Molecular Mechanisms of Exocytosis:  
Role of Cdc42 and Actin in late steps of Regulated Exocytosis

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Abstract

The general aim of this Master Thesis is to understand the molecular mechanism by which actin intervenes on regulated exocytosis of secretory granules (SG), in neuroendocrine cells.

Rho GTPases are key regulators of the actin cytoskeleton. Cdc42 was shown to facilitate exocytosis in neuroendocrine cells probably by promoting actin polymerization in the subplasmalemmal region. In order to determine the importance of Cdc42 action in regulated secretion, its gene was silenced in BON and PC12 cells and the consequences were carefully analyzed. The silencing efficiencies on endogenous Cdc42 expression were estimated at 60 ± 8% in BON cells and 67 ± 22% compared to cells transfected with control siRNA. Morphological effects can be seen in BON cells exhibiting these silencing levels, as well as the formation of interesting actin structures. Furthermore, a slight decrease on the number of cells presenting peripheral SG enrichment was observed in silenced cells (~20%). However, by Total Internal Reflection Fluorescence Microscopy (TIRFM), we observe an increase of SG density near the plasma membrane in Cdc42 silencing conditions, so Cdc42 must play a role on the number of SG in enriched areas. Furthermore, secretion assays were realized and did not indicate any noteworthy inhibition on tritiated serotonin ([3H]-5HT) secretion on cells deficient in Cdc42 (15% at most). However, these findings seem to disagree with the paper of Gasman et al. (2006) that states a significant diminution in growth hormone secretion. Once we are studying the secretion of a small molecule instead of a macromolecule (as did Gasman laboratory). Therefore, Cdc42 could be involved in the mechanism of pore extension, during exocytosis, that allows macromolecules to be released.

Keywords: exocytosis, secretory granule, actin, Cdc42 GTPases, BON cells, PC12 cells.

Introduction

Many hormones and neuropeptides are stored in secretory granules (SG) and released by calcium-triggered exocytosis. SG are transported along microtubules from the trans-Golgi network to the cell periphery (Rudolf et al., 2001; Varadi et al., 2002) where they accumulate in the actin-rich cortex (Trifaro et al., 2000). It has been recently demonstrated that the GTPase Rab27 recruits the actin-based motor Myosin Va (Myo Va) onto SG via the adaptor protein Myrip. This complex mediates the interaction of SG with actin filaments, controls the mobility of SG beneath the plasma membrane and SG docking (Desnos, 2003). Whether actin is involved in exocytosis is yet not known once its role in exocytosis must still be determined.

Most secretory cells display a dense subplasmalemmal actin network. Since its mesh size is smaller than a vesicle diameter (Nakata and Hirokawa, 1992), the first main idea was that actin should function as a barrier for SG traffic. However, SGs must cross the actin cortex in order to reach and interact with the plasma membrane. Consequently, it is considered that activation of secretion and exocytosis are accompanied by a reorganization of the peripheral actin filaments.

Actin dynamics is intrinsically related with Rho GTPase family. Its members are well known to regulate cell polarity and motility through their effects on the cytoskeleton, membrane trafficking and cell adhesion. They consist of about 20 members which the best studied are RhoA, Rac1 and Cdc42.

To affect actin dynamics in the cortex, Rho GTPases are recruited and activated on the plasma membrane by GEFs proteins (Rossman et al., 2005). Then, they can activate two different types of molecules: WASP/WAVE proteins (Wiskott-Aldrich syndrome proteins and WASP family homologous WASP proteins) and DFRs (Diaphanous-related formins). WASP/WAVE proteins activate Arp2/3 complex that binds to the sides of existing actin filaments and induces polymerization of actin to origin a branching filament network. Cdc42 is upstream this cascade once it binds directly to WASP and N-WASP proteins. Therefore, Cdc42 indirectly stimulates Arp2/3 activation (Stradal et al., 2006). The Rho
GTPase Rac was also shown to interact indirectly with Arp2/3. However the intermediate molecule is WAVE and not N-WASP, and the morphological structures created are not the same. When Cdc42 activates Arp2/3, it leads to the formation of filopodia (broad sheet-like protrusions containing a network of branching actin filaments) while, with Rac activation, it leads to lamellipodia (finger-like membrane protrusions that contain parallel bundles of actin filaments) (Ridley, 2006).

Several studies revealed the positive effects of members of the Rho GTPase family on exocytosis. For instance:

1) N-WASP and the Arp2/3 complex mediate actin-dependent propulsion of secretory vesicles (Mullins, 2000)
2) Cdc42 actively participates in insulin secretion in pancreatic-β-cells (Nevins, 2003).
3) In PC12 cells, Arp2/3 is associated with SG and follows them until they reach docking sites at the plasma membrane upon cell activation (Gasman et al., 2004);
4) Cdc42 mutant or its silencing inhibit exocytosis (Malacombe et al., 2006).

The last statement suggests that secretagogue-evoked stimulation induces the sequential ordering of Cdc42, N-WASP and Arp2/3 in order to specifically target local actin filament polymerization at docking sites.

Cdc42 clearly exhibits a major signaling role in providing specific actin structures required for efficient exocytosis. What remains to be established is the relation between the reported effects of Cdc42 on exocytosis and actin dynamics. Following this purpose, the aim of this work is to clarify the role of actin in late steps of secretion, using Cdc42 as an intermediate.

**Material and Methods**

**Culture of PC12 and BON cells**

To properly track SG trafficking, the neuroendocrine cell lines used were:
- PC12 cells – cells from a tumor of the adrenal rat gland (pheochromocytoma cells) that secrete neurotransmitters like adrenaline and neuropeptides.
- BON cells – human carcinoid cells that secrete serotonin (5-HT) and other peptides.
- stable clone of BON cells, called BC21, that express neuropeptide-Y tagged with GFP, a marker of SG.

PC12 cells were grown in DMEM supplemented with glucose (1g/l) and containing 5% fetal bovine serum, 10% horse serum, 100U/ml penicillin, 100 µg/ml streptomycin, at 37°C under 5% CO2.

BON cells were grown in HAM’s F12 DMEM containing 10% fetal bovine serum, 100U/ml penicillin, 100µg/ml streptomycin, at 37°C under 5% CO2.

**Plasmids and short interference RNA**

The plasmid PEG-NPY-GFP was constructed by the host laboratory and the Cdc42 tagged GFP was gently ceded from Gasman Laboratory.

Three sequences of siRNA against Cdc42-GTPase were used: GGGCAAGAGGATTATGACATT (from Gasman Laboratory), GACUGCUUUCUUGCUUGUUTT or GAUAACUCACCAUCCATT (Dharmacon). For control conditions three duplexes were tested: one against lamin (Dharmacon), one against EGFP (Sigma-Proligo) and one against luciferase (Dharmacon).

**Transfection**

To transfect plasmids in cells the lipofectant Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions. To transfect short siRNA duplexes, two transfectants were used, Dharmafect (Dharmacon) and Interferin (Polyplus transfection), according to manufacturer’s instructions. Under these conditions, the transfection efficiency reached 80-90%.

**Antibodies and Immunobloting**

The following antibodies were used: rabbit polyclonal α-chromogranine A/B (abcam); mouse monoclonal α-Actin (Sigma); mouse monoclonal α-Cdc42 (Transduction Laboratory); mouse monoclonal α-GFP (Sigma); alexa-labeled secondary antibodies; rabbit R-HRP (sigma); mouse M-HRP (sigma).

Cells extracts were prepared for electrophoresis and Western Blot analysis of Cdc42. The blots were scanned and quantified using Image J software. Levels of actin were used to normalize the results.
**Immunofluorescence**

Cells grown on collagen-coated coverslips were washed with PBS+ and fixed for 15min in 4% paraformaldehyde in PBS+. They were rinsed before being permeabilized with PBS+ 0.2% BSA 0.05% saponin 0.25% Triton X-100 for 15min. Afterwards, cells were exposed to rhodamin phalloidin for 30min. After extensive washing, the coverslips were mounted on slides using mowiol.

Stained cells were visualized using an inverted microscope Nikon TE 2000E and a coolSNAPES CCD camera. Using the software MetaVue, it was possible to acquire and quantify digital images.

**TIRFM (Total Internal Reflection Fluorescence Microscopy)**

BON cells that were transfected with a fluorescent marker (NPY-mRFP or NPY-GFP) were checked with the TIRF microscope. The NPY positive SGs in the closest proximity to the glass-slide (close to the cell periphery) get excited by the laser beam and emit fluorescence, which can be detected under the microscope. Since only the area of the cell, which is directly adherent to the glass gets excited, it is possible to get fluorescence with a very low background. Under these conditions, films at 10Hz were taken at a depth of 100nm.

**Secretion**

Cells were incubated with radioactive serotonin $[^3]$H]5-HT, a physiological neurotransmitter, that accumulate inside the cell into the secretory granules. Then, cells were rinsed with culture medium and chassed for 2h at 37ºC. Afterwards, cells were submitted to two consecutive 10min washes with supplemented Locke’s solution (containing 5.5 mM glucose, 3.5 mM HCO$_3^-$, 163.7 mM Cl$, 154.5$ $\text{mM Na}^+$, 5.5 $\text{mM K}^+$, 15 $\text{mM}$ Hepes, 2.5 $\text{mM}$ Ca$^{2+}$, 1.2$mM$ Mg$^{2+}$). Locke’s solution was aspirated in a seriated way and was substituted with Locke +/- secretagogues: 1 $\mu$M ionomycine or 1.9 $\text{mM}$ barium, in presence of calcium. The ionomyicine is responsible for the creation of small pores inside the plasma membrane that allow the entrance of calcium, the exocytosis trigger. After 10 min, the supernatants were recovered, in order to measure the radioactivity. The cells were also recovered in Triton 100X 1% and the radioactivity levels were determined and expressed in percentage of ratio between supernatant and cell contains.

**Results**

**Cdc42 Silencing**

To determine the effect of the siRNA duplex used to silence Cdc42, BON and PC12 cells were transfected with siRNA against Cdc42 or control siRNA (against lamin, GFP or luciferase). 72h after transfection, cell extracts were recovered and prepared for electrophoresis and immunoblotting with monoclonal antibodies against Cdc42 and actin. The blots were quantified using actin levels to normalize the results.

With only one transfection, the blots showed that the siRNA targeting Cdc42 has a silencing effect on the expression of endogenous Cdc42 of about 60 ± 8% in BON cells (4 experiments) and 67 ± 22% in PC12 cells (2 experiments). With a two-fold transfection (24h between transfections), the results for BON cells were 87% using dharmafect or 100% using interferin, as transfectants. For PC12 cells, the percentage of silencing reached is 56% (see Figure 1).
Figure 1: Silencing results from single and double transfection with siRNA against Cdc42. (A) Histogram presenting the silencing effect on BON and PC12 cells after one single transfection with siRNA targeting Cdc42. (B) Histogram presenting the silencing effect on BON and PC12 cells after two-fold transfection with siRNA targeting Cdc42.

Silencing Effects on Cell Morphology

Cells were transfected with Cdc42 siRNA or Lamin siRNA (control assay) for 72h. Then, as described above, the cell containing coverslips were observed using an inverted microscope Nikon TE 2000E and a coolSNAPES CCD camera (see Figure 2).

Figure 2: Observation of NPY-GFP in BC21 cells transfected with siRNA Cdc42 or lamin. The digital images were taken 72h after transfection. a and b refer to cell extensions. Bar, 15µm.

A typical BON cell exhibits a spread-out triangular shape with SG enriched peripheral regions. The preliminary observations indicated that cells, after Cdc42 silencing, present a changed morphology under lower density (their extensions were longer and slimmer than in control cells) and a smaller cellular body. The term extension is used to refer to the plasma membrane prolongations that grow from the main cell body (see Figure 2). To quantify these observations, pictures were taken with a 10x objective of both control and silenced cells. Firstly, the number of cells with slim extensions was compared with the number of present cells per field. Secondly, the average number of extensions per cell was determined and, thirdly, the length of each observable extension was measured. The results can be seen in Table 1, Table 2 and Table 3.

<table>
<thead>
<tr>
<th>Table 1: Determination of the percentage of cells with slim extensions.</th>
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<tr>
<td>517 cells transfected with siRNA Lamin and 560 cells transfected with Cdc42 were observed.</td>
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<tr>
<td></td>
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<tr>
<td>Cells transfected with siRNA Lamin</td>
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<tr>
<td>Cells transfected with siRNA Cdc42</td>
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<table>
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<th>Table 2: Average number of extensions per cell.</th>
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<tr>
<td>30 cells transfected with siRNA Lamin and 42 cells transfected with Cdc42 were observed.</td>
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<tr>
<td></td>
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<tr>
<td>Cells transfected with siRNA Lamin</td>
</tr>
<tr>
<td>Cells transfected with siRNA Cdc42</td>
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Table 3: Average extension length.
145 cells transfected with siRNA Lamin and 141 cells transfected with Cdc42 were observed.

<table>
<thead>
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<th>Average extension length (µm)</th>
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<tr>
<td>Cells transfected with siRNA Lamin</td>
<td>2.97</td>
</tr>
<tr>
<td>Cells transfected with siRNA Cdc42</td>
<td>5.86</td>
</tr>
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</table>

From the results shown above, it is possible to notice that cells subjected to Cdc42 silencing have fewer extensions but thinner and longer. These morphological effects confirm the efficacy of the Cdc42 knockdown in transfected cells.

We decided to look at the actin cytoskeleton inside BON cells after Cdc42 silencing. Actin can be visualized after fixation of the cells and labelling with the rhodamin phalloidin (in red). First, we made two observations: (i) presence of thin and dense labelling like compact cable structures in close vicinity of the plasma membrane; (ii) less small filopodia that emerge from cell cytoplasm.

The first observation is difficult to quantify, however it is easily seen by imaging.

![Cell transfected with siRNA against Lamin](image1.png)

![Cell transfected with siRNA against Cdc42](image2.png)

Figure 3: Observation of microfilaments marked with rhodamine phaloidin in BON AOC cells.

To quantify the number of filopodia present in cells, photos were taken and divided in smaller fields. Then, the number of visible filopodia was counted in each field and an average was determined (see Table 4)

Table 4: Average number of filopodia per field in cells transfected with siRNA against Cdc42 or lamin.

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>siRNA</th>
<th>Filopodia/field</th>
<th>Number of fields</th>
</tr>
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<tbody>
<tr>
<td>BC21</td>
<td>Lamin</td>
<td>4.8 ± 0.7</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Cdc42</td>
<td>3.4 ± 0.4</td>
<td>46</td>
</tr>
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Cells transfected with siRNA against Cdc42 present lower number of filopodia (see Table 4). The decrease is estimated at 30%.

However, in PC12 cells, no main morphological effect is observable.

Secretory Granules (SG) distribution within cells

SGs are generally enriched at the cell periphery and in cell extensions. Frequently, they can also be scattered in the cell and, under certain conditions, concentrate in the perinuclear region, particularly, in Golgi area.

An important issue was to observe the influence of the decrease of endogenous Cdc42 on SG localization within the cells, so we decided to verify the position of SG enrichment in silenced cells. BON cells expressing the NPY-GFP granule marker were visualized using an inverted microscope Nikon TE 2000E and a coolSNAPES CCD camera. To acquire digital images the software MetaVue was used. Two experiments were performed: one in BC21 cells and another in BON AOC transfected with NPY-GFP. The number of cells with peripheral SG enrichment was counted and compared with the number of cells per field The results pointed to a slight decrease on peripheral distribution in silenced cells (~30%), however, the difference is not significant (see Table 5).
Table 5: Quantification of cells with peripheral SG distribution

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>siRNA</th>
<th>Cells with SG at periphery (%)</th>
<th>Number of observed cells</th>
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<tbody>
<tr>
<td>BC21</td>
<td>Lamin</td>
<td>58 ± 10</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Cdc42</td>
<td>41 ± 12</td>
<td>181</td>
</tr>
<tr>
<td>BON</td>
<td>Luciferase</td>
<td>61</td>
<td>44</td>
</tr>
<tr>
<td>AOC</td>
<td>Cdc42</td>
<td>40</td>
<td>88</td>
</tr>
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Furthermore, we also looked for granules in the slim extensions of cells and we noticed their presence. Thus, it is thought that the absence of Cdc42 doesn’t prevent granule migration to the periphery of cells.

**Secretory Vesicles (SV) density in cell periphery**

In order to determine whether the Cdc42 silencing has an effect on the number of VS close to plasma membrane, the most suitable technique is the Total Internal Reflection Fluorescence Microscopy (TIRFM). Therefore, BC21 cells expressing the fluorescent NPY-GFP as a SG masker and transfected with siRNA lamin or Cdc42 were checked with TIRF microscope.

Films at 10Hz were taken at a depth of 100nm. In those films it is possible to visualize the SG in a well defined cell footprint (see Figure 4). Therefore, the number of vesicles was determined using Multidimensional Image Analysis (MIA) software, and expressed as the density of SG for 100µm² of print size.

![Figure 4: SG peripheral density in BON cells at 100nm. (A) et (B) BC21 cellular print observer by TIRFM. (A) Cell transfected with siRNA targeting lamin. (B) Cell transfected with siRNA targeting Cdc42. (C) Histogram illustrating SG quantification of data. For each cell, SG density was determine by dividing the number of SG by the size of the cell print. The results are expressed as the number of VS per 100µm² of print size. Single experiment: 16 cells transfected with siRNA Lamin and 16 cells transfected with Cdc42 were observed.](image)

This experiment shows an increase in the number of NPY-GFP positive granules at the periphery of cells after Cdc42 silencing. This increase is estimated at 44%.

**Secretion assays**

**Serotonin Secretion**

In order to determine the effect of Cdc42 on exocytosis, we measured secretion on cells after Cdc42 silencing, in both stimulated and non-stimulated conditions. Therefore, assays of secretion were made in non-transfected cells (NT cells), cells transfected with siRNA control (control cells) and cells transfected with Cdc42 siRNA. The cell lines used were BON, BC21 and PC12. A first series of experiments was done 72h after one or two transfection with siRNA duplex. The corresponding Cdc42 silencing level goes around 60%.

Radioactivity levels are expressed in percentage of [³H]5-HT in the supernatant compared to the total contents (supernatant and intracellular medium). In the absence of Ca²⁺ ionophore, unstimulated cells
illustrate the experiment noise. As small is the corresponding signalling value, as low is the noise. To determine the ratio signal-to-noise is fundamental for a proper result interpretation.

Four independent secretion experiments were made in BON cells (three in BON AOC and one in BC21) using siRNA against lamin as siRNA control. The results are summarized in Figure 5:

![Figure 5](image)

In Figure 5, the results for control cells are much smaller than in non-transfected cells. The decrease is about 40%. This observation questions the validity of siRNA Lamin as a control because, normally, control conditions should be similar to non-transfected conditions. It is not possible to know if the secretion decrease is due to off-target effects or not. Thus, to overcome this obstacle, we tried secretion assays with another siRNA control: two secretion tests were realized using siRNA targeting GFP. The correspondent results can be seen in Figure 6.

![Figure 6](image)

The results presented in Figure 6 show very similar results between control and non-transfected cells. This fact infers that siRNA against GFP is a better siRNA control than against lamin.

In order to determine the effect of Cdc42 knockdown in secretion assays using lamin siRNA, it has been considered a good option to use non-transfected cells as reference. Notice that, even if it is not the current procedure, the similarity between GFP siRNA and non-transfected results points to its validity. In this way, it is possible to use all secretion tests realized in order to take conclusions.

Surprisingly, by observing Figure 5 and Figure 6, no difference in secretion stimulation can be detected in BON cells exposed to Cdc42 silencing (16 ± 3 %) or reference cells (16 ± 3 %) (non-transfected or transfected siRNA targeting GFP).

Three secretion experiments were realized also in PC12 cells. These cells present a better response to Ca\textsuperscript{2+} stimulation and do not exhibit a decrease in secretion when transfected with siRNA lamin. Therefore, the two experiments done using lamin siRNA as control and the single experiment using GFP siRNA, will be treated together. Results are shown in Figure 7.
Figure 7: Secretion results in PC12 cells using siRNA lamin and GFP as siRNA control. (A) Table summarizing the results from the two secretion experiments. (B) Histogram illustrating percentage of Specific secretion. NT – non-transfected cells; CTRL – cells transfected with siRNA lamin; GFP – cells transfected with siRNA GFP; Cdc42 – cells transfected with siRNA Cdc42; Stim – Ca2+ stimulation condition; NStim – non-stimulation condition.

From Figure 7, it is possible to notice a small decrease in stimulated secretion in cells transfected with siRNA targeting Cdc42 of 15% which means nothing. However, this phenomenon is reproducible and observed in the three independent experiments; it cannot be neglected.

The published paper of Gasman et al (2006) states that reduction of endogenous Cdc42 inhibited the secretion of growth hormone stimulated by a depolarizing concentration of K+ (about 70% reduction in secretion). One possible explication for the difference on our results is that, in their paper, they analyse the secretion of a macromolecule, the human growth hormone, and here, it is measured the secretion of the monoamine serotonin. The size of the molecule secreted can be the cause of this incompatibility of results. As it is currently known, there are different mechanisms by which exocytosis can occur. For instance, after vesicle docking at plasma membrane, two phenomena can take place:

- a small pore is formed between the vesicle and plasma membranes and only small molecules can pass through and be released in the extracellular medium.
- both vesicle and plasma membranes fuse completely and there is a total release of the vesicle contains in the extracellular medium (notice that, beside peptides and other small molecules, granules are rich in macromolecules that form a dense matrix);

Thus, one hypothesis would be that Cdc42 is involved in the process of complete fusion between vesicular and plasma membranes and so, in its absence, the secretion of macromolecules is inhibited but not the secretion of smaller molecules, as monoamines.

Chromogranin and GFP Secretion

To test this hypothesis, the following step was to study the secretion of a macromolecule, endogenous chromogranin (70kDa). This protein comes from the intragranular matrix and is released upon secretion.

Western Blotting assays were realized with the supernatant of cells stimulated and non-stimulated, in order to detect chromogranin secretion. In parallel, it has also been observed NPY-GFP secretion. This GFP tagged protein is a granule marker that is also released upon secretion. It exhibits, with the GFP tag, an intermediate size between serotonin and chromogranine (~43kDa).

A signal is clearly detected in both chromogranine and NPY-GFP. However, there is no main difference between stimulated and non-stimulated conditions, neither between the three siRNA transfection conditions (NT, CTRL and Cdc42). These observations lead to the idea that maybe there could be lysed cellular contents in the supernatants. Therefore, to test if the detected signals are secreted molecules and not intracellular content, we immunoblot an intracellular protein not secreted: actin. A strong actin signal is detected in the supernatants which means that, effectively, they are contaminated with cellular debris. The main conclusion to be taken is that, for studying stimulated secretion of chromogranine and NPY-GFP in BON cells, Western blot is not the more suitable technique.
**Discussion**

The several silencing tests done in this work allowed us to guarantee at least 60% of reduction in Cdc42 expression within cells (both BON and PC12 cell lines). Under these silencing conditions, BON cells present a changed morphology; their extensions were longer and slimmer than in control cells and the cellular body appeared to be smaller. These effects confirm knockdown Cdc42 efficacy in BON cells, and indicate whether there is silencing before doing Western Blot. However, in PC12 cells, no main morphological effect is observable which brings evidence that different cell lines may respond distinctly to the same treatment.

The analysis of photos taken to BC21 cells indicates a decrease of 30% on the number of filopodia between silenced and control cells. It must be noticed that a more substantial decrease was expected under our silencing conditions. We think that the complexity inherent to all these regulation pathways must be behind this phenomenon. For instance, it is known that Rif/RhoF, RhoD and Wrch1 can all induce filopodium extensions, and so, might substitute Cdc42 (Aspenstrom et al., 2004; Ellis et al., 2005; Pellegrin et al., 2005).

Another interesting observation in silenced cells morphology is the presence of dense bundles of actin in cell periphery, mainly parallel to the plasma membrane. More investigations have to be done to determine the origin of these structures, like using TIRFM with actin tagged GFP.

To study the effect of Cdc42 silencing in SG docking at the plasma membrane, we choose to do a preliminary observation of SG density only in this region. With this purpose, we used single images taken from films shot by TIRFM, of BC21 cells at 100nm of membrane penetration depth. We noticed that Cdc42 silenced cells exhibit a little increase in granular density (44%). These results support the idea that the absence of Cdc42 in cells does not prevent SG migration to the periphery, but indicate that Cdc42 can have a role in controlling the accumulation towards the plasma membrane. Furthermore, SGs are even able to reach the subplasmalemmal region where, maybe, they will dock.

After confirming the effectiveness of the Cdc42 silencing conditions, we tried to confirm the effect on secretion with our experimental approach. Therefore, assays of secretion on release of serotonin were made in cells transfected with siRNA control and cells transfected with Cdc42 siRNA. The cell lines used were BON and PC12. The secretory assays worked satisfactorily, we have around 15% secretion in BON and 40% in PC12 (results coherent with previous tests realized by the host laboratory).

Surprisingly, probably due to the size of the secreted molecules, our results are distinct from the ones published in Malacombe et al. (2006). We do not see a significant inhibition in secretion (15% at most compared to their 70%). The main hypothesis is that Cdc42 interferes with one of the mechanisms that involve the formation of a fusion pore through which molecules can be released. This idea was born from the assumption that the opening and/or closure of the fusion pore is based on the observable formation of microfilaments around the granules at docking sites. These filaments may be produced by the combined action of Cdc42, N-WASP and Arp2/3 (Gasman, 2004). Additionally, it has been demonstrated that both full fusion and “kiss and run” occur to varying membrane extents in neuroendocrine cells and may depend on stimulation conditions. Consequently, if the absence of Cdc42 inhibits the secretion of macromolecules but not of small molecules, it is very probable that Cdc42-triggered actin filaments may grant a mechanism to activate full fusion exocytosis.

To test the validity of this hypothesis is extremely interesting. For that reason, the following step is to study the secretion of a macromolecule, chromogranin (70kDa), once it is a protein from the intragranular matrix that is released upon secretion. Unfortunately, we realized that Western Blot is not a suitable technique to detect secreted proteins in BON cells. The contamination with lysed cellular contents prevents us to distinguish the levels of secretion. Therefore, in next studies, stimulated secretion of chromogranine tagged GFP and NPY-GFP will be analysed by imaging, a current procedure in the host laboratory.

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