Regulated Exocytosis in Neuroendocrine Cells: A Role for Subplasmalemmal Cdc42/N-WASP–induced Actin Filaments

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In neuroendocrine cells, actin reorganization is a prerequisite for regulated exocytosis. Small GTPases, Rho proteins, represent potential candidates coupling actin dynamics to membrane trafficking events. We previously reported that Cdc42 plays an active role in regulated exocytosis in chromaffin cells. The aim of the present work was to dissect the molecular effector pathway integrating Cdc42 to the actin architecture required for the secretory reaction in neuroendocrine cells. Using PC12 cells as a secretory model, we show that Cdc42 is activated at the plasma membrane during exocytosis. Expression of the constitutively active Cdc42^{L61} mutant increases the secretory response, recruits neural Wiskott-Aldrich syndrome protein (N-WASP), and enhances actin polymerization in the subplasmalemmal region. Moreover, expression of N-WASP stimulates secretion by a mechanism dependent on its ability to induce actin polymerization at the cell periphery. Finally, we observed that actin-related protein-2/3 (Arp2/3) is associated with secretory granules and that it accompanies granules to the docking sites at the plasma membrane upon cell activation. Our results demonstrate for the first time that secretagogue-evoked stimulation induces the sequential ordering of Cdc42, N-WASP, and Arp2/3 at the interface between granules and the plasma membrane, thereby providing an actin structure that makes the exocytotic machinery more efficient.

INTRODUCTION

Rapid remodeling of the actin cytoskeleton is required for a variety of cellular processes, including cell morphology, locomotion, polarity, and adhesion (Borisy and Svitkina, 2000). Over the past years, actin has emerged as a key player in membrane trafficking, especially in exo- and endocytotic events (Doussau and Augustine, 2000; Jeng and Welch, 2001). Neuroendocrine cells, like most secretory cells, display a dense network of filamentous actin beneath their plasma membrane. This actin web is classically viewed as a physical barrier to exocytosis by restricting the access of secretory granules to their release sites at the plasma membrane (Aunis and Bader, 1988; Vitale *et al.*, 1995). However, evidence is emerging that actin is not simply a physical barrier but could be responsible for an active step in exocytosis. For example, agents that completely depolymerize actin filaments have been shown to inhibit secretagogue-induced exocytosis in various secretory cells, including PC12 cells (Matter *et al.*, 1989), HIT insulin-secreting cells (Li *et al.*, 1994), and mast cells (Pendleton and Koffer, 2001). Moreover, a recent report in PC12 cells, illustrated that actin can both hinder and mediate movements of green fluorescent protein (GFP)-labeled dense-core granules in the sub-

plasmalemmal region (Lang *et al.*, 2000). Thus, the actin cytoskeleton seems to be necessary for regulated exocytosis to occur. However, to what extent secretion in neuroendocrine cell requires de novo actin polymerization and the regulatory mechanisms underlying this event remains to be elucidated.

Dynamic regulation of the actin cytoskeleton has been shown to involve the Rho GTPase family (Hall, 1998). Belonging to the small GTP-binding proteins of Ras superfamily, mammalian Rho GTPases consist of at least 20 distinct members, of which RhoA, Rac1, and Cdc42 are the best characterized (Etienne-Manneville and Hall, 2002). Several recent studies have demonstrated the involvement of Rho proteins in a wide range of membrane trafficking aspects (Ridley, 2001). Indeed, RhoB and RhoD regulate endosomal trafficking (Gampel *et al.*, 1999; Gasman *et al.*, 2003), whereas RhoA and Rac are involved in receptor internalization (Lamaze *et al.*, 1996), phagocytosis (Caron and Hall, 1998), and neurotransmitter release (Doussau *et al.*, 2000). In chromaffin cells, we previously proposed that reorganization of the actin cytoskeleton underlying membrane trafficking at the site of exocytosis is under the combined control of two members of the Rho family. RhoA bound to secretory granules was found to maintain actin filaments in the vicinity of secretory granules by modulating the activity of a granule-associated phosphatidylinositol-4 kinase (Gasman *et al.*, 1997; Gasman *et al.*, 1998). However, inactivation of RhoA did not modify secretagogue-evoked secretion, excluding the active participation of RhoA in mediating actin structures required for the exocytotic machinery (Gasman *et al.*,

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1999). Conversely, in mast cells, RhoA has been involved in secretion but through a pathway that does not involve actin (Sullivan *et al.*, 1999). Further investigations led us to the idea that Cdc42 might play an actin-dependent function in chromaffin cell secretion (Gasman *et al.*, 1999). The active participation of Cdc42 in the exocytotic reaction in mast cells and pancreatic β cells has also been described (Kowluru *et al.*, 1997; Brown *et al.*, 1998). In view of these observations, Cdc42 seems to be a good candidate to coordinate the actin architecture to the molecular machinery underlying exocytosis. To date, several functionally distinct Cdc42 effectors have been identified (Cotteret and Chernoff, 2002). Among them, the neural Wiskott-Aldrich syndrome protein (N-WASP) links Cdc42 to actin polymerization through the actin-related protein-2/3 (Arp2/3) complex, which promotes actin nucleation and polymerization (Carlier *et al.*, 1999; Rohatgi *et al.*, 1999; Higgs and Pollard, 2001).

The aim of the present work was to investigate the role of Cdc42 in the exocytotic process in PC12 cells. We report that in secretagogue-stimulated cells, subplasmalemmal Cdc42 switches to its active GTP-bound form and facilitates secretion by promoting actin polymerization in the cell periphery. Our results reveal that N-WASP and Arp2/3 are the molecules that bridge Cdc42 signaling to the actin cytoskeleton and the exocytotic machinery.

MATERIALS AND METHODS

Antibodies and DNA Constructs

The following antibodies were used: rabbit polyclonal anti-Cdc42 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-chromogranin B (CGB) antibodies (kindly provided by Dr. M.H. Metz-Boutigue, INSERM U-575, Strasbourg, France), monoclonal anti-hemagglutinin (HA) antibodies (Babco, Richmond, CA), polyclonal anti-p34-Arc antibodies (Upstate Biotechnology, Euromedex, France), and monoclonal anti-SNAP25 antibodies (Sternberger Monoclonals, Lutherville, MD). Cyanine-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Amersham Biosciences (Les Ulis, France).

The N-terminally GFP-tagged human Cdc42^{N17}, Cdc42^{L61} (G25 isoform), and all the WASP constructs were described previously (Moreau *et al.*, 2000). GFP-Cdc42^{L61A124} was generated by site-directed mutagenesis with a QuikChange mutagenesis kit from Stratagene (La Jolla, CA). Cdc42^{L61C40} was a gift from V. Moreau (INSERM U-441, Pessac, France). Myc-tagged PAK1 constructs (wild-type PAK1, PAK1^{T423E} and PAK1^{K299R} mutants; Sells *et al.*, 1999) were given by N. Vitale (CNRS UPR-2356, Strasbourg, France). The GFP-tagged TC10^{L75} and TCL^{L79} were gifts from A. Blangy (CNRS UPR-1086, Montpellier, France).

Culture, Transfection, and Assay of Growth Hormone (GH) Release from PC12 Cells

PC12 cells were grown in DMEM supplemented with glucose (4500 mg/l) and containing 30 mM NaHCO₃, 5% fetal bovine serum, 10% horse serum, and 100 U/ml penicillin/streptomycin. Mammalian expression vectors were introduced into PC12 cells together with the GH plasmid pXGH5 (six-well dishes, 80% confluence, 4 μ g/well of each plasmid) by using GenePorter (Gene Therapy Systems) according to the manufacturer's instruction.

GH release experiments were performed 48 h after transfection. PC12 cells were washed twice with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.2) and then incubated for 10 min with calcium-free Locke's solution (basal release) or stimulated with an elevated K⁺ solution (Locke's containing 59 mM KCl and 85 mM NaCl). The supernatant was collected and the cells harvested by scraping in 10 mM phosphate-buffered saline. The amounts of GH secreted into the medium and retained in the cells were measured using a radioimmunoassay kit (Nichols Institute, San Juan, Capistrano, CA). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

[³H]Noradrenaline Release from Chromaffin Cells

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase, purified on self-generating Percoll gradients, and cultured as monolayers on 24 multiple 16-mm Costar plates at a density of 2.5×10^5 cells/well. For release experiments, cells were labeled with [³H]noradrenaline (14,68 Ci/mmol; PerkinElmer Life Sciences, Boston,

MA) for 60 min, washed four times with Locke's solution, and subsequently stimulated for 10 min with Locke's solution containing 10 μ M nicotine. [³H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium and in cells after precipitation with 10% (wt/vol) trichloroacetic acid. Release of [³H]noradrenaline is expressed as a percentage of total radioactivity present in the cells before Ca²⁺-induced stimulation.

Pull Down Assay for Cdc42-GTP

Activated Cdc42 was precipitated using a Rac/Cdc42 activation assay kit (Upstate Biotechnology). After stimulation, PC12 cells were immediately lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 5 mM EDTA, 1% NP-40, 10% glycerol, 0.1 mM Na₃VO₄, 4 mM NaF and mammalian protease inhibitor cocktail [1:100 dilution]; Sigma, Saint Quentin Fallavier, France). GTP-bound Cdc42 was pulled down by incubating lysates containing equal amounts of proteins (700 μ g) with the Cdc42-interacting domain (CRIB) domain of PAK1 (p21 activated kinase) for 2 h at 4°C. Lysates loaded with guanosine 5'-O-(3-thio)triphosphate and GDP served, respectively, as positive and negative controls. The precipitated GTP-bound Cdc42 was resolved on 12% polyacrylamide-SDS gels and immunoblotted with antibodies specific for Cdc42 (1:400). Blots were processed using the Western-Light Plus chemiluminescent detection system (Tropix, Bedford, MA).

Immunoblotting, Immunofluorescence, Confocal Microscopy, and Image Analysis

One dimensional SDS gel electrophoresis was performed on 12% acrylamide gels in Tris-glycine buffer. The proteins were transferred to nitrocellulose sheets at a constant current of 100 mA for 2 h. Blots were processed using the Western-Light Plus chemiluminescent detection system. Immunoreactive bands from Western blot experiments were quantified using ImageJ 1.29 $\times$ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

For immunocytochemistry, PC12 cells grown on poly D-lysine-coated glass coverslips were maintained in Locke's solution or stimulated with elevated K⁺. The cells were then fixed for 20 min in 4% paraformaldehyde in 0.12 M sodium/phosphate buffer, pH 7.0, and for a further 10 min in fixative containing 0.1% Triton X-100. Actin filaments were stained by incubation with rhodamine-conjugated phalloidin (Sigma) at concentration of 0.2 μ g/ml in phosphate-buffered saline for 15 min. Immunostaining was performed as described previously (Vitale *et al.*, 2001), and stained cells were visualized using a confocal microscope LSM 510 (Carl Zeiss, Jena, Germany). Using the Zeiss CLSM instrument software 2.8, the proportion of Cdc42/p34-Arc colocalized with SNAP-25 or p34-Arc colocalized with chromogranin B was estimated from the double-labeled pixels, expressed as an average fluorescence intensity normalized to the corresponding surface area, and calculated as a percentage of the total Cdc42/p34-Arc fluorescence detected in each cell. The amount of F-actin present in PC12 cells was measured and expressed as an average fluorescence intensity normalized to the corresponding surface area and divided by the total surface of each cell. This allows a comparison of the F-actin content in different cells.

Subcellular Fractionation

Subcellular fractionation of PC12 cells was performed as described previously (Vitale *et al.*, 2002). Briefly, cells were washed twice with Locke's solution and then incubated for 10 min with Locke's solution (resting condition) or stimulated with an elevated K⁺ solution. Medium was removed and cells immediately scrapped in 1 ml of sucrose 0.32 M (20 mM Tris-HCl, pH 8.0). Cells were broken in a Dounce homogenizer and centrifuged at 800 $\times$ g for 15 min. The supernatant was centrifuged at 20,000 $\times$ g for 20 min. The resulting supernatant was further centrifuged for 60 min at 100,000 $\times$ g to obtain the cytosol (supernatant) and microsomes (pellet enriched in endosomes). The 20,000 $\times$ g pellet containing the crude membrane fraction was resuspended in sucrose 0.32 M (20 mM Tris-HCl, pH 8.0), layered on a cushion sucrose density gradient (sucrose 1–1.6 M, 20 mM Tris-HCl, pH 8.0) and then centrifuged for 90 min at 100,000 $\times$ g. The upper fractions containing the plasma membrane and the pellet containing secretory granules were collected.

RESULTS

Cdc42 Is Activated in Response to a Secretagogue and Modulates Regulated Exocytosis in PC12 Cells

If Cdc42 is implicated in the control of dense-core granule exocytosis, then it is reasonable to think that it should be activated in response to a secretagogue. PC12 cells were maintained under resting conditions or stimulated for various periods of time with depolarizing concentration of potassium, and Cdc42 activation was measured using the PAK1-binding domain as a bait to trap the GTPase in its GTP-bound form (Figure 1A). We found that the amount of

Figure 1. Peripheral Cdc42 is activated in stimulated PC12 cells and expression of the GTP-loaded mutant enhances the secretory response. (A) GTP-loaded Cdc42 pull down assay in resting and stimulated PC12 cells. PC12 cells were stimulated with 59 mM K⁺ for the indicated periods of time and/or maintained in Locke's solution for 10 min (resting). Cells were then immediately lysed by addition of ice-cold lysis buffer, and the lysate (700 μ g) was used for affinity precipitation of GTP-loaded Cdc42. Pulled down Cdc42-GTP and Cdc42 in lysates (1/30 of the total) were detected by immunoblotting by using anti-Cdc42 antibodies. The histogram illustrates a semiquantitative analysis of Cdc42 activation upon cell stimulation. Values obtained by scanning densitometry analysis are given as the mean values $\pm$ SEM ($n = 3$) and expressed as the percentage of GTP-loaded Cdc42 relative to the total Cdc42. (B) Localization of endogenous Cdc42 in PC12 cells. Confocal immunofluorescent images obtained by labeling resting and K⁺-stimulated PC12 cells with anti-Cdc42 antibodies visualized with Cy2-conjugated secondary antibodies and monoclonal anti-SNAP25 antibodies visualized with Cy3-conjugated secondary antibodies. Masks representing the regions of Cdc42/SNAP25 colocalization were generated by selecting the double-labeled pixels. Bar, 5 μ m. The histogram represents a semiquantitative analysis of the percentage of Cdc42 signal colocalized with SNAP25 in resting (R) and stimulated cells (S). Data are given as the mean values $\pm$ SEM ($n = 10$). (C) Distribution of Cdc42 in PC12 cell subcellular fractions. Resting or K⁺-stimulated PC12 cells were lysed and processed for subcellular fractionation on sucrose gradients. Fractions containing the cytosol (Cy), plasma membrane (PM), secretory granules (Gr), and endosomes (Endo) were collected, separated by gel electrophoresis (15 μ g protein/fraction), and

GTP-bound Cdc42 is relatively low in resting cells. However, 2–10 min of stimulation with 59 mM K⁺ increased the level of cellular Cdc42-GTP by three- to fourfold, respectively (Figure 1A, histogram). Importantly, Cdc42-GTP rapidly decreased to basal levels as cells returned to the resting condition, revealing a tight coupling between membrane depolarization and Cdc42 activation. Because the immediate consequence of membrane depolarization is calcium influx and secretion, these findings indicate that Cdc42 activation accompanies the exocytotic process.

Next, we examined the distribution of Cdc42 in resting and stimulated PC12 cells. As illustrated in Figure 1B, Cdc42 immunoreactivity was essentially detected in the cell periphery. Double labeling with antibodies against the plasma membrane marker SNAP25 revealed a partial association with the plasma membrane that markedly increased upon stimulation with 59 mM K⁺ (Figure 1B, mask). Quantification of the relative proportion of Cdc42 colocalizing with SNAP25 indicated that 23% of the detected Cdc42 immunoreactivity was found associated to the plasma membrane marker in stimulated cells compared with 5% in resting cells (Figure 1B, histogram). When the distribution of Cdc42 was examined in subcellular fractions obtained from resting and stimulated PC12 cells, most of the protein was found in the soluble fraction (Figure 1C). However, stimulation with K⁺ induced also a reproducible threefold increase in the amount of Cdc42 detected in the fraction containing the plasma membrane (Figure 1C, histogram).

To directly establish whether Cdc42 plays a role in exocytosis, we examined the effect of expressing a GFP-tagged Cdc42 mutant defective in GTP hydrolysis (Cdc42^{L61}) and its corresponding dominant inactive mutant preferentially binding GDP (Cdc42^{N17}) on exocytosis by using GH as a secretory reporter (Vitale *et al.*, 2001). We found that Cdc42 mutants modified GH secretion in response to high potassium (Figure 1D). Expression of the constitutively active Cdc42^{L61} increased GH release by ~60%, whereas the dominant negative Cdc42^{N17} slightly but significantly decreased it (~25% inhibition). Thus, Cdc42 is able to play a positive role in the exocytotic pathway of large dense-core granules, raising the question about the underlying mechanism(s).

Cdc42 Facilitates Exocytosis through a Pathway Implicating the Actin Cytoskeleton

Many downstream targets for Cdc42 have been described, including phospholipase D (PLD) (Walker *et al.*, 2000), a

protein recently proposed as a key factor for the exocytotic machinery in PC12 cells (Vitale *et al.*, 2001). To test whether PLD might be the effector by which Cdc42 activates the exocytotic process, we superimposed the S124A mutation onto the constitutively active Cdc42^{L61}, leading to a mutant of Cdc42 defective in PLD activation but still able to activate other effectors (Walker and Brown, 2002). Expression of Cdc42^{L61A124} in PC12 cells stimulated GH release to a similar extent as that of Cdc42^{L61}, suggesting that PLD is not required for Cdc42-mediated exocytosis (Figure 2A). Given the well established role of actin in exocytosis, we next tested the double mutant Cdc42^{L61C40}, which is unable to interact with N-WASP and PAK (Owen *et al.*, 2000), two major Cdc42 effectors involved in actin organization (Cotteret and Chernoff, 2002). Interestingly, superimposing the Y40C mutation onto the constitutively active Cdc42^{L61} prevented its ability to stimulate exocytosis and instead, Cdc42^{L61C40} behaved as a dominant negative, inhibiting secretion to a similar extent as the inactive GDP-bound Cdc42^{N17} (Figure 2A). This observation suggests that N-WASP and/or PAK play a major role in the pathway by which Cdc42 regulates exocytosis.

To further probe the idea that Cdc42 regulates the secretory response by controlling actin dynamics, we overexpressed GFP-tagged mutants of Cdc42 and examined their effect on the cortical actin network in stimulated PC12 cells. As shown in Figure 2B, Cdc42^{N17} displayed a diffuse staining pattern, indicating a predominant localization to the cytosol. In contrast, the active Cdc42^{L61} mutant exhibited a major staining in the cell periphery consistent with the plasma membrane localization of endogenous Cdc42 (Figure 2B). In chromaffin and PC12 cells, the majority of actin filaments are concentrated in the subplasmalemmal region, forming a continuous cortical actin network that is partially disassembled upon activation of exocytosis (Gasman *et al.*, 1997; Vitale *et al.*, 2001). Hence, rhodamine-phalloidin staining in PC12 cells expressing Cdc42^{N17} revealed a classical disrupted cortical actin network commonly observed in stimulated cells (Figure 2B). Surprisingly, expression of Cdc42^{L61} enhanced the rhodamine-phalloidin fluorescence in the cell periphery, indicating that the GTP-bound form of Cdc42 triggers actin polymerization in stimulated cells (Figure 2B). These observations suggest that activated Cdc42 facilitates exocytosis through the de novo formation of actin filaments in the subplasmalemmal region. This implies that cortical actin, classically viewed as a barrier that hinders the recruitment of secretory granules to the plasma membrane, may also play a positive role in the exocytotic process. Accordingly, treatment of cultured chromaffin cells with latrunculin B, an actin-filament-disrupting molecule, produced a dual effect on secretion: it increased catecholamine release at a low concentration but progressively inhibited it at higher doses (Figure 2C). Similar results were observed in PC12 cells (Matter *et al.*, 1989), in agreement with the idea that a minimal actin structure is necessary for exocytosis to occur.

N-WASP Stimulates Secretion and Promotes Actin Polymerization in PC12 Cells

To discriminate between N-WASP and PAK as the molecular effectors that link Cdc42 to the actin cytoskeleton and exocytosis, we expressed in PC12 cells wild-type and mutated N-WASP or PAK proteins and examined their effect on K⁺-evoked GH secretion. As shown in Figure 3A, N-WASP expression resulted in a stimulation of GH secretion as efficient as that effected by Cdc42^{L61}. In contrast, expression of the Myc-tagged wild-type PAK1 (our unpublished data), the

Figure 1 (cont). transferred to nitrocellulose for immunoblotting analysis with anti-Cdc42 antibodies. Similar results were obtained in three independent fractionation performed with different cell cultures. The histogram represents a semiquantitative analysis of the Cdc42 immunoreactivity detected in the plasma membrane under resting (R) and stimulating conditions (S). Data are given as the mean values $\pm$ SEM ($n = 3$) and expressed as the percentage of plasma membrane-bound relative to the total Cdc42. (D) Cdc42 stimulates dense-core granule exocytosis in PC12 cells. PC12 cells were transfected with pCB6-GFP (control), pCB6-GFP-Cdc42^{L61}, or pCB6-GFP-Cdc42^{N17} along with the plasmid encoding GH. At 48 h posttransfection, cells were washed and subsequently incubated for 10 min in calcium-free Locke's solution (LN) or stimulated for 10 min with 59 mM K⁺ (K59). GH secreted into the medium and retained in the cells was then estimated by radioimmunoassay. GH release is expressed as the percentage of total GH present in the cells before stimulation. Data are given as the mean values $\pm$ SEM ($n = 3$). * $p < 0.05$ when tested by Student's t test. Similar results were obtained in three independent experiments performed with different cell cultures.

Figure 2. Effect of various Cdc42 mutated proteins on secretagogue-evoked GH secretion and actin reorganization in PC12 cells. (A) Effect of dominant active Cdc42^{L61} constructs mutated in their effector domain on GH release in PC12 cells. PC12 cells cotrans-

constitutively active PAK1^{E423} or the kinase-dead PAK1^{R299} mutants produced no effect on GH secretion (Figure 3A), electing N-WASP as the best partner for Cdc42 in the exocytotic process. Note that expression of an N-WASP mutant containing only the CRIB and lacking the other functional motifs inhibited exocytosis by ~35% (Figure 3A). This effect could be due to the partial sequestration of endogenous Cdc42 because expression of CRIB^{D208} that is unable to interact with Cdc42 (Miki *et al.*, 1998), had no effect on GH release (Figure 3A). On the other hand, the CRIB domain-induced inhibition could be assigned to the sequestration of other Cdc42-related GTPases, which are known to interact with N-WASP like TC10 and/or TCL (Vignat *et al.*, 2000). To probe this idea, we examined the effect of expressing the constitutively active TC10^{L75} and TCL^{L79} mutants on GH release in PC12 cells. Both TC10^{L75} and TCL^{L79} localized in cell periphery as seen by fluorescent confocal microscopy (our unpublished data). TC10^{L75} inhibited GH secretion by ~30% (Figure 3B), an effect that might be related to a possible competition with endogenous Cdc42 for N-WASP interaction leading to an inhibition of secretion. TCL^{L79} did not modify K⁺-evoked secretion but it increased the basal release (Figure 3B). We have currently no explanation for this effect; however, endogenous TCL has not been detected in PC12 cells (Abe *et al.*, 2003). Together, these results indicate that, in contrast to Cdc42, neither TC10 nor TCL are able to act as a positive regulator of exocytosis in PC12 cells.

Next, we examined the exocytotic activity and actin filament organization in PC12 cells transfected with cDNAs encoding a series of truncated GFP-tagged N-WASP mutants differentially lacking important structural determinants, namely, ΔWH1-N-WASP, ΔWA-N-WASP, or the WA domain alone (Figure 4). The WH1 domain has an important function in N-WASP targeting (Moreau *et al.*, 2000). The confocal images presented in Figure 5 show that overexpressed N-WASP and ΔWH1-N-WASP displayed a predominant cytosolic staining in resting cells but partially translocated to the cell periphery in stimulated cells. This

fects with GH and the indicated Cdc42 constructs were incubated for 10 min in calcium-free Locke's solution or stimulated for 10 min with 59 mM K⁺. The net secretory response was obtained by subtracting the basal release from the release evoked by 59 mM K⁺. In the different condition tested, basal released ranged from 4.2 to 7.5%. As control, GH release was measured in PC12 cells overexpressing empty pBR6 vector. Data are given as the mean values ± SEM (n = 3). Similar results were obtained in three independent experiments performed with different cell cultures. (B) Active GTP-bound Cdc42 localizes in cell periphery and stimulates actin formation. PC12 cells expressing pCB6-GFP-Cdc42^{L61} or pCB6-GFP-Cdc42^{N17} (GFP) were stimulated for 10 min with 59 mM K⁺ and subsequently fixed and stained with rhodamine-conjugated phalloidin to visualize actin filaments (actin). The asterisk indicates a nontransfected cell displaying a classical disruption of the cortical actin network in response to K⁺-stimulation. Peripheral actin depolymerized similarly in cells expressing Cdc42^{N17}. In contrast, the presence of Cdc42^{L61} in the subplasmalemmal region triggered the formation of actin filaments in the cell periphery. Bar, 5 μm. (C) Actin filaments are essential components of regulated exocytosis. Cultured chromaffin cells were preincubated for 45 min with [³H]noradrenaline and then exposed 1 h to the indicated concentration of latrunculin B. Cells were subsequently washed and stimulated for 10 min with 10 μM nicotine. The net secretory response was obtained by subtracting the basal release from the release evoked by nicotine. In the different condition tested, basal released ranged from 3.4 to 5.5%. Data are given as the mean values ± SEM (n = 3). Similar results were obtained in two independent experiments performed with different cell cultures.

Figure 3. Cdc42 but not TC10/TCL, and N-WASP but not PAK1, enhance the exocytotic release of GH from PC12 cells. (A and B) PC12 cells coexpressing GH and the indicated proteins were incubated for 10' min in calcium-free Locke's solution or stimulated for 10 min with 59 mM K⁺. Control cells were transfected with the empty pBR6 vector. In A, basal release (~8%) was unchanged and was subtracted from the release evoked by 59 mM K⁺ to obtain the net secretory response. Data are given as the mean values $\pm$ SEM (n = 3). Similar results were obtained in three independent experiments performed with different cell cultures.

observation suggests that activation of exocytosis triggers the recruitment of N-WASP to the cell periphery and that the WH1 domain is not involved in this event. Similarly to Cdc42, these two constructs enhanced GH release by ~60 and ~75%, respectively (Figure 5C) and induced the formation of actin filaments in the subplasmalemmal region in high K⁺-stimulated cells (Figure 5, A and B). Compared with the full-length N-WASP, the Δ WH1 protein is more efficiently recruited to the cell periphery, induces more cortical actin filaments, and triggers a stronger secretory response (Figure 5, A and B). N-WASP is maintained in an autoinhibited conformation by intramolecular bounds that are relieved upon binding of Cdc42 or phosphoinositides

Figure 4. Schematic representation of N-WASP protein depicting the position of the various functional domains. WH1, WASP homology 1 domain; IQ, calmodulin-binding motif; PRD, proline-rich SH3-adaptor-binding domain; WH2, WASP homology 2 domains also called V for Verprolin-homology; C, cofilin homology region; and A, acidic domain. The WH2, C and A domains constitute the WA region (delineated by an arrow). The N-terminally GFP-tagged truncated mutants used in this study are indicated.

(Kim *et al.*, 2000; Prehoda *et al.*, 2000; Rohatgi *et al.*, 2000). Deleting the WH1 domain could weaken these intramolecular interactions and result in a protein that is unregulated or less regulated than the autoinhibited full length as already suggested (Moreau *et al.*, 2000).

To determine whether the stimulatory effect of N-WASP on secretion is dependent on its ability to promote actin polymerization in the cell periphery, we used a mutant lacking the functional WA domain interacting with G-actin and Arp2/3 (Δ WA-N-WASP). As previously described in adipocytes (Jiang *et al.*, 2002), Δ WA-N-WASP displayed a predominant nuclear localization in PC12 cells. Some Δ WA-N-WASP was nevertheless recruited to the cell periphery during cell stimulation, but this mutant was clearly less efficient in promoting the formation of actin filaments (Figure 5, A and B), and it did also not modify the secretory response (Figure 5C). Conversely, expression of the WA domain (WA) alone highly stimulated actin polymerization throughout the whole cell (Figure 5A) but failed to stimulate exocytosis (Figure 5C), most likely because WA was not recruited to the sites of exocytosis in stimulated cells. Together, these data indicate that the stimulatory effect of N-WASP on secretion is closely related to its ability to be recruited to the subplasmalemmal area and its capacity to induce actin filaments at the plasma membrane. These results reinforce also the idea that exocytosis requires to some extent the formation of actin filaments at the site of granule docking and fusion with the plasma membrane.

Cdc42 Recruits N-WASP at the Plasma Membrane

To strengthen the idea that activated Cdc42 is involved in the recruitment of N-WASP at the plasma membrane upon cell stimulation, we coexpressed the constitutively active Cdc42^{L61} or the inactive Cdc42^{N17} with the GFP-tagged N-WASP and examined their distribution by confocal microscopy (Figure 6A). In resting PC12 cells, N-WASP was predominantly cytosolic (Figure 5A). Hence, when individually coexpressed with the GDP-bound Cdc42^{N17}, N-WASP was also distributed into the cytosol (Figure 6A). In contrast, in cells expressing the active Cdc42^{L61}, N-WASP was found to colocalize with Cdc42 at the plasma membrane (Figure 6A), indicating that it was efficiently recruited by the GTP-bound form of Cdc42. To identify which domain is required for the recruitment of N-WASP, we coexpressed the active Cdc42^{L61} with the various N-WASP truncated mutants. As shown in Figure 6B, Cdc42 recruited similarly Δ WH1- and Δ WA-N-WASP but not the WA domain, suggesting that the recruitment of N-WASP was mediated through its CRIB domain. Accordingly, the CRIB domain was recruited to the plasma membrane by Cdc42^{L61}, whereas CRIB^{D208}, unable to interact with Cdc42, was not (Figure 6B). Together, these results are in line with the idea that GTP-loaded Cdc42 in secretagogue-activated cells is able to directly interact with N-WASP and thereby to recruit it at the plasma membrane.

To further demonstrate that the function of N-WASP lies downstream of Cdc42, we examined the effect of the dominant negative Δ WA-N-WASP mutant on the stimulation of exocytosis induced by the active Cdc42^{L61} (Figure 6C). Co-expression of Δ WA-N-WASP completely abolished the stimulatory effect of Cdc42^{L61} on GH release, confirming that N-WASP acts as a downstream effector of Cdc42 in the exocytotic pathway.

Presence of Arp2/3 on Large Dense-Core Secretory Granules

The carboxy terminus of WASP proteins requires the Arp2/3 complex to stimulate actin polymerization (Prehoda

Figure 5. N-WASP-induced actin polymerization in the subplasmalemmal region increases the exocytotic response in PC12 cells. PC12 cells were transfected with plasmids encoding the indicated GFP-tagged N-WASP mutants alone (A) or along with the plasmid encoding GH (C). Forty-eight hours after transfection, cells were stimulated for 10 min with 59 mM K^+ (stimulated) or maintained in calcium-free Locke's solution (resting) and then processed for immunocytochemistry or GH release assay. (A) Confocal images representing the distribution of the various GFP-tagged N-WASP mutants (GFP) and the actin filaments visualized with rhodamine-conjugated phalloidin. Nontransfected cells are indicated by asterisks. Masks representing the regions of colocalization (N-WASP constructs/actin filaments) were generated by selecting the double-labeled pixels. Bar, 5 μ m. (B) Semi-quantitative analysis of the F-actin content in K^+ -stimulated cells expressing the indicated N-WASP proteins. Data are given as the mean values $\pm$ SEM ($n = 10$) and expressed as arbitrary units (AU). (C) K^+ -evoked GH-secretion from cells expressing the indicated N-WASP constructs. Similar results were obtained in three independent experiments performed with different cell cultures. Data are given as the mean values $\pm$ SEM ($n = 3$). Note that the N-WASP stimulatory effect on GH secretion correlates with its ability to trigger actin filaments in the cell periphery.

Figure 6. GTP-loaded Cdc42 recruits cytosolic N-WASP to the cell periphery in resting PC12 cells. (A and B) PC12 cells expressing GFP-tagged N-WASP with either HA-tagged Cdc42^{L61} or HA-tagged Cdc42^{N17} (A) or PC12 cells expressing the indicated GFP-tagged N-WASP mutants along with HA-tagged Cdc42^{L61} (B). Cells were fixed, labeled with anti-HA antibodies (α -HA), and analyzed by confocal microscopy. HA antibodies were visualized with Cy3-conjugated secondary antibodies. Bar, 5 μ m. (C) K⁺-evoked GH-secretion from cells expressing Δ WA-N-WASP or Cdc42^{L61} alone or coexpressing these two mutants together. Similar results were obtained in three independent experiments performed with different cell cultures. Data are given as the mean values $\pm$ SEM (n = 3). Note that coexpression of Δ WA-N-WASP prevented the stimulatory effect of Cdc42^{L61}.

et al., 2000). We examined the intracellular distribution of Arp2/3 in PC12 cells, by using antibodies against p34-Arc, a subunit of the Arp2/3 complex. Double-labeling experiments with SNAP-25 revealed that p34-Arc was not found on the plasma membrane in resting cells but it displayed a punctuate pattern of distribution, suggesting an association with some intracellular membrane-bound compartment (Figure 7A). To probe an eventual association of Arp2/3 with secretory granules, double-labeling experiments were performed with antibodies against p34-Arc and against chromogranin B (CGB), a specific marker for secretory granule in PC12 cells. As shown in Figure 7A, p34-Arc and CGB fluorescence partially overlapped, indicating that Arp2/3 complexes were associated to large dense-core granules. Arp2/3 complex or its subcomponents have not been pre-

viously localized to secretory granules, although they have been found on phagosomes (May *et al.*, 2000) and intracellular vacuoles in yeast (Eitzen *et al.*, 2002). However, it is interesting to remember that isolated secretory granules do contain actin and actin-binding sites (Burrige and Phillips, 1975; Meyer and Burger, 1979; Bader and Aunis, 1983) and are able to induce the assembly of actin filaments (Wilkins and Lin, 1981). Stimulation of PC12 cells with 59 mM K⁺ triggered a partial movement of secretory granules to the cell periphery and the docking of a portion of the granules at the plasma membrane (Vitale *et al.*, 2002). p34-Arc and CGB remained colocalized (Figure 7A, mask p34-Arc/CGB), indicating that Arp2/3 complexes accompany the secretory granules to the periphery. Note the increased colocalization of p34-Arc with SNAP-25 in stimulated cells (Figure 7, A

Figure 7. Intracellular distribution of Arp2/3 in resting and stimulated PC12 cells. (A) PC12 cells were stimulated for 10 min with 59 mM K^+ (stimulated) or maintained in calcium-free Locke's solution (resting). Cells were subsequently fixed and stained with anti-p34-Arc antibodies revealed by secondary Cy3-labeled anti-rabbit antibodies (p34). Cells were then either stained with monoclonal anti-SNAP25 antibodies revealed with secondary Cy2-labeled anti-mouse antibodies or postfixed and stained with anti-chromogranin B (CGB) antibodies revealed with Cy2-labeled anti-rabbit antibodies. Masks representing the region of colocalization are obtained by selecting the pixels double-labeled with Cy2 and Cy3. Bar, 5 μ m. (B) Histogram representing a semiquantitative analysis of the percentage of p34 colocalized with CGB or with SNAP25 in resting (R) and stimulated cells (S). Data are given as the mean values $\pm$ SEM ($n = 10$).

and B), consistent with the presence of Arp2/3 at the granule docking sites on the plasma membrane.

DISCUSSION

In most neuroendocrine cells, actin filaments form a cortical network that separates secretory granules into a small release-ready pool and a larger reserve pool (Trifaro *et al.*, 2000). Thus, the transit of granules to fuse with the plasma membrane requires a subtle remodeling of the subplasmalemmal actin filaments. Ideal candidates to orchestrate such regulation are the small GTPases Rho family members that have emerged as important players in coupling actin dynamics to membrane trafficking events in eukaryotic cells (Ridley, 2001). Investigating the function of Rho GTPases in chromaffin cell secretion, we previously proposed that, although the granule-associated RhoA maintains actin filaments at the vicinity of the secretory granules (Gasman *et al.*, 1998), Cdc42 might play an active role in exocytosis by mediating some actin rearrangements preceding secretion (Gasman *et al.*, 1999). The aim of the present work was to further probe the function of Cdc42 in neuroendocrine cell secretion and to dissect the downstream effector pathway integrating Cdc42 to the actin architecture required for exocytosis. Using PC12 cells, a well established model for neuroendocrine secretion studies (Vitale *et al.*, 2001), we have demonstrated a causal relationship between activation of Cdc42, actin, and elicitation of exocytosis and proposed a molecular mechanism involving N-WASP and the Arp2/3 complex.

Intracellular Distribution of Cdc42

We previously studied the distribution of endogenous Cdc42 in cultured chromaffin cells and found that Cdc42

localizes in the subplasmalemmal region (Gasman *et al.*, 1999). Our current observations confirm that Cdc42 is peripherally localized and partially associated to the plasma membrane in PC12 cells. Yet, upon subcellular fractionation most of the protein is recovered in a soluble fraction. A possible explanation is that cytosolic Cdc42 could not be detected by immunofluorescence because the protein is complexed to RhoGDI. Alternatively, Cdc42 might be loosely bound to the plasma membrane and detached during cell fractionation. It is interesting to note that, in stimulated cells, Cdc42 is activated and that a larger amount associates to the plasma membrane. This suggests that stimulation with a secretagogue might induce a modification in the binding of Cdc42 with some plasma membrane-bound protein, i.e., a nucleotide exchange factor and/or an effector. Thus, it is conceivable that in this set up, activation of Cdc42 does not result in a classical cytosol-membrane translocation but rather in subtle changes in the interaction of Cdc42 with its molecular partners present at the plasma membrane. Using GFP-tagged proteins, we examined the localization of the constitutively active Cdc42^{L61} and the dominant negative Cdc42^{N17} mutants. The localization of Cdc42^{L61} paralleled that of the endogenous protein, validating the use of this mutant for functional assays. In contrast, the dominant negative Cdc42^{N17} was largely present in the cytosol. This distribution was unexpected because in resting cells, endogenous Cdc42, which seems to be mostly in its GDP-bound form (as shown here), was localized in the cell periphery. Moreover, it has been recently reported that N17/19 dominant negative mutants of the Rho family fail to bind RhoGDI, most likely because the mutants are locked in a nucleotide-free state (Strassheim *et al.*, 2000; Michaelson *et al.*, 2001). This results in localization to membrane compartments in epithelial cell lines (Michaelson *et al.*, 2001), a

distribution that has been proposed to play a significant role in their dominant negative activity, by permitting the N17/19 mutants to sequester membrane-associated GEFs. We have currently no explanation for the basis of the cytosolic distribution of Cdc42^{N17} in PC12 cells. Nevertheless, the modest inhibitory effect on secretion observed in cells expressing Cdc42^{N17} might be related to the mislocalization of this mutant unable to efficiently behave as a dominant negative at the sites of exocytosis.

Cdc42 Regulates Exocytosis by Recruiting N-WASP

We show here that overexpression of the constitutively active Cdc42^{L61} mutant significantly enhanced GH secretion from PC12 cells, revealing the participation of Cdc42 in large dense-core granule exocytosis. Accordingly, mast cells, pancreatic β cells, and yeast require the active participation of Cdc42 for secretion (Kowluru *et al.*, 1997; Brown *et al.*, 1998; Adamo *et al.*, 2001). How might plasma membrane-associated GTP-loaded Cdc42 facilitate exocytosis in PC12 cells? In yeast, Cdc42 has been shown to promote docking and/or fusion of secretory vesicles with the plasma membrane through an interaction with the exocyst, a conserved eight-subunit complex implicated in tethering vesicles to specific sites on the plasma membrane (Zhang *et al.*, 2001). To date, there are no published reports linking Cdc42 to the mammalian exocyst. However, a connection between Cdc42 and syntaxin has been described previously (Daniel *et al.*, 2002), suggesting that Cdc42 may have functions related to the SNARE complex formation. PLD is also an attractive candidate to be a downstream partner of Cdc42 in the exocytotic pathway. Cdc42 is an activator of PLD1 (Walker *et al.*, 2000), and PLD1 located at the plasma membrane has been recently described as a key factor for exocytosis in neuroendocrine cells (Vitale *et al.*, 2001). On the other hand, it is possible that the action of Cdc42 on exocytosis is related to its ability to promote the formation of actin filaments in the cell periphery as shown here in cells expressing the GTP-bound form of Cdc42. In a first approach to decipher the cascade of events initiated by Cdc42, transfection experiments with various cDNA encoding Cdc42 mutated in the effector loop ruled out the participation of PLD1 but revealed that effectors of the WASP/PAK branch linked to the actin cytoskeleton might be involved. We have demonstrated a causal role for Cdc42-regulated N-WASP in PC12 cell exocytosis by establishing that 1) upon activation of exocytosis, overexpressed N-WASP is recruited to the cell periphery where it enhances F-actin polymerization; 2) overexpression of N-WASP stimulates exocytosis in PC12 cells as efficiently as Cdc42, whereas a truncated mutant unable to polymerize actin has no effect; 3) N-WASP is actively recruited to the plasma membrane by Cdc42; and 4) coexpression of Cdc42 with a dominant negative N-WASP mutant completely abolishes the effect of active Cdc42 on exocytosis. Moreover, overexpressing PAK1 had no effect on GH secretion, electing N-WASP as the downstream effector by which Cdc42 modulates exocytosis. So far, the participation of N-WASP in the neuroendocrine exocytotic pathway has been suggested once in PC12 cells but in a manner that was independent of Cdc42 (Frantz *et al.*, 2002). A possible explanation for this discrepancy is that the Cdc42^{V12} mutant used by Frantz *et al.* (2002) was not localized on plasma membrane but rather on some intracellular punctuated structures.

The Actin Cytoskeleton at the Sites of Exocytosis

Actin plays a central role in regulated exocytosis but its exact mode of action remains unknown. Based on studies in a variety of secretory model systems, including neurons and

Figure 8. Hypothetical model for the role of Cdc42/N-WASP-induced actin filaments in regulated exocytosis. In resting cells, N-WASP is in the cytosol, Arp2/3 associated to secretory granules, and Cdc42 located at the plasma membrane. Stimulation of exocytosis triggers the activation of Cdc42 at the plasma membrane, which in turn recruits N-WASP. In parallel, secretory granules are mobilized from the reserve pool, and they bring their associated Arp2/3 complexes to the granule docking site of the plasma membrane; actin filament polymerization occurs then at the site of exocytosis. Note that the precise actin structures required for exocytosis are currently unknown. This part of model remains purely speculative.

endocrine cells, many potential roles have been proposed. Using evanescent wave microscopy in PC12 cells, Lang *et al.* (2000) were able to illustrate a dual function for actin by demonstrating that it can both hinder and mediate movements of GFP-labeled secretory granules in the subplasmalemmal region. In neurons, synaptic activity induces de novo actin polymerization (Colicos *et al.*, 2001; Sankaranarayanan *et al.*, 2003). In pancreatic acinar cells, actin polymerizes around secretory granules during exocytosis, and it has been proposed that this actin coating facilitates the movement of granules across the actin network toward their fusion site (Valentijn *et al.*, 2000). Hence, actinomyosin-based motility has also been shown to play an important role in vesicular trafficking, because myosin II and/or myosin V are able to drive synaptic vesicles in neurons and secretory granules in neuroendocrine cells (Langford, 2002; Neco *et al.*, 2002; Rose *et al.*, 2003; Rudolf *et al.*, 2003). On the other hand, recent data suggest that actin filaments may act as a scaffold in synaptic terminals by concentrating regulatory molecules near the releasable pool of synaptic vesicles (Sankaranarayanan *et al.*, 2003). In yeast, Cdc42p triggers actin remodeling through a molecular pathway involving Las17p (the yeast WASP homolog) and the Arp2/3 complex. Of particular interest to the present study is that Cdc42p-induced actin filaments accumulate on docked vacuoles and are required for the terminal step leading to homotypic membrane fusion (Eitzen *et al.*, 2002). Finally, actin filaments could also be important in secretory membrane recycling by providing the force for the membrane invagination or the pinching of the endocytic vesicles (Jeng and Welch, 2001).

How do the present data fit with these current views of the actin machinery underlying regulated exocytosis? N-WASP initiates the growth of actin filaments by bringing together actin monomers and the Arp2/3 complex (Prehoda *et al.*, 2000). Although we did not directly demonstrate the functional importance of the Arp2/3 complex in secretion, we show here that Arp2/3 is present on secretory granules but not on the plasma membrane, in resting PC12 cells. An exciting speculation relates to the differential localization of N-WASP and Arp2/3 in resting versus stimulated cells (see hypothetical model in Figure 8). In resting cells, N-WASP is

in the cytosol and Arp2/3 associated to secretory granules. Secretagogue-induced activation stimulates Cdc42, which in turn recruits N-WASP to the plasma membrane, and in parallel mobilizes secretory granules to the docking sites at the plasma membrane. Thus, the interaction between N-WASP, Arp2/3, and the actin monomers would take place only at the granule docking sites, providing a way to specifically target local actin filament polymerization at the interface between the granule and the plasma membrane. Recent work has established that for dense-core granules, the fusion pore opening-closing time can be modulated by various signaling pathways, allowing a control of the amount of hormones released per granule (Elhamedani *et al.*, 2001; Taraska *et al.*, 2003). The possibility that actin filaments formed at the granule docking site regulate expansion and/or closure of the fusion pore, thereby providing a molecular basis for control of quantal release, will be an interesting future issue.

To conclude, activation of secretion in neuroendocrine cells does not simply trigger the disassembly of the cortical actin barrier but rather induces a fine remodeling of the peripheral actin network into structures required for exocytosis. The present results provide a molecular support for the de novo formation of actin filaments in the course of exocytosis by sequentially ordering Cdc42, N-WASP, and Arp2/3 in a signaling pathway dedicated for the first time to the secretion of hormones. Further studies are now required to investigate whether the local production of actin filaments during exocytosis would serve for docking, scaffolding, fusion, and/or membrane retrieval.

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