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Biogenesis of Von Willebrand factor-containing organelles in heterologous transfected CV-1 cells

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Von Willebrand factor (vWF) is a multimeric protein involved in the adhesion of platelets to an injured vessel wall. vWF is synthesized by the endothelial cell and the megakaryocyte as a precursor protein (pro-vWF) that consists of four repeated domains, denoted D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. Previously, we have defined the domains on the pro-vWF molecule involved in dimerization as well as the domains involved in multimer assembly of vWF dimers. In the endothelial cell, part of the vWF multimers is stored in specialized organelles, the Weibel-Palade bodies. By using immuno-electron microscopy, we demonstrate that upon expression of full-length vWF cDNA, vWF-containing organelles are encountered in monkey kidney CV-1 cells that are morphologically similar to the endothelial-specific Weibel-Palade bodies. Expression in CV-1 cells of a series of vWF cDNA deletion mutants, lacking one or more domains, revealed that only those vWF mutant proteins that are able to assemble into multimers are encountered in dense-cored vesicles. Our data show that this process is independent of a particular domain on vWF and indicate that a 'condensed', multimeric vWF is required for targeting to the Weibel-Palade body.

Key words: basal secretion/CV-1/multimer assembly/von Willebrand Factor/Weibel-Palade body

Introduction

In the eukaryotic cell, multiple mechanisms exist that direct secretory proteins to their correct intracellular or extracellular destination. During the last few years, evidence has been obtained for the selective removal of proteins from the 'bulk flow' along the secretory pathway by receptor-mediated mechanisms. It has been shown that proteins, harbouring the amino acid sequence Lys-Asp-Glu-Leu (KDEL), interact with a specific receptor, termed ERD-2, that redirects these proteins to the endoplasmic reticulum (ER) (Pelham, 1990). For proteins destined for the lysosomes, a similar ligand-receptor interaction has been defined between mannose-6-phosphate moieties on the lysosomal protein and mannose-6-phosphate receptors present in the Golgi apparatus (Kornfeld, 1986). Contrary to these well-characterized sorting events, little information is available on the signals present on proteins destined for secretory granules (for a review, see Burgess and Kelly, 1987). It has been proposed that, similar to sorting of proteins to the ER and to the lysosomes, targeting of proteins to the regulated pathway is a receptor-mediated event. Alternatively, it has been put forward that targeting of proteins to secretory granules occurs as a consequence of selective condensation of proteins in the trans-Golgi. The latter theory is supported by immunocytochemical studies, using either the mouse pituitary AtT-20 cell line or rat pancreatic B cells, which reveal the presence of condensed secretory proteins in maturing secretory granules budding from the trans-Golgi network (TGN) (Tooze and Tooze, 1986; Orci *et al.*, 1987; Tooze *et al.*, 1987). Furthermore, a detailed study of the routing of a variant of insulin, containing a substitution of a particular histidine residue, postulated to be involved in Zn^{2+}-dependent hexamer formation, revealed that this mutant protein is less efficiently targeted to secretory granules (Gross *et al.*, 1989). Additional studies, however, provided evidence that hexamer formation per se is not required for targeting of insulin to the secretory granule, although hexamer formation of these molecules was not addressed in the transfected cells studied (Quinn *et al.*, 1991). Using secretogranin II as a model, Huttner and co-workers have demonstrated a pH- and calcium-dependent condensation of proteins destined for secretory granules (Gerdes *et al.*, 1989; Chanat and Huttner, 1991; Tooze *et al.*, 1991). Together with morphological data, these observations suggest a close relationship between the condensation of proteins in the TGN and the subsequent targeting to secretory granules.

The precursor protein von Willebrand factor (vWF) provides an excellent model to study the latter proposal for the biogenesis of secretory granules. During biosynthesis of vWF in the endothelial cell, the vWF protein is assembled into multimers which are packaged in secretory granules, the Weibel-Palade bodies (Weibel and Palade, 1964; Wagner *et al.*, 1982; Reinders *et al.*, 1984; Ewenstein *et al.*, 1987). The assembly of vWF multimers consists of two distinct processes, namely dimerization of pro-vWF subunits and, subsequently, these dimers act as protomers for multimer assembly. Dimerization occurs in the ER and involves intermolecular disulphide bonding of vWF precursors (pro-vWF) at their carboxyl-terminus, resulting in pro-vWF dimers. In the Golgi apparatus, intermolecular disulphide bonding of pro-vWF dimers takes place at the amino-terminus of the vWF protein, yielding high molecular weight vWF multimers (Wagner and Marder, 1984). In the TGN, part of the vWF multimers is packaged into secretory vesicles and released from the endothelial cell via the constitutive pathway. In addition, another part of the vWF multimers is stored in Weibel-Palade bodies, which are released upon stimulation of the endothelial cell with agents that activate the protein kinase C pathway (Loesberg *et al.*, 1983; Sporn *et al.*, 1986).

The pro-vWF subunit consists of an array of four repeated domains, termed A, B, C and D, as deduced from determination of the primary amino acid sequence of the vWF protein (Bonthron *et al.*, 1986; Shelton-Inloes *et al.*, 1986; Titani *et al.*, 1986; Verweij *et al.*, 1986). The pro-polypeptide of the vWF protein consists of the domains D1 and D2, while the domains D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2 constitute mature vWF. Previously, we have defined the domains involved in dimerization and multimer assembly by expressing vWF cDNA and mutants derived thereof in COS-1 cells (Verweij *et al.*, 1987, 1988; Voorberg *et al.*, 1990, 1991). An important implication of these studies is the finding that multimer assembly and dimerization are independent events, mediated by distinct domains on the vWF protein (Voorberg *et al.*, 1990, 1991). To study the relation between assembly of vWF multimers and the targeting of the vWF protein to the Weibel–Palade body, we expressed 'full-length' vWF cDNA and mutant cDNAs in CV-1 cells. It is shown that, upon expression of full-length vWF cDNA in CV-1 cells, the vWF is encountered in dense-cored vesicles strongly resembling the endothelial cell-specific Weibel–Palade bodies. Similar results have been obtained upon expressing vWF cDNA in the mouse pituitary AtT-20 cells, known to allow storage of hormones (Wagner *et al.*, 1991; Mayadas and Wagner, 1992). By employing several derivatives of the vWF cDNA, these authors indicate that the pro-polypeptide of vWF directs the formation of these vWF granules. Interestingly, in those studies multimer assembly was not required for the formation of vWF-containing organelles (Mayadas and Wagner, 1992). Here, we show, using a set of well-defined mutant vWF proteins, that the pro-polypeptide is not sufficient for the formation of Weibel–Palade-like organelles in CV-1 cells. In addition, our data reveal that proteolytic processing of vWF is not required for granule formation in CV-1 cells. Furthermore, it is shown that the pro-polypeptide, when provided *in trans*, can redirect a mutant vWF protein lacking the pro-polypeptide to the dense-cored vesicles observed in transfected CV-1 cells. Based on a strict correlation between the biogenesis of vWF-containing organelles and the ability of a vWF protein to assemble into multimers, it is argued that a 'condensed', multimeric vWF is required for the formation of a Weibel–Palade body in the endothelial cell.

Results

Expression of vWF in non-endothelial CV-1 cells

Previously, we have expressed 'wild-type' vWF cDNA and mutants lacking specific domains of the vWF molecule in monkey kidney COS-1 cells. Those studies allowed us to assign the different domains on the vWF molecule which are responsible for dimer and multimer assembly (Verweij *et al.*, 1987; Voorberg *et al.*, 1990, 1991). Furthermore, we demonstrated, both by biochemical methods and by immunolocalization, that vWF and vWF mutant proteins are present mainly in the ER of the transfected COS-1 cells. In endothelial cells, vWF is stored in post-Golgi organelles, the Weibel–Palade bodies (Wagner *et al.*, 1982; Reinders *et al.*, 1984; Ewenstein *et al.*, 1987). Here, we expressed wild-type vWF cDNA in monkey kidney CV-1 cells. Analysis of transiently expressed wild-type vWF cDNA by immunofluorescence, using both a polyclonal and monoclonal antibodies raised against plasma vWF, specifically revealed the presence of rod-shaped organelles, strongly resembling the Weibel–Palade bodies present in endothelial cells (Figure 1; compare panels A and B with panel C). Besides the particulate pattern of staining observed in the transfected cell, a reticular background was frequently observed reminiscent of vWF present in the ER of the cell (Figure 1A).

Biochemical characterization of wild-type vWF expressed in CV-1 cells

Having found initial evidence for the occurrence of dense-cored vesicles in transfected CV-1 cells, a stable cell line expressing wild-type vWF under the control of the RSV promoter was constructed (clone CV-1/vWF3). Morphological analysis by immunofluorescence revealed the presence of vWF-containing organelles similar to the ones found in transiently transfected cells (data not shown). In the

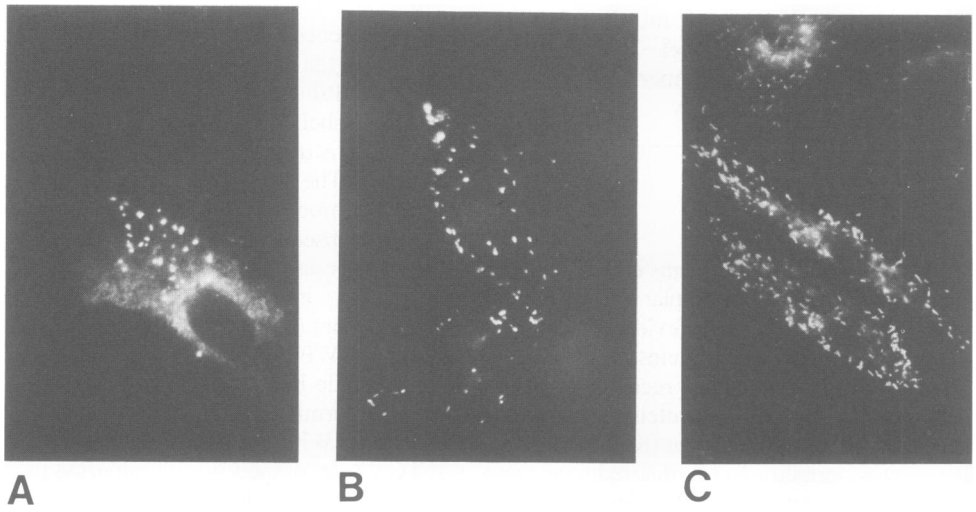

Fig. 1. Immunolocalization of wild-type vWF in transfected CV-1 cells by immunofluorescence. (**A**) CV-1 transfected with wild type vWF cDNA. (**B**) CV-1 cells transfected with wild-type vWF cDNA. (**C**) Human umbilical vein endothelial cell. Note the diffuse staining in panel A, reminiscent of the presence of wild-type protein in the ER. Panels A and C are stained with a polyclonal anti-vWF serum. Panel B is stained with monoclonal CLB-Rag 35 against vWF. Magnification, 1250×.

endothelial cell, the vWF protein present in the Weibel–Palade bodies is present in a completely processed form and consists of high molecular weight multimers (Sporn *et al.*, 1986). We and others have shown that the vWF protein, upon expression of the vWF cDNA in cells without a regulated pathway, is present in the cell in the unprocessed form (Verweij *et al.*, 1988; Wise *et al.*, 1988; Kaufman *et al.*, 1989; Voorberg *et al.*, 1990, 1991; Wagner *et al.*, 1991; Lyons *et al.*, 1992). In addition, inspection of the multimeric pattern inside cells exclusively harbouring a constitutive pathway revealed predominantly dimers (Voorberg *et al.*, 1991; Wagner *et al.*, 1991; Lyons *et al.*, 1992).

To establish the intracellular subunit structure of the vWF protein in the transfected CV-1 cell, clone CV-1/vWF3 was metabolically labelled with [^{35}S]methionine and 'chased' for various times in the presence of an excess of unlabelled methionine. Analysis of cell extracts revealed that, at 1 h after the chase, most of the labelled vWF is present in the non-processed form. However, at longer chase periods a small but significant part of the newly synthesized vWF is proteolytically processed (Figure 2). Surprisingly, most of the labelled vWF has left the cell at a chase period of 8 h, although the processed vWF persists somewhat longer. This decrease in intracellular vWF is counterparted by an increase in secreted material (Figure 2B). The observed rate of secretion of vWF in CV-1/vWF3 cells is different from that observed in endothelial cells, where a significant intracellular pool of vWF is encountered for extended periods (Wagner *et al.*, 1986). In the endothelial cell, secretion of vWF protein from the Weibel–Palade bodies can be induced by agonists that activate protein kinase C (Loesberg *et al.*, 1983). Attempts to stimulate vWF secretion from CV-1/vWF3, both by several protein kinase A and C agonists, were unsuccessful (data not shown). This observation is compatible with the turnover rate of the processed form of the vWF molecule.

In the endothelial cell, the vWF protein encountered in storage organelles consists of high molecular weight multimers (Wagner *et al.*, 1985). In order to investigate the multimers encountered in the cell line CV-1/vWF3, cell extracts were analysed on a 2% SDS–agarose gel, followed by staining of the gel with ^{125}I-labelled anti-vWF antibodies (Figure 3). Inspection of the multimeric pattern revealed the presence of two intense bands migrating at the molecular weight of the unprocessed and processed vWF dimer. In addition, the presence of high molecular weight multimers is clearly observed (Figure 3, lane 1). Most of the extremely high molecular weight material is not separated in the agarose gel used and is encountered at the top of the running gel. This observation shows that, similar to endothelial cells, transfected CV-1 cells contain an intracellular pool of high molecular weight multimers. In the endothelial cell, the presence of weak bases, e.g. chloroquine and ammonia, has been shown to interfere both with multimer assembly and targeting of vWF to the Weibel–Palade bodies (Wagner *et al.*, 1986). Similarly, inspection of cell extracts of CV-1/vWF3 cells incubated with 100 μM chloroquine prevents the formation of high molecular weight multimers (Figure 3, lane 2).

Immunoelectron microscopy of vWF-containing organelles in transfected CV-1 cells

Immunoelectron microscopy was performed on ultrathin frozen sections and Lowicryl K4M-embedded sections of

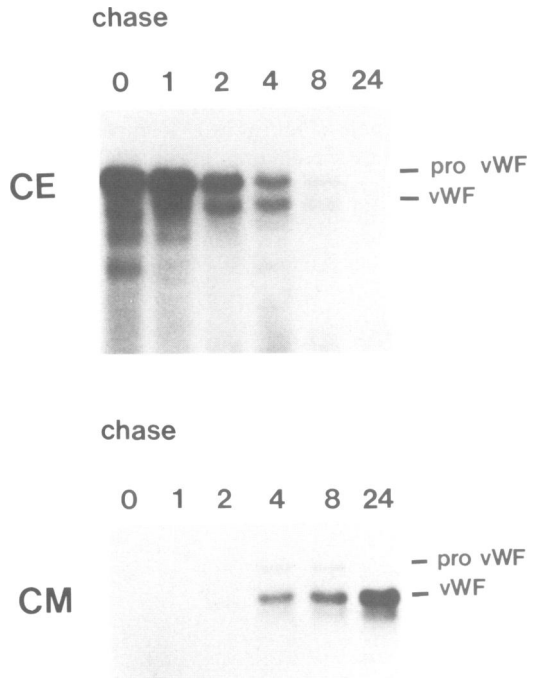

Fig. 2. Pulse–chase analysis of clone CV-1/vWF3, stably transfected with wild-type vWF cDNA. Cells were metabolically labelled with [^{35}S]methionine for 1 h and chased for 0, 1, 2, 4, 8 and 24 h. Radiolabelled vWF was immunopurified from both cell extract and conditioned medium at the different chase periods, and analysed under reducing conditions on a 6% (w/v) SDS–polyacrylamide gel. The different chase periods are indicated at the top of the figure. The pro-vWF and the vWF subunit are indicated at the left of the figure. Molecular weight markers are indicated.

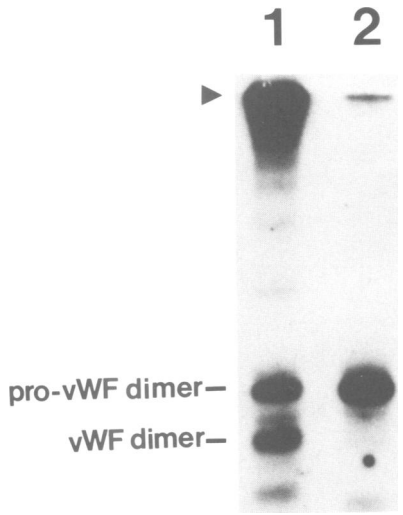

Fig. 3. Multimeric composition of CV-1/vWF3 cells stably transfected with wild-type vWF cDNA. Cell extracts of CV-1/vWF3 were harvested in IPB and analysed under non-reducing conditions on a 2% SDS–agarose gel. **Lane 1**, clone CV-1/vWF3; **lane 2**, clone CV-1/vWF3 grown for 16 h in 100 μM chloroquine. The position of the pro-vWF dimer and the vWF dimer are indicated. For the untreated CV-1/vWF3 cells, a substantial part of the high molecular weight multimers does not enter the gel. The top of the running gel is indicated by an arrowhead.

CV-1 cells transfected with the wild-type vWF cDNA. In accordance with the data obtained by immunofluorescence, described above, rod-shaped organelles were observed which strongly labelled with anti-vWF antibody (Figure 4). These dense-cored vesicles, clearly enclosed by a unit membrane, were mainly located near the plasma membrane (Figure 4A); some of them were in the process of being extruded into the medium or cytoplasmic vacuoles (Figure 4B). Further inspection of a transiently transfected CV-1 cell showed association of vWF with the Golgi stack and ER (Figure 5). Faintly electron-dense material labelled with anti-vWF antiserum was also observed within cytoplasmic vacuoles and extracellularly near the cell membrane, and sometimes the elongated pattern of a secretory granule dissolving into the medium could be recognized.

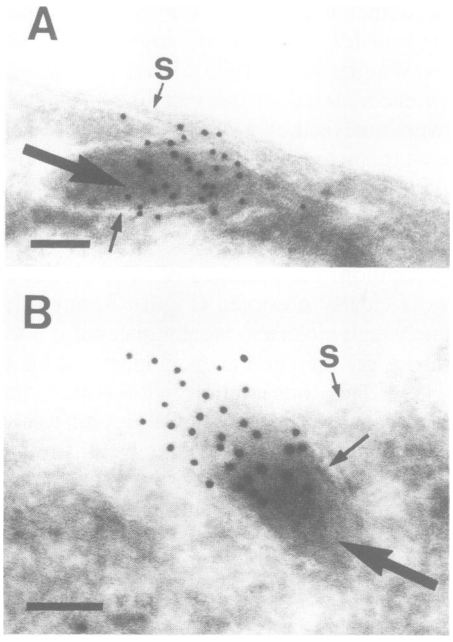

Fig. 4. Ultrathin frozen sections of CV-1 cells transfected with vWF cDNA and immunostained with anti-vWF antiserum. (**A**) and (**B**) Rod-shaped bodies (large arrows) surrounded by a unit membrane (small arrows) are labelled, the gold particles were found only at the planes where these granules had been sectioned. In (A) the body is underneath the cell surface (s), whereas in (B) the organelle is in the process of being released from the cell. Bar 0.1 μm.

Thin sections of the cell line CV-1/vWF3 embedded in LX112/Araldite showed organelles similar to the Weibel–Palade bodies present in the endothelial cell. In CV-1 cells, the organelles consisted of small tubules, 15–20 nm thick, disposed parallel to the long axis of the rod and enveloped by a membrane (Figure 6). These organelles were not present in CV-1 cells that did not express the vWF protein. The Weibel–Palade bodies are found only in the endothelial cells and cell fractionation studies have indicated that these organelles are the storage organelles for vWF (Weibel and Palade, 1964; Ewenstein *et al.*, 1987; Reinders *et al.*, 1988).

Subsequently, we performed a morphological comparison between the vWF-containing organelles, encountered in transfected CV-1 cells, and the Weibel–Palade bodies present in endothelial cells. Originally, immunoelectron microscopy with anti-vWF antisera has been used to define the Weibel–Palade body of the endothelial cell as the storage granule for vWF (Wagner *et al.*, 1982; Ewenstein *et al.*, 1987; Reinders *et al.*, 1988). After incubation of ultrathin frozen sections and Lowicryl K4M sections of cultured endothelial cells with anti-vWF antiserum, labelling was found associated with the rod-shaped Weibel–Palade bodies of the endothelial cell (Figure 7). In contrast to the organelles found in CV-1 cells, these entities were dispersed all over the cytoplasm and seldom was an organelle observed being extruded into a vacuole (Figure 7A). Comparison of the Weibel–Palade bodies encountered in endothelial cells and the vWF-containing granules in CV-1 cells revealed a close similarity, although in the endothelial cells the organelles

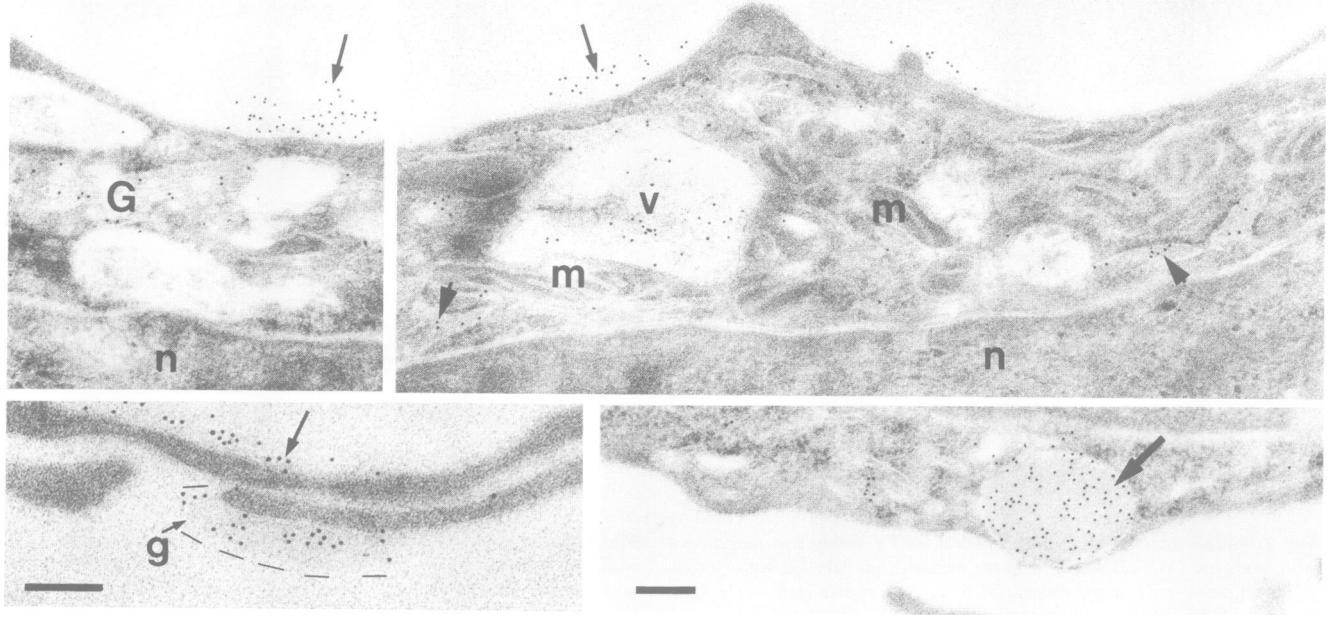

Fig. 5. Lowicryl K4M thin section of CV-1 cells transfected with vWF cDNA. Different areas of the same cell showing labelling of: rough endoplasmic reticulum (arrowheads); Golgi stack (G); faintly electron-dense material in a cytoplasmic vacuole (v); a transverse section of a rod-shaped body (large arrow) and extracellular discrete areas located close to the cell membrane (small arrows). Sometimes the elongated pattern of a vWF-containing granule dissolving in the medium can be recognized (g). Bar 0.2 μm.

tend to be longer and more slender than the organelles encountered in CV-1 cells. Besides labelling of the Weibel–Palade bodies, labelling was also observed in cisternae of the ER of the endothelial cell (not shown) and some Golgi stack. Occasionally a vesicle, strongly labelled with anti-vWF antiserum, was seen in connection with the TGN, suggesting that in the endothelial cell the vWF protein accumulates in this compartment of the endothelial cell prior to organelle formation (Figure 7B).

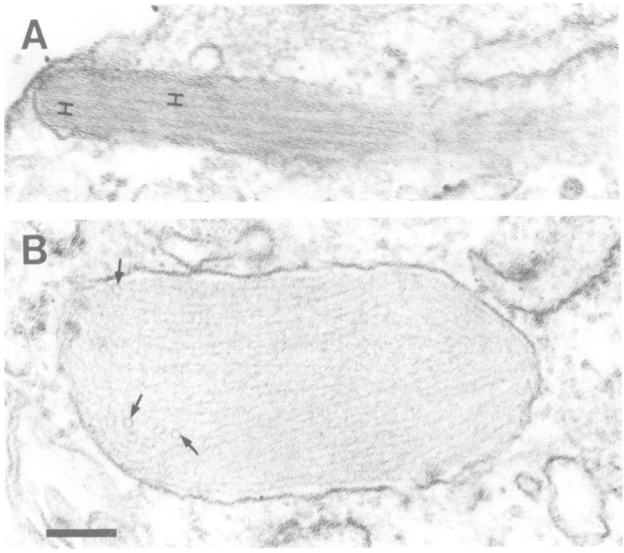

Fig. 6. Thin sections of clone CV-1/vWF3 embedded in LX112/Araldite. (**A**) Longitudinal section of a Weibel–Palade body-like structure showing parallel arrangement of internal tubules (H). (**B**) Oblique section of a Weibel–Palade body-like structure, some of the tubules are cross-sectioned (arrows). Bar 0.1 μm.

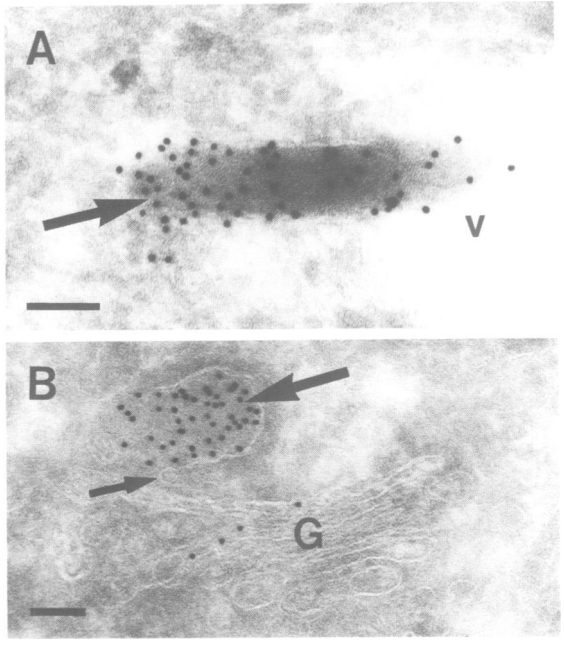

Fig. 7. Ultrathin frozen sections of endothelial cells immunostained with anti-vWF antiserum. (**A**) Labelled Weibel–Palade body (arrow) in the process of release into cytoplasmic vacuole (v). Note the similarity with the bodies shown in Figure 2. (**B**) Strongly labelled vesicle (large arrow) with the limiting membrane still connected with the TGN (small arrow). The Golgi stack (G) is slightly labeled. Bar 0.1 μm.

Expression of wild-type and mutant vWF proteins in CV-1 cells

In this paragraph, and in the subsequent ones, we have addressed the issue of whether targeting of vWF to organelles in transfected CV-1 cells is directed by (i) one or more defined domain(s) of the vWF molecule or (ii) proteolytic processing of pro-vWF or (iii) multimerization of vWF. To that end, we expressed in CV-1 cells wild-type vWF and a set of vWF cDNA derivatives, which either lack specific domains of the molecule or cannot be proteolytically processed in CV-1 cells (Figure 8). The choice of vWF mutants was based on our current knowledge on the properties of vWF mutant proteins synthesized in transfected COS-1 cells, particularly on their ability to multimerize and to be proteolytically processed correctly (Verweij *et al.*, 1987, 1988; Voorberg *et al.*, 1990). Consequently, we first established whether these biochemical features of the various vWF proteins synthesized by CV-1 cells are comparable to those synthesized by COS-1 cells. Proteolytic processing was analysed as follows. CV-1 cells, transiently transfected with these mutant cDNAs, were metabolically labelled with [^{35}S]methionine. The radiolabelled material was subsequently immunoprecipitated from the conditioned media and, finally, analysed by 5% (w/v) SDS–PAGE under reducing conditions (Figure 9). Wild-type vWF was encountered almost exclusively in the processed form, demonstrating that intracellular proteolytic processing of pro-vWF into the pro-polypeptide and mature vWF subunit was virtually complete. The mutant protein vWFgly763, harbouring a mutation (arg763 → gly763) at the cleavage site between the pro-polypeptide and mature part of vWF, is secreted in the uncleaved form, in agreement with data obtained upon expression of this mutant protein in COS-1 cells (Verweij *et al.*, 1988). As expected for the mutant protein vWFdelpro, lacking the pro-polypeptide, only the mature subunit can be detected in the conditioned medium. All other selected mutant vWF proteins, i.e. vWFdelD', vWFdelD3 and vWFdelD'D3 (see Figure 8), are secreted almost exclusively in a proteolytically processed form similar to wild-type vWF. The multimeric properties of the mutant vWF proteins expressed in CV-1 cells were determined by employing 2% (w/v) SDS–agarose gel electrophoresis,

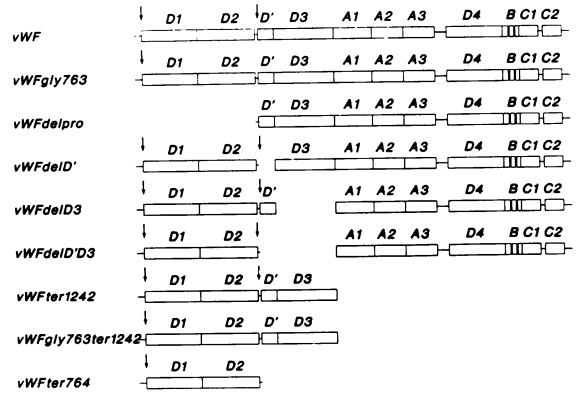

Fig. 8. Domain structure of the vWF protein and the mutant proteins used in this study. Both the proteolytic cleavage site between the signal peptide and the pro-polypeptide, and the cleavage site between the pro-polypeptide and the mature vWF subunit, are indicated by vertical arrows. Mutant proteins lacking specific domains of the vWF were constructed as outlined in Materials and methods.

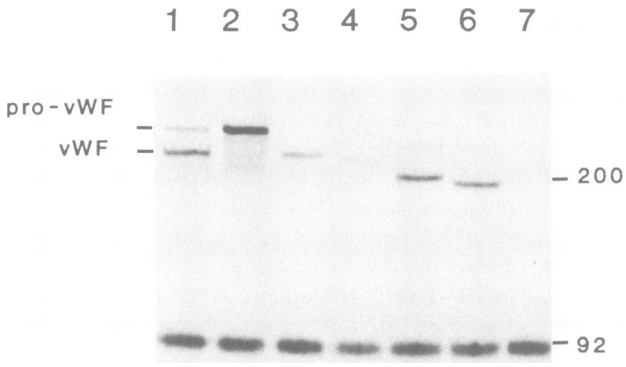

Fig. 9. Subunit composition of wild-type and mutant proteins expressed in CV-1 cells. Transfected cells were metabolically labelled with [^{35}S]methionine and the labelled vWF was immunopurified from the conditioned medium and analysed under reducing conditions on 5% (w/v) SDS–PAGE. CV-1 cells transfected with pSV2t-PA were treated identically and used as a control. **Lane 1**, wild-type vWF; **lane 2**, vWFgly763; **lane 3**, vWFdelpro; **lane 4**, vWFdelD′; **lane 5**, vWFdelD3; **lane 6**, vWFdelD′D3; **lane 7**, t-PA control. Molecular weight markers of 200 000 and 92 000 are indicated on the right side of the figure.

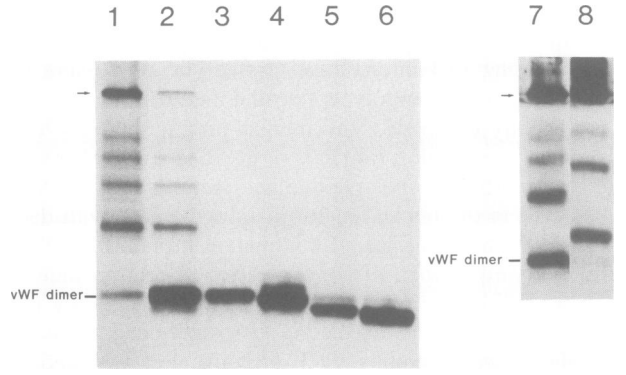

Fig. 10. Multimeric composition of wild-type vWF and mutant proteins. Medium was harvested from transfected CV-1 cells and analysed by electrophoresis under non-reducing conditions on a 2% (w/v) SDS–agarose gel. **Lane 1**, wild-type vWF; **lane 2**, vWFdelpro and vWFter764; **lane 3**, vWFdelpro; **lane 4**, vWFdelD′; **lane 5**, vWFdelD3; **lane 6**, vWFdelD′D3; **lane 7**, wild-type vWF; **lane 8**, vWFgly763. The position of the vWF dimer is indicated. The mutant protein vWFgly763 was analysed and compared to the wild-type protein in a separate experiment. The top of the running gel is indicated by an arrow.

followed by staining of the gel with ^{125}I-labelled anti-vWF antibodies (Figure 10). The results are in full agreement with the data obtained upon expression of these mutant cDNAs in COS-1 cells: multimerization is observed with wild-type vWF and the mutant vWFgly763, whereas none of the other mutant proteins is assembled beyond the dimer stage (Verweij *et al.*, 1987, 1988; Voorberg *et al.*, 1990).

Previously, we have described the expression of the mutant proteins vWFter1242 and vWFgly763ter1242 in COS-1 cells (Voorberg *et al.*, 1990). These mutant proteins lack the carboxyl-terminal part of vWF and, consequently, do not dimerize. However, owing to the presence of the domains D1, D2, D′ and D3, these proteins do form intermolecular disulphide bonds at their respective amino-termini. Here, we expressed these mutant proteins in transfected CV-1 cells and analysed their properties by SDS–PAGE both under

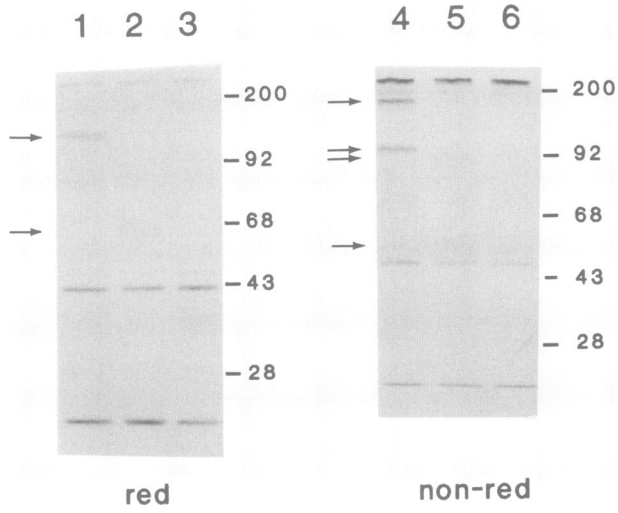

Fig. 11. Multimeric and subunit composition of vWFter1242 and vWFgly763. Transfected CV-1 cells were metabolically labelled and the labelled vWF was immunopurified from the conditioned medium and analysed under both reducing (lanes 1–3) and non-reducing conditions (lanes 4–6). **Lane 1**, vWFgly763-ter1242; **lane 2**, vWFter1242; **lane 3**, pSV2t-PA control; **lane 4**, vWFgly763-ter1242; **lane 5**, vWFter1242; **lane 6**, pSV2t-PA. Molecular weight markers are indicated at the right side of the figure. Arrows indicate the position at which both mutant proteins migrate under either reducing or non-reducing conditions.

reducing and non-reducing conditions (Figure 11). Our data clearly show that also upon expression in CV-1 cells these proteins form intermolecular disulphide bonds at their amino-termini, similar to transfected COS-1 cells. As exemplified by the behaviour of the mutant protein vWFgly763ter1242, which under non-reducing conditions gives rise to two bands, while only one band is observed for this mutant protein under reducing conditions. In conclusion, we have expressed a set of vWF derivatives which differ in proteolytic processing and in the ability to multimerize. The properties of these proteins, as synthesized in CV-1 cells, are not significantly different from those produced in transfected COS-1 cells. Subsequently, we have employed these vWF mutant proteins to address the requirements for targeting to the vWF-containing dense-cored vesicles in CV-1 cells.

Immunofluorescence of CV-1 cells transfected with vWF cDNA derivatives

The ability of the different vWF derivatives to direct the formation of dense-cored vesicles in transfected CV-1 cells was recorded by immunofluorescence (Figure 12). The mutant protein vWFgly763, present exclusively in the uncleaved form, displays a morphological appearance similar to that of wild-type vWF protein (Figure 12B). Clearly, the elongated pattern of a vWF-containing granule is observed in the transfected CV-1 cell. This observation allows us to conclude that proteolytic processing of the pro-vWF subunit does not affect the formation of dense-cored vesicles in CV-1 cells. In contrast, the mutant protein vWFdelpro, lacking the pro-polypeptide (consisting of the domains D1, D2), is not encountered in these organelles. The staining observed for this mutant protein is indicative of the presence of vWF in the ER (Figure 12C). Similarly, no organelles were found in CV-1 cells transfected with cDNA encoding the mutant proteins vWFdelD′, vWFdelD3 and vWFdelD′D3 (Figure

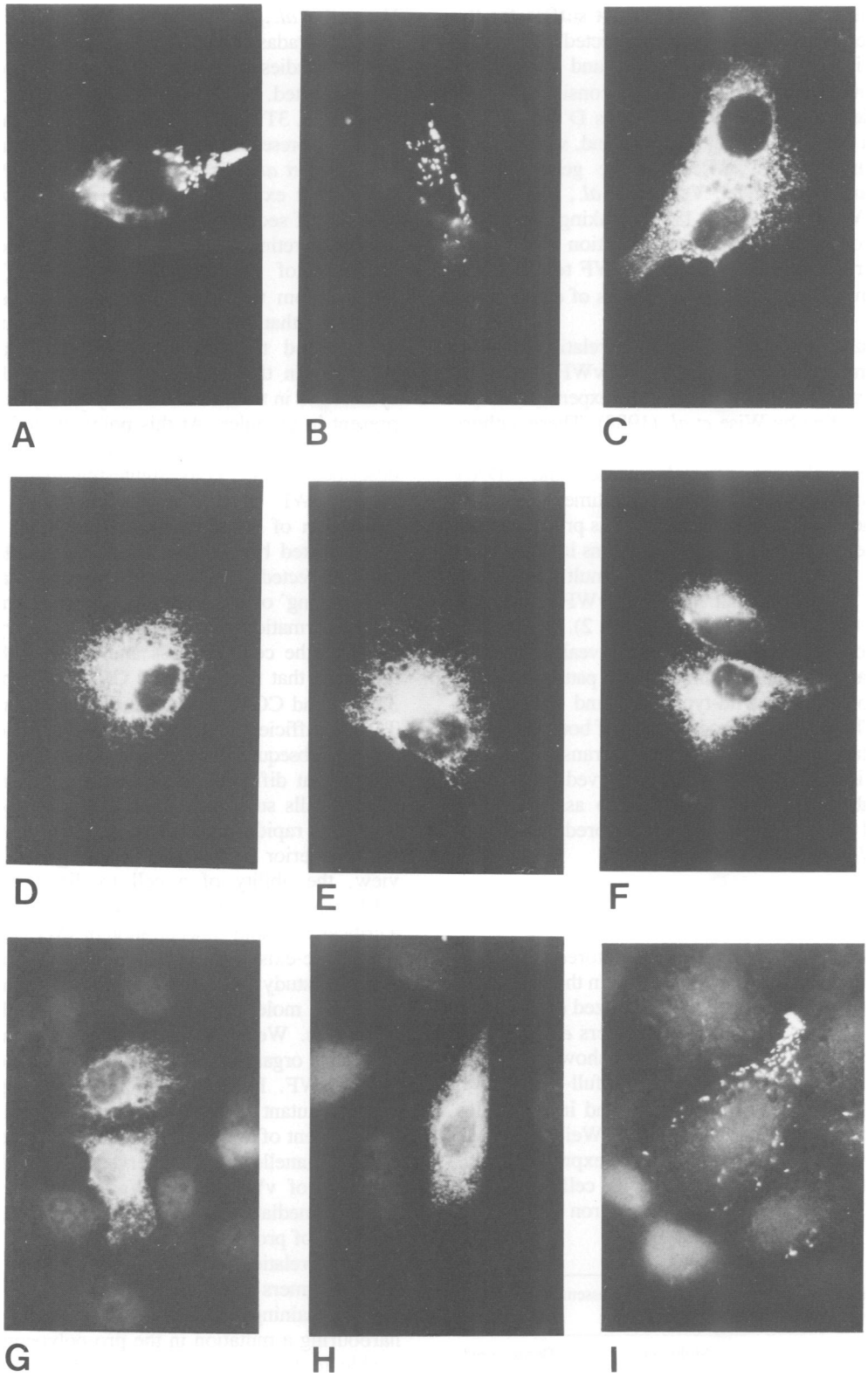

Fig. 12. Immunolocalization of mutant vWF proteins in CV-1 cells. Transfected CV-1 cells were prepared for immunofluorescence as described in Materials and methods. (**A**) Wild-type vWF; (**B**) vWFgly763; (**C**) vWFdelpro; (**D**) vWFdelD′; (**E**) vWFdelD3; (**F**) vWFdelD′D3; (**G**) vWFter1242; (**H**) vWFgly763-ter1242; (**I**) vWFdelpro and vWFter764.

12D, E and F). Rather, a morphological pattern is observed which is identical to that found for the mutant protein vWFdelpro. From these results, it might be deduced that the presence of each of the domains D1, D2, D′ or D3 is required for the biogenesis of vWF-containing organelles. However, transfection of CV-1 cells with cDNA encoding vWFgly763ter1242 and vWFter1242 (containing D1, D2, D′ and D3, but lacking the domains beyond D3) did not reveal the typical morphological pattern obtained with either wild-type vWF or vWFgly763 (Figure 12G and H).

Apparently, the domains D1 to D3 do not suffice for the formation of dense-cored vesicles in transfected CV-1 cells. At this point, it should be noted that we and others have previously shown that this very region, consisting of the pro-polypeptide (D1, D2) and the domains D′ and D3, is obligatory for a correct multimer assembly and, significantly, that these four domains suffice for the generation of multimers (Marti *et al.*, 1987; Verweij *et al.*, 1987; Wise *et al.*, 1988; Voorberg *et al.*, 1990). Taking these data together, we conclude that a strict correlation is observed between the structural requirements of vWF to ultimately assemble into multimers and the biogenesis of dense-cored vesicles in CV-1 cells (Table I).

To further substantiate the observed correlation between multimer assembly and the presence of vWF-containing organelles, we performed a co-transfection experiment based on a previous finding by Wise *et al.* (1988). These authors showed that transfection of COS-1 cells with both cDNA encoding exclusively the pro-polypeptide, and cDNA encoding only mature vWF, resulted in multimer formation, conceivably due to *in trans* promotion of this process by the pro-polypeptide. Similar to these observations in transfected COS-1 cells, we also detected vWF multimers upon co-expression of the mutant proteins vWFdelpro and vWFter763 in CV-1 cells (Figure 10, lane 2). A morphological inspection by immunofluorescence revealed immunoreactive material in organelles, similar to the pattern obtained upon expression of both wild-type vWF and vWFgly763 (Figure 12I). The concomitant occurrence of both multimers and vWF-containing granules in the co-transfected cells provides additional support for the observed correlation between the ability of a vWF protein to assemble into multimers and its localization in dense-cored vesicles in transfected CV-1 cells.

Discussion

In the endothelial cell, the vWF protein is stored in specific organelles, termed Weibel–Palade bodies. In the past, these organelles have been extensively characterized (Weibel and Palade, 1964; Wagner *et al.*, 1982; Reinders *et al.*, 1984, 1988; Ewenstein *et al.*, 1987). Here, we show that in the non-endothelial CV-1 cell, transfected with full-length vWF cDNA, the expressed vWF protein is found in organelles similar to the endothelial cell-specific Weibel–Palade bodies. Previously, we and others have expressed vWF cDNA in a number of other non-endothelial cells, including COS-1, CHO, 3T3 and mouse L cells (Bonthron *et al.*, 1986; Verweij *et al.*, 1987; Kaufman *et al.*, 1989; Wagner *et al.*, 1991; Mayadas *et al.*, 1992; Meulien *et al.*, 1992). In none of these studies has the presence of vWF protein in granules been reported. Moreover, it has been demonstrated both in CHO cells, 3T3 cells and in COS-1 cells that the expressed vWF was present in the ER of the cell (Kaufman *et al.*, 1989; Voorberg *et al.*, 1991; Wagner *et al.*, 1991). It has been shown that expression of vWF in cells with a regulated pathway of secretion (i.e. mouse pituitary AtT-20 and rat insulin-secreting B-cell line RIN 5F) does result in the formation of vWF-containing granules (Wagner *et al.*, 1991). From the latter observations, these authors have concluded that the formation of vWF-containing granules is restricted to cells which accommodate a regulated pathway. In this paper, we present evidence that vWF synthesized in transfected monkey kidney cells (CV-1) is also present in granules. At this point, it should be mentioned that, to our knowledge, no secretory granules have been described in CV-1 cells, suggesting that the mere presence of the vWF protein in this particular cell directs the generation of dense-cored vesicles. This view is further substantiated by the absence of the tubular organelles in non-transfected CV-1 cells. Previously, it has been proposed that 'sorting' of a protein to a secretory granule is initiated by the formation of a condensed, secretory product in the TGN of the cell (Burgess and Kelly, 1987). It could be advanced that in transfected CV-1 cells, but not in CHO, 3T3, L and COS-1 cells, the concentration of vWF in the TGN is sufficiently high to govern the condensation of vWF and the subsequent packaging in organelles. Indeed, we have shown that different conditions are present in transfected COS-1 cells such that, upon exit from the ER, the vWF protein is rapidly transported through the Golgi apparatus to the exterior of the cell (Voorberg *et al.*, 1991). In this view, the ability of a cell to direct the formation of vWF-containing granules would be primarily determined by a critical threshold concentration of vWF in the TGN, rather than a pre-existing regulated secretory pathway.

In this study, we have investigated the requirements on the vWF molecule for targeting to Weibel–Palade-like organelles. We have shown that the formation of vWF-containing organelles is independent of proteolytic processing of pro-vWF. Furthermore, by employing a set of well-defined mutant vWF proteins, we were able to exclude the involvement of a particular domain in targeting the protein to the organelles. This observation strongly indicates that targeting of vWF to the Weibel–Palade body is not a receptor-mediated event, as has been demonstrated for targeting of proteins to the lysosomes. Rather, we observe a strict correlation between the assembly of a (mutant) protein into multimers and its ability to direct the generation of vWF-containing organelles. Recently, a vWF molecule harbouring a mutation in the pro-polypeptide, rendering it unable to form multimers, was shown to be capable of granule induction in AtT-20 cells (Mayadas *et al.*, 1992). This observation does not point towards the assembly of multimers as the discriminatory step in organelle formation of the vWF protein. Several reasons can be advanced to explain the apparent discrepancy between the two studies. First, it cannot be excluded that the sorting mechanism of the vWF protein is dependent on the cell employed. However by both biochemical and morphological criteria, the organelles observed in transfected CV-1 cells and transfected AtT-20 cells seem very similar. Alternatively, it can be put

Table I. Ability of the different mutant proteins to assemble into multimers and to induce dense-cored vesicles

vWF protein	Multimer assembly	Dense-cored vesicle
vWF	+	+
vWFgly763	+	+
vWFdelpro	–	–
vWFdelD′	–	–
vWFdelD3	–	–
vWFdelD′D3	–	–
vWFter1242	–	–
vWFgly763ter1242	–	–
vWFdelpro + vWFter764	+	+

forward that not the ultimate multimer assembly itself, but an event prior to multimer formation, determines granule formation of vWF molecules.

Despite extensive morphological similarities between the vWF-containing organelles found in transfected CV-1 cells and the endothelial Weibel–Palade bodies, our experimental data reveal an essential difference between vWF present in these organelles. In the endothelial cell, vWF is released from Weibel–Palade bodies upon treatment of the cell with agonists such as phorbol myristate acetate (PMA) or Ca-ionophore A23187 (Loesberg *et al.*, 1983). Attempts to induce the release from the vWF-containing organelles of transfected CV-1 cells, employing various stimuli (PMA, Ca-ionophore A23187, forskolin and 8-bromo-cyclic AMP) were unsuccessful (data not shown), similar to the lack of stimulated release in transfected AtT-20 cells, which do contain a regulated pathway of secretion (Voorberg *et al.*, unpublished observations; Wagner *et al.*, 1991). At present, no satisfactory explanation can be advanced which accounts for the inability of organelles in both cell types to release their contents upon administration of the agonists. However, pulse–chase analysis of CV-1/vWF3 reveals that a small amount of the secreted vWF is found in the processed form inside the cell. In contrast to the endothelial cell, the turnover of this population of vWF molecules is relatively fast in the transfected CV-1 cell. Furthermore, the dense-cored vesicles observed in the transfected CV-1 cells are located mainly near the plasma membrane and immunoreactive material is often encountered outside the cell. These observations are consistent with a fast turnover of organelles, as suggested by pulse–chase analysis. Apparently CV-1 cell, as well as AtT-20 cells, lack the mechanism to retain these 'vWF granules' inside the cell. It cannot be excluded that, in both cell types, the observed dense-cored organelles follow the constitutive pathway of secretion. In our view, the observed organelles cannot therefore be termed 'storage organelles', despite their almost complete identity with the endothelial cell-specific Weibel–Palade bodies. Matsuuchi and Kelly (1991) have proposed an alternative pathway for the secretion of proteins destined for the regulated pathway. This pathway has been termed 'basal secretion' and is thought to arise from the fusion of secretory granules with the plasma membrane in the absence of a stimulus. Our morphological and biochemical data are consistent with the existence of such a 'basal secretion pathway' in transfected CV-1 cells. Interestingly, electron microscopy of endothelial cells from the alveolar vessel from rabbit lungs revealed a pattern of secreting Weibel–Palade bodies similar to that observed in transfected CV-1 cells (McNiff and Gil, 1983). The observed similarity suggests that in transfected CV-1 cells a constitutive release of vWF-containing dense-cored vesicles occurs that is independent of the presence of a stimulus.

Materials and methods

Materials

Restriction and DNA-modifying enzymes were from Bethesda Research Laboratories (Rockville, MD). The Sequenase kit was from the USB Corporation (Cleveland, OH). The plasmid pcRSV was obtained from In Vitro Gen (San Diego, CA). Immunoreagents were either from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) or from Dakopatts (Glostrup, Denmark). Radioactive chemicals were purchased from the Radiochemical Centre (Amersham, UK). Synthetic oligonucleotides were prepared with an automated DNA synthesizer type 381A (Applied Biosystems, Foster City, CA). Culture media and antibiotics were purchased from Gibco (Paisley, UK). Gelatin–Sepharose, Protein A–Sepharose and cyanogen bromide-activated Sepharose-4B were obtained from Pharmacia LKB (Uppsala, Sweden).

Plasmid constructions

The plasmids pSVLE, pSVLvWF, pSVLvWFdelpro, pSVLvWFdelD', pSVLvWFdelD3, pSVLvWFdelD'D3, pSVLvWFter1242, pSVLvWFgly-763-ter1242 and pSVLvWFgly763 have been described previously (Van de Ven *et al.*, 1990; Voorberg *et al.*, 1990, 1991). In addition, a mutant cDNA, encoding solely the domains D1 and D2 of vWF (the propolypeptide), was constructed as follows. First, a 482 base pair (bp) *Hin*dIII–*Bam*HI fragment (position 2239–2721) was subcloned into double-stranded M13mp8 phage DNA, digested with both *Hin*dIII and *Bam*HI (Messing and Vieira, 1982). The translation termination codon was introduced at amino acid position 764 by using the M13 gapped duplex mutagenesis procedure (Kramer *et al.*, 1984). To that end, the following synthetic oligonucleotide was used: [5'GGA.TAG.GCT.T*CA*.TTT. GCT.GCG.3']. A clone which contained the two desired substitutions (indicated with an asterisk) was selected and the complete nucleotide sequence of the *Hin*dIII–*Bam*HI insert was determined by dideoxy sequencing employing the Sequenase protocol (Tabor and Richardson, 1987). Subsequently, the mutated *Hin*dIII–*Bam*HI fragment was inserted into pSVLvWF, yielding pSVLvWFter764. The plasmid pcRSVvWFneo was constructed as follows. An *Eco*RI fragment harbouring the wild-type vWF cDNA was inserted in the *Eco*RI site of pcRSV and, after transformation, a construct with the vWF cDNA in the correct orientation was isolated.

Tissue culture and transfection

Monkey kidney CV-1 cells were maintained in Iscove's modified minimal medium, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal calf serum. Twenty-four hours after seeding, the semi-confluent cells were transfected by the calcium phosphate protocol (Graham and Van der Eb, 1973). The calcium phosphate precipitate contained 10 μg of DNA and was incubated on the cells for 16–18 h. The cells were then washed twice with serum-free Iscove's modified minimal medium and subsequently incubated in the same medium, supplemented with 10% (v/v) fetal calf serum. A stable cell line expressing wild-type vWF was established employing a similar protocol with the following modifications: 24 h after transfection cells were treated with trypsin and replated. Selection was applied 24 h after splitting and consisted of 1000 μg/ml G418 in Iscove's medium supplemented with 10% (v/v) fetal calf serum. After 2 weeks, individual clones were selected and propagated in the same medium containing 500 μg of G418/ml. One of the clones, CV-1 cells/vWF3, positive for vWF, was used throughout this study. Endothelial cells were isolated from human umbilical veins and cultured as described previously (Reinders *et al.*, 1984).

Metabolic labelling of transfected cells

Transfected cells were maintained in Iscove's minimal medium for 48 h post-transfection. The cells were then washed with PBS [10 mM sodium phosphate (pH 7.4), 0.14 M NaCl] and, subsequently, starved for 1 h in RPMI medium lacking methionine. The cells were labelled for 4 h in RPMI medium, supplemented with [^{35}S]methionine (50 μCi/ml, specific radioactivity > 800 Ci/mmol), followed by a chase of 16 h with unlabelled methionine (30 μg/ml). After radiolabelling, the medium was collected and centrifuged for 5 min at 1500 r.p.m. Pulse–chase analysis of CV-1/vWF3 was performed using a similar protocol, employing a labelling time of 1 h. To obtain cell extracts, cells were washed twice with PBS and subsequently lysed in IPB [10 mM Tris–HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 10 mM benzamidine, 5 mM *N*-ethylmaleimide and 1 mM PMSF, and 10 mM Tris–HCl (pH 7.8)]. Conditioned media and cell extracts obtained from metabolically labelled cells were stored at −80°C or used immediately for immunoprecipitation.

Immunoprecipitation

Preclearing of media was performed by two successive incubations at room temperatures with gelatin–Sepharose and, subsequently, with preformed complexes between rabbit serum IgG and Protein A–Sepharose. Immunoprecipitation of radiolabelled vWF was carried out with preformed complexes of an IgG preparation, derived from rabbit anti-vWF serum (Dakopatts, Glostrup, Denmark), coupled to Protein A–Sepharose. Immunoprecipitated material was extensively washed with IPB and 10 mM Tris–HCl (pH 7.8), respectively, and analysed by SDS–PAGE (Laemmli, 1970).

vWF multimer analysis

Conditioned medium of transfected cells was harvested 3 days after transfection. After centrifugation for 5 min at 1500 r.p.m., the conditioned medium was adjusted to 0.5 × IPB and concentrated by Centricon-30

(Amicon, Danvers, MA) filtration. Cellular debris was removed by centrifugation for 30 min at 15 000 r.p.m. and the resulting supernatant was analysed by discontinuous SDS–agarose gel electrophoresis as previously described (Ruggeri and Zimmerman, 1981). An affinity-purified ^{125}I-labelled rabbit anti-vWF IgG preparation was used for staining of the gel. Multimeric analysis of the mutant proteins vWFter1242 and vWFgly763ter1242 was performed by analysing immunoprecipitated material of metabolically labelled CV-1 cells, transfected with the mutant cDNAs on SDS–PAGE, under non-reducing conditions. Multimeric analysis of cell extracts of the stable cell line CV-1/vWF3 was performed as described previously (Voorberg *et al.*, 1991).

Immunofluorescence

CV-1 cells, grown on glass coverslips coated with fibronectin, were transfected as described, followed by incubation for 72 h in serum-containing medium. The transfected cells were then washed twice with PBS and fixed with methanol for 15 min at room temperature. After fixation, the cells were washed twice with PBS and incubated for 1 h with polyclonal anti-vWF antiserum (Dakopatts, Glostrup, Denmark) in PBS, supplemented with 3% (v/v) bovine serum albumin (Organon Technika, Oss, The Netherlands). In several experiments, cells were stained with any of the monoclonal antibodies CLB-Rag 35, CLB-Rag 201, or 506, directed against the mature subunit of vWF. Subsequently, the coverslips were washed twice with PBS and incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or FITC-conjugated goat anti-mouse antiserum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), which was diluted 100-fold in PBS, supplemented with 3% (v/v) bovine serum albumin (BSA). Coverslips were prepared for microscopy, after extensive washing with PBS, by embedding them in mounting fluid: 80% (v/v) glycerol, 1 mg/ml *o*-phenylenediamine (Sigma, St Louis, MO), 1 mM sodium phosphate (pH 8.8), 0.014 M NaCl and were viewed with a Leitz Ortholux II fluorescence microscope.

Electron microscopy

Transfected CV-1 cells were maintained in serum-containing medium for 72 h post-transfection. Cells were fixed in a mixture of 4% (v/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 0.1 M phosphate (pH 7.2), scraped from the dishes and embedded in 10% (w/v) gelatin. Ultrathin frozen sections were incubated at room temperature with rabbit anti-vWF antiserum (Dakopatts, Glostrup, Denmark; dilution 1:100) and gold-conjugated goat anti-rabbit (Janssen Pharmaceutica, Beerse, Belgium; dilution 1:40; particle size 10 nm). Both incubations were for 1 h at room temperature. The sections were stained with uranyl acetate and embedded in methyl cellulose. Other gelatin blocks were embedded at low temperature with Lowicryl K4M (Bio-Rad, Veenendaal, The Netherlands). Thin sections were incubated with anti-vWF antiserum for 2 h, followed by a gold-conjugate incubation for 1 h, and stained with uranyl acetate and lead citrate. This procedure allowed us to make many sections of different parts of the pellet, thus facilitating the identification of a transfected cell among the population of non-transfected cells. Labelling, however, was stronger on ultrathin frozen sections and in general the membranes around the organelles were better resolved with this technique. Cells from clone CV-1/vWF3 were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed in 15 osmium tetroxide in the same buffer, stained en bloc with uranyl acetate and embedded in a mixture of LX112/Araldite. All preparations were examined with a Philips CM 10 electron microscope.

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References

Bonthron,D.T., Handin,R.I., Kaufman,R.J., Wasley,L.C., Orr,E.C., Mitsock,L.M., Ewenstein,B., Loscalzo,J., Ginsburg,D. and Orkin,S.H. (1986) *Nature*, **324**, 270–273.

Burgess,T.L. and Kelly,R.B. (1987) *Annu. Rev. Cell Biol.*, **3**, 243–293.

Chanat,E. and Huttner,W.B. (1991) *J. Cell Biol.*, **115**, 1505–1519.

Ewenstein,B.M., Warhol,M.J., Handin,R.I. and Pober,J.S. (1987) *J. Cell Biol.*, **104**, 1423–1433.

Gerdes,H.-H., Rosa,P., Phillips,E., Bauerle,P.A., Frank,R., Argos,P. and Huttner,W.B. (1989) *J. Biol. Chem.*, **264**, 12009–12015.

Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456–467.

Gross,D.J., Halban,P.A., Kahn,R., Weir,G.C. and Villa-Komaroff,L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4107–4111.

Kaufman,R.J., Wasley,L.C., Davies,M.V., Wise,R.J., Israel,D.I. and Dorner,A.J. (1989) *Mol. Cell. Biol.*, **9**, 1233–1242.

Kornfeld,S. (1986) *J. Clin. Invest.*, **77**, 1–6.

Kramer,W., Drutsa,V., Jansen,H.-W., Kramer,B., Pflugleder,M. and Fritz,H.-J. (1984) *Nucleic Acids Res.*, **12**, 9441–9456.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Loesberg,C., Gonsalves,M.D., Zandbergen,J., Willems,Ch., Van Aken,W.G., Stel,H.V., Van Mourik,J.A. and De Groot,Ph.G. (1983) *Biochim. Biophys. Acta*, **763**, 160–168.

Lyons,S.E., Bruck,M.E., Walter Bowie,E.J. and Ginsburg,D. (1992) *Proc. Natl. Acad. Sci. USA*, **267**, 4424–4430.

Marti,T., Rosselet,S.J., Titani,K. and Walsh,K.A. (1987) *Biochemistry*, **26**, 8099–8109.

Matsuuchi,L. and Kelly,R.B. (1991) *J. Cell Biol.*, **112**, 843–852.

Mayadas,T.N. and Wagner,D.D. (1989) *J. Biol. Chem.*, **264**, 13947–13503.

Mayadas,T.N. and Wagner,D.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3531–3535.

McNiff,J.M. and Gil,J. (1983) *J. Appl. Physiol.*, **54**, 1284–1286.

Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269–276.

Meulien,P., Nishino,M., Mazurier,C., Dott,K., Pietu,G., Jorieux,S., Pavirani,A., Girma,J.P., Oufkir,D., Courtney,M. and Meyer,D. (1992) *Thromb. Haem.*, **67**, 154–160.

Orci,L., Ravazzola,M., Amherdt,M., Perrelet,A., Powell,S.K., Quinn,D.L. and Moore,H.-P.H. (1987) *Cell*, **51**, 1039–1051.

Pelham,H.R.B. (1990) *Trends Biochem. Sci.*, **15**, 483–486.

Quinn,D., Orci,L., Ravazzola,M. and Moore,H.-P.H. (1991) *J. Cell Biol.*, **113**, 987–996.

Reinders,J.H., De Groot,Ph.G., Gonsalves,M.D., Zandbergen,J., Loesberg,C. and Van Mourik,J.A. (1984) *Biochim. Biophys. Acta*, **804**, 223–226.

Reinders,J.H., De Groot,Ph.G., Sixma,J.J. and Van Mourik,J.A. (1988) *Haemostasis*, **18**, 246–261.

Ruggeri,Z.M. and Zimmerman,T.S. (1981) *Blood*, **57**, 1140–1143.

Shelton-Inloes,B.B., Titani,K. and Sadler,J.E. (1986) *Biochemistry*, **25**, 3164–3171.

Sporn,L.A., Marder,V.J. and Wagner,D.D. (1986) *Cell*, **46**, 185–190.

Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.

Titani,K., Kumar,S., Takio,K., Ericsson,L.H., Wade,R.D., Ashida,K., Walsh,K.A., Chopek,W., Sadler,J.E. and Fujikawa,K. (1986) *Biochemistry*, **25**, 3171–3184.

Tooze,J. and Tooze,S.A. (1986) *J. Cell Biol.*, **103**, 839–850.

Tooze,J., Tooze,S.A. and Fuller,S.D. (1987) *J. Cell Biol.*, **105**, 1215–1226.

Tooze,S.A., Flatmark,T., Tooze,J. and Huttner,W.B. (1991) *J. Cell Biol.*, **115**, 1491–1503.

Van de Ven,W.J.M., Voorberg,J., Fontijn,R., Pannekoek,H., Van den Ouweland,A.M.W., Van Duijnhoven,H.L.P., Roebroek,A.J.M. and Siezen,R.J. (1990) *Mol. Biol. Rep.*, **14**, 265–275.

Verweij,C.L., Diergaarde,P., Hart,M. and Pannekoek,H. (1986) *EMBO J.*, **5**, 2885–2890.

Verweij,C.L., Hart,M. and Pannekoek,H. (1987) *EMBO J.*, **6**, 2885–2890.

Verweij,C.L., Hart,M. and Pannekoek,H. (1988) *J. Biol. Chem.*, **263**, 7921–7924.

Voorberg,J., Fontijn,R., Van Mourik,J.A. and Pannekoek,H. (1990) *EMBO J.*, **9**, 797–803.

Voorberg,J., Fontijn,R., Calafat,J., Janssen,H., Van Mourik,J.A. and Pannekoek,H. (1991) *J. Cell Biol.*, **113**, 195–205.

Wagner,D.D. and Marder,V.J. (1984) *J. Cell Biol.*, **99**, 2123–2130.

Wagner,D.D., Olmsted,J.B. and Marder,V.J. (1982) *J. Cell Biol.*, **95**, 355–360.

Wagner,D.D., Mayadas,T., Urban-Pickering,M., Lewis,B.H. and Marder,V.J. (1985) *J. Cell Biol.*, **101**, 112–120.

Wagner,D.D., Mayadas,T. and Marder,V.J. (1986) *J. Cell Biol.*, **102**, 1320–1324.

Wagner,D.D., Saffaripour,S., Bonfanti,R., Sadler,J.E., Cramer,E.M., Chapman,B. and Mayadas,T.N. (1991) *Cell*, **64**, 403–413.

Weibel,E.R. and Palade,G.E. (1964) *J. Cell Biol.*, **23**, 101–112.

Wise,R.J., Pitman,D.D., Handin,R.I., Kaufman,R.J. and Orkin,S.H. (1988) *Cell*, **52**, 229–236.

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