Weibel-Palade bodies (WPBs) are large rod-shaped secretory granules found in endothelial cells (1–4). Regulated exocytosis of WPBs delivers the adhesive protein von Willebrand factor (VWF) and the leukocyte adhesion molecule P-selectin to the endothelial cell surface, where they play important roles in vascular hemostasis and inflammation (1, 5, 6). Correct targeting of VWF into WPBs requires the propolypeptide of VWF (proregion) (7, 8). The cleaved proregion is co-packaged with VWF in WPB in a 1:1 stoichiometry and released along with VWF during WPB exocytosis (9). The extracellular function of proregion is not clear. Previous biochemical studies have shown that although proregion does associate transiently with VWF inside the secretory pathway (10), it does not associate with extracellular VWF, matrix components, or the endothelial cell surface, indicating that it is unlikely to play an important role in endothelial-platelet adhesion (9). A putative role in regulating collagen-induced platelet-platelet aggregation has been suggested (11, 12), and it may also act as a ligand for the VLA-4 (Very Late Antigen-4) integrin present on monocytes (13). VLA-4 plays an important role in the recruitment of monocytes into the blood vessel wall and is implicated in the vascular accumulation of monocytes that marks the early development of atherothrombotic lesions (14). These data indicate that WPB-derived VWF and proregion perform distinct extracellular functions, VWF on the endothelial surface and proregion at sites distant from the endothelial surface. Little is known about rates of dispersal of these proteins from the endothelial cell surface following WPB exocytosis, a process that will influence the effectiveness of these molecules at their sites of action. A recent study using viral infection of human umbilical vein endothelial cells (HUVECs) with a fusion protein of VWF and green fluorescent protein (GFP), in which the GFP replaced the A2 domain of VWF, has shown that WPBs can be fluorescently labeled and that stimulation results in the formation of extracellular patches of VWF-GFP that remained visible on the cell surface for up to 20 min (15). A detailed analysis of the rate of loss of surface-associated VWF was not made. In this study, we constructed chimeras of VWF and its proregion, fused to enhanced green fluorescent protein (EGFP) (16). We show that both constructs are correctly targeted to WPBs when expressed in HUVECs and that the resulting fluorescent WPBs undergo exocytosis in response to the WPB secretagoge histamine. Using these constructs, we have determined the time course of VWF-EGFP and proregion-EGFP loss from individual WPBs following exocytosis.

**Experimental Procedures**

**Antibodies and Reagents**

Rabbit polyclonal anti-human VWF was purchased from Dako Ltd. (Cambridgeshire, UK). Mouse monoclonal anti-P-selectin (clone AK-6) was from Serotec (Oxon, UK). The anti-CD63 monoclonal antibody (clone H5C6) developed by J. T. August and J. E. K. Hildreth was obtained from The Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa. Secondary antibodies coupled to fluorophores were purchased from Jackson ImmunoResearch. All other reagents were purchased from Sigma-Aldrich, unless stated otherwise.
Primary HUVECs were purchased from TCS Cellworks and grown as previously described (17) or in the TCS Cellworks HUVEC culture medium. HUVECs were transfected using 5–10 μg of expression vector DNA using the Nucleofection device and buffers according to the manufacturer’s instructions (Amaxa GMBH). Cells were used between 1 and 3 days after Nucleofection.

Expression Vectors

pVWF-EGFP—A 228-bp PCR fragment flanked by EcoRI and AgeI restriction sites was generated from the carboxyl terminus of human VWF using primers 5′-ctgagctggaagatgcttc-3′ and 5′-ctgagctggaagatgcttc-3′ and the vector pMT2-ADA-VWF (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pVWF-tern-N1, which encodes a fusion of the VWF carboxyl terminus in-frame with the EGFP cDNA. This cloning strategy gave rise to a joining sequence of four extraneous amino acids (Pro-Val-Ala-Thr) preceding the carboxyl-terminal glycine of VWF (mutated from Stop) and the amino-terminal methionine of EGFP. The entire 8442-bp human VWF coding sequence minus the 204-bp 3′ of the unique EcoRV site (and with some additional 5′-UTR sequence) was cut out of pMT2-ADA-VWF using EcoRI and EcoRV and cloned into EcoRI/EcoRV-digested pVWF-tern-N1, producing pVWF-FL-N1. A 350-bp EcoRI/XbaI fragment encoding the amino-terminal ATG of human VWF without any additional 5′-UTR was generated from pMT2-ADA-VWF using PCR with the primers 5′-gagaacttgtatggtggttttag-3′ and 5′-ctgggaaccttgtac-3′. This fragment was ligated into EcoRI/XbaI-digested pVWF-FL-N1 to give pVWF-EGFP. All PCR products were verified by sequencing. It should be noted that VWF is actually synthesized as a precursor protein (pre-pro-VWF) that has to undergo proteolytic processing in the secretory pathway to give rise to the mature (circulating) VWF (1). After post-translational modifications, the predicted product of the pre-pro-VWF is EGFP-fused to the carboxyl terminus of mature VWF. For simplification, we refer to this construct as pVWF-EGFP, and we refer to the protein to which it gives rise as VWF-EGFP (rather than pre-pro-VWF-EGFP).

pProregion-EGFP—The proregion of VWF refers to the 741-amino acid protein that is generated from the amino terminus of pre-pro-VWF by its cleavage at a tetra-basic, furin-like cleavage site (19, 20). To manufacture a proregion-EGFP fusion protein, we took advantage of the presence of a unique HindIII restriction site just 50 bp 5′ of the proregion-VWF cleavage site. The HindIII site in the multiple cloning site of pEGFP-N1 (Clontech) was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The reverse primer and the vector pMT2-ADA-VWF (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pProregion-EGFP—pProregion-EGFP—pProregion-EGFP—pProregion-EGFP—pProregion-EGFP (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pProregion-EGFP—pProregion-EGFP (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pProregion-EGFP—pProregion-EGFP (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pProregion-EGFP—pProregion-EGFP (18) (American Type Culture Collection) as template. This fragment included a unique EcoRV site 204 bp from the VWF stop codon. The reverse primer was designed to mutate the VWF Stop codon (tga) into a glycine (gga). The PCR product was digested with EcoRI and AgeI and cloned into the EcoRI/AgeI sites of pEGFP-N1 (Clontech), giving rise to plasmid pProregion-EGFP (rather than pre-pro-VWF-EGFP).

Immunocytochemistry

Nucleofected HUVECs grown on gelatin-coated glass coverslips were processed for immunofluorescence as described previously (21).

Fluorescence Imaging of WPB Exocytosis in Living Cells

Nucleofected HUVECs were grown on poly-d-lysine-coated glass-bottomed chambers (MatTek Corp.). Time-lapse images of EGFP fluorescence were recorded in release medium (medium 199 supplemented with 10 mM HEPES, pH 7.2, and 0.2% bovine serum albumin), at room temperature or at 37 °C as indicated, on a Deltavision Imaging system (Applied Precision Inc., Seattle, WA) housed within a temperature-controlled incubator. Images were obtained using either an Olympus U-Plan-Apo ×100 objective 1.35 numerical aperture or PLANAPO ×60 1.4 numerical aperture objective in conjunction with a Prinston Instru-
an extracellular Alexa-546-conjugated anti-VWF antibody was applied at the same time as histamine to detect newly secreted VWF. The rapid binding (within a few seconds of WPB collapse) and co-localization of the anti-VWF antibody with patches of fluorescent VWF-EGFP confirm the extracellular nature of the newly formed patches of fluorescent VWF-EGFP. Exposure of non-stimulated cells to the fluorescent VWF antibody alone did not evoke exocytosis or formation of extracellular patches of VWF (data not shown). Histamine also evoked exocytosis of proregion-EGFP-containing WPBs (Movie 3). Fig. 2B and Movie 4 show the exocytosis of a single proregion-EGFP-containing WPB evoked by histamine and in the presence of an extracellular Alexa-546-conjugated anti-VWF antibody. In this case, proregion-EGFP was seen to rapidly disperse from the release site and did not form extracellular patches. The fluorescent VWF antibody detected extracellular VWF (co-packaged in the WPBs with the proregion-EGFP) at the release site within a few seconds of WPB exocytosis.

**VWF-EGFP but Not Proregion-EGFP Is Lost Slowly from Release Site after WPB Exocytosis—** Experiments were carried out at 37 °C. When imaged with subsecond time resolution, an abrupt increase in the fluorescence of intra-WPB-EGFP is seen prior to or coinciding with release of contents (e.g., Figs. 2B and 3). This increase in fluorescence is due to the collapse of the WPB intra-granule pH to that of the extracellular medium (pH 7.4) following WPB fusion to the plasma membrane, and it is described in more detail elsewhere (25). WPB exocytosis can occur on both the upper and lower surfaces of the HUVEC cell membrane, and the time course data analyzed here represent a mixture of both cases. Fig. 3 shows the total fluorescence of a WPB containing proregion-EGFP (Fig. 3A) or VWF-EGFP (Fig. 3B) during exocytosis. The data are normalized to the peak fluorescence seen at the point of fusion. Exocytosis of proregion-EGFP-containing WPBs (Fig. 3A) resulted in an abrupt increase in fluorescence (mean, 2.7 ± 0.7-fold, n = 32 WPBs) following a rapid decline to background levels. No persistent extracellular patches of fluorescence could be observed. The time taken for fluorescence to decline to 50% of the initial peak increase was 2.98 ± 1.88 s (±S.D., n = 32 WPBs). Exocytosis of VWF-EGFP-containing WPBs also resulted in an abrupt increase in fluorescence (mean, 2.6 ± 0.6-fold, n = 20 WPBs) (Fig. 3B). Following the peak of the fluorescence increase, the fluorescence of newly formed extracellular patches of VWF-EGFP declined slowly toward background levels. The decline in fluorescence of extracellular patches of VWF-EGFP was interpreted as the loss of VWF-EGFP from the cell surface. The time taken for fluorescence to decline to 50% of the initial peak increase was 323.5 ± 146.2 s (±S.D., n = 20 WPBs). Extracellular fluorescent patches of VWF-EGFP at sites of WPB exocytosis could be observed 30–40 min after exocytosis. The dramatic difference in the rate of dispersal of proregion-EGFP compared with VWF-EGFP is highlighted in Fig. 3C. For easier comparison, the normalized fluorescence data from Fig. 3A (black trace) and Fig. 3B (gray trace) were aligned at the point of WPB fusion and plotted on an expanded time scale to show the period immediately following fusion (Fig. 3C, iii). Images corresponding to the time points indicated by open circles in the traces in Fig. 3C, iii are shown in Fig. 3C, i (proregion-EGFP) and ii (VWF-EGFP).

**DISCUSSION**

Circulating VWF comprises predominantly dimers and low molecular weight multimers of VWF derived from constitutive secretion from vascular endothelial cells (see Ref. 1). These forms of soluble VWF do not readily adhere to the surface membrane of platelets or endothelial cells but can bind to exposed extracellular matrix components and promote platelet adhesion following vessel damage (26). VWF stored in WPBs consists of a highly multimeric form of VWF (27, 28). Stimulation of cultured endothelial cells with thrombin or histamine results in WPB exocytosis and the appearance of complex patches of this form of VWF on the cell surface (15, 27). The function of WPB-derived VWF on the endothelial surface is not clear. Basolateral secretion (toward the basement membrane) has been suggested to increase endothelial adhesion to the extracellular matrix components through interactions between VWF, endothelial integrin receptors, and basement membrane components (see Ref. 29). In vivo experiments have shown that apical secretion of VWF elicited by histamine supports platelet attachment and rolling on the endothelial cell membrane (6).
during exocytosis evoked by histamine (100 μM) of a proregion-EGFP (in total fluorescence (normalized to the peak fluorescence on WPB fusion) of a proregion-EGFP (A) or VWF-EGFP (B) containing WPB during exocytosis evoked by histamine (100 μM, 37 °C). The half-time for the decline in fluorescence from the peak of the initial increase was 2.8 s for proregion-EGFP and 338.4 s for VWF-EGFP. C, iii shows the data in A and B aligned at the point of WPB fusion and on an expanded time scale. i and ii show a montage of images, corresponding to the time points indicated by open circles in iii, for proregion-EGFP (i) and VWF-EGFP (ii) containing WPBs. Scale bars: 1 μm, i; 2 μm, ii.

The time course and extent of VWF-dependent platelet-endothelial surface interactions will depend in part on the kinetics of WPB exocytosis, VWF release, and the rate of its dispersal or loss from the endothelial cell surface. The time course for VWF-dependent platelet adhesion and rolling on the endothelial surface determined in vivo (6) includes a delay between endothelial stimulation and platelet adhesion of 15–20 s before an abrupt increase in the numbers of adherent and rolling platelets is seen, peaking 30–60 s after stimulation, and followed by a slow decline in the numbers of adherent and rolling platelets over 5–6 min to a lower but significantly elevated level (6). In previous studies, we have shown that WPB exocytosis in response to elevated [Ca^{2+}]_{i} includes a delay of 10–20 s (25, 30), which may account in part for the delay in platelet adhesion observed following endothelial activation in vivo (6). In this study, we have shown that WPB exocytosis in living HUVECs leads to the formation of complex extracellular patches of VWF-GFP identical to those observed in histological studies following hormone stimulation (27) and in live HUVECs (15). In the latter study (15), a time resolution of 60 s did not allow a detailed study of the formation and loss of VWF-EGFP from extracellular patches on the cell surface. Here we describe in more detail the slow, complex time course for loss of VWF-EGFP from extracellular patches with improved time resolution. There was some variability in the time course of VWF-EGFP loss from WPBs. This may reflect both a complex interplay of adhesive interactions between VWF-EGFP and components of endothelial surface and differences in the local environment in which the exocytotic events occurred (e.g. those that might exist on upper or lower surfaces of the cell). The overall slow time course for VWF-GFP dispersal seen here occurs over a similar time scale to the slow decrease in platelet adhesion and rolling observed in vivo (6), suggesting that the latter may be due in part to the slow loss of VWF from the endothelial surface membrane following WPB exocytosis. In contrast to VWF-EGFP, proregion-EGFP is rapidly lost from the endothelial surface interactions will depend in part on the kinetics of WPB exocytosis and adhesion and rolling observed in vivo (6), suggesting that the latter may be due in part to the slow loss of VWF from the endothelial surface membrane following WPB exocytosis.