Small GTP-binding proteins and regulated secretation of von Willebrand factor by endothelial cells

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CHAPTER 5

SMALL GTPASE RALA MEDIATES REGULATED EXOCYTOSIS OF VON WILLEBRAND FACTOR BY ENDOTHELIAL CELLS


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ABSTRACT

Weibel-Palade bodies are endothelial cell-specific organelles, which contain von Willebrand factor (vWF), P-selectin and a number of other proteins. Upon stimulation of endothelial cells, Weibel-Palade bodies fuse with the plasma membrane and release their contents into the blood stream. Recently, we found that the small GTP-binding protein RaLA is present in a subcellular fraction containing Weibel-Palade bodies. In the present study, we investigated whether RaLA is functionally involved in regulated exocytosis of Weibel-Palade bodies. Activation of endothelial cells by thrombin resulted in transient cycling of RaLA from its inactive GDP- to its active GTP-bound state. The amount of GTP-bound RaLA reached a maximum after 2 min and returned to basal levels approximately 10 min following stimulation with thrombin. Exocytosis of Weibel-Palade bodies was initiated 30 sec following stimulation with thrombin. In time, a gradual increase in release of vWF was observed. RaLA activation and exocytosis of Weibel-Palade bodies was significantly reduced by incubation with trifluoperazine, an inhibitor of calmodulin, prior to thrombin stimulation. Together, these findings indicate that activation of RaLA coincides with exocytosis of Weibel-Palade bodies from endothelial cells. Functional involvement of RaLA in exocytosis was further investigated by expression of constitutively active and dominant negative RaLA variants in primary endothelial cells. Introduction of active RaLA G23V resulted in disappearance of Weibel-Palade bodies from endothelial cells. In contrast, expression of the dominant negative RaLA S28N did not affect the distribution or amount of Weibel-Palade bodies in transfected cells significantly. These results indicate that RaLA serves a role in controlling exocytosis of Weibel-Palade bodies by endothelial cells.

INTRODUCTION

Von Willebrand factor (vWF) is a multimeric glycoprotein involved in adhesion of platelets to a damaged vessel wall (Ruggeri, 1997). Synthesis of vWF is confined to endothelial cells and megakaryocytes. During its biosynthesis in endothelial cells, vWF is segregated from the bulk flow of proteins and stored in rod-shaped organelles, the Weibel-Palade bodies (Wagner, 1990; Hop and Pannekoek, 1996). A number of other components have been identified in Weibel-Palade bodies which include P-selectin, CD63, endothelin and interleukin-8 (McEver et al., 1989; Vischer and Wagner, 1993; Russell et al., 1998; Wolff et al., 1998, Utgaard et al., 1998). Upon stimulation with agonists such as thrombin and histamine, Weibel-Palade bodies release their contents into the blood (Levine et al., 1982; de Groot et al., 1984; Hamilton and Sims, 1987). The mechanism of thrombin-induced exocytosis of Weibel-Palade bodies has only been partially elucidated (Birch et al., 1992; Carew et al., 1992; van der Eijnden-Schrauwen et al., 1997). Thrombin induces elevation of intracellular Ca²⁺-levels which appears crucial for release of vWF from Weibel-Palade bodies (Birch et al., 1994; van den Eijnden-Schrauwen et al., 1997). Inhibition studies have shown that intracellular Ca²⁺ exerts
its effect on regulated secretion of vWF via calmodulin (Birch et al., 1992; van den Eijnden-Schrauwen et al., 1997). Recently, we identified the small GTP-binding protein RalA in a subcellular fraction containing Weibel-Palade bodies suggesting a role for this GTPase in regulated exocytosis of these organelles (de Leeuw et al., 1999). RalA has also been identified on dense granules in platelets and on synaptic vesicles in nerve terminals (Mark et al., 1996; Bielniski et al., 1993). These findings suggest a general role for RalA in regulated exocytosis. Interestingly, RalA has been proposed to interact with calmodulin in Ca\(^{2+}\)-dependent manner (Wang et al., 1997). Binding to calmodulin enhances GTP-binding to RalA 2-3 fold (Wang and Roufogalis, 1999). These observations suggest a regulatory role for RalA in calmodulin-mediated release of vWF from endothelial cells.

In this study, we investigated the functional involvement of RalA in the secretion of Weibel-Palade bodies by endothelial cells. We show that activation of RalA correlates with thrombin induced secretion of vWF from Weibel-Palade bodies. Expression of constitutively active RalA in endothelial cells results in exocytosis of Weibel-Palade bodies whereas expression of a dominant negative RalA variant did not show this effect. Together, these findings suggest that RalA is involved in regulated exocytosis of Weibel-Palade bodies from endothelial cells.

**MATERIALS AND METHODS**

*MATERIALS*

Culture media, trypsin, penicillin, streptomycin and fungizone were from Gibco BRL (Rockville, MD). Human serum was from healthy donors. Heparin (5000 IE/ml) was purchased from Leo Pharmaceutical Products (Weesp, The Netherlands). Bovine fibroblast growth factor and soybean trypsin inhibitor were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Chemiluminescence blotting substrate was from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibody CLB-RAg 35 directed against vWF has been described previously (Stel et al., 1984). Peroxidase-conjugated polyclonal rabbit IgG against human vWF was obtained from Dakopatts a/s (Glostrup, Denmark). Monoclonal anti-RalA antibody was from Transduction Laboratories (Lexington, KY). Hybridoma cell line 9E10 was from ATCC (USA). Protease inhibitors cocktail, complete™ mini, was from Boehringer Mannheim (Mannheim, Germany). Vectashield was from Vecta Laboratories (Burlington, CA, USA). All chemicals used were of analytical grade.

*Cell culture*

Endothelial cells were isolated from human umbilical veins and cultured as described previously (de Leeuw et al., 1999). Stimulation of endothelial cells by thrombin, Ca\(^{2+}\)-ionophore A23187 and phorbol myristic acetate (PMA) was performed as follows. Endothelial cells were washed three times with PBS and cultured for 2 h in M199 medium supplemented with 1% human serum albumin. At the onset of stimulation, the culture medium
was replaced by medium containing thrombin, Ca$^{2+}$-ionophore, PMA or no agonist. To study the effect of the calmodulin inhibitor trifluoperazine (TFP) on thrombin induced secretion, cells were precultivated for 30 min with 40 μM TFP prior to stimulation by thrombin.

**Construction of epitope tagged RalA variants**

Total cDNA of HUVEC and plasmid pGEM-T-Myc-Rab30 were used to construct epitope tagged human RalA (de Leeuw et al., 1998). Oligonucleotide primers RalAsense 5'- ATC TCC GAG GAG GAC CTG ATG GCT GCA AAT AAG CCC and RalAAS 5'- AAT GAA TTC GAG CTC TTA TAA AAT GCA GCA TCT TTC were used to amplify the coding sequence of RalA. Oligonucleotide primers MS 5'-AAT GGA TCC CTC GAG ATG GAG CAG AAG CTG and MAS 5'-CAG GTC CTC CTC GGA GAT were used to amplify a fragment encoding the first 11 amino acids of c-myc. Myc-RalA was obtained by a second PCR using primers MS and RalAAS. The amplified fragment was cloned into vector pGEM-T yielding pGEM-T-Myc-RalA. The construct was digested with BamHI and EcoRI and cloned into vector pCDNA3.1 yielding pCDNA3.1-Myc-RalA. In the resulting construct the coding sequence of RalA is preceded by 11 amino acids that comprise the epitope of monoclonal antibody 9E10 (Evan et al., 1985). Constructs pCDNA3.1-Myc-RalA G23V and S28N were constructed by overlap PCR using pCDNA3.1-Myc-RalA as template. Fragments were amplified using primers MS and G23Vrev 5'-GCC CAC GCC AAC ACT GCC CAC or S28Nrev 5'-AGT CAG AGC ATT CTT GCC CAC and RalAAS with G23Vfor 5'-GTG GGC AGT GTT GGC GTG GGC or S28Nfor 5'-GTG GGC AAG AAT GCT CTG ACT. The second PCR was performed using primers MS and RalAAS. Amplified DNA was digested with EcoRI and cloned into EcoRI digested pCDNA3.1-Myc-RalA wild type yielding pCDNA3.1-Myc-RalA G23V and S28N, respectively. The sequences of the constructs were verified using an automatic sequencer ABI100 (Perkin-Elmer, Norwalk, USA).

**RalA activation assay**

The GTP-bound form of RalA was isolated from total cell lysates by incubating the cell lysate with GST-RalBD coupled to glutathione Sepharose essentially as described previously (Wolthuis et al., 1998). Vector pGEX4T3-GST-RalBD was kindly provided by dr. J.L. Bos (Utrecht University, The Netherlands). GST-RalBD was purified from IPTG-induced bacteria as described previously (Wolthuis et al., 1997). HUVEC were cultured in 6 wells dishes and grown to confluency. Stimulation of endothelial cells was performed as described in the previous paragraph. At indicated time periods HUVEC were lysed in Ral buffer (15% (v/v) glycerol, 1% NP-40, 50 mM Tris (pH 7.4), 200 mM NaCl, 2.5 mM MgCl$$_2$$, 1 mM PMSF and 0.1 μM Trasylol). Cell lysates were incubated with 15 μg GST-RalBD precoupled to glutathione Sepharose for 60 min at 4 °C. Beads were washed and analyzed by 12.5% SDS-PAGE and Western blotting with a monoclonal anti-RalA antibody.
Transient expression of RalA variants in HUVEC

HUVEC were transfected by electroporation using a Genepulser equipped with a RF module (Biorad, Veenendaal, The Netherlands). Confluent HUVEC were trypsinized and 2 million cells were resuspended in 350 μl HEPES-buffered media. Five μg CsCl-purified plasmid was added to the cell suspension and incubated for 5 min at room temperature. Electroporation was performed in 2 mm cuvets at 240 V. Following transfection, cells were seeded on coverslips and cultured for 48 h. Cells were fixed with 3.7 % formaldehyde for 10 min and permeabilized with 0.02% saponin in PBS supplemented with 1% BSA. Cells were then stained with monoclonal anti-myc antibody 9E10 and polyclonal anti-vWF antibody in PBS/0.02% saponin/1% BSA. Secondary antibodies used were FITC-labelled goat anti-mouse (CLB, Amsterdam, The Netherlands) and Texas Red-labelled horse anti-rabbit antibodies (Vector Laboratories, Burlingame, CA). Cells were embedded in Vectashield mounting medium and viewed by confocal microscopy using a Leica TCS NT (Leica Microsystems, Heidelberg, Germany). Results of two independent experiments are given. The number of Weibel-Palade bodies present in endothelial cells expressing RalA wild type, G23V or S28N was determined. For each construct 20-30 individual transfected cells were evaluated.

RESULTS

Thrombin-induced secretion of von Willebrand factor coincides with activation of RalA

Stimulation of endothelial cells by agents such as thrombin results in exocytosis of Weibel-Palade bodies (Levine et al., 1982; de Groot et al., 1984). Recently, we have shown the presence of RalA in a subcellular fraction containing Weibel-Palade bodies (de Leeuw et al., 1999). To investigate whether secretion of Weibel-Palade bodies coincides with activation of RalA, HUVEC were stimulated with thrombin for various periods of time (Figure 1). Exocytosis of Weibel-Palade bodies was determined by measuring release of vWF in the medium. Secretion of vWF initiated 30 sec after the addition of 1 U/ml of thrombin and gradually increased in time (Figure 1A). Activation of RalA was measured by determining the binding of active GTP-bound RalA to GST-tagged Ral binding domain coupled to glutathione beads (Figure 1B). A transient activation of RalA was observed which reached a maximum after 2 min of stimulation with thrombin. After 10 min the amount of GTP-bound RalA had decreased significantly. No increase in activation of RalA could be detected in unstimulated cells and the total amount of RalA was similar in all samples analyzed (Figure 1C). These results show that activation of RalA coincides with release of vWF in endothelial cells following stimulation by thrombin.

Role of calmodulin in activation of RalA and regulated secretion of vWF:

In endothelial cells, calmodulin has been implicated in thrombin-induced exocytosis of Weibel-Palade bodies (Birch et al., 1992; van den Eijnden-Schrauwen, 1997). Recently, a binding site for calmodulin on RalA has been detected and calmodulin has been shown to
enhance the binding of GTP to RalA (Wang et al., 1997; Wang and Roufogalis, 1999). We investigated whether thrombin-induced activation of RalA is affected by antagonists of calmodulin. Endothelial cells were stimulated with thrombin in the presence and absence of calmodulin inhibitor TFP. Two min after the addition of thrombin, TFP inhibited secretion of vWF by 70%. A slightly lower inhibition was observed at 5 and 10 min following incubation with TFP (Figure 2A). In the same experiment the effect of TFP on activation of RalA was determined. In the absence of TFP, transient activation of RalA was observed (Figure 2B).

Figure 1. Thrombin-induced secretion of vWF coincides with RalA activation in endothelial cells.

Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199 1% (v/v) HSA. Subsequently, cells were stimulated with thrombin (1 U/ml) for the indicated periods of time. Medium was collected and cells were lysed in Ral binding buffer. The concentration of vWF in culture medium was determined by ELISA as described previously (Borchelli et al., 1996). Activation of RalA was measured as described in Materials and Methods. (A) Concentration of vWF in medium (white bar, control; black bar, stimulated with thrombin); (B) Activation of RalA in endothelial cells stimulated with thrombin; (C) Activation of RalA in non-stimulated endothelial cells.

Thrombin-induced activation of RalA was strongly inhibited by TFP (Figure 2B). Inhibition was most pronounced 2 min following addition of thrombin. At later time points, a slight increase in the amount of GTP-bound RalA was observed when compared to control cells that were not incubated with TFP. Our findings suggest that TFP partially inhibits both activation of RalA and release of vWF in endothelial cells stimulated with thrombin. Apparently, RalA acts downstream of calmodulin after thrombin stimulation.

The role of calcium and protein kinase C in activation of RalA in endothelial cells

Previous studies have shown that agents like Ca2+-ionophore A23187 and PMA can induce exocytosis of Weibel-Palade bodies from endothelial cells (Loesberg et al., 1983). We tested whether RalA was also activated upon stimulation of endothelial cells with these agents. Incubation of endothelial cells with 1 μM Ca2+-ionophore A23187 resulted in exocytosis of Weibel-Palade bodies, as determined by measuring the concentration of vWF in medium (Figure 3A). A rapid, transient activation of RalA was observed (Figure 3B). The amount of
Figure 2. Effect of the calmodulin inhibitor TFP on thrombin-induced secretion of Weibel-Palade bodies and activation of RalA.

Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199/1 % HSA. Then medium was replaced and TFP was added to a final concentration of 50 μM. In control cells no TFP was added. After 30 min, cells were stimulated with thrombin (1 U/ml) for indicated periods of time in the presence/absence of TFP and analysed as described in Materials and Methods. (A) Concentration of vWF in culture medium: non stimulated endothelial cells (white bar); cells stimulated with thrombin in absence (black bar) or presence (grey bar) of TFP. (B) Activation of RalA in non-stimulated endothelial cells (left panel) and cells stimulated with thrombin in absence (second panel) or presence (right panel) of TFP.

GTP-bound RalA at 2 min following stimulation with 1 μM Ca²⁺-ionophore A23187 was compared to that observed after incubation of endothelial cells with 1 U/ml of thrombin. Slightly less RalA was activated upon incubation of endothelial cells with Ca²⁺-ionophore A23187 than with thrombin (Figure 3B). Our findings indicate that increased intracellular Ca²⁺ levels result in rapid activation of RalA. Subsequently, we determined activation of RalA in cells that were treated with 100 ng/ml PMA. Incubation with PMA resulted in a time-dependent increase in the level of GTP-bound RalA (Figure 4B). Levels of GTP-bound RalA under these conditions were significantly lower than observed following stimulation of endothelial cells for 2 min by thrombin (Figure 4B). The amount of vWF released increased gradually in time upon incubation with PMA (Figure 4A). It should be noted that the amount of vWF released at 10 min following addition of PMA, was similar to that released upon incubation of endothelial cells for 10 min with thrombin. Our analysis indicates that PMA does not induce rapid activation as observed for thrombin and Ca²⁺-ionophore A23187. Apparently, Ca²⁺-ionophore A23187 and thrombin act differently on activation of RalA and release of vWF than PMA, an activator of protein kinase C.

Overexpression of RalA in endothelial cells affects exocytosis of Weibel-Palade bodies

In the previous paragraphs we showed that RalA activation coincides with thrombin-induced release of vWF. To study the functional role of RalA in exocytosis of Weibel-Palade bodies, we expressed RalA wild type, constitutively active (GTP-bound) RalA G23V or dominant negative (GDP-bound) RalA S28N in primary human endothelial cells by electroporation. Expression of wild type myc-tagged RalA revealed that in the majority of transfected cells the
Figure 3. Effect of Ca²⁺-ionophore A23187 on the activation of RalA in endothelial cells.

Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199/1 % (v/v) HSA. Subsequently, cells were stimulated with Ca²⁺-ionophore A23187 (1 μM) for indicated periods of time. As a control, cells were stimulated with thrombin (1 U/ml) for 2 min. Cells were analysed as described in Materials and Methods. (A) Cells stimulated with Ca²⁺-ionophore A23187. Concentration of vWF was determined in medium: non-stimulated cells (white bars); cells stimulated with Ca²⁺-ionophore A23187 (black bars). (B) Activation of RalA in endothelial cells stimulated with Ca²⁺-ionophore A23187.

Figure 4. Effect of PMA on the activation of RalA in endothelial cells.

Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199/1 % (v/v) HSA. Subsequently, cells were stimulated with PMA (100 ng/ml) for indicated periods of time. As a control, cells were stimulated with thrombin (1 U/ml) for 2 min. Cells were analysed as described in Materials and Methods. (A) Cells stimulated with PMA. Concentration of vWF was determined in medium of non-stimulated cells (white bars) or cells stimulated with PMA (black bars). (B) Activation of RalA in endothelial cells stimulated with PMA.

The number of Weibel-Palade bodies was greatly reduced (Figure 5A,B,C). In some of the transfected cells, a residual number of Weibel-Palade bodies could be detected (Table 1). Quantification of a large number of transfected cells revealed that 48% of cells expressing RalA wild type contained a limited number (<5) Weibel-Palade bodies (Table 1). A similar phenotype was observed in cells overexpressing constitutively active RalA G23V (Figure 5D,E,F). In 48% of cells expressing RalA G23V the number of Weibel-Palade bodies is strongly decreased compared to non-transfected cells. In cells transfected with dominant negative mutant RalA S28N normal amounts of Weibel-Palade bodies were observed. (Figure 5G,H,I). Quantitative analysis revealed that in only 8% of cells transfected with RalA S28N reduced numbers of Weibel-Palade bodies were present. Similarly, 4% of non-transfected primary endothelial cells also contained reduced numbers of Weibel-Palade bodies. Overall, these results indicate that overexpression of RalA in the GTP-bound form induces exocytosis.
of Weibel-Palade bodies from endothelial cells.

**Figure 5.** Wild type and constitutively active RalA induce exocytosis of Weibel-Palade bodies in endothelial cells. Primary endothelial cells were transfected with RalA variants and cultured on coverslips for 48 hours. Cells were fixed with 3.7% formaldehyde and immunofluorescence was performed as described in Materials and Methods. Exogenous RalA was visualized using monoclonal antibody 9E10 directed to Myc-tag and a FITC-labelled secondary goat anti-mouse antibody (A, D, G). vWF was visualized using polyclonal antibody directed to vWF and a Texas Red-labelled secondary horse anti-rabbit antibody (B, E, H). (A, B, C) Cells transfected with pCDNA3.1-Myc-RalA wildtype; (D, E, F) Cells transfected with pCDNA3.1-Myc-RalA G23V; (G, H, I) Cells transfected with pCDNA3.1-Myc-RalA S28N.
TABLE 1. Quantitative analysis of the number of Weibel-Palade bodies in transfected and non-transfected human endothelial cells.

Primary endothelial cells were transfected with RalA wild type, RalA G23V, and RalA S28N as described in Materials and Methods. For each construct the number of Weibel-Palade bodies was determined in 20-30 transfected cells. Results are expressed in percentages and are compared to the number of Weibel-Palade bodies present in non-transfected cells.

<table>
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<th>Construct</th>
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</tr>
<tr>
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<td>48</td>
</tr>
<tr>
<td>RalA G23V</td>
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<tr>
<td>RalA S28N</td>
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DISCUSSION

Stimulation of endothelial cells with agents such as thrombin results in release of high molecular weight multimers of vWF and translocation of P-selectin to the plasma membrane. Both these events are an immediate consequence of the thrombin-induced exocytosis of endothelial cell-specific storage organelles, the Weibel-Palade bodies. In this report, we provide evidence that the small GTP-binding protein RalA is transiently activated following stimulation of endothelial cells by thrombin. A previous study has shown that activation of RalA occurs also upon stimulation of human platelets with thrombin (Wolthuis et al., 1998). In platelets, thrombin-induced activation of RalA reached a maximum 1 minute following stimulation with thrombin whereas in endothelial cells maximal levels were reached after 2 min (Figure 1; Wolthuis et al., 1998). Furthermore, the amount of activated RalA decreased rapidly (between 5 and 10 min) in endothelial cells whereas in platelets significant levels of activated RalA were still present at 10 min following stimulation. Similar to what has been observed for platelets, elevation of intracellular calcium levels resulted in a rapid activation of RalA. Our results suggest that activation of RalA by thrombin proceeds via a similar mechanism in platelets and endothelial cells. Incubation with PMA, however, did result in a relatively slow activation of RalA in endothelial cells whereas in platelets no activation of RalA was observed following treatment with PMA (Wolthuis et al., 1998). The significance of activation of RalA under these conditions is presently not clear. Activation of RalA by agonists that activate the protein kinase C has so far not been reported (Wolthuis et al., 1998; Bos, 1998). Prolonged incubation with PMA for up to 60 min resulted in a further increase in RalA activation. Massive secretion of vWF was observed under these conditions. The molecular mechanism by which protein kinase C activates RalA in endothelial cells is currently unknown. Birch and co-workers have shown that the calmodulin antagonist W-7
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inhibits both thrombin and PMA-stimulated release of vWF from endothelial cells (Birch et al., 1992). The observed effect on PMA-induced release of vWF was attributed to the limited specificity of W-7 but may also indicate involvement of calmodulin in this process. In this study, we show that thrombin-induced activation of RalA is inhibited by the calmodulin antagonist TFP. This observation indicates that RalA functions downstream of calmodulin in endothelial cells. It should be noted that in the presence of TFP a slight increase in activation of RalA is observed at 5 and 10 min following addition of thrombin (Figure 2). These findings suggest that the calmodulin antagonist TFP delays but does not completely inhibit activation of RalA by thrombin. Interestingly, also the release of vWF is less inhibited by TFP at 5 and 10 min following activation by thrombin compared to 2 min of stimulation. These findings lend additional support to a close correlation between activation of RalA and regulated secretion of vWF.

Functional involvement of RalA in regulated exocytosis of Weibel-Palade bodies by endothelial cells is suggested by the absence of Weibel-Palade bodies in endothelial cells overexpressing wild type and 'constitutively active' RalA. In some transfected cells, a residual number of Weibel-Palade bodies can still be detected (Table 1). This may relate to variability in expression levels of RalA variants among individual primary endothelial cells. A large variability of number of Weibel-Palade bodies is also observed in non-transfected primary human endothelial cells (Table 1). Finally, our analysis does not allow for direct monitoring of exocytosis of Weibel-Palade bodies since only 10% of the endothelial cells were transfected (data not shown). A reduced number of Weibel-Palade bodies in a particular cell does not always result from exocytosis but may also be caused by cell-to-cell variability within primary cultures. Therefore, we determined the number of Weibel-Palade bodies in a large number of transfected primary endothelial cells. Our results suggest that both constitutively active RalA (G23V) and wild type RalA can induce exocytosis of Weibel-Palade bodies. The number of Weibel-Palade bodies in endothelial cells transfected with dominant negative RalA (S28N) is similar to control, non-transfected cells. This suggests that activation of RalA induces exocytosis of Weibel-Palade bodies by endothelial cells. In a previous study, we have reported that RalA associates with Weibel-Palade bodies in endothelial cells (de Leeuw et al., 1999). In the present study, expression of RalA variants did not clearly reveal colocalization of RalA with vWF in Weibel-Palade bodies. Several reasons maybe forwarded for this apparent discrepancy. First, RalA may only transiently associate with Weibel-Palade bodies. Secondly, only limited part of the intracellular amount of RalA may participate in the exocytosis of Weibel-Palade bodies. Cell fractionation studies revealed that RalA is not exclusively present in subcellular fractions that contain Weibel-Palade bodies. A significant amount of RalA was detected in other subcellular fractions derived from endothelial cells (data not shown).

Multiple functional roles for RalA have been proposed. RalA interacts in a GTP-dependent manner with filamin inducing filopodia (Ohta et al., 1999). Furthermore, RalA interacts with
RLIP76, a RalA effector protein with GTPase protein activity for cdc42 (Jullien-Flores et al., 1995). Both these observations suggest a role for RalA in cytoskeleton dynamics. Another target for RalA is phospholipase D. A number of studies have suggested that a complex of RalA with phospholipase D and Arf is involved in vesicle budding from the Golgi apparatus (Luo et al., 1997, 1998). Recently, RalA has also been implicated in endocytosis (Nakashima et al., 1999). Interestingly, both constitutively active and dominant negative forms of RalA inhibited endocytosis. These observations suggest that GTP hydrolysis of active RalA is required for endocytosis. The results of our study indicate that the presence of activated RalA, generated upon activation of endothelial cells, suffices to induce release the contents of Weibel-Palade bodies into the circulation.

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LITERATURE


