Molecular mechanism of secretory vesicle docking

Heidi de Wit1

Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, Neuroscience Campus Amsterdam, Vrije Universiteit (VU) Amsterdam and VU Medical Center, De Boelelaan 1085, 1081 HV, Amsterdam, The Netherlands

Abstract
Docking, the stable association of secretory vesicles with the plasma membrane, is considered to be the necessary first step before vesicles gain fusion-competence, but it is unclear how vesicles dock. In adrenal medullary chromaffin cells, access of secretory vesicles to docking sites is controlled by dense F-actin (filamentous actin) beneath the plasma membrane. Recently, we found that, in the absence of Munc18-1, the number of docked vesicles and the thickness of cortical F-actin are affected. In the present paper, I discuss the possible mechanism by which Munc18-1 modulates cortical F-actin and how it orchestrates the docking machinery via an interaction with syntaxin-1. Finally, a comparison of Munc18’s role in embryonic mouse and adult bovine chromaffin cell model systems will be made to clarify observed differences in cortical F-actin as well as docking phenotypes.

Introduction
In electron micrographs of synapses and chromaffin cells, many synaptic and secretory vesicles are found morphologically docked at the target membrane. Morphologically docked vesicles are traditionally defined as those vesicles that have no measurable distance between vesicle and plasma membrane, but some authors use less stringent criteria (for a review, see [1]). Docking is generally considered to be a necessary first step before vesicles gain fusion-competence, but it is unknown how vesicles dock. Bovine adrenal medullary chromaffin cells have been for many years a major model for the study of the molecular machinery underlying exocytosis [2]. We have studied docking and fusion of secretory vesicles in mouse embryonic chromaffin cells as a preferred docking model, because docking phenotypes are typically more evident than in other systems studied so far (for a review, see [1]). Previously, we showed that deficiency of the SM (Sec1/Munc18) gene munc18-1 [3] or the protein syntaxin-1 [4] not only abolished exocytosis, but also produced robust docking phenotypes in chromaffin cells. We also observed that manipulating Munc18-1 expression levels affected the thickness of subplasmalemmal F-actin (filamentous actin) [5]. Yet, it is well accepted that the density of these F-actin filaments beneath the plasma membrane determines the access of secretory vesicles to fusion sites [6] and that F-actin thickness is controlled by at least two pathways [7]: PKC (protein kinase C) activation mediated by DAG (diacylglycerol)/phorbol esters [8], and scinderin mediated by Ca2+[9], but it is unknown whether Munc18-1 acts in the same or parallel pathways. In addition, Munc18-1 can bind to both ‘closed’ and ‘open’ syntaxin-1 [10–13], but it is unclear what binding mode is essential to perform its function in both cortical F-actin modulation as well as docking. Binding of Munc18-1 to assembled SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) complexes containing syntaxin-1 in an ‘open’ conformation seems to be involved in executing membrane fusion [10,11,13,14], whereas there are only a few observations that can explain the importance of Munc18-1 binding to ‘closed’ syntaxin-1. Recently, we have shown that binding to ‘open’ syntaxin-1 is not sufficient for docking, since a docking phenotype similar to syntaxin-1- and munc18-1-null was observed in chromaffin cells from knockin mice that express a mutant syntaxin-1 which only allows the ‘open’ conformation [12]. Moreover, it seems that both the cortical F-actin thickness and number of docked vesicles is developmentally controlled because, in adult bovine chromaffin cells, cortical F-actin seems thicker, whereas the number of docked vesicles seems lower compared with embryonic chromaffin cells ([5,15–17], and H. de Wit, unpublished work), but the reason for this developmental difference is unclear.

The present review summarizes findings on docking and cortical F-actin modulation by Munc18-1 in embryonic chromaffin cells and discusses whether Munc18-1 must bind to ‘open’ or ‘closed’ syntaxin-1 to perform its function. These data will be compared with published data in mature bovine chromaffin cells to explain Munc18’s function during the development of the cortical F-actin and docking machinery.

Munc18 promotes ‘morphological’ docking
Previously, we found that deletion of the munc18-1 gene in embryonic chromaffin cells produces a 10-fold reduction of ‘morphological’ docked secretory vesicles in vivo [3] and

Key words: docking, electron microscopy, exocytosis, filamentous actin (F-actin), Munc18-1, syntaxin-1.

Abbreviations used: F-actin, filamentous actin; Lat A, latrunculin A; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C; M18-1, non-PKC-phosphorylated Munc18-1 mutant; SNARE, soluble N-ethylmaleimide-sensitive factor-attachment protein receptor.

1 email heidi.de.wit@cncr.vu.nl
in vitro [5], and explains the severe secretion phenotype. Gene-dose reductions in munc18-1 expression reduce docking, whereas gene-dose enhancements increase docking [5], indicating that Munc18-1 promotes docking. The positive function of Munc18-1 in docking is not limited to embryonic chromaffin cells, because Munc18-1 overexpression also results in increased docking in mature bovine chromaffin cells [16].

In addition to neuronal Munc18-1, we also expressed two ubiquitous Munc18 isoforms, Munc18-2 and Munc18-3, in munc18-1-deficient chromaffin cells. Munc18-2 and Munc18-3 have relatively low expression levels in neuroendocrine cells [18,19]. Munc18-2 is involved in histamine secretion in mast cells [20] and apical vesicle trafficking in epithelial cells [21], whereas Munc18-3 regulates cell-surface expression of GLUT4 (glucose transporter 4) in adipocytes [22,23]. The amino acid sequence homology between Munc18-1 and Munc18-2 is more (60%) compared with Munc18-3 (50%), and both Munc18-1 and Munc18-2 show strong affinity for syntaxin-1, syntaxin-2 and syntaxin-3, but not syntaxin-4 [19], whereas Munc18-3 shows high affinity only for syntaxin-2 and syntaxin-4 [22]. We found that Munc18-2 rescues the severe docking phenotype of munc18-1-null chromaffin cells identical with that of Munc18-1, whereas the more downstream vesicle-priming steps are still impaired [24]. On the other hand, docking is rescued only partially after Munc18-3 expression in munc18-1-null chromaffin cells (Figure 1). The phenotypic difference of the ubiquitous Munc18 isoforms cannot be caused by a lack of PKC phosphorylation because all homologues can be phosphorylated by PKC [21,25–27]. Together, our observations show that Munc18-1 and Munc18-2 promote docking by binding to syntaxin-1, whereas a distinct interaction mode seems to be required to regulate the consecutive priming step.

**Binding of Munc18-1 to ‘closed’ syntaxin-1 is essential for docking**

Proteolytic cleavage of syntaxins by botulinum toxin C in mouse chromaffin cells produces a docking phenocopy of munc18-1-null cells, without affecting the total number of vesicles [4]. Therefore we consider that binding of Munc18 to syntaxin-1 orchestrates a docking complex involving syntaxin-1 on the plasma membrane. Munc18-1 can interact with both ‘closed’ and ‘open’ syntaxin-1 [28], but it is unclear which binding mode is essential to perform its function in docking. Munc18-1 binding to ‘open’ syntaxin-1 involves an interaction with the N-terminal H(abc) domain of syntaxin-1 and the four-helical bundle of the assembled SNARE complex [11,13,29]. We have made two independent observations to believe that Munc18-1 binding to the N-terminus of syntaxin is not sufficient for docking. First, analysis of morphological docking in chromaffin cells from knockin mice that express a mutant syntaxin-1 which only allows N-terminal interaction showed a robust docking defect similar to munc18-1-null cells [12]. Secondly, when we expressed the D34N/M38V mutant of Munc18-1 that is known to perturb the interaction with ‘closed’ syntaxin [30,31], this resulted in a partial rescue of the docking defect in munc18-1-null cells [24]. Other researchers have shown that Munc18-1 binding to ‘open’ syntaxin is essential to execute fusion [10,11,13,14]. Taken together, it seems that the dual binding modes of Munc18-1 to syntaxin-1 are important to regulate subsequent steps in the secretory pathway: docking (‘closed’ syntaxin) and fusion (‘open’ syntaxin).

**Munc18-1 controls access of vesicles to docking sites via cortical F-actin modulation**

Bovine chromaffin cells possess a dense mesh of cortical F-actin underneath the plasma membrane which is thought to provide a barrier for secretory vesicles to access exocytotic sites under resting conditions [32]. Consistent with this view, perturbations of the submembrane F-actin modulate docking and secretion (see [33] for a review). Upon encountering a secretory stimulus, it seems that two major cortical F-actin-severing pathways are activated. The first pathway is controlled by Ca²⁺ entry that results in activation of scinderin [9], whereas the second pathway depends on PKC activation and phosphorylation of the MARCKS (myristoylated alanine-rich C-kinase substrate) [17]. Both scinderin and MARCKS are F-actin-severing proteins, and their activation induces a local disruption of the cortical F-actin architecture to facilitate recruitment of secretory vesicles towards release sites; however, their involvement in docking remains unresolved.

We observed that the F-actin organization is severely disturbed in the absence of the docking factor Munc18-1 in embryonic mouse munc18-1-null chromaffin cells [5]. Munc18-1 is rapidly phosphorylated by PKC upon depolarization [34], and is an important downstream target in PKC-dependent potentiation of secretory vesicle release [35]. Together, this makes Munc18-1 a likely candidate as a downstream target for PKC-dependent reorganization of the F-actin network to regulate docking. However, we found that either expression of a constitutively PKC-phosphorylated Munc18-1 (M18Δ) mutant or a non-PKC-phosphorylated (M18ΔPKC) mutant had a similar effect on the cortical F-actin intactness (H. de Wit, unpublished work). This PKC-independent function of Munc18-1 in cortical F-actin reorganization suggests that Munc18-1 acts via a novel pathway in parallel with PKC/MARCKS to recruit new vesicles to fusion sites. Further experiments are required to see whether Munc18-1 alone is sufficient to rearrange cortical F-actin or whether additional factors are required. For example, it was found previously that Munc18-1 co-localizes with cytoskeleton proteins [36] and is phosphorylated by Cdk5 (cyclin-dependent kinase 5), which is found in connection with neurofilaments [37], but it remains to be tested whether Munc18-1 acts directly as an actin-severing protein.Irrespective of the mechanism it seems that Munc18-1’s function does not depend on binding to syntaxin-1.
Figure 1 | Munc18-3 cannot rescue docking in munc18-1-null chromaffin cells

The number of docked secretory vesicles is reduced after expression of Munc18-3 compared with Munc18-1 and Munc18-2 in munc18-1-null chromaffin cells as shown on electron micrographs in a subregion of the plasma membrane (A). Scale bar, 100 nm. Reprinted from [24] with permission. This significant difference in vesicle docking is not caused by a lower biogenesis of secretory vesicles, because the total number of vesicles is unchanged in the presence of Munc18-3 (B). Results are means ± S.E.M. ***P < 0.001 (Student’s t test). E18, embryonic day 18.

Developmental aspects of docking in chromaffin cells

Mature bovine chromaffin cells have a denser cortical F-actin network [8,32] compared with embryonic wild-type mouse chromaffin cells [5]. Because of this difference, the number of docked secretory vesicles is higher under non-stimulatory conditions in embryonic chromaffin cells [5] compared with bovine cells [16] (Figure 2 and Table 1). We estimated that in bovine chromaffin cells, only ~1% of total secretory vesicles are docked, which is in line with results from Plattner et al. [15], whereas in embryonic chromaffin cells, we estimated that ~33% of the vesicles are docked (see Table 1). Munc18-1 overexpression increases the number of docked vesicles in both mature [16] and immature cells [5], and this increased docking seems highly correlated with reduced cortical F-actin (H. de Wit, unpublished work); however, the differences in mature chromaffin cells are more robust. We also found that Lat A (latrunculin A) can cause potentiation of secretion in mature chromaffin cells, but not in embryonic chromaffin cells and that also phorbol esters only affected cortical F-actin in mature chromaffin cells (H. de Wit, unpublished work). Thus, although it is still unclear why immature and mature chromaffin cells show these differences, it seems that the accessibility of vesicles to the plasma membrane becomes more highly controlled during maturation. In addition, compared with embryonic wild-type cells, munc18-null chromaffin cells contain a denser cortical F-actin network, and only a few vesicles are docked [5] (Figure 2 and Table 1). Strikingly, we observed that phorbol esters can reduce the cortical F-actin and partially restore docking (H. de Wit, unpublished work) and secretion [24]. Our observation that phorbol esters partially restores the docking/secretion defect can be explained by the fact that reduction of the cortical F-actin network alone, as shown previously after Lat A application [5], is not sufficient for functional docking/secretion. An alternative explanation is that other Munc18 isoforms, either Munc18-2 [24] or Munc18-3 [22], are phosphorylated by PKC activation. However, the fact that we did not observe

because syntaxin-1 deletion by botulinum toxin C does not affect the cortical F-actin [4].
Figure 2 | Comparison of cortical F-actin thickness and docking in embryonic and adult chromaffin cells

(A) Upper panel: bovine (adult) chromaffin cells have a thicker cortical F-actin network compared with wild-type (E18, embryonic day 18) chromaffin cells as visualized by rhodamine-phalloidin staining. Scale bar, 2 μm. (B) In the absence of Munc18-1, the percentage of intact cortical F-actin is not different from bovine chromaffin cells and significantly increased compared with wild-type (E18) chromaffin cells. (A) Lower panel: the more intact cortical F-actin in both munc18-1-null (E18) and bovine (adult) chromaffin cells might explain why a lesser amount of secretory vesicles are docked, as observed by electron microscopy. Scale bar, 100 nm. This docking phenotype is the opposite for wild-type (E18) chromaffin cells (A and B). As published previously, munc18-1-null (E18) and wild-type chromaffin cells show no difference in the total number of vesicles [5]; however, in adult bovine chromaffin cells, the total number of vesicles is increased compared with embryonic chromaffin cells (B; see also Table 1). Notice that secretory vesicles in bovine chromaffin cells have a larger diameter compared with mouse chromaffin cells (see also Table 1 for quantification of vesicle diameters). Results are means ± S.E.M. ***P < 0.001 (Student’s t-test). (C) Putative cartoon model summarizing the role of Munc18-1 and cortical F-actin in docking. In mature chromaffin cells, most of the secretory granules form a reserve pool that is trapped in the cortical F-actin cytoskeleton. In embryonic chromaffin cells the cortical F-actin is less developed and more vesicles are docked. Upon depolymerization of cortical F-actin stimulated by PKC, Munc18-1 or Ca^{2+} (e.g. scinderin) granules have relatively more access to docking sites in mature chromaffin cells, because we hypothesize that these sites are already occupied in embryonic cells. PM, plasma membrane.
Table 1  Overview of secretory vesicle electron microscopy parameters in adult bovine and wild-type and munc18-1-null embryonic chromaffin cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Genotype</th>
<th>Number of total vesicles (Per section)</th>
<th>Number of docked vesicles (Per section)</th>
<th>Number of docked vesicles (Per cell)</th>
<th>Number of total vesicles (Per cell)</th>
<th>Proportion (%)</th>
<th>n</th>
<th>Vesicle diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>Adult</td>
<td>Wild-type</td>
<td>225.2 ± 22.2 18</td>
<td>22.5 ± 2.5 18</td>
<td>16</td>
<td>314.2 ± 22.2 18</td>
<td>25.6 ± 2.5 18</td>
<td>26</td>
<td>83.9 ± 2.5 18</td>
</tr>
<tr>
<td>Mouse</td>
<td>E18</td>
<td>Wild-type</td>
<td>34.8 ± 22.2 18</td>
<td>3.8 ± 2.5 18</td>
<td>10</td>
<td>160.6 ± 22.2 18</td>
<td>10.5 ± 2.5 18</td>
<td>25</td>
<td>89.3 ± 2.5 18</td>
</tr>
<tr>
<td>Mouse</td>
<td>E18</td>
<td>munc18-1-null</td>
<td>3.8 ± 22.2 18</td>
<td>0.6 ± 0.3 18</td>
<td>8</td>
<td>32.9 ± 16.5 18</td>
<td>0.6 ± 0.3 18</td>
<td>23</td>
<td>86.9 ± 2.5 18</td>
</tr>
</tbody>
</table>

See [15] for details of calculation per cell. Our estimated docked and total vesicle number per cell in bovine chromaffin cells corresponds with previous calculations by others [15]. The Proportion column reflects the number of docked vesicles as a percentage of the total number of vesicles. No difference was observed in this ratio when the ratio was calculated per section or per cell. The proportion of vesicles docked corresponds with previous findings [15]. Values are means ± S.E.M.; E18, embryonic day 18.

any effect after phorbol ester application in embryonic wild-type chromaffin cells makes this latter assumption unlikely. Therefore a more likely explanation for these differences is that no additional vesicle recruitment upon PKC activation is possible due to a limited number of available docking sites.

Actin cytoskeleton and docking in synapses

Similarly to chromaffin cells, synapses seem to have a cortical F-actin network [38,39]. F-actin is abundantly distributed throughout the presynaptic terminal and is often associated with the synapsin filaments [40] to link synaptic vesicles [41]. Biochemical [42], ultrastructural [43] and functional [44] studies indicate that actin also constitutes an important component of the active zone. In hippocampal synapses, Lat A treatment promotes neurotransmitter release, but the readily releasable pool size and its rate of refilling are not altered by Lat A [44]. This suggests that Lat A exerts its effect on vesicles that are already docked at the active zone. Similarly, actin-depolymerizing agents are ineffective in increasing neurotransmitter release in hippocampal slices of mouse harbouring a homozygous deletion of the LIM kinase 1 gene (LIMK1), a kinase that modifies actin dynamics by phosphorylating and thereby inactivating coflin [45]. Taken together, these observations suggest that the actin cytoskeleton negatively regulates neurotransmitter release by forming a structural barrier for exocytosis at or near release sites. However, it can be expected that the organization of the actin cytoskeletal network at release sites in chromaffin cells might be different from synapses, probably because their release sites do not contain a subcellular specialization like the active zones in synapses and expression of certain synaptic actin-associated proteins (e.g. synapsins, LIM kinases) is absent [38,39]. These molecular and cellular differences might also explain why we observed an additive effect on secretion in M18PKC-expressing embryonic chromaffin cells after phorbol ester application (H. de Wit, unpublished work), whereas in neurons the response to phorbol esters was absent in the presence of M18PKC [35]. The difference observed can also be explained by the fact that no additional vesicle recruitment upon phorbol ester administration is possible due to a limited number of available docking sites at synaptic active zones (for a review, see [1]). This assumption is also in line with our previous observations in mature chromaffin cells that have only a few vesicles docked at steady state and after stimulation more vesicles can be replenished compared with embryonic chromaffin cells [16].

Conclusions

We have shown that Munc18-1 is capable of modulating the cortical F-actin cytoskeleton and the number of docking sites in mature and immature chromaffin cells (Figure 2C). Munc18-1’s function seems to be independent of PKC activation as shown previously in neurons [35]. Therefore we propose that Munc18-1 and PKC (via MARCKS),...
together with scinderin, act in three parallel pathways to modulate cortical F-actin and determine the number of docked vesicles. In addition, we observed that the cortical F-actin barrier increases and the number of docked vesicles decreases during development (Figure 2C). This difference has probably evolved to keep docking sites available during certain stimulatory demands.

Finally, future experiments are required to unravel further why differences between embryonic and mature chromaffin cells exist and determine whether the same actin signalling pathways are used during neurotransmitter release at neuronal active zones. These studies will help to unravel the synaptic structural changes caused by actin remodelling associated with learning and memory.

Since the present paper was submitted, my co-workers and I have identified four proteins that together constitute the minimal docking machinery using knockout and (cross-) rescue experiments in chromaffin cells [46]. Ultrastructural morphometry and electrophysiology together show that vesicles stably dock at the target when synaptotagmin-1 binds to syntaxin–SNAP-25 (25 kDa synaptosome-associated protein) acceptor complexes, whereas Munc18-1 promotes or stabilizes these acceptor complexes at the target and also facilitates full SNARE complex formation by promoting association of synaptobrevin [46].

**Acknowledgements**

I thank Ruud F.G. Toonen and Matthijs Verhage for fruitful discussions and critical comments on the manuscript.

**Funding**

This work was supported by the Netherlands Organization for Scientific Research (NWO) [grant number VENI 916-36-043].

**References**


Received 30 March 2009
doi:10.1042/BST0380192