Molecular mechanisms involved in Weibel-Palade body exocytosis

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General discussion
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Weibel-Palade bodies are unique rod shaped vesicles that are exclusively present in endothelial cells. The multimeric glycoprotein von Willebrand factor (VWF) is the major component of these organelles. Besides VWF a large number of other bioactive proteins are stored within these organelles. Basal release of WPBs occurs in the absence of a stimulus and has been recently suggested to result from spontaneous fusion of WPBs with the plasma membrane [1,2]. Regulated secretion of WPBs occurs upon stimulation with different secretagogues. At present, two intracellular signaling pathways have been described that promote release of WPBs [3]. Agonists like histamine and thrombin mediate WPB release by raising intracellular Ca\(^{2+}\) levels, whereas cAMP-dependent signaling pathways control epinephrine and vasopressin-induced release of WPBs. A large number of accessory proteins has been implicated in the biogenesis, intracellular trafficking and exocytosis of WPBs. In this thesis we studied the molecular mechanisms contributing to the regulated release of WPBs.

Cyclic AMP-mediated Weibel Palade body exocytosis

A large number of agonist have been described that promote fusion of WPBs with the plasma membrane [3,4]. In general agonists can be classified in two distinct categories; agonists like histamine, thrombin and sphingosine-1-phosphate raise intracellular Ca\(^{2+}\) levels whereas epinephrine and vasopressine promote WPB exocytosis in a cAMP-dependent manner [5]. In response to cAMP-raising agonists, a subset of WPBs clusters at the microtubule organizing center thereby maintaining a critical level of secretable WPBs [5,6]. The kinetics of Ca\(^{2+}\)- and cAMP-mediated WPB exocytosis are not completely identical. Agonists like thrombin and histamine induce a rapid release whereas cAMP-dependent WPB exocytosis proceeds relatively slow and occurs in a more sustained manner [7]. This is also reflected by the activation kinetics of the small GTP binding protein RalA which mediates assembly of the exocyst complex a crucial late step in WPB exocytosis [7,8]. Thrombin induces a rapid transient activation of RalA which peaks at 2 minutes whereas epinephrine induces a slower more gradual activation of RalA [7]. The physiological importance of cAMP-mediated WPB release is illustrated by the clinical use of the vasopressin analog DDAVP in patients with von Willebrand disease and mild hemophilia.
Chapter 6

A [9]. An elegant study by Kaufmann and co-workers revealed that DDAVP targets the vasopressin V2 receptor which is exclusively expressed on lung endothelial cells [10]. The vasopressin V2 receptor is not expressed in human umbilical vein endothelial cells (HUVEC) [10]. In Chapter 2 and 3 of this thesis we focused on the mechanisms underlying cAMP-mediated WPB release. Exchange protein activated by cAMP (Epac) and holoenzyme PKA have a similar affinity for cAMP and parallel activation of both PKA and Epac is observed in several exocytotic pathways such as the acrosome reaction in sperm, synaptic vesicle release in neurons and insulin secretion in pancreatic β-cells [11,12,13,14]. The results documented in Chapter 2 clearly show that WPB release in endothelial cells is also dependent on Epac1 [15]. The results in Chapter 3 position the phosphatidylinositol-3,4,5-triphosphate-dependent Rac1 exchange factor 1 (PREX1) as a crucial downstream target of Epac-Rap1 [16] (Figure 1). Phospholipase Cε has evolved as a downstream target of Epac/Rap1/2 that induces release of Ca²⁺ from intracellular stores [17,18]. Epinephrine-induced WPB release has been not linked to elevation of intracellular Ca²⁺ suggesting that phospholipase Cε-mediated Ca²⁺ does not contribute to epinephrine-induced WPB release [19]. In Chapter 2 we show that 8-CPT-2’-O-Me-cAMP a compound specifically activating Epac can induce release of WPBs. We have previously shown that 8-CPT-2’-O-Me-cAMP-induced WPB release can be blocked by the Ca²⁺-chelator BAPTA-AM [7] potentially implicating phospholipase Cε as a downstream effector of Rap1 under these conditions. The lack of Ca²⁺ release in the presence of epinephrine suggests that PKA-mediated phosphorylation events may prevent release of Ca²⁺ from intracellular stores. Vascular endothelial cells express Epac1 and not Epac2 [20]. Epac2 has been implicated in cAMP-dependent release of insulin in pancreatic β cells [14,21]. In pancreatic β cells Epac2 binding to the Rab3A effector Rim2 and the Ca²⁺-binding protein Piccolo has been shown to promote insulin secretion [21]. Also, Munc13-1 has been identified as binding partner of Rim2 in pancreatic β cells [22]. The Rab3A/Munc13-1/Rim2 complex mediates docking of insulin granules at the plasma membrane [22,23]. Glucose stimulation dissociates the Munc13-1/RIM2 complex and released Munc13-1 activates syntaxin-1 resulting in exocytosis of insulin granules [23]. Interestingly, Munc13-4 a binding partner of Rab27A has recently been localized to WPBs [24,25]. Knockdown of Munc13-4 resulted in an impaired release of VWF suggesting a functional role of Munc13-4 in WPB exocytosis [24,25]. Also, Rab3B and Rab3D have been localized to WPBs but a functional role for these small
GTPases in WPB exocytosis has not yet been uncovered [26]. Overall, it appears that the mechanism involved in regulation of insulin release are similar to those observed for WPB exocytosis. In our studies we did not explore whether members of the Rim or Munc-13 family are linked to Epac1. Both in pancreatic β-islets as well as in nerve terminals Epac2 through its binding partner Rim2 orchestrates Ca\(^{2+}\)-dependent exocytosis by spatially coordinating Ca\(^{2+}\)-channels and docking/priming of secretory vesicles [27,28]. Epac1-mediated cAMP-dependent release of WPBs proceeds in a Ca\(^{2+}\)-independent manner. It is presently unknown whether Epac1 also coordinates positioning of exocytotic machinery linked to WPB release in endothelial cells.

**Rabs and Rap effectors involved in WPB release.**

Different Rab proteins have been described to play an important role in WPB exocytosis including Rab3D and Rab27A [29,30]. Rab3D plays a role in WPB formation, dominant negative Rab3D and siRNA-mediated down-regulation of Rab3D interfere with the formation of WPBs [29]. Overexpression of wild-type or activated Rab3D causes an inhibition of WPB release, suggesting a negative regulatory role of Rab3D in exocytosis [29]. Beside the small GTPase Rab3D also small GTPases Rab3B and Rab27A are expressed on WPBs [26]. Rab27A has multiple effector proteins [31,32,33] which include Myosin Va Rab interacting protein (MyRIP), synaptotagmin-like protein 4-a (Slp4-a) and Munc13-4. MyRIP acts as a negative regulator of WPB exocytosis and in cooperation with the actin motor protein myosin Va links WPBs to the actin cytoskeleton [34,35]. Slp4-a as well as Munc13-4 are Rab27A effectors that promote WPB exocytosis [24,25,26]. In Chapter 3 we identified Rab33A as a possible downstream target of Rap1-GTP [16]. Rab33A and to a lesser extent also Rab33B have been shown to interact to the RUN domain containing protein RUFY3 [36]. The RUN domain RUFY3 has also been shown to bind to Rap2B-GTP but not Rap1A-GTP [37]. These observations raise the possibility that RUFY3 provides a link between activated Rap1/2 and Rab33A. Indeed we observed that mEGFP-RUFY3 can interact with components of a signaling complex that binds in a stimulation-dependent manner to the Ras binding domain of RaLGDS (Chapter 3; Figure 2 and 3). As expected binding of RUFY3 to the Rap binding domain of RaLGDS is mediated by its RUN domain (Figure 3). These data suggest that RUFY3 provides a link between active Rap1/2 and Rab33A. It should be noted that our data do not specify how RUFY3 is recruited to the Ras binding domain of RaLGDS. Both Rap1A, Rap1b, Rap2A,
Rap2B and Rap2C are expressed in endothelial cells [38]. The RBD domain of RalGDS has been shown to bind to Rap1 and Rap2B, but probably also binds to the other Rap1/2 isoforms [39,40,41]. The RUN domain of RUFY3 has been shown to bind Rap2A but not Rap1A [37]. Future studies need to show whether Rap1/2 is needed for binding of RUFY3 to the Ras binding domain of RalGDS. Subsequently, specific Rap1/2 isoforms involved in this process will need to be identified. RUFY3 has been shown to interact with the p85 and P110α subunit of phosphatidylinositol-3 kinase (PI3K) [42]. The p85 subunit of PI3K was also identified as a potential downstream target of Rap1 in endothelial cells [16]. The RUN domain containing protein UNC-14 indirectly associated with the motor protein kinesin-1 in Caenorhabditis elegans [43]. Current data suggest that RUFY3 promotes anterograde transport (Figure 1); as yet it is not known whether RUFY3 is also part of a scaffolding complex that is linked to kinesins but this will be an interesting direction for future studies.

**Involvement of Rac and Rho in WPB exocytosis.**

In Chapter 3 we show that Rac1 is required for epinephrine-induced WPB exocytosis through the exchange factor PREX1. In agreement with these findings epinephrine results in a upregulation of levels of active Rac1 (Chapter 3). In contrast, thrombin-mediated WPB release is not affected by depletion or inhibition of Rac1. In Chapter 5 we shown that S1P-mediated release is inhibited by the Rho kinase (p160ROCK) inhibitor Y27632. Apparently, S1P-mediated WPB release is dependent on RhoA whereas epinephrine-induced release requires Rac1. Extensive crosstalk between members of the Rho family in the regulation of cytoskeletal changes has been reported [44]. The crucial role of cytoskeletal dynamics in WPB release has been well established and is illustrated by the recent observation of the transient formation of actin rings on WPBs that expel the content of these organelles following their fusion with the plasma membrane [45]. The formation of these actin rings is critically dependent on the phosphorylation of myosin II by myosin light chain kinase [45]. In endothelial cells both ROCK which acts downstream of RhoA and p21 associated kinase (PAK) acting downstream of Rac1 have been shown to promote phosphorylation of myosin light chain by myosin light chain kinase [46]. As yet the regulation of the transient formation of actin rings on fused WPBs has not been dissected. Cdc42 and Arp2/3 has been suggested as potential candidates for regulation of actin ring formation [45]. In this respect it is interesting to note that dominant negative Cdc42
has been shown to negatively affect both Ca\textsuperscript{2+} and cAMP-mediated WPB release [47]. It will be of interest to address whether dominant negative Cdc42 interferes with the assembly of WPB associated actin rings. Evidence has been obtained for spatio-temporal organization of Rho GTPase activity in so-called “activity zones” (reviewed in [48]). We speculate that these Rho GTPase activity zones are also involved in the regulation of agonist-induced WPB release.

**Figure 1. Model depicting signaling pathways regulating exocytosis of WPBs.** Thrombin and S1P stimulate WPB exocytosis by increasing the intracellular concentration of its second messenger Ca\textsuperscript{2+} in a phospholipase C (PLC)-dependent manner. In contrast, cAMP-mediated agonists such as epinephrine induce WPB exocytosis by upregulation of intracellular cAMP levels through the activation of adenylate cyclase (AC). In this thesis we show that beside protein kinase A (PKA), also Epac1 is essential for cAMP-mediated WPB exocytosis. Epac1 is able to activate Rap1 which via PI3K and PREX1 activates Rac1 what is suggested to induce WPB exocytosis by cytoskeletal remodeling. We also showed interaction between activated Rap1 and RUFY3; which was described to bind to Rab33A. Rab33A has been shown to be able to localize to WPBs. Unexpectedly, WPBs were depleted in mEGFP-RUFY3 expressing cells. S1P induces release of WPBs through its binding to the S1P receptor 3 (S1PR3). To induce secretion, both PKA and intracellular Ca\textsuperscript{2+} (via calmodulin, CaM) induce the activity of the Ral guanine nucleotide exchange factor RaLGDS, which leads to activation of the small GTPase RalA. Activated RalA mediates the interaction between members of the exocyst complex, which allows WPBs to tether to the plasma membrane, finally resulting in the fusion of WPBs with the plasma membrane during exocytosis.
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Final remarks and remaining issues.

In this study several novel players involved in the regulation of WPB exocytosis are described. Our findings show that cAMP-dependent WPB exocytosis is dependent on an Epac-Rap1-PREX1-Rac1 signaling pathway. We also show that Rab33A and Rab33B as well as RUFY3 are novel candidate proteins involved in the regulation of WPB release. Future studies are needed to define the precise role of both Rab33 isoforms in WPB exocytosis. The modular domain structure of RUFY3 positions this molecule as a potential scaffold for spatio-temporal integration of Rap1/2-mediated signaling events and Rab-mediated vesicular transport. Another novel observation documented in this thesis is the lack of WPBs in endothelial cells expressing mEGFP-RUFY3. We do not know the underlying mechanism for this phenotype; it would be very interesting to study and determine whether this phenomenon is due to a defect in WPB biogenesis or an increase in WPB exocytosis or degradation. We also showed that the S1P receptor 3 is responsible for S1P-mediated WPB exocytosis (Figure 1). S1P-dependent WPB exocytosis proceeds in a Ca\(^{2+}\)-dependent and Rho kinase-dependent fashion. Apparently, different members of the family of Rho GTPases can modulate WPB release. The precise role of individual members of the family of Rho GTPase family members as well as their possible crosstalk during WPB exocytosis will be interesting topics for future studies.

References


