWF-GFP and LZRS-hTERT-NGFR virus (1:1 v/v). In terms of the expression and localization of VWF-GFP in cells and trafficking of WPbs, we observed no differences between cells transduced by either method.

In this study results shown are both from VWF-GFP/hTERT-NGFR- and VWF-GFP-transfected HUVEC. The respective cell line used is indicated. The transduction efficiency was tested by determining the percentage of GFP positive cells using flow cytometry analysis with a FACSscan (Becton Dickinson, San Jose, CA, USA).

Multimer analysis
HUVEC and VWF-GFP-infected HUVEC were grown in fibronectin coated 80 cm\(^2\) flasks. At confluence, cells were incubated for 60 min in 6 ml serum-free medium containing 1U/ml thrombin (Sigma). Cell supernatants were collected and cleared of cell debris by centrifugation. The medium containing VWF was concentrated by incubation with S-Sepharose Fast Flow (Pharmacia; Uppsala, Sweden) and subjected to SDS-agarose gel electrophoresis and Western blotting as described \(^1\). Blots were incubated with anti-GFP (PoAb A11122, Molecular Probes, Eugene, Oregon, U.S.A) and HRP-labeled horse-anti-rabbit antibodies or HRP-labeled anti-VWF (DAKO, Denmark A/S, Glostrup,
Denmark) polyclonal antibodies. Multimers were visualized by ECL (Roche Diagnostics Nederland B.V., Almere, The Netherlands).

**Immunofluorescence microscopy**

Cells were grown to confluence on 1 cm-diameter gelatin-coated glass coverslips, fixed in 3.7% (v/v) formaldehyde and permeabilized for 30 min with PBS buffer containing 0.02% saponin (Sigma) and 1% bovine serum albumin. The cells were then incubated with unconjugated primary antibodies in the permeabilizing buffer for 1 h at 37°C, washed three times, and incubated with conjugated secondary antibodies, as indicated in the figure legends. VWF was visualized with a monoclonal antibody directed against either the A1 domain (CLB-RAg 35) or the A2 domain (CLB-RAg 50) of VWF. A rabbit anti-human cathepsin D polyclonal antiserum (a generous gift from Dr. J.M.F.G. Aerts, Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands) was used as a lysosomal marker. The monoclonal antibody to P-selectin (RUU 1.18) was kindly provided by Dr H. K. Nieuwenhuis, University Medical Center Utrecht, Utrecht, The Netherlands. As secondary antibody, we used Texas-Red -conjugated goat-anti-mouse IgG or horse-anti-rabbit IgG (Vector Laboratories, Burlington, CA, USA). Cells were embedded in Vectashield mounting medium (Vector Laboratories) and viewed on a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Heidelberg, Germany).

**VWF secretion**

Regulated secretion of VWF-GFP was investigated either by determining the amount of secreted VWF-GFP antigen or by assessing the decrease of the number of WPbs in living cells after stimulation. HUVEC were grown in 35 mm-diameter fibronectin-coated wells and were used at confluence. Cells were washed three times with PBS and were stimulated for 60 min either with serum-free medium alone or with medium containing 50 ng/ml PMA. VWF antigen concentrations (nM) were measured by ELISA as described previously. VWF-GFP levels (expressed as absorbance at 550 nm) were measured by ELISA using a monoclonal antibody raised against GFP (MoAb 3E6 from Molecular probes) as coating antibody and an HRP-labeled anti-VWF polyclonal antibodies (DAKO) as conjugate. Results are expressed in relative values, i.e. as a percentage of release from unstimulated control cells from the same cell preparation. To measure the decrease of the number of WPbs, real time imaging was performed on living cells (see next paragraph). Cells were stimulated with different secretagogues (1 U/ml thrombin, 50 ng/ml PMA, or 10 μM forskolin / 100 μM IBMX), Z-stacks were taken before and 60 min after stimulation and the number of WPbs was counted. The number obtained at t=0 was set at 100%.

**Real-time imaging and microscopy**

HUVEC-VWF-GFP were grown on 35 mm-diameter gelatin-coated glass coverslips. At confluence, cells were mounted in an incubation chamber (LaCon, Germany) in HEPES medium consisting of 20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂ and 1.2 mM K₂HPO₄, supplemented with 1 mg/ml glucose and 2% human serum albumin. Confocal images were recorded.
with the Zeiss LSM510 using standard settings for GFP and Texas-Red detection. When required, images were recorded in the multi-track mode (sequential scanning) to exclude interchannel crosstalk. During real-time analysis, the sample chamber was maintained at 37°C using a thermostatted chamber holder. Time-lapse images of WPbs in living HUVEC-VWF-GFP were generated by repetitive imaging at the same focal plane or by making series of optical sections (Z-stacks, 400 nm interval/thickness, 12-15 slices/cell) every 2-5 min. Selected cells were first imaged for 10 min in their initial medium. Subsequently, 10 µl of the secretagogue of interest (final concentrations 1 U/ml thrombin, 50 ng/ml PMA, 10 µM forskolin / 100 µM IBMX or 10 µM epinephrine / 100 µM IBMX; all from Sigma) was carefully added to the medium in order not to disturb the cells or change the focal plane. The recording was continued for another 60 min. Top view of the cells was performed using the XY axis. Three-dimensional analysis was performed using depth-coding software that allowed the monitoring of WPbs translocation along the Z-axis. This software enabled division of cells in three areas (the apical, the central and the basal area) by encoding each part with a color. Lateral analysis of WPbs was visualized by plotting the cells as YZ diagrams. The fluorescence intensity of single WPbs was measured using the LSM510 function “Mean Region of Interest”.

Real-time movies
All movies are real-time series of fluorescent images recorded by confocal laser scanning microscopy using standard settings for GFP detection. Three-dimensional analysis was performed on video1 and video3 using depth-coding software. This software enabled division of cells in three areas (the apical, the central and the basal area) by encoding each part with a color. Red-coded vesicles are located at the bottom of the cell, green in the middle region and blue at the top of the cell. All movies are in QuickTime format and are compressed. All movies are played at 2 frames per sec (fps).
REFERENCES


**Table I.** Decrease of WPb number from VWF-GFP-infected HUVEC induced by different secretagogues

<table>
<thead>
<tr>
<th>secretagogue</th>
<th>decrease (± SD)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>control</td>
<td>3.6 ± 9.4</td>
<td>NS</td>
</tr>
<tr>
<td>thrombin</td>
<td>24.3 ± 13.3</td>
<td>0.001</td>
</tr>
<tr>
<td>PMA</td>
<td>30.4 ± 11.3</td>
<td>0.001</td>
</tr>
<tr>
<td>forskolin</td>
<td>40.4 ± 12.3*</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
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Cells were incubated with 1 U/ml thrombin, 50 ng/ml PMA, 10 µM forskolin / 100 µM IBMX or medium alone (control), and monitored in real-time. The number of WPbs was counted in individual cells before and after 60 min of incubation. The relative decrease (in %) of vesicle number after 60 min is depicted in this table. The number of WPbs was difficult to assess due to strong perinuclear clustering (cf. Fig. 9). NS, not significant.
Figure I. HUVEC transduced with VWF-GFP.
(A) Schematic representation of the primary structure of the pro-VWF-GFP chimera used for the expression of VWF-GFP. The location of conserved structural domains of the propeptide and mature VWF moiety is indicated. The A2 domain of VWF was replaced by GFP (green) allowing direct visualization of hybrid VWF-GFP.
(B) Localization of VWF-GFP-containing vesicles in transduced HUVEC. The VWF-GFP vector shown in A was retrovirally transduced in wild-type HUVEC. VWF-GFP was only observed in rod-shaped vesicles. The distribution of VWF-GFP-expressing vesicles in the cell was determined by three-dimensional analysis of the cells using depth-coding software (see Materials and Methods). This software enabled division of cells in three areas (the apical, the central and the basal area) by encoding each part with a color. Red-coded vesicles are located at the bottom of the cell (upper left panel), green in the middle region (upper right panel) and blue at the top of the cell (lower left panel). The merged image of all parts is shown in the lower right panel. Scale bars, 10 μm.
Figure II. VWF-GFP is co-localized with endogenous VWF and P-selectin in WPbs. VWF-GFP-infected HUVEC were fixed and examined by immunofluorescence microscopy. VWF-GFP-containing vesicles were directly visualized (a,d,g,j). Cells were stained either with a monoclonal antibody, CLB-RAg 35, directed against the VWF-A1 domain (b,c) or an antibody, CLB-RAg 50, against the VWF-A2 domain (e,f), followed by Texas-red-conjugated goat anti-mouse IgG. Monoclonal anti-P-selectin antibody RUU 1.18, and Texas Red-labeled goat-anti-mouse IgG were used for P-selectin detection (h,i). Co-localization of VWF-GFP containing vesicles with P-selectin (i) is shown in yellow. Localization of cathepsin D was determined with anti-human cathepsin D polyclonal antiserum, followed by Texas-red-conjugated horse-anti-rabbit IgG (k,l). VWF-GFP was not co-localized with the lysosomal marker cathepsin D (l). Scale bars, 10 µm.
Figure III. Multimer analysis of VWF-GFP. Wild-type HUVEC (lanes 1 and 3) as well as VWF-GFP-infected HUVEC (lanes 2 and 4) were stimulated for 60 min with 1U/ml thrombin. Released VWF was concentrated by S-Sepharose, run on a 1.4% agarose gel, and blotted. Blots were incubated either with anti-VWF (left panel) or with anti-GFP (right panel). VWF multimers produced by HUVEC-VWF-GFP (lane 2) are similar to wild-type multimer forms (lane 1). VWF-GFP multimers also react with the anti-GFP antibody (lane 5), in contrast to wild-type VWF (lane 3). Slight differences in mobility most likely reflect differences in size between the A2 domain (191 amino acids) present in wild-type VWF and the inserted GFP (239 amino acids).

Figure IV. Regulated secretion of VWF and GFP by VWF-GFP-transfected cells. Confluent VWF-GFP-infected HUVEC grown in 35 mm-diameter wells were incubated for 60 min with medium containing 50 ng/ml PMA (closed bars) or with medium alone (open bars). Released VWF-GFP was assessed by measuring VWF (nM) and GFP (A550) antigen (see Materials and Methods). Results are expressed in relative values. VWF and GFP release from unstimulated cells was defined as 100%. Results are the mean ± SEM of 3 experiments.
Figure V. Rapid patch formation induced by thrombin. A VWF-GFP/hTERT-NGFR-transduced cell was stimulated with 1 U/ml thrombin for 60 min. Time-lapse images were taken at intervals of 2 min. The depicted time points (0, 4, 16 and 46 min) show the appearance of patches very soon after stimulation (WPb 1-3, 4 min). After 10 min of stimulation, disappearance of the patches coincided with the appearance of clouds (WPb 2 and 3, 16 min). N, Nucleus. Scale bar, 10 μm.
VIDEO LEGENDS

**Video1. Dynamics of WPbs in resting cells.** Living VWF-GFP/hTERT-NGFR transduced HUVEC were monitored in real-time at intervals of 2 min during 54 min of incubation with medium. Localization of WPbs was visualized with a color scale. Red-coded vesicles are located at the bottom of the cell, green in the middle region and blue at the top of the cell (see also Online Fig.I). Note the random localization of WPbs. Some vesicles seem motionless whereas others seem to move in a stochastic manner. Note that among the moving WPbs, some travel long distances, notably toward the periphery, whereas others return to their original position. Several WPbs seem round because they are positioned perpendicularly to the plasma membrane. The whole sequence consists of 27 frames (54 min) and is played at 2 frames per sec (13 sec).

**Video2. Dynamics of WPbs upon stimulation of HUVEC-VWF-GFP with PMA.** A single VWG-GFP HUVEC is monitored in real-time at intervals of 1 min during 54 min of incubation with 50 ng/ml PMA. During the first 10 min WPbs show the same behavior as that in resting cells (video1). However, after 10 min of incubation with PMA, WPbs start to transform into highly GFP-condensed patches. Patch formation does not occur in phase. However, most of the patches are observed 20 min after stimulation with PMA. Patches signals last on average 20 min. Some patches form a cloud before completely disappearing. 60 min after stimulation the cell is devoid of WPbs. The whole sequence consists of 54 frames (54 min) and is played at 2 frames per sec (27 sec).

**Video3. Perinuclear clustering of WPbs induced by epinephrine.** A VWF-GFP/hTERT-NGFR-transduced cell stimulated with 10 µM epinephrine / 100 µM IBMX for 60 min. Time-lapse images are taken at intervals of 5 min. Remarkable is the perinuclear recruitment of WPbs that is observed about 30 min after stimulation. They seem to be docked at a distinct site proximal to the nucleus, forming a starlike structure. Formation of patches is also observed after about 45 min of stimulation. Patches disappear within 5 min of stimulation or formed a cloud. The whole sequence consists of 12 frames (60 min) and is played at 2 frames per sec (6 sec). Note that red-coded vesicles are located at the bottom of the cell, green in the middle region and blue at the top of the cell (see also Online Fig.I).