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Kalirin/Trio Rho Guanine Nucleotide Exchange Factors Regulate a Novel Step in Secretory Granule Maturation[D]

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The molecular mechanisms involved in the maturation of secretory granules, organelles that store hormones and neuropeptides, are poorly understood. As granule content proteins are processed, the composition of granule membranes changes, yielding constitutive-like secretion of immature content proteins and producing secretagogue-responsive mature granules. Constitutive-like secretion was not previously recognized as a process subject to regulation. We show that Kalirin and Trio, homologous Rho guanine nucleotide exchange factors (GEFs), which interact with a secretory granule resident protein, modulate cargo secretion from immature granules. Some of the Kalirin and Trio isoforms expressed in neuroendocrine cells colocalize with immature granules. Overexpression of their N-terminal GEF domain (GEF1) enhances secretion from immature granules, depleting cells of secretory cargo in the absence of secretagogue. This response requires GEF1 activity and is mimicked by Kalirin/Trio substrates Rac1 and RhoG. Accordingly, selective pharmacological inhibition of endogenous GEF1 activity decreases secretagogue-independent release of hormone precursors, accumulating product peptide in mature secretory granules. Kalirin/Trio modulation of cargo secretion from immature granules provides secretory cells with an extra layer of control over the sets of peptides released. Control of this step enhances the range of physiological responses that can be elicited, whereas lack of control could have pathological consequences.

INTRODUCTION

Neuroendocrine cells store classical transmitters, bioactive peptides and hormones in secretory granules or large dense core vesicles, specialized organelles that differ from synaptic vesicles in dimensions and biogenesis (Mains and Eipper, 2006). On stimulation, neuroendocrine cells release the substances stored in secretory granules into the extracellular medium, initiating signaling either locally or in distant tissues. Calcium has long been recognized as a second messenger able to trigger exocytosis in neuroendocrine cells (Douglas and Rubin, 1961). In the 1980s, a role for guanosine trisphosphate (GTP) as a secretory messenger was identified, suggesting that G-proteins mediate some of the processes involved in regulated exocytosis (Pinxteren *et al.*, 2000). Further investigation identified Rho GTPases as molecular mediators of regulated exocytosis from mast cells (Norman *et al.*, 1996). Rho GTPases act as molecular switches, inactive if GDP-bound, active and able to interact with their effectors when GTP-bound. The guanine-nucleotide bound state of G-proteins is tightly regulated by activators, the guanine-nucleotide exchange factors (GEFs), which catalyze GTP loading, and inactivators, the GTPase activating proteins (GAPs), which enhance the rather poor enzymatic activity of GTPases, speeding up hydrolysis of bound GTP, thus returning G-proteins to their inactive GDP-bound state (Rossman *et al.*, 2005).

Peptidylglycine α-amidating monooxygenase (PAM) is an essential enzyme in the processing of many bioactive peptides and hormones (Prigge *et al.*, 2000). To carry out this function, PAM is targeted to the regulated secretory pathway in neurons and neuroendocrine cells. PAM, a type one membrane protein, has two enzymatic domains within the lumen of the secretory pathway, a transmembrane region and a cytosolic domain that governs its cellular routing (Milgram *et al.*, 1996). Among the proteins able to interact with the cytosolic domain of PAM, we previously identified two RhoGEFs, Kalirin and Trio, the mammalian homologues of *Drosophila* Trio and *Caenorhabditis elegans* UNC-73 (Alam *et al.*, 1996; Xin *et al.*, 2004). In mammals, Kalirin and Trio are encoded by separate genes, but invertebrates have a single homologous gene (Steven *et al.*, 1998; Bateman *et al.*, 2000). Kalirin and Trio share the same complex multidomain structure and display several splice variants (Debant *et al.*, 1996; Johnson *et al.*, 2000; McPherson *et al.*, 2005). The longest Kalirin and Trio proteins have a Sec14 domain, a stretch of spectrin repeats, a GEF domain (GEF1), an SH3 domain, a second GEF domain (GEF2), a second SH3 domain, Ig/FNIII domains, and a Kinase domain. The GEF1 domain of Kalirin and Trio is the region of highest homology (McPherson *et al.*, 2002).

Recent observations demonstrating that Rho proteins play a role in regulated exocytosis by neuroendocrine cells, coupled with the fact that both Kalirin and Trio interact with a secretory granule resident protein, make these two GEFs obvious candidates to control Rho-mediated processes related to the secretory granule physiology. However, to date the role of Kalirin and Trio in this context has been only

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modestly explored (Alam *et al.*, 1997; Mains *et al.*, 1999; Xin *et al.*, 2004). In this study we aimed to investigate the role of Kalirin/Trio in secretory granule function.

Secretory cargos destined for storage and regulated secretion are initially packaged into immature secretory granules at the *trans*-Golgi network (TGN). They are sorted from the bulk of constitutively trafficked proteins, which are rapidly transported by small vesicles from the TGN to the plasma membrane. During a process defined as maturation, small vesicles bud from immature secretory granules, remodeling its protein composition and at the same time, removing from the granule a fraction of cargo proteins that are then released at the plasma membrane. This process is called constitutive-like secretion because, unlike constitutive secretion from the TGN, it occurs from post-TGN immature secretory granules, only in cells with a regulated pathway (Arvan *et al.*, 1991). Although release of soluble products from mature secretory granules results from the fusion of this organelle with the plasma membrane in response to secretagogues, secretion from the TGN and immature secretory granules does not require exocytic stimuli. However, it is noteworthy that use of the terms constitutive and constitutive-like for these pathways does not mean that these routes are not subject to control. Indeed, it is clear that the flow of secretory molecules through the constitutive pathway can be modulated by tyrosine phosphorylation of Golgi proteins (Webb *et al.*, 2005) and retrieval of specific membrane proteins from maturing secretory granules is modulated by phosphorylation-dependent recruitment of adaptor proteins (Eaton *et al.*, 2000; Hinners *et al.*, 2003; Kakhlon *et al.*, 2006).

Here, we show that the activity of the GEF1 domain of Kalirin and Trio modulates constitutive-like secretion of secretory cargo from immature secretory granules, a step previously not recognized as subject to control.

MATERIALS AND METHODS

Antibodies and Reagents

Antisera to the spectrin regions of Kalirin (JH2582) and Trio (CT233) were previously described (Penzes *et al.*, 2000; McPherson *et al.*, 2005). Antiserum Kathy, directed to the exposed C-terminus of ACTH (1–39), recognizes mature ACTH and ACTH biosynthetic intermediate (Sobota *et al.*, 2006). JH189 antiserum, directed to the γ_3-melanocyte–stimulating hormone (MSH) region of pro-opiomelanocortin (POMC), recognizes intact POMC, intermediate, 18- and 16-kDa fragments and γ_3-MSH (Cullen and Mains, 1987). Rac1 mAb was from BD Biosciences (San Jose, CA). Polyclonal antibodies to Myc (ab1906) and hemagglutinin (HA; ab9110) were from Abcam (Cambridge, MA). Monoclonal antibodies to GM130, SNAP25, and γ-adaptin were from BD Biosciences. Antibodies to Myc (9E10), TGN38, Chromogranin A (CgA), and Carboxypeptidase E (CPE) were described previously (Eskeland *et al.*, 1996; Varlamov *et al.*, 1996; Milgram *et al.*, 1997). Secondary Cy3- and fluorescein isothiocyanate–conjugated antibodies were from Jackson ImmunoResearch (West Grove, PA). 1-(3-nitrophenyl)-1H-pyrrol- 2,5-dione (NPPD) was from ChemBridge (San Diego, CA).

Constructs

His_6-Myc tagged Kal-GEF1, Kal-GEF2, and Kal-GEF1(ND/AA) in the pEAK10 vector were described previously (Schiller *et al.*, 2005). His_6-Myc tagged Trio-GEF1 and Trio-GEF2 (G^{1211}-K^{1550} and D^{1187}-G^{2234} of human Trio, respectively) were inserted into the pEAK10 vector. The same tagged constructs were subcloned into the dual promoter pCMS.EGFP vector and fused in-frame to enhanced green fluorescent protein (EGFP) in the pEGFP-C3 vector (Clontech, Mountain View, CA). The GEF domain of mouse TIAM1 was cloned from mouse cerebral cortex cDNA by PCR, using the forward primer 5′-aaagaattc**tcggatgcggataagctgcgc**-3′ with an EcoRI site and the reverse primer 5′-tttgcggccgctcatca**gacatactgctgggctgacgg**-3′ with a NotI site (restriction sites are underlined and TIAM1 sequences are boldface). The purified PCR product was digested with EcoRI and NotI and inserted into a pCI.neo vector engineered to encode tandem HA epitopes with an EcoRI site in reading frame at the 3′ end; the construct encoded HA_2-Glu-Phe-TIAM1(Ser^{1036}-Val^{1415}). The GEF domain of mouse DBS was cloned from the same source using a similar strategy: forward primer 5′-aaagaattc**ggccgcaccagctccacaggg**-3′; reverse primer 5′-tttgcggccgctcatca**gtgggactgctccagggctcg**-3′; the construct encoded HA_2-Glu-Phe-DBS(Gly^{577}-His^{926}). pEGFP-Rac1(Q61L), pEGFP-RhoG(Q61L), and pCGN.VAV2 GEF (191-518) were described previously (Booden *et al.*, 2002; Wennerberg *et al.*, 2002).

Cell Culture and Transfections

AtT-20, PC12, GH3, and pEAK Rapid cells were maintained as described (Sobota *et al.*, 2006). Transfections for imaging utilized Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cells were plated onto glass coverslips coated with poly-L-lysine and NuSerum (Collaborative Research, Bedford, MA). At 24 h after transfection, cells were fixed and processed for imaging by confocal or deconvolution microscopy as described (Sobota *et al.*, 2006).

Subcellular Fractionation

AtT-20 cells were homogenized in 20 mM HEPES-NaOH, pH 7.5/5 mM Na_2EDTA/300 mM sucrose (with protease inhibitor mix) by five passes through a 25-gauge (5/8) needle and six passes through a ball bearing cell cracker. Homogenates were centrifuged at 900 × *g* for 5 min. Postnuclear supernatants were fractionated by velocity gradient centrifugation (Tooze and Huttner, 1990; Eaton *et al.*, 2000). Protein (0.5–1.0 mg in 200 μl) was loaded onto a sucrose step gradient formed by layering 200 μl of the following solutions: 0.640, 0.725, 0.810, 0.895, 0.980, 1.065, 1.150, 1.235, 1.320, 1.405, and 1.490 M sucrose in 20 mM HEPES-NaOH, and 5 mM Na_2EDTA, pH 7.5. Samples were centrifuged at 35,000 rpm in a TLS55 rotor (107,000 × *g* max) for 15 min. Fractions (200 μl each) were collected from the top and analyzed by immunoblotting to identify regions of the gradient containing immature (fractions 1 and 2) and mature (fractions 4 and 5) secretory granules. The immature granule pool was diluted with 1 volume of homogenization buffer and the mature granule pool was diluted with 1 volume of 20 mM HEPES-NaOH, 5 mM Na_2EDTA, pH 7.5 buffer. The diluted samples were loaded onto two identical gradients formed by layering 125 μl of the following solutions: 0.980, 1.065, 1.150, 1.235, 1.320, 1.405, 1.490, 1.575, 1.660, 1.745, and 1.830 M sucrose in 20 mM HEPES-NaOH, 5 mM Na_2EDTA, pH 7.5. Fifteen fractions were collected from the top and analyzed with the indicated antibodies.

Secretion Experiments

Where required, transfection was performed with an Amaxa Nucleofector II (Amaxa, Gaithersburg, MD). The standard protocol (10 μg plasmid plus 1–2 × 10^6 cells in 100 μl Nucleofector solution L with programs X-001 or T-005) resulted in transfection of 70–90% of the cells (not shown). Cells were plated in 12- or 24-well dishes and grown overnight in growth medium (Sobota *et al.*, 2006). Cells were fed with complete serum-free medium (Sobota *et al.*, 2006) containing 10 μg/ml bovine serum albumin (BSA) and 10 μg/ml lima bean trypsin inhibitor (Sigma-Aldrich) at 24 h. Media were collected at the indicated times, and cells were extracted with SDS-lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5% SDS, 1 mM dithiothreitol, 1 mM Na orthovanadate, 2 mM Na_2EDTA, 50 mM NaF, and protease inhibitor mix). Lysates and media were concentrated by cold acetone addition to 80% final volume and incubation overnight at −20°C. The samples were centrifuged (18,000 × *g*, 30 min at 4°C) and the air-dried pellets were solubilized by boiling with Laemmli sample buffer, fractionated by SDS-PAGE (4–20% polyacrylamide gels, Invitrogen), transferred to PVDF membranes, and probed with the indicated antibodies. Immunosignals were quantified using ImageJ software (NIH).

GST-Rac1-GDP Binding Assay

pEAK Rapid cells were transfected with plasmids pCMS.EGFP.His_6-Myc-Kal-GEF1, pCI.neo.HA_2-TIAM1GEF, pCI.neo.HA_2-DBSGEF, and pCGN.VAV2GEF with Lipofectamine 2000 (Invitrogen). After serum starvation, at 48 h after transfection, cells were lysed in 25 mM HEPES, pH 7.5, 250 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM Na_2-EDTA, 10% glycerol, and 1 mM orthovanadate and protease inhibitor mix. Equal aliquots from each lysate were incubated with 1 μg of GST-Rac1, 1 mM GDP, 10 mM Na_2-EDTA (added from a 250 mM stock), and dimethyl sulfoxide (DMSO) or the indicated concentration of NPPD in the same volume of DMSO at 30°C for 15 min and then chilled on ice. $MgCl_2$ from a 1 M stock was added to a final concentration of 50 mM to allow loading of GDP onto Rac, followed by glutathione-Sepharose beads. After tumbling at 4°C for 1 h, the beads were rinsed three times with 400 μl of lysis buffer and eluted with Laemmli sample buffer.

RESULTS

Kalirin and Trio Isoforms Are Expressed in Neuroendocrine Cells

To play a role in the regulated secretory pathway, Kalirin and Trio must be expressed in the relevant secretory tissues. We probed for the presence of these two RhoGEFs in rat anterior pituitary and adrenal gland and in AtT-20 mouse corticotrope tumor cells, rat GH3 somatomammotrope cells, and PC12 pheochromocytoma cells, using adult rat cerebral

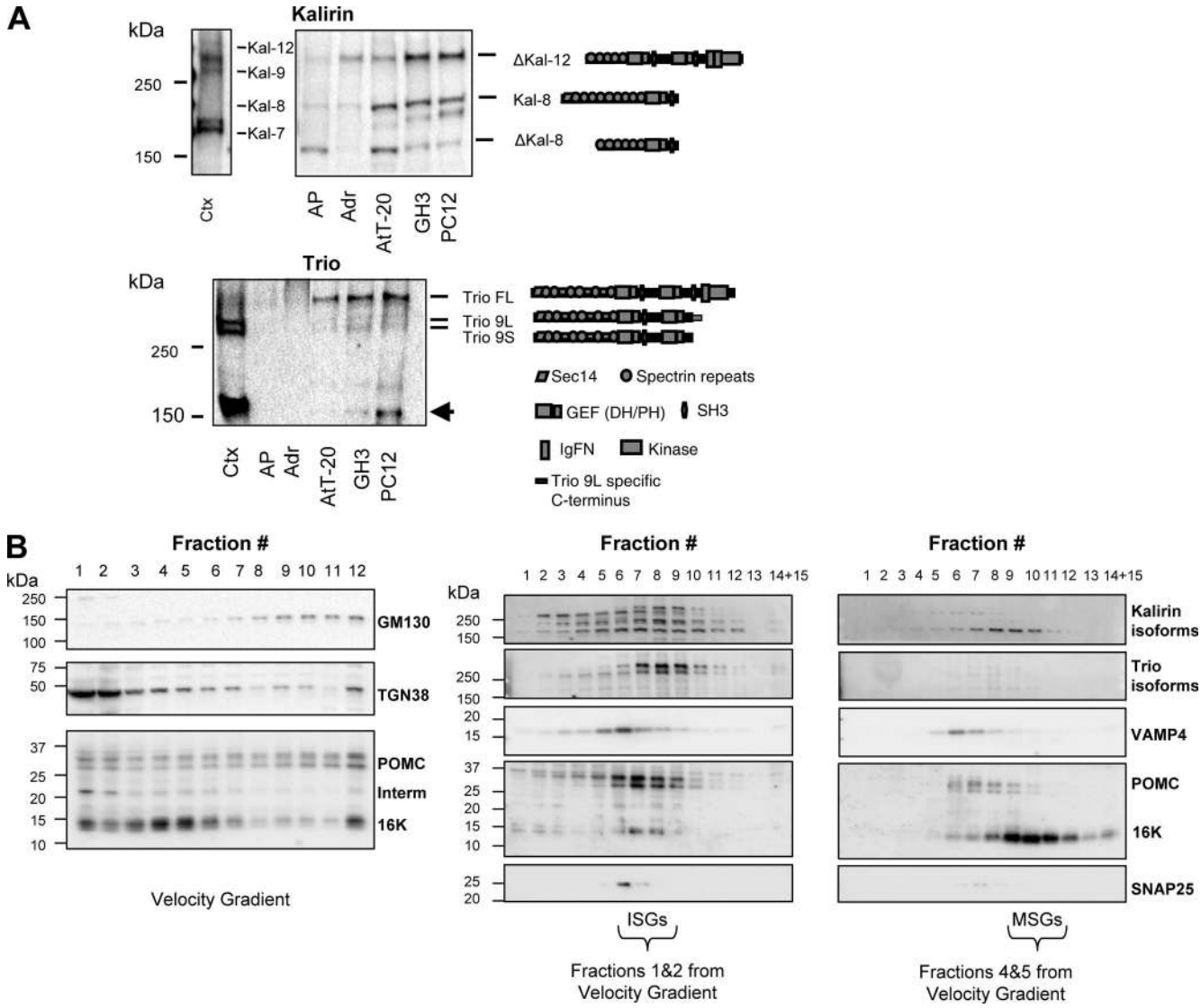

Figure 1. Kalirin and Trio isoforms are expressed in secretory tissues and cell lines. (A) The indicated lysates (100 μg protein) were fractionated on 5% polyacrylamide gels and probed for Kalirin (top panel) or Trio (bottom panel). The mobility of recombinant Kalirin isoforms fractionated in parallel is indicated. The domain structures of the various Kalirin and Trio isoforms are depicted. Ctx, adult rat parietal cortex; AP, adult rat anterior pituitary; Adr, adult rat whole adrenal gland; AtT-20, mouse corticotrope tumor cells; GH3, rat somatomammotrope tumor cells; PC12, rat pheochromocytoma cells. The arrow indicates a nonspecific cross-reactive band. (B) AtT-20 postnuclear supernatant was subjected to velocity centrifugation (left panel); samples collected from the top were fractionated on 4–20% polyacrylamide gels and probed as indicated. Fractions enriched in unprocessed POMC and intermediate (1 and 2) were pooled as a source of immature secretory granules (ISGs). Fractions enriched in the 16-kDa fragment (4 and 5) were pooled as a source of mature secretory granules (MSGs). Pooled ISGs (middle) and MSGs (right) were subjected to equilibrium centrifugation. Fractions from these gradients were probed for Kalirin, Trio, TGN/immature granule/endosome marker VAMP4, POMC products, and the plasma membrane marker SNAP25.

cortex as a positive control (Johnson *et al.*, 2000; McPherson *et al.*, 2005). In adult rat cortex, the major Kalirin isoform is Kalirin-7, but Kalirin-9 and -12 or ΔKalirin-12 are also found (Figure 1A, top; Johnson *et al.*, 2000). Delta-isoforms of Kalirin are generated by an alternative transcriptional start site, producing proteins missing the Sec14 and first four spectrin repeats (Johnson *et al.*, 2000). At least three Kalirin isoforms are expressed in all the tissues and cell lines probed (Figure 1A, top). Based on the relative mobility of recombinant Kalirin isoforms, we identified these isoforms as ΔKalirin-12, Kalirin-8 and ΔKalirin-8 (Figure 1A, top).

We previously established that Trio, like Kalirin, is subject to alternative splicing, yielding several protein products; the most abundant brain variants are Trio-9L and -9S (Figure 1A, bottom; McPherson *et al.*, 2005). The secretory tissues and cell lines examined express Trio isoforms the size of full-length (FL) Trio and the Trio-9L and -9S variants (Figure 1A, bottom), with FL Trio being the most abundant. Interestingly, both RhoGEFs show consistently higher levels of expression in tumor cell lines compared with tissues (based on equal amounts of protein).

Using indirect immunofluorescence, our Kalirin and Trio antisera are unable to visualize endogenous Kalirin and Trio in AtT-20 cells, preventing determination of their localization. To overcome this limitation, we performed a two-step subcellular fractionation. First, we separated immature and mature secretory granules using velocity centrifugation (Figure 1B, left panel; Eaton *et al.*, 2000). Analysis of these

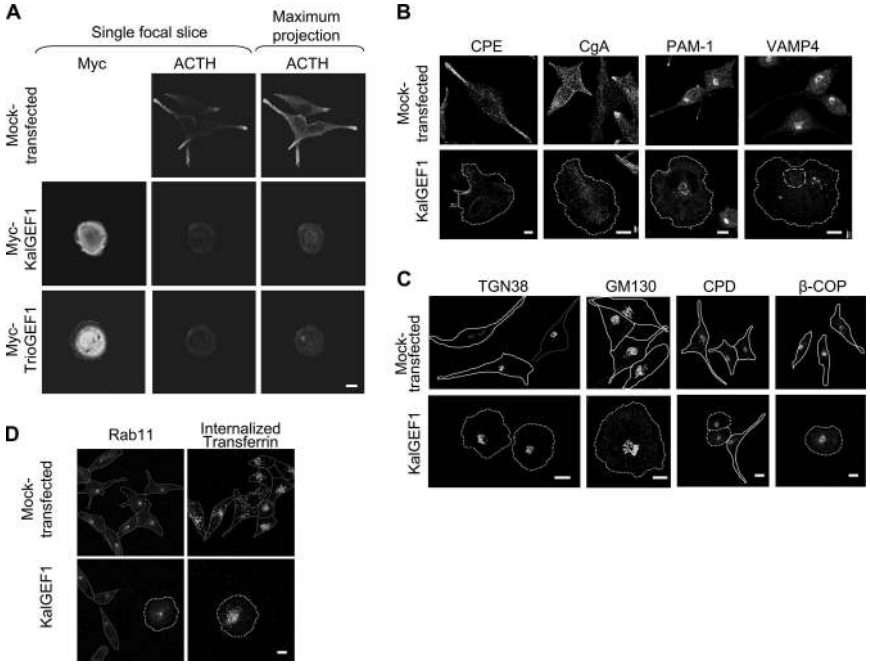

Figure 2. Kal- or Trio-GEF1 overexpression causes depletion of cell hormone content. AtT-20 cells were mock-transfected (i.e., treated with Lipofectamine 2000 in the absence of plasmid) or transfected with myc-tagged Kal- or Trio-GEF1 constructs. Stacks of images encompassing the entire thickness of the cells were acquired (by confocal or deconvolution microscopy); maximum projections in the z-dimension are shown in B–D and the right-side panels in A; the middle and left panels in A display single focal planes acquired close to the substrate. (A) Recombinant proteins (Myc) and mature ACTH were visualized. (B) Secretory granule markers were visualized: Carboxypeptidase E (CPE), Chromogranin A (CgA), PAM-1 (in PAM-1 AtT-20 cells), and VAMP4. (C) Golgi/TGN markers were visualized: TGN38, GM130, Carboxypeptidase D (CPD), and β-COP. (D) Cells were fixed and stained for Rab11 or incubated with Alexa-Fluor 546-transferrin (Molecular Probes) for 15 min and then chased in nonfluorescently labeled transferrin for 15 min before fixation. Dotted and solid lines outline GEF1- and nontransfected cells, respectively. Scale, 10 μm.

fractions using antisera to TGN38 and GM130 indicated that TGN and Golgi were effectively separated. Analysis of POMC products identified mature granules (enriched in terminally processed the 16-kDa fragment) in fractions 4 and 5 and immature granules (enriched in unprocessed POMC and intermediate) in fractions 1 and 2 (Figure 1B, left panel).

Pools enriched in immature and mature secretory granules were then subjected to equilibrium centrifugation (Figure 1B, middle and right panels). Because of the higher protein load, compared with total lysates in Figure 1A, in addition to ΔKal-12, Kal-8 and ΔKal-8, Kal-12 could be detected in these granule-enriched fractions. POMC staining identified immature granules in fractions 6–9. Kal-12, Trio-FL, Trio-9L, and Trio-9S showed the greatest overlap with immature granules; ΔKal-12, Kal-8, and ΔKal-8 exhibited partial overlap. The sample loaded onto the immature granule gradient also contains TGN and endosomes. VAMP4, a SNARE protein that cycles through immature granules and endosomes but largely localizes to the TGN at steady-state (Steegmaier *et al.*, 1999) was used as a TGN, endosome, and immature granule marker. VAMP4 shows partial overlap with the immature granules. Based on the localization of SNAP25, the plasma membrane is largely recovered in a single fraction; although several Kalirin and Trio isoforms are present in this fraction, their distribution is much broader.

Mature granules were recovered in fractions 9–11 of the second equilibrium gradient (Figure 1B, right panel). The only Kalirin or Trio isoform associated with mature secretory granules was ΔKal-8, which was readily detectable in fractions enriched in the 16-kDa fragment. These data, together, indicate that Kalirin and Trio splice variants are expressed in secretory tissues and are differentially associated with TGN, endosomes, and immature granules.

Overexpression of Kalirin or Trio GEF1 Domain Depletes Secretory Granule Cargo

The multidomain structure of Kalirin and Trio (Debant *et al.*, 1996; Johnson *et al.*, 2000) is suggestive of complex regulation. Such an articulate architecture may serve as a molecular platform to integrate multiple incoming signals (Rossman *et al.*, 2005). The regions of the RhoGEFs N-terminal to the GEF domain often have an inhibitory effect on enzymatic activity; these regions are deleted in mutant RhoGEFs that display unregulated activation of their Rho substrates, leading to cell transformation (Schmidt and Hall, 2002; Rossman *et al.*, 2005). For these reasons we explored the effects of Kalirin and Trio by overexpressing their isolated GEF domains in AtT-20 corticotrope tumor cells, a well-characterized system to study regulated secretion.

Rho GTPases are established regulators of actin cytoskeletal dynamics (Schmidt and Hall, 1998), thus changes in cell shape were expected upon overexpression of the isolated GEF1 domain of Kalirin or Trio. AtT-20 cells are normally spindle-shaped, with secretory granules concentrated at the tips of processes (Figure 2A). AtT-20 cells expressing Kalirin or Trio GEF1 were markedly flattened and lacked processes. GEF1-expressing cells were most often round, with thin membrane protrusions (lamellipodia) at the periphery (Figure 2A, Myc staining). However, C-shaped and flat pancake-like morphologies were not uncommon (Supplementary Figure S1). The GEF1 domain of Kalirin and Trio was largely cytosolic (Figure 2A and Supplementary Figure S1), with some protein localized at the edges of lamellipodia (Figure 2A, and Supplementary Figure S1, insets) and dorsal ruffles (not shown). These shape changes were accompanied by rearrangement of the actin cytoskeleton (Supplementary Figure S1). Comparison of the area in cells expressing catalytically active and inactive Kalirin GEF1 showed that GEF activity was required for this change in shape (Supplementary Figure S2B). The morphological changes induced by Kalirin and Trio GEF1 were indistinguishable, likely due to the fact that these domains are very similar (91% identity and 97% conservation at the amino acid level for rat Kalirin GEF1 and human Trio GEF1; see also McPherson *et al.*, 2002).

AtT-20 cells synthesize POMC and process it into mature products (such as adrenocorticotropic hormone [ACTH]) that are stored in secretory granules. In mock-transfected

(Figure 2A, top) and control-transfected (not shown) cells, ACTH was localized at the tips of processes. When ACTH was visualized in cells overexpressing GEF1, levels were greatly reduced (Figure 2A, middle and bottom panels). This effect was also observed with an antiserum that recognizes intact POMC and mature ACTH (not shown). Hormone staining in Kal-GEF1 transfectants was reduced to ~60% of control-transfected cells (Supplementary Figure S2C). No significant effect on total cellular content of POMC and its products was seen in cells overexpressing the catalytically inactive Kal-GEF1 (ND/AA) mutant (Supplementary Figure S2C), demonstrating enzymatic activity is necessary for depletion of hormone. Overexpression of the GEF2 domain of Kalirin or Trio did not affect cell shape or ACTH content (not shown).

We next determined whether other secretory granule cargo proteins were depleted after expression of GEF1. Both the content and localization of soluble (Chromogranin A and Carboxypeptidase E) and membrane (PAM-1 and VAMP4) granule proteins were altered by GEF1 overexpression (Figure 2B). In contrast, Golgi (GM130, β-COP) and TGN (TGN38, CPD) markers were unaltered (Figure 2C). Based on visualization of BiP and LAMP-1, endoplasmic reticulum and lysosomes, respectively, were unaffected (not shown).

TGN38 cycles through the plasma membrane, serving as a marker for the constitutive secretory pathway; impairment of its endocytic trafficking results in its accumulation in early endosomes (Chapman and Munro, 1994; Reaves and Banting, 1994). The fact that GEF1 overexpression did not affect the steady-state localization of TGN38 in the TGN (Figure 2C), suggested that GEF1 did not have a dramatic effect on the endocytic pathway. To explore this possibility, the effect of GEF1 expression on transferrin internalization and the steady-state localization of Rab11, a marker of recycling endosomes, was determined (Figure 2D). Based on both parameters, endocytic pathways were not dramatically affected by GEF1 expression.

As in AtT-20 cells, GEF1 overexpression in PC12 cells produced cell flattening and alterations in the actin cytoskeleton (not shown). GEF1 expression also caused a reduction in soluble (Chromogranin B) and membrane-associated (Synaptotagmin−1) secretory cargo in PC12 cells (Supplementary Figure S3, insets). Thus GEF1 effects on the regulated secretory pathway are not unique to AtT-20 cells and are a general feature in secretory cells. Together, these data suggest that GEF1 acts at a post-TGN location, specifically affecting compartments of the regulated secretory pathway.

Kalirin and Trio Rho Substrates Reproduce the GEF1 Phenotype

On the basis of the fact that GEF1 activity is required for production of this phenotype (Supplementary Figure S2), we reasoned that its effects should be mimicked by overexpression of the appropriate activated Rho substrate(s). Trio and Kalirin GEF1 activate RhoG, Rac1, and Rac2 (Debant *et al.*, 1996; Blangy *et al.*, 2000; Schiller *et al.*, 2005). AtT-20 cells expressing Rac1 or RhoG (Q61L) mutants, locked in the GTP-bound active state, adopt a flattened morphology (Figure 3). When viewing the entire thickness of the cell, we observed that expression of Rac1(Q61L) consistently produced flat, round AtT-20 cells with dorsal ruffles; activated Rac1 localized on ridges at the dorsal plasma membrane. RhoG(Q61L) produced a more varied morphology. Cells expressing high levels of RhoG(Q61L) resembled those expressing GEF1, with extensive lamellipodia (Supplementary Figure S4); moderate expressors did not (Figure 3). RhoG(Q61L) localized preferentially in the perinuclear region (Figure 3, GFP asterisks and Supplementary Figure S4). At similar levels of expression (Supplementary Figure S4), Rac1 and RhoG (Q61L) cause similar, but not identical, morphological modifications in AtT-20 cells.

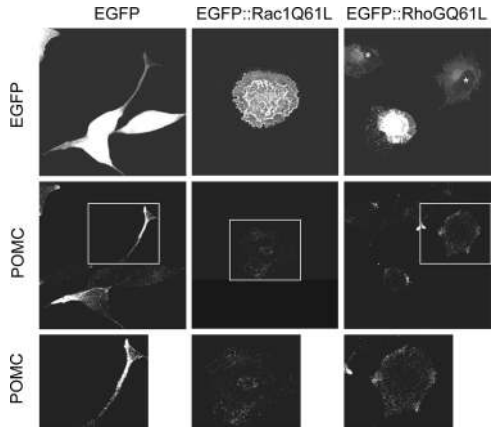

Figure 3. Activated Kalirin/Trio GEF1 substrate Rho proteins produce a phenotype similar to that of GEF1 overexpression. EGFP (control) and EGFP-fusion proteins of Rac1(Q61L) and RhoG(Q61L) were expressed in AtT-20 cells. Hormone content was visualized with antiserum that recognizes POMC and mature ACTH. To assess cellular content of hormone, maximal projection images are shown, as in Figure 2. Note the perinuclear localization of RhoG(Q61L) in moderate expressors (*). For POMC, magnifications of the insets are shown in the bottom panel.

As observed for GEF1, overexpression of active Rac1 or RhoG led to reduction of cellular hormone content (Figure 3, POMC; Supplementary Figure S4, ACTH). Although Kalirin and Trio GEF1 may activate GTPases other than Rac and RhoG, our data demonstrate that sustained Rac1 or RhoG activity induces a phenotype similar to that produced by GEF1.

GEF1-induced Depletion of Regulated Secretory Cargo Is Caused by Enhanced Constitutive-like Secretion from Immature Secretory Granule

Depletion of secretory cargo could result from three processes: reduced synthesis, increased degradation, or enhanced secretion. We found that reduced synthesis and increased degradation of secretory cargo did not account for GEF1-induced depletion (Supplementary Figure S5). AtT-20 cells are ideal for studying the regulated secretory pathway because the sequence of events leading from unprocessed POMC to mature peptides and their unstimulated release or storage is known in detail (Figure 4A). Newly synthesized POMC is packaged into immature secretory granules (ISGs) that bud from the TGN and is then endoproteolytically processed (Zhou *et al.*, 1993). In AtT-20 cells, PC1 cleaves POMC in an orderly manner, first producing a 23-kDa intermediate plus β-lipotropin. The former is cleaved to yield an 18-kDa fragment plus ACTH; the 18-kDa fragment is further cleaved to yield a 16-kDa fragment plus a joining peptide. The terminal products of processing are stored in mature secretory granules and released upon stimulation (Mains and Eipper, 1976; Cullen and Mains, 1987; Fernandez *et al.*, 1997; Figure 4A).

Immediately after budding from the TGN, ISGs are not responsive to secretagogues, and a fraction of their content is secreted through the constitutive-like route (Noel and Mains, 1991; Fernandez *et al.*, 1997). During maturation, secretory granules acquire responsiveness to secretagogues,

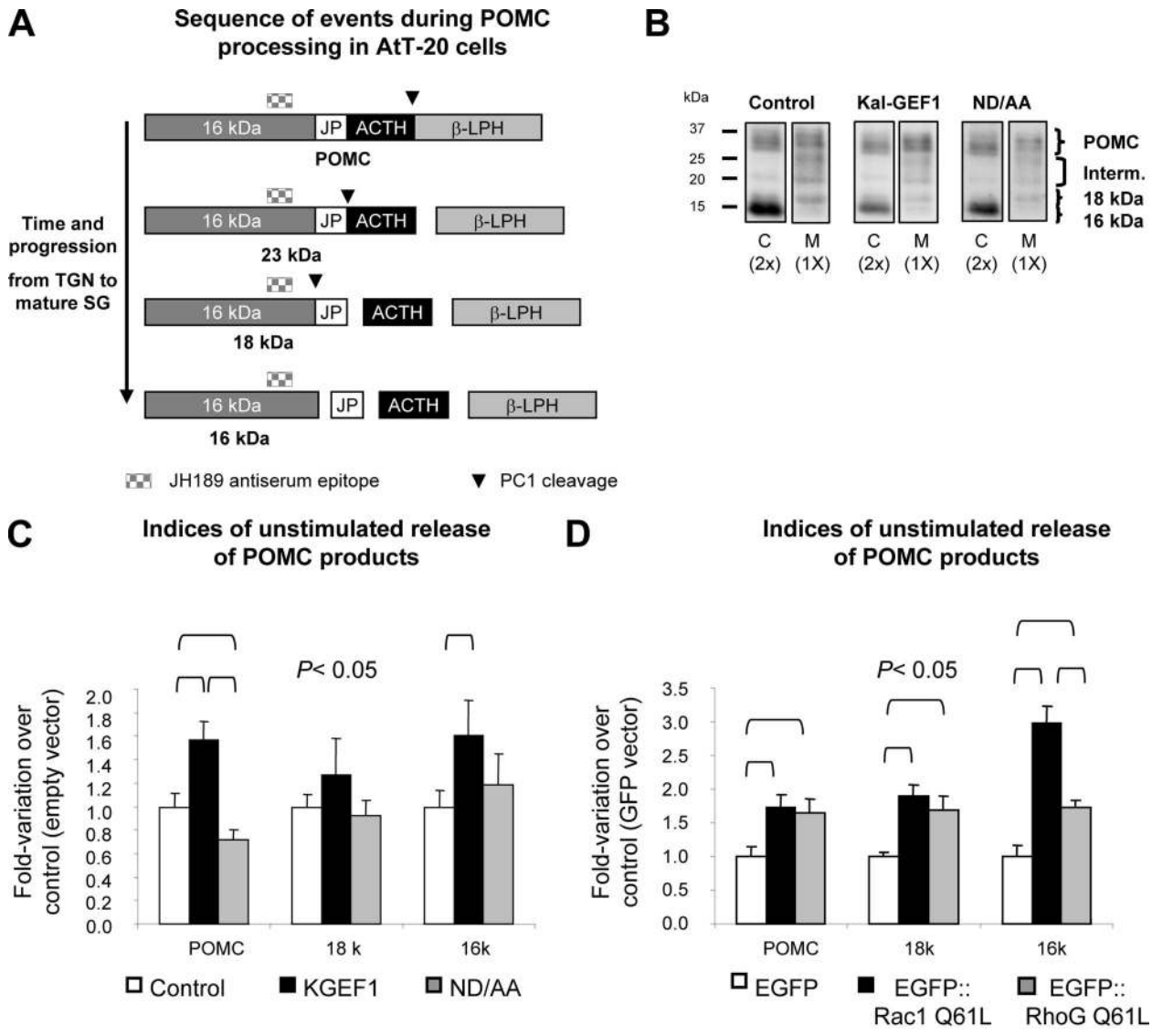

Figure 4. Overexpression of GEF1 or its activated Rho substrates enhances release of unprocessed and partially processed secretory cargo. AtT-20 cells were nucleofected with the indicated plasmids. Media (from 24 to 48 h after transfection) and cells were collected and analyzed by immunoblotting. (A) Schematic of POMC processing. PC1 cleavage sites and antibody specificity are indicated. (B) Representative blots from control-KalGEF1 and ND/AA-transfected cells. Two volumes of lysate and one volume of medium (2× and 1×) were loaded to yield comparable signal intensities; cells shown release this amount of POMC products in 12 h. Although POMC product signals in cells and media for each vector are comparable, Control, Kal-GEF1 and ND/AA cells were not normalized and are not comparable. (C) Secretion indices from blots obtained as in B were determined by dividing the signal intensities of the media by those of the corresponding lysates for each POMC product, after normalization to the volumes loaded. Indices, normalized to the corresponding value in controls, are expressed as means ± SEM; significant differences are shown (paired *t* test; n = 6). (D) Cells nucleofected with the indicated plasmids were treated and processed as in B; n = 6. (E) Cells were nucleofected with the indicated plasmids (empty pCMS EGFP vector was used as control) as in B and C. Secretagogue challenge was for 30 min with 2 mM $BaCl_2$. Release of the 16-kDa product was assessed by immunoblotting and expressed as percent of cell content (means ± SEM; n = 3).

a step that coincides with removal of VAMP4 and Synaptotagmin-IV (Eaton *et al.*, 2000). GEF1 activity could enhance either unstimulated secretion from mature secretory granules (MSGs) or constitutive-like secretion from ISGs; either effect would lead to the reduced cargo content we observe morphologically (Figure 2).

Using an antibody that detects intact and processed POMC (Cullen and Mains, 1987; Figure 4A), we measured the relative amount of each product in cells and media after a 24-h collection period in the absence of secretagogue. Partial glycosylation leads to multiple size variants of POMC and intermediate, which were grouped together for quantification (Figure 4B). Secretion of POMC and each product was measured as the ratio of its signal in medium to its signal in cells (secretion index). Kal-GEF1 transfected cells released more intact POMC than control cells. Secretion of intact POMC was inhibited in cells expressing the inactive GEF1 ND/AA mutant (Figure 4C). To cause a higher rate of secretion of unprocessed POMC, GEF1 must act early during secretory granule maturation, when the fraction of intact POMC is still high. A higher fraction of the 16-kDa product found in the mature granules that remain in Kal-GEF1 transfected cells was also released in resting cells. This observation can be explained by the GEF1-mediated activation of Rac, a known facilitator of secretion from mature granules in presence of cytosolic calcium (Li *et al.*, 2003); the spontaneous electrical activity of AtT-20 cells is known to cause transient increases in cytosolic calcium (Surprenant, 1982).

Both Rac1(Q61L) and RhoG(Q61L) increased unstimulated release of POMC and the 18-kDa fragment (Figure 4D). Unlike Rac1(Q61L), RhoG(Q61L) had a less dramatic effect on secretion of the 16 kDa than the 18-kDa product. The fact that active RhoG, like GEF1, is more effective in the parts of the secretory pathway proximal to the TGN may reflect the localization of wild-type and active RhoG to the Golgi/TGN region in AtT-20 cells (Figure 3, asterisks, Supplementary Figure S4), where ISGs form. RhoG could thus be the actual effector of endogenous Kalirin/Trio. The ability of Kalirin and Trio GEF1 to interact with other proteins (e.g., filamin, TrkA) through its PH domain may contribute to its cellular localization, thus explaining why its Rho substrates have similar but not identical phenotypes.

Although it has been reported that Rac1 activation enhances stimulated secretion from chromaffin cells (Li *et al.*, 2003), other studies suggested that inactivation of Rac1 and other Rho proteins lead to increased stimulated release of regulated secretory cargo (Frantz *et al.*, 2002). Our previous data are consistent with a positive modulatory role for Rac1 in stimulated secretion from anterior pituitary cells (Xin *et al.*, 2004). Therefore, we tested the effects of Kal-GEF1 and Rac1(Q61L) overexpression on the stimulated release of POMC products by AtT-20 cells. Both Kal-GEF1 and Rac1 overexpression induced an increase in the amount of 16-kDa product secreted upon barium challenge over that of control cells (control ~5-fold; Kal-GEF1 ~7–8-fold; Rac1(Q61L) ~11-fold over unstimulated rates; Figure 4E). Although depleted, in GEF1- and Rac1-transfected cells, the residual pool of mature granules is more sensitive to secretagogue challenge.

Inhibition of Endogenous GEF1 Activity Blocks Constitutive-like Release of Regulated Secretory Cargo

If the results obtained by overexpression reflect a physiological phenomenon, impairment of endogenous GEF1 should result in visible effects on both constitutive-like and stimulated release of granule content. A means to test this expectation would be the targeted down-regulation of Trio and Kalirin expression. However, a major drawback of this approach is ablation of the entire molecule, not just a single domain. Specific inhibition of GEF1 activity would be a more reliable method to test the role of endogenous Kalirin and Trio GEF1 domains in the regulated pathway without disrupting the functions of other domains of these complex molecules.

Screening of a chemical library using a yeast growth assay recently led to identification of small molecules that efficiently inhibit the exchange activity of Trio GEF1 in vitro (Blangy *et al.*, 2006), with no effect on the activity of Trio GEF2, ArhGEF17, or ARNO. We tested one of these compounds, NPPD, in mammalian cells. In HEK293-derived pEAK-RAPID cells transfected with vectors encoding GFP, GFP-KalGEF1, or GFP-TrioGEF1, we measured Rac activation after incubation with NPPD or vehicle. As expected, overexpression of Kalirin or Trio GEF1 led to activation of endogenous Rac (Figure 5A). NPPD inhibited Rac1 activation in control- and Kal/Trio GEF1-transfected cells by 50% (Figure 5A). Thus, NPPD is effective in living cells, but does not discriminate between Kalirin and Trio GEF1. Importantly, NPPD reduced basal activation of Rac in control cells, presumably due to inhibition of endogenous Trio, Kalirin, or a similar unidentified GEF. In fact, we know that Trio is expressed in pEAK-RAPID cells (not shown).

The effect of NPPD on other RhoG and Rac GEFs was not reported (Blangy *et al.*, 2006). To investigate specificity, we performed in vitro experiments with three additional Rho GEFs. Because GEFs interact with Rho proteins in their inactive state (Rossman *et al.*, 2005), binding of each GEF to immobilized GST-Rac1 loaded with GDP was measured. The binding of DBS, TIAM1, and VAV2 to Rac1-GDP was not affected by NPPD (Figure 5B). In contrast, NPPD enhanced Kal-GEF1 association with Rac1-GDP 5–6-fold. This suggests that NPPD inhibits Kalirin and Trio GEF1 activity by stabilizing the GEF1-Rac/RhoG-GDP complex, thus preventing GTP loading and activation of Rac/RhoG (Figure 5B). Besides providing information on the mechanism of inhibition, this experiment shows that NPPD selectively affects the activity of Kal/Trio GEF1, providing a tool to examine the effects of inhibition of Kalirin/Trio GEF1 activity on secretory pathways.

AtT-20 cells were incubated with vehicle or 100 μM NPPD for 3 h, and secretion was measured. NPPD reduced the secretion indices of all POMC products by 80–90% (Figure 6A; top and middle). Normalizing the signal for the 16-kDa product to the corresponding γ-adaptin signal, we found an approximately threefold increase in the amount of mature 16 kDa stored in cells (Figure 6A; bottom). We performed similar experiments with a lower concentration of NPPD for a longer time (10 μM for 24 h). NPPD decreased the secretion indices of intact POMC, intermediate and the 18-kDa fragment by 50–60%, with no significant change in the release of the mature 16-kDa fragment (not shown). This may indicate involvement of a GEF1 differing in NPPD sensitivity at the plasma membrane; in AtT-20 cells, the presence of different Kalirin and Trio isoforms, which may vary in their ability to be activated, may account for these differences between constitutive-like secretion and secretion from mature granules at lower NPPD concentrations. Overexpression experiments suggested a role for GEF1 in the response of AtT-20 cells to secretagogues (Figure 4E). Consistent with this, NPPD reduced the stimulated secretion of the mature 16-kDa fragment by ~70% (Figure 6B).

We wanted to determine whether overexpression of Kal-GEF1 or treatment with NPPD affected the constitutive delivery of proteins to the plasma membrane and extracellular

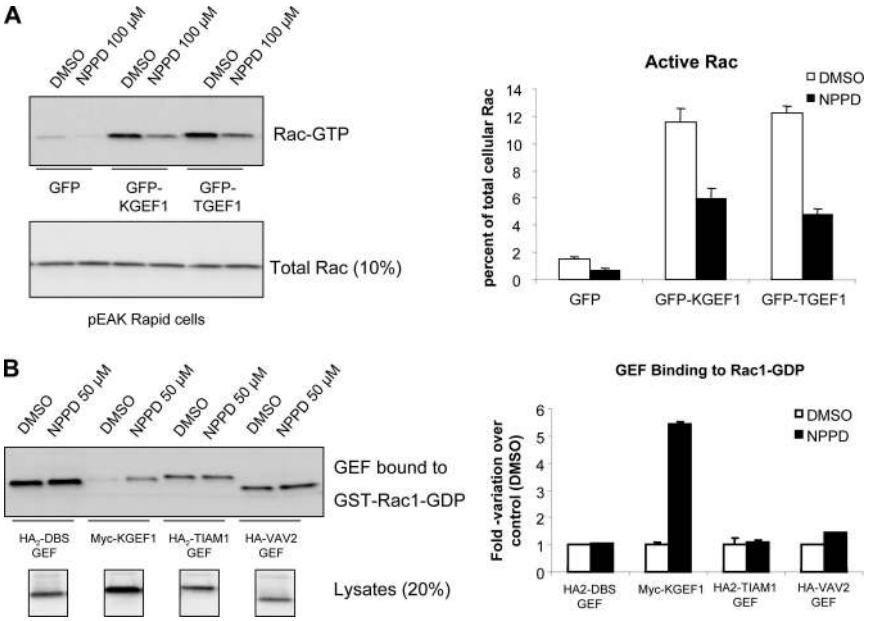

Figure 5. NPPD is a selective inhibitor of Kal/Trio GEF1 activity. (A) NPPD inhibits Kalirin and Trio GEF1 activity in cells. HEK293-derived pEAK Rapid cells were transfected with vectors encoding GFP, GFP-Kal-GEF1, or GFP-Trio-GEF1. For each transfection, duplicate wells were incubated with vehicle or 100 μM drug for 1 h, after which cells were lysed and processed for effector binding Rac activation assays (Schiller *et al.*, 2005). Active (GTP-bound) and total Rac1 in lysates were detected by immunoblotting. (B) Lysates of pEAK rapid cells transfected with constructs encoding the indicated isolated GEF domains were incubated with GST-Rac1 loaded with GDP in the presence of 50 μM NPPD or an equal amount of vehicle (DMSO). GEFs bound to GST-Rac1 immobilized to glutathione-Sepharose beads were eluted and detected by immunoblotting with anti-Myc and anti-HA antibodies. In both A and B, signals (left panels) were densitized and plotted (right panels). One experiment performed in duplicate is shown (bars, range of variation; where not visible, the variation was small). The experiments were repeated with similar results.

matrix. When expressed in AtT-20 cells, GFP with an appended signal sequence is targeted to the lumen of the secretory pathway but is stored in mature granules relatively inefficiently (El Meskini *et al.*, 2001). Overexpression of KalGEF1 did not significantly increase the unstimulated GFP secretion, which occurs via the constitutive and constitutive-like pathways (Figure 6C). NPPD potently inhibited GFP secretion from control cells and from cells overexpressing GEF1 (Figure 6C). This result suggests that Kalirin and/or Trio play a more general role as modulators of post-TGN secretory routes.

NPPD Treatment Affects the Actin Cytoskeleton and Rescues the Depletion of Regulated Cargo Induced by GEF1 Overexpression

The effects of NPPD on secretion of POMC products by nontransfected cells suggested that it might rescue the phenotype produced by GEF1 overexpression. We therefore tested the effects of NPPD treatment on the actin cytoskeleton and secretory granules in control- and KalGEF1-transfected cells (Figure 7). In both control and GEF1-expressing cells, NPPD treatment greatly reduced phalloidin staining intensity (Figure 7A). Control cells lost their prominent subplasma membrane filamentous actin, retracted their processes, and adopted a round shape. Although Kal-GEF1–expressing cells remained flat, lamellipodial protrusions were lost, and cortical filamentous actin was disrupted. Cells were stained for POMC to evaluate the effects of NPPD on granule cargo (Figure 7B). As expected (Figure 6A), POMC immunoreactivity increased in NPPD-treated control cells (Figure 7B). Most strikingly, NPPD treatment resulted in the accumulation of POMC products at the margins of GEF1-overexpressing cells (Figure 7B). We confirmed this observation biochemically. Kal-GEF1–expressing cells treated with NPPD showed an increase in the cellular content of 16 kDa compared with DMSO-treated Kal-GEF1–expressing cells (Figure 7C).

The results from these pharmacological inhibition studies agree with the predictions made from overexpression experiments and confirm a physiological role for Kalirin/Trio GEF1 activity at multiple stages in the regulated secretory pathway. As a whole, our findings support the observations by others that Rho proteins and their GEFs modulate the exocytic response to secretagogues. More importantly, we show that constitutive and constitutive-like release during the maturation of secretory granules is subject to regulation by Kalirin/Trio GEF1 activity.

DISCUSSION

Kalirin/Trio RhoGEF Subfamily and Regulated Secretory Pathway

Mammalian Kalirin and Trio and their *Drosophila* and *C. elegans* homologues are essential for neuronal development (Steven *et al.*, 1998; Bateman *et al.*, 2000; Newsome *et al.*, 2000; O'Brien *et al.*, 2000; Chakrabarti *et al.*, 2005). However, Kalirin was identified based on its interaction with trafficking determinants in the cytosolic domain of PAM (Alam *et al.*, 1996; Milgram *et al.*, 1996). Trio also interacts with PAM (Xin *et al.*, 2004). PAM catalyzes one of the final steps in peptide biosynthesis and is retrieved from immature secretory granules and from the plasma membrane for re-entry into secretory granules (Ferraro *et al.*, 2005). Increased expression of PAM-1 causes changes in cytoskeletal organization, accumulation of both POMC and PAM in the TGN, decreased secretory granule accumulation at process tips, and decreased regulated release of ACTH. Kalirin overexpression in AtT-20 cells stably expressing PAM-1 enhances targeting of PAM to the regulated pathway and restores secretagogue sensitivity (Mains *et al.*, 1999). We used AtT-20 cells to explore Kalirin/Trio functions related to secretory granule physiology.

Rho Proteins and Their GEFs in Regulated Secretion

Rac, Cdc42, and RhoA regulate mast cell degranulation (Price *et al.*, 1995; Norman *et al.*, 1996; Hong-Geller and Cerione, 2000) and neuroendocrine secretion, though, in the latter case, there is no consensus as to the family members or guanine nucleotide–bound state involved (Gasman *et al.*, 1999; Frantz *et al.*, 2002; Li *et al.*, 2003). Rho proteins modulate actin dynamics, and their effects on stimulated secretion

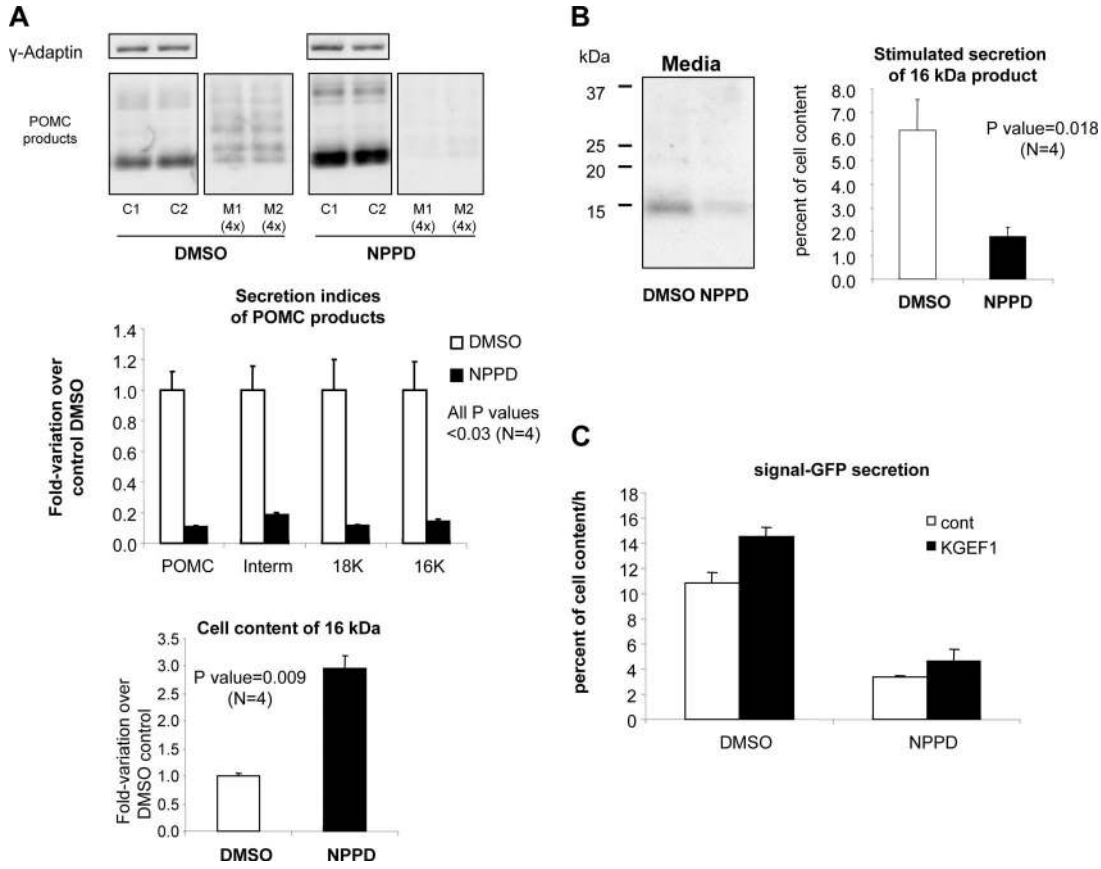

Figure 6. Inhibition of endogenous GEF1 activity blunts release of secretory cargo. (A) AtT-20 cells were incubated with medium containing vehicle (DMSO) or 100 μM NPPD for 3 h. Cells and media were analyzed as described in Figure 4. Blots of lysates (C1, C2) and media (M1, M2) from two samples for each treatment are shown (top panel). Secretion indices were determined (means ± SEM), middle panel. From the same blots, the signal intensity of the cellular 16-kDa product was normalized to the corresponding γ-adaptin signal (means ± SEM; bottom panel). (B) AtT-20 cells were pretreated for 2.5 h in CSFM with 100 μM NPPD or the same amount of vehicle (DMSO) and then challenged with 2 mM $BaCl_2$ for 30 min (with DMSO or NPPD). A representative immunoblot from media is shown (top panel). The amount of mature 16-kDa product secreted was determined (means ± SEM; bottom panel). (C) AtT-20 cells were cotransfected by nucleofection with signalGFP vector and control vector pEAK10 or pEAK10 vector encoding Kal-GEF1. Cells were either treated with DMSO or NPPD (100 μM) for 2 h. Cell lysates and media separated by SDS-PAGE were probed for GFP; the signals were densitized (n = 3).

have been attributed to changes in the subplasmalemmal actin cytoskeleton (Trifaro and Vitale, 1993; Li *et al.*, 2003). The identity of RhoGEFs involved in the regulated secretory pathway and their regulation have not been extensively explored. To date, roles for β-Pix and Intersectin-1L, activators of Rac and Cdc42, respectively, in chromaffin cells (Audebert *et al.*, 2004; Malacombe *et al.*, 2006) and for Kalirin and Trio, activators of Rac and RhoG, in pituitary cells (Mains *et al.*, 1999; Xin *et al.*, 2004) have been established. These studies focused on stimulated exocytosis, hence on events at the plasma membrane. A more general role for RhoGEFs in the regulated secretory pathway has not been explored. Our finding that Kalirin and Trio cofractionate with different secretory organelles suggests that these RhoGEFs function at multiple stages in the secretory pathway.

GEF1 Activity Regulates Constitutive-like Secretion

Studies of the oncogenic capabilities of the first identified GEFs revealed both the strong biological potency of these GEFs and the modulatory effects of adjacent regions in the larger, proto-oncogene forms (Schmidt and Hall, 2002; Rossman *et al.*, 2005). In this context, we set out to study the effects of the GEF1 and GEF2 domains of Kalirin and Trio on the regulated secretory pathway in AtT-20 cells. The isolated GEF1 domains of Kalirin and Trio cause dramatic changes in cytoskeletal organization and depletion of secretory cargo cell content in the absence of secretagogue; release comes primarily from immature secretory granules. Accordingly, selective pharmacological inhibition of endogenous Kalirin/Trio GEF1 strongly reduces secretion from early compartments during granule maturation and increases accumulation of mature product in the cells. In contrast, the GEF2 domains of Kalirin and Trio have no obvious effect on cytoskeletal organization or secretion. A morphologically similar GEF1 phenotype was observed in PC12 cells, suggesting that the activity of Kalirin/Trio GEF1 has a general effect on secretory granule physiology.

Model

Our results are consistent with a model in which constitutive traffic from the TGN and constitutive-like secretion from immature granules are regulated by the activity of endogenous Kalirin/Trio (Figure 8). Endoproteolytic cleavage of newly synthesized POMC begins in ISGs and continues during secretory granule maturation, allowing unprocessed POMC and immature products to be secreted through the

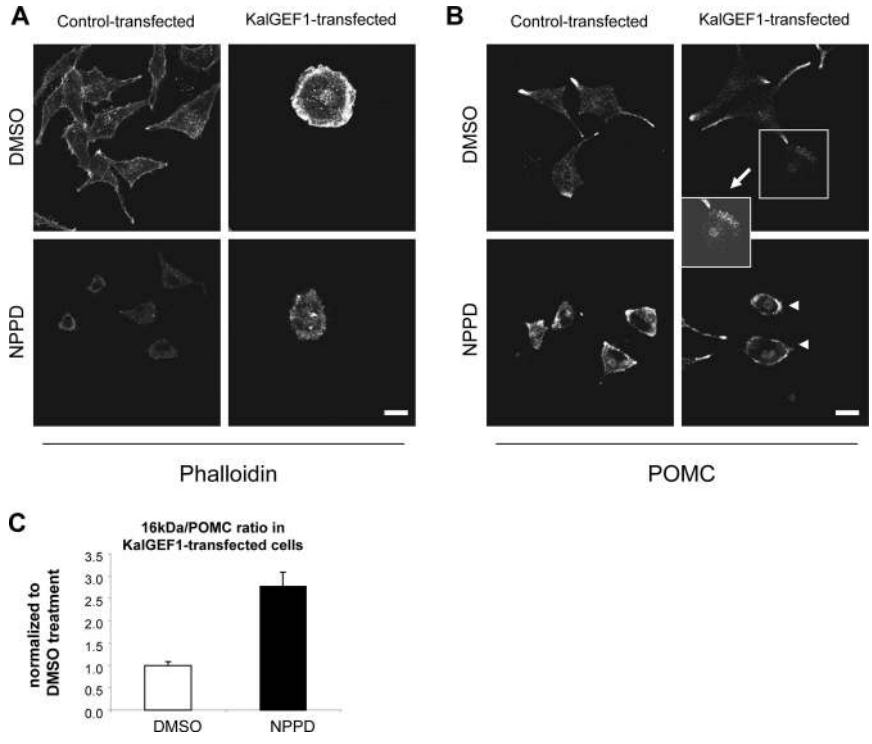

Figure 7. NPPD disrupts filamentous actin and reverses the effects of GEF1 overexpression on the secretory pathway. Cells transfected with plasmid encoding Kal-GEF1 or its parental vector (control) were treated for 90 min with DMSO or NPPD (100 μM). Filamentous actin (A) and POMC (B) were visualized. In B GEF1 expressors are indicated by arrowheads. The inset region is shown with enhanced luminosity to show the GEF1 expressor; scale bar, 20 μm. (C) Lysates from cells treated as in A and B were extracted and probed for POMC product by immunoblotting. Enrichment of mature 16 kDa is measured relative to POMC (n = 3).

constitutive-like pathway (Noel and Mains, 1991; Fernandez *et al.*, 1997).

Constitutive-like release of partially processed secretory products is thought to involve the budding of vesicular intermediates from immature secretory granules (Figure 8A; Arvan *et al.*, 1991; Kuliawat and Arvan, 1992; Dittie *et al.*, 1997; Klumperman *et al.*, 1998). A subset of granule membrane proteins is removed by this process. Although GEF1 expression alters the steady-state distribution of PAM-1 and VAMP4, localization of TGN38 and CPD shows little change. At least in some cell types, these vesicular intermediates fuse with an endosomal compartment, from which membrane proteins are retrieved and soluble cargo secreted (Turner and Arvan, 2000).

Activation of endogenous Kalirin/Trio GEF1 leads to enhanced constitutive-like secretion, possibly through increased budding of carrier vesicles from ISGs; as a result, the cargo content of mature granules is reduced (Figure 8B). Conversely, when GEF1 activity is diminished, constitutive-like release is inhibited, resulting in the accumulation of mature cargo (Figure 8C). After budding from the TGN, as processing of secretory cargo starts in the lumen, the membrane of immature secretory granules undergoes remodeling, with removal of selected components that are either missorted (mannose 6-phosphate receptors, furin) or absent from mature granules (Syntaxin 6, VAMP4, and Synaptotagmin IV; Fernandez *et al.*, 1997; Wendler *et al.*, 2001).

Overexpression of GEF1 alters the steady-state localization of VAMP4, suggesting an effect on ISGs. In AtT-20 cells, inhibition of Arf recruitment to ISGs by brefeldin A allows POMC processing in ISGs (Fernandez *et al.*, 1997) but blocks VAMP4 and Synaptotagmin-IV removal from the ISG membrane, a step coinciding with acquisition of stimulus responsiveness by secretory granules (Eaton *et al.*, 2000). Phosphorylated VAMP4 recruits PACS-1 and AP1 to ISGs, modulating selective removal of membrane components via clathrin (Hinners *et al.*, 2003). The complexity of secretory granules is emerging from

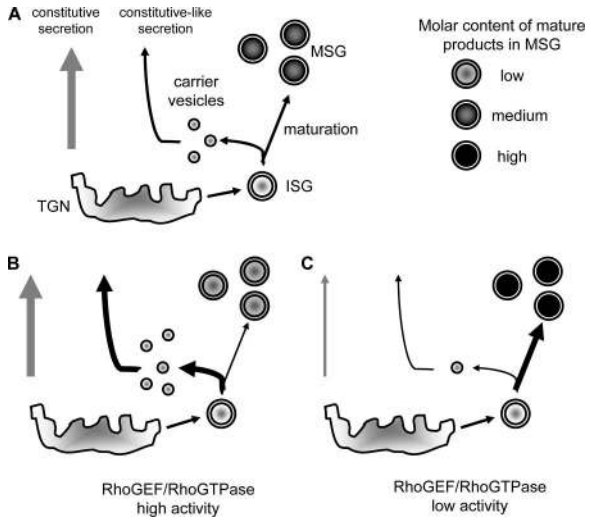

Figure 8. Effects of GEF1-like activity modulation on the regulated secretory pathway. (A) During secretory granule maturation, a fraction of the soluble cargo is secreted through the constitutive-like route while the rest proceeds to full maturation and storage in mature granules. Constitutive-like release is thought to occur through vesicles that bud from ISGs (carrying with them partially processed cargo) and fuse with the plasma membrane either directly or through endosomal intermediates (not drawn) as suggested by experimental evidence from insulinoma cells (Turner and Arvan, 2000). The direct route from the TGN to the plasma membrane (constitutive secretion) is also depicted. (B) Activation of GEF1-like enzymes and their Rho substrates leads to increased constitutive-like release from immature granules, resulting in a reduction of secretory cargo content. (C) The opposite result is produced when endogenous GEF1-like activity is reduced; both constitutive-like and constitutive release may be inhibited (see effects on secreted GFP, Figure 6C), resulting in an increase in secretory cargo content.

proteomic analyses (Muth *et al.,* 2004; Chen *et al.,* 2006). Hundreds of proteins are associated with these organelles, including Kalirin with atrial granules, which are relatively immature granules containing unprocessed proatrial natriuretic factor (Muth *et al.,* 2004). Some associate with granules only transiently (e.g., Rab GTPases), suggesting that protein composition is dynamic and that granules exist in different immature and possibly mature functional states, each with its particular complement of proteins.

GEF1 overexpression has a major effect on POMC secretion, which in theory can be secreted directly from the TGN via the constitutive pathway. The ability of NPPD to inhibit release of GFP from AtT-20 cells expressing signal GFP is consistent with an effect on constitutive release (Figures 6C and 8C). Subcellular fractionation certainly suggests that isoforms of Kalirin could localize to TGN and endosomes (Figure 1B). Overexpression of active Rac or RhoG has a more general effect, increasing secretion of partially processed POMC products (18 kDa) and identifying the immature granule and constitutive-like secretion as targets. Our experiments with NPPD, which inhibits constitutive-like secretion of all partially processed POMC products, support this conclusion.

Many constitutively released proteins transit through endosomal compartments en route to the plasma membrane (Rodriguez-Boulan and Müsch, 2005). The fact that Kal/Trio GEF1 affect signal-GFP but not TGN38 and CPD, which cycle through the TGN (Figure 2C), may indicate an important role for Kalirin and/or Trio interactors in recruiting the GEF to specific subdomains of this compartment. In addition to effects on ISGs and at the plasma membrane, Kalirin and Trio isoforms may be involved in vesicular traffic events in additional compartments.

Although NPPD is a valuable new tool for selectively inhibiting Kalirin and Trio GEF activity, it cannot distinguish these two GEFs. Additional studies will be required to determine whether Kalirin or Trio is the actual effector of constitutive-like secretion in any given cell type. The subcellular localization of the relevant endogenous GEF is critical to function. Immunohistochemical studies on Kalirin expression at various stages of embryonic rat development identified Kalirin in the perinuclear/TGN region, consistent with a function early in the secretory pathway (Hansel *et al.,* 2001). The signals acting upon the Kalirin/Trio that regulate constitutive-like release from ISGs remain to be identified.

What Rho Effectors Are Involved in This Cellular Process?

Some of the remodeling events at the immature granule membrane are mediated by Arf1, likely through AP1 and GGAs (Fernandez *et al.,* 1997; Eaton *et al.,* 2000; Hinners *et al.,* 2003; Kakhlon *et al.,* 2006). A role for localized actin dynamics in clathrin-mediated vesicle budding has been established (Carreno *et al.,* 2004), suggesting that it may also be involved in maturation of secretory granules. However, not all Rho signaling involves actin dynamics. Phospholipase D (PLD) activity stimulates secretory vesicle budding from the TGN (Chen *et al.,* 1997). PLD requires Rho proteins as cofactors for activation (Siddhanta and Shields, 1998; Jones *et al.,* 1999). Another lipid-modifying enzyme, phosphatidylinositol 4-phosphate 5-kinase (PI4P5K), binds Rho proteins and regulates secretion in neuroendocrine cells (Tolias *et al.,* 1995; Holz *et al.,* 2000; Aikawa and Martin, 2003; Weernink *et al.,* 2004).

What Is the Biological Significance of Regulating Constitutive and Constitutive-like Release by Peptidergic Secretory Cells?

We propose that Kalirin/Trio modulate signaling during development and in pathological states. Because of the temporal sequence of events during processing (see Figure 4A), the constitutive and constitutive-like routes release sets of peptides that differ from those stored in mature granules. For a secretory cell that produces several peptides from a common propeptide, the ability to regulate secretion from TGN and endosomes, (constitutive release), immature granules (constitutive-like release), and mature granules allows a different spectrum of biological responses. For example, ACTH precursors appear to play a role during late stages of development by regulating the levels of fetal plasma cortisol (Schwartz *et al.,* 1995). Neurotrophins, which can be sorted to the regulated pathway, serve as another example (Mowla *et al.,* 1999; Wu *et al.,* 2004). Nerve growth factor (NGF) promotes neuronal differentiation and survival, whereas pro-NGF is proapoptotic (Lee *et al.,* 2001). Shifting release from mature granules, where processed NGF is stored, toward the constitutive or constitutive-like route, with increased pro-NGF secretion, may represent a mechanism for fine tuning and coordination of survival and apoptosis during neuronal development.

In addition, dysregulation of the balance between constitutive/constitutive-like secretion and maturation could lead to pathology. For instance, in Alzheimer disease, high levels of pro-NGF are thought to contribute to neuronal apoptosis (Pedraza *et al.,* 2005). Another example is the hypersecretion of precursors, a characteristic of secretory tumors. Enhanced constitutive/constitutive-like secretion of peptides that act as autocrine growth factors (galanin, ghrelin, and insulin) by adenomas (Invitti *et al.,* 1999; Ohsugi *et al.,* 2005; Martinez-Fuentes *et al.,* 2006) may play an important role in the establishment and maintenance of these tumors.

In summary, we report the identification of a novel regulatory step during secretory granule maturation. Kalirin/Trio RhoGEF signaling modulates the extent to which regulated cargo enter and remain in the regulated secretory pathway. Regulation at these steps would allow fine tuning of the set of peptides released by a single secretory cell type. Impaired signaling leading to increased Kalirin/Trio RhoGEF activity could contribute to the development of pathological states.

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