Dynamin II Regulates Hormone Secretion in Neuroendocrine Cells*

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The dynamin family of GTP-binding proteins has been implicated as playing an important role in endocytosis. In Drosophila shibire, mutations of the single dynamin gene cause blockage of endocytosis and neurotransmitter release, manifest as temperature-sensitive neuro-muscular paralysis. Mammals express three dynamin genes: the neural specific dynamin I, ubiquitous dynamin II, and predominantly testicular dynamin III. Mutations of dynamin I result in a blockade of synaptic vesicle recycling and receptor-mediated endocytosis. Here, we show that dynamin II plays a key role in controlling constitutive and regulated hormone secretion from mouse pituitary corticotropic (AtT20) cells. Dynamin II is preferentially localized to the Golgi apparatus where it interacts with G-protein βγ subunit and regulates secretory vesicle release. The presence of dynamin II at the Golgi apparatus and its interaction with the βγ subunit are mediated by the pleckstrin homology domain of the GTPase. Overexpression of the pleckstrin homology domain, or a dynamin II mutant lacking the C-terminal SH3-binding domain, induces translocation of endogenous dynamin II from the Golgi apparatus to the plasma membrane and transformation of dynamin II from activity in the secretory pathway to receptor-mediated endocytosis. Thus, dynamin II regulates secretory vesicle formation from the Golgi apparatus and hormone release from mammalian neuroendocrine cells.

Dynamin is a polypeptide with a modular structure comprising a GTP-binding domain in the N-terminal third, a middle domain of unknown function, a pleckstrin homology (PH)1 domain and a C-terminal proline-rich or Src homology 3- (SH3-) binding domain (for reviews, see Refs. 1–8). Mammals have at least three dynamin genes which code for dynamin I, II, and III. Although the homology between dynamin I and II proteins is 79% and between dynamin I and III proteins is 89%, significant variation occurs at the C-terminal regions. In addition, dynamin I is specifically expressed in neural tissues, whereas dynamin II is ubiquitous and dynamin III is predominantly testicular. These developmentally divergent dynamins may thus represent a large protein family apparently performing a range of functions in association with distinctive sites in mammals (1, 2).

While dynamin I has been implicated as playing an important role in mediating synaptic vesicle recycling, little is known of the physiological function of dynamin II. Since dynamin I complexes a large ring-like structure surrounding the necks of clathrin-coated endocytic pits on the cytoplasmic surface of presynaptic plasma membrane during synaptic vesicle recycling (3, 6), it is thought that dynamin II plays a similar role to that of dynamin I in receptor-mediated endocytosis in non-neuronal cells (4–8). In support of this hypothesis are the findings that the GTPase activities of both dynamin I and II, and of their SH3-binding domain truncation mutants, are similarly stimulated in vitro by phospholipids, grb2, and microtubules (9), suggesting that the modes of interaction of the PH domains from dynamin I and II with phosphatidylinositol (4,5)-bisphosphate and of the SH3-binding domain with microtubules and grb2 are similar in vitro (9). However, it is not yet established why dynamin I and II are concomitantly present in neurons (4), and why rapid endocytosis in adrenal chromaffin PC12 cells is significantly inhibited by the microinjected PH domain of dynamin I but not by the PH domain of dynamin II (10). Given the previous reports that dynamin II associates with the trans-Golgi network in human hepatic (HepG2) cells (11), and that dynamin I and/or II immunoreactivity is present in the Golgi apparatus of human foreskin melanocytes and fibroblasts (12), it is conceivable that dynamin II might have a different function in membrane trafficking from the endocytic role of dynamin I in mammalian cells (4). To establish a biological function of dynamin II, the present study was undertaken to explore the activity of dynamin II in mouse pituitary corticotropes (AtT20 cells). We found that dynamin II plays a key role in controlling both constitutive and regulated hormone secretions at the Golgi apparatus of these neuroendocrine cells.

MATERIALS AND METHODS

Chemicals and Peptides—ATP, GTP, creatine phosphate, and creatine kinase were from Roche Molecular Biochemicals Australia Pty. Ltd. (New South Wales, Australia). Na125I was from PerkinElmer Life Sciences Australia Pty. Ltd. (NSW, Australia). The peptide hormones rat CRHb, Tyr-Oct-CRH, and β-endorphin were from Peninsula Laboratories, Inc. (San Carlos, CA). The dynamin II peptides (607CDSQED-VDSWKASFLRA and 593PIRPAEFLLD) were synthesized by Chiron Technologies Pty. Ltd. (Victoria, Australia). Gel electrophoresis reagents were from Bio-Rad. Human transferrin (T2252), myelin basic protein, protein A-Sepharose 4B, and all other chemicals were from Sigma.

Preparation of Dynamin Expression Constructs and Expression of Recombinant Proteins—Expression vectors for wild-type, GTP-binding mutation, SH3-binding mutation, and PH domain alone of dynamin I and II were generated by subcloning PCR recombinant cDNA fragments into pcDNA3HA plasmids with CMV and SV40 promoters flank-
Dynamin II and Hormone Secretion

ing the multiple cloning site to drive expression of the cDNA. pcDNA3HA was derived from pcDNA3 (Invitrogen, NV, Leek, The Netherlands) by subcloning a double-stranded oligonucleotide sequence containing a BamHI restriction site and a downstream sequence coding for HA peptide (YPYDVPDYA). Dynamin II PCR fragments were made with primers (Promega, Madison, WI), which were used to verify the sequence of the cDNA inserts and the molecular sizes of the expressed proteins. The expression vectors were then transfected into monolayer cultures of AtT20 cells (D16-16, kindly provided by Ken Sheppard) with LipofectAMINE (10 μl/μg of DNA/ml). After about 4 weeks of selection with G418 (400 μg/ml), cells were tested for expression of recombinant dynamin cDNAs, protein localization, receptor-mediated endocytosis, vesicular production, and hormone release. In all cases, we used either normally cultured AtT20 cells or stably transfected cells after G418 selection as indicated in the individual experiments. For the glutathione S-transferase dynamin II PH domain fusion protein a DNA fragment, generated by specific PCR using dynamic IIa cDNA template and the synthetic oligonucleotides (ggatggtgttgcgcgctctggcc (jp22), gctgccggcgctctggcc (jp19), accgagctttgcgccggcgctctggcc (jp22), cggactctccggcccagctcgcag (jp4)) as primers, was subcloned into the pGex5X-3 plasmid and after sequence verification transformed into Escherichia coli BL21 to produce the fusion proteins under isopropyl-1-thio-galactopyranoside induction.

Antibodies—Dynamin II antibodies were raised in rabbits by multiple immunization with the synthetic peptides with the PH domain peptide (MILLEDVPRPMPARRPLQG) (Promega) and the HA peptide (YPYDVPDYA). The affinity-purified antibodies were affinity-purified against the PH domain peptide or the C-terminal peptide, respectively, as described previously (15). Other antibodies used to detect proteins included anti-HA epitope monoclonal antibody (12CA5 hybridoma), anti-endothelin antibody (12CA5 hybridoma), anti-ACTH antibody (R72) (17), anti-beta-COP antibody (Sigma), anti-human p230 antiserum (18), anti-Golgi antibody (Sigma), anti-peripherin antibody (R56) that detects both β-endorphin and its precursor POMC peptide (17). For cellular signaling pathways underlying cellular growth and differentiation, mitogen-activated protein (MAP) kinase activity and DNA synthesis were determined by phospho-specific antibodies. For immunoprecipitation, 100 μg of protein were passed through the 22-gauge needle 5 times, and the protein samples were then analyzed by immunoblotting and immunoprecipitation. In some experiments, highly purified Golgi fractions were used by diluting the fractions 3–5 with 1.8 μg/ml sucrose followed by sequential overlays with 1.18 and 0.96 μg/ml sucrose and centrifugation as described (11). For immunoblotting, proteins in 15 μl of each fraction were separated by electrophoresis on a 10% acrylamide minigel, transferred to 0.45-μm nitrocellulose membranes, and detected by incubation with specific primary antibodies followed by further incubation with a peroxidase-conjugated anti-IgG and then with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). All steps were performed at room temperature. The membranes were subjected to an incubation at 4 °C for 1 h with specific primary antibodies in the presence or absence of glutathione S-transferase-PH domain fusion protein (10 μg) followed by another incubation with Gammabind plus Sepharose (Amersham Pharmacia Biotech) and extensive washing. Immunoprecipitated proteins were resolved in SDS-PAGE followed by immunoblotting with different antibodies. Immuno-fluorescence Microscopy—AtT20 cells or PC12 were grown on glass coverslips and after washing in PBS subjected to immunofluorescence staining as described previously (22). Briefly, cells were prefixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min, free aldehyde groups quenched in 50 mM NH4Cl in PBS, and the cells permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After washing and blocking for 30 min with 1% bovine serum albumin, cells were incubated for 1 h at room temperature with the primary antibodies diluted in 1% bovine serum albumin in PBS, followed by washing and another incubation with fluorescein isothiocyante-conjugated sheep anti-rabbit IgG (Silenus, Australia) for 1 h at room temperature. After a further wash in PBS, the coverslips were mounted onto glass slides with 2.6% DABCO (1,4-diazabicyclo[2.2.2]octane; Pierce) in 90% dioxane (pH 6.5). Slides were then analyzed in a confocal laser scanning imaging system (Bio-Rad MRC 1024 imaging system). The confocal images were captured by a 60X/1.4 Nikon oil lens (Nikon ECLIPSE E600). For dual labeling experiments, cells were incubated with affinity purified rabbit anti-dynamin II antibody (1:2000 dilution in 1% bovine serum albumin/PBS) for 1 h at room temperature, washed, and then incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG. After a further wash and further incubation with human anti-p230 antibody. After washing in PBS, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated sheep anti-human IgG (Dako Corp., Botany, Australia). After a further wash, the coverslips were mounted as above. Control incubations demonstrated no cross-reactivity between the conjugated secondary antibodies and between the primary antibodies and unrelated second antibodies.

Secretory Vesicle Production—Nascent vesicular formation from the Golgi was assessed according to published methods with minor modifications (20, 21). Briefly, 10 × 10⁶ cells stably expressing wild-type or mutated dynamin II were treated with lysis buffer (0.25 mM sucrose, 10 mM HEPES-KOH, pH 7.2, 1 mM EDTA) or swelling buffer (10 mM HEPES-KOH, pH 7.2, 10 mM NaCl) at 4 °C for 5 min for vesicle formation analysis in vitro and in intact cells, respectively. Cells in the lysis buffer were further lysed by passing them through a 22-gauge needle 5 times, followed by centrifugation at 600 × g for 10 min to remove the nuclei, and the post-nuclear supernatant at 14,000 × g for 10 min at 4 °C to obtain the Golgi-rich membrane fraction. In contrast, cells in swelling buffer were passed through the 22-gauge needle once to generate semi-permeable membranes, which were then incubated in 5 volumes of breaking buffer (100 mM KCl, 1 mM HEPES-KOH, pH 7.2, 100 units/ml Trasylol). The Golgi-rich membranes and semi-permeable cells were incubated in the nascent vesicle buffer (10 mM HEPES-KOH, pH 7.3, 0.5 mM CaCl₂, 2.5 mM MgCl₂, 110 mM KCl, 35 mM KOAce, 10 mM creatine phosphate, 80 μM/μl creatine phosphate kinase, 0.5 mM phenylisothiourea fluoride, and 5 μM/ml Trasylol) for different times at
RESULTS

Expression of Dynamin II Regulates β-Endorphin Secretion from AtT20 Cells—To explore the cellular function of dynamin II, dynamin II mutants were generated and stably expressed in AtT20 cells. While transfection of cultured AtT20 cells with wild-type or mutated dynamin II showed no effect on clathrin-dependent and -independent receptor internalization (data not shown), both basal and CRH-stimulated hormone secretion were modulated over 2–48 h of observation by overexpression of either wild-type or mutated dynamin II (Fig. 1), with the pattern of release of β-endorphin triggered by KCl (not shown) or CRH from the different transformants remaining similar. Overexpression of wild-type dynamin II significantly enhanced both basal and CRH-stimulated secretion of β-endorphin, whereas the single amino acid mutation in dynamin II GTP-binding domain (K44A) lower basal and CRH-induced β-endorphin release (30–60% reduction relative to plasmid-only control cells, and 50–75% to the wild-type). To determine whether these effects of dynamin II on hormone secretion were due to alterations in β-endorphin synthesis, cellular content of β-endorphin was examined. Cellular content of β-endorphin was not significantly changed between the different groups of cells transfected with control plasmids, wild-type, or GTP-binding domain-mutated dynamin II over a period from 2 to 24 h (not shown). To determine whether the effects of dynamin II on hormone secretion might have resulted from nonspecific changes in cellular proliferation or signaling, basal DNA synthesis and MAP kinase activity in response to epidermal growth factor and the phorbol ester, phorbol 12-myristate 13-acetate were measured in the different transformants. No significant difference was observed for either DNA synthesis or MAP kinase activity between control and dynamin II mutants (Fig. 1C). Given that changes in hormone release induced by dynamin II variants are accompanied by no change in cellular content and increased total synthesis of the hormone, it is likely that dynamin II may be involved in regulating secretory vesicle transport with the coupling between hormone secretion and synthesis not being affected; enhanced effects on secretion may lead to enhanced levels of synthesis to replenish the released stores of β-endorphin.

To further explore the physiological role of dynamin II in hormone secretion, two dynamin II antisense expression plasmids were generated and transfected into AtT20 cells. Transfection of the cells with either antisense construct resulted in a decrease of endogenous dynamin II by 50–60% (Fig. 2A) and a reduction of β-endorphin secretion at rest as well as after stimulation by CRH at different concentrations (Fig. 2B). To determine the specificity of the effect of dynamin II β-endorphin secretion, we examined whether the dynamin I GTP-binding domain mutant might have such an effect. As shown in Fig. 2B, the dynamin I GTP-binding mutant had no significant effect on β-endorphin secretion. On the other hand, transfection with the dynamin II antisense constructs showed no effect on transferrin receptor-mediated endocytosis, whereas transfection with the dynamin I GTP-binding domain mutant showed about 40% inhibition compared with the control (Fig. 2C). These findings, that decreasing or increasing cellular expression of dynamin II inhibits or stimulates hormone release from neuroendocrine cells, suggest that the dynamin II levels regulate the level of hormone secretion in vivo.

Dynamin II Resides in the Golgi Apparatus—To further characterize the action of dynamin II during hormone secretion, the subcellular distribution of dynamin II was studied by fractionation of cultured AtT20 cells followed by immunoblotting. As shown in Fig. 3, dynamin II immunoreactivity was predominantly found in fractions 3–5 from the top of a continuous sucrose gradient (0.8–2 M). These were confirmed as Golgi containing fractions by the presence of the Golgi marker proteins Rab6 and β-COP (Fig. 3), and the trans-Golgi network (TGN) protein p230 (18, 22) (data not shown). In contrast, no immunoreactive dynamin II was found in the plasma membrane fractions containing plasma membrane receptors for transforming growth factor β R1 (Fig. 3). Confocal immunofluorescence microscopy was also employed.
to localize dynamin II in cultured AtT20 cells, using the antibody against dynamin II C-terminal tail sequence (858PTIIR-PAEPSLLD). Significant immunofluorescent staining was observed in the juxtanuclear regions of cultured AtT20 (Fig. 4). When the staining was compared with that seen with an antibody against the TGN protein p230 (18, 22), dynamin II was found to colocalize with p230 in cultured AtT20 cells, either nontransfected or transfected with control plasmids (Fig. 4). In contrast, in cells transfected with dynamin II antisense plasmids, staining for p230 was clearly visible but little staining for dynamin II was observed, suggesting that levels of endogenous dynamin II were significantly lowered by dynamin II antisense RNA. To corroborate the Golgi localization of dynamin II in another type of neuroendocrine cells, subcellular localization of dynamin II and p230 was determined in adrenal chromaffin PC12 cells. As shown in Fig. 5, dynamin II and p230 colocalized in the perinuclear regions of PC12 cells with or without treatment by nerve growth factor (NGF-7S, 1 μg/ml) for 24 h. These data suggest that dynamin II is preferentially associated with the Golgi apparatus in both AtT20 and PC12 cells. Given that the antibody used to localize dynamin II is against the C-terminal tail region that does not differentiate dynamin II alternative splicing product (13), it is formally possible that the dynamin II associated with the Golgi may be a dynamin II splice variant that is a predominant form in the neuroendocrine cells.

Overexpression of Dynamin II PH Domain, or a Dynamin II Mutant Lacking the SH3-binding Domain, Induces Translocation of Endogenous Dynamin II from the Golgi Apparatus to the Plasma Membrane—

To explore roles for the C-terminal PH-domain.

C, underexpression of dynamin II does not inhibit transferrin receptor-mediated endocytosis. Cells transfected with empty vectors (○), dynamin II antisense 1 (▲), dynamin II antisense 2 (▼), or dynamin I GTP-binding mutant (▲) were incubated with or without various concentrations of CRH as indicated for 8 h and β-endorphin release was determined. While the dynamin I mutant does not significantly affect hormone secretion, underexpression of dynamin II inhibits β-endorphin release which is similar to that observed with the dynamin I GTP-binding mutant seen in Fig. 1. The letter B denotes basal condition without CRH. Data are mean ± S.D. from one of two similar experiments.

| Proteins | Control | Antisense 1 | Antisense 2 |
|----------|---------|-------------|-------------|
| + + + +  | - - - -  | - - - -     | - - - -     |
| MW (kDa) | 20 40 60 80 100 120 140 160 180 200 | 20 40 60 80 100 120 140 160 180 200 |

**FIG. 2.** Expression of endogenous dynamin II plays an important role in hormone secretion. A, expression of two dynamin II antisense constructs inhibits endogenous dynamin II synthesis in AtT20 cells. Cells were stably transfected with empty vectors (lanes 1–3), dynamin II antisense construct 1 (lanes 4–6), or dynamin II antisense construct 2 (lanes 7–9), and 5, 10 and 20 μg of cellular extract analyzed for each transformant by SDS-PAGE followed by immunoblotting with anti-dynamin II antibody. B, underexpression of dynamin II inhibits hormone secretion. Cells transfected with empty vectors (E), dynamin II antisense 1 (Œ), dynamin II antisense 2 (), or dynamin I GTP-binding mutant (l) were incubated with or without various concentrations of CRH as indicated for 8 h and β-endorphin release was determined. While the dynamin I mutant does not significantly affect hormone secretion, underexpression of dynamin II inhibits β-endorphin release which is similar to that observed with the dynamin II GTP-binding mutant seen in Fig. 1. The letter B denotes basal condition without CRH. Data are mean ± S.D. from one of two similar experiments.

**FIG. 3.** On subcellular fractionation dynamin II colocalizes with the Golgi proteins Rab6 and β-COP. Total cell lysates were subjected to continuous sucrose gradient (0.8–2 M) centrifugation followed by fractionation from the top of the centrifugation column. Fractions from 1 to 13 were used to immunoblot for different proteins with specific antibodies. While receptors for transforming growth factor β (TGFβR) were found in fraction 9-11 (A), dynamin II (B), Rab6 (C), and β-COP (D) were detected in the Golgi-enriched fractions (fractions 3–5; sucrose 1.1–1.2 M). Results are representative of four similar experiments.
and SH3-binding domains of dynamin II in subcellular localization, constructs coding for wild-type dynamin II, dynamin II GTP-binding-domain mutant, dynamin II PH domain only mutant, and the dynamin II mutant lacking the SH3-binding domain were prepared and stably transfected into AtT20 cells. On confocal microscopy these transfected cells showed a striking alteration of the subcellular localization of endogenous dynamin II visualized with antibody against the C-terminal tail region of dynamin II. As shown in Fig. 6, in cells transfected with wild-type dynamin II (panel A series) or dynamin II GTP-binding domain mutant (panel B series) dynamin II colocalized with the TGN protein p230 at the Golgi apparatus. In cells transfected with the dynamin II SH3-binding domain deletion mutant, however, staining for p230 remained un-
changed, with the majority of staining for dynamin II found on the plasma membrane, and the Golgi apparatus almost completely devoid of dynamin II staining (Fig. 6, panel C series). An identical pattern of staining for dynamin II was also seen in cells transfected with the dynamin II PH domain only construct (Fig. 6, panel D series). These findings suggest that overproduction of either the dynamin II mutant missing the C-terminal SH3-binding domain or the dynamin II PH domain only mutant induces the translocation of endogenous dynamin II from the Golgi apparatus to the plasmalemma. The predominant staining of dynamin II at the plasma membrane showed a discrete punctate distribution to regions close to the Golgi apparatus in these mutant-transfected cells (Fig. 6, panel C and D series).

**Dynamin II Translocation from the Golgi Apparatus to Plasma Membrane Is Associated with a Decrease of Hormone Release and an Increase in Transferrin Receptor-mediated Endocytosis**—Given that overexpression of the dynamin II mutant without the SH3-binding domain or of the dynamin II PH domain only mutant induces translocation of authentic dynamin II from the Golgi apparatus to plasma membrane, it is clearly of interest to determine whether secretion of β-endorphin and transferrin receptor-mediated endocytosis are affected by the dynamin II variants. Overexpression of the dynamin II mutant lacking the SH3-binding domain significantly reduced β-endorphin release from the cells, with 60–70% of reduction in both basal and CRH-stimulated β-endorphin release (Fig. 7A). Similarly, overexpression of the PH domain also inhibited both basal and stimulated β-endorphin secretion, although the extent of inhibition was less than that with the SH3-binding domain deletion mutant (Fig. 7A).

What was unexpected was that transferrin receptor-mediated endocytosis was significantly enhanced by either the dynamin II mutant lacking the SH3-binding domain or the dynamin II PH domain only mutant (Fig. 7B). Overexpression of the SH3-binding domain deletion mutant almost doubled the levels of receptor internalization in control cells. Although less potent than the SH3-binding domain deleting mutant, overexpression of the PH domain also stimulated receptor internalization. Thus, in addition to the requirement of the GTP-binding domain, the SH3-binding domain also plays an obligatory role in dynamin II-regulated hormone release at the Golgi apparatus. Once translocated by the overproduction of dynamin II mutants, the endogenous dynamin II can then apparently functionally mimic dynamin I in terms of receptor-mediated endocytosis at the plasma membrane.

**Interaction between Dynamin II and G-protein βγ Subunit at the Golgi Apparatus**—In an attempt to determine the proteins with which dynamin II interacts at the Golgi apparatus, we immunoprecipitated dynamin II from purified Golgi fractions and then probed the immunoprecipitates with various antibodies. Since dynamin I has been shown to interact with α-adaptin of AP-2 adaptor protein at plasma membrane, we determined whether γ-adaptin of AP-1 might coinmunoprecipitate with dynamin II from the Golgi fraction, and found no evidence for such an interaction (data not shown). Significant levels of immunoreactive G-protein β subunit were, however, found in the dynamin II immunoprecipitates by immunoblotting analysis.
while no G-protein β subunit was detected in the immunoprecipitates obtained with antibodies against the Golgi proteins β-COP or Rab6 (Fig. 8A). In addition, immunoreactive dynamin II was found in the immunoprecipitates obtained with anti-G-protein β subunit antibody, but not in the immunoprecipitates obtained with anti-β-COP antibody. Interestingly, immunoprecipitated dynamin II was observed as a single major band under denatured conditions (not shown), but as multiple oligomers when partially denatured in 0.5 mM dithiothreitol in SDS-PAGE (Fig. 8B). Furthermore, the coimmunoprecipitation of dynamin II with G-protein βγ subunits was blocked by addition of a PH domain glutathione S-transferase fusion protein to the lysates before immunoprecipitation (Fig. 8B). These data suggest that dynamin II interacts with the βγ subunits of G-proteins through its PH domain at the Golgi apparatus.

Dynamin II Regulates β-ENDorphin Release from the Golgi Apparatus in Vitro and in Semi-permeabilized AtT20 Cells—To explore the biological function(s) of dynamin II at the Golgi apparatus, we have determined the effects of differential expression of dynamin II constructs on the production of secretory vesicles from the Golgi apparatus by assessing the release of β-endorphin through nascent vesicular budding from the Golgi apparatus in vitro and in semi-intact cells. As shown in the bottom panel of Fig. 9, incubation of semi-permeabilized cells in the presence of ATP, GTP, cytosolic proteins, Mg2+, Ca2+, creatine phosphate, and creatine phosphate kinase led to increases in β-endorphin immunoreactivity in the medium, reflecting presumptive de novo biosynthesis from the Golgi appa-
Dynamin II regulates β-endorphin secretory vesicle formation from the Golgi of neuroendocrine AtT20 cells. Golgi-enriched membrane preparations (A) and permeabilized cells (B) were incubated with or without cytosol (1 mg/ml) or both ATP (1 mM) and GTP (0.05 mM) in a buffer containing Ca\(^{2+}\) (0.5 mM), Mg\(^{2+}\) (2.5 mM), K\(^{-}\) (110 mM), creatine phosphate (10 mM), and creatine phosphate kinase (80 μg/ml) at 37 °C for different times as indicated. Samples were subsequently separated into pellet and nascent vesicle-containing supernatant fractions and β-endorphin immunoreactivity determined after dilution with lysis buffer. Percentages of the cytosol- and nucleotide hydrolysis-dependent β-endorphin release from the Golgi are presented and compared between the cells transfected with empty vector (open bars), wild-type dynamin II (hatched bars), or dynamin II carrying a mutation in its GTP-binding domain (cross bars). The results are mean ± S.D. of triplicate determinations from one of five similar experiments.

**FIG. 9.** Dynamin II regulates β-endorphin secretory vesicle formation from the Golgi of neuroendocrine AtT20 cells. Golgi-enriched membrane preparations (A) and permeabilized cells (B) were incubated with or without cytosol (1 mg/ml) or both ATP (1 mM) and GTP (0.05 mM) in a buffer containing Ca\(^{2+}\) (0.5 mM), Mg\(^{2+}\) (2.5 mM), K\(^{-}\) (110 mM), creatine phosphate (10 mM), and creatine phosphate kinase (80 μg/ml) at 37 °C for different times as indicated. Samples were subsequently separated into pellet and nascent vesicle-containing supernatant fractions and β-endorphin immunoreactivity determined after dilution with lysis buffer. Percentages of the cytosol- and nucleotide hydrolysis-dependent β-endorphin release from the Golgi are presented and compared between the cells transfected with empty vector (open bars), wild-type dynamin II (hatched bars), or dynamin II carrying a mutation in its GTP-binding domain (cross bars). The results are mean ± S.D. of triplicate determinations from one of five similar experiments.

DISCUSSION

The present study defines for the first time dynamin II as a Golgi membrane trafficking protein required for both constitutive and regulated hormone secretion from neuroendocrine cells. A striking consequence of loss of dynamin II function in neuroendocrine cells is lowered hormone secretion with both basal and CRH-stimulated secretion of β-endorphin being affected. A similar phenotype is also found when antisense mRNA is used to block the synthesis of endogenous dynamin II. By contrast, an increase in β-endorphin secretion is observed when wild-type dynamin II is overexpressed. Thus, the extent of both constitutive and regulated hormone secretions from these neuroendocrine cells may be limited by the expression levels of endogenous dynamin II under physiological conditions. This finding is in line with previous findings that dynamin II is associated with the Golgi vesicular budding in vitro (23) and in cultured rat epithelial cells (24), and that microinjection of dynamin II does not mimic that of dynamin I on rapid endocytosis in adrenal chromaffin cells (10). Since dynamin II has also been implicated in caveolae endocytosis in hepatocytes (25) and receptor endocytosis in adipocytes (26), HeLa cells and Madin-Darby canine kidney cells without affecting exocytotic pathways (27, 28), it is possible that the cellular functions of dynamin II are largely determined by its specifically targeted subcellular localization in a cell specific fashion. Subcellular localization studies in neuroendocrine cells suggest the presence of dynamin II at the Golgi apparatus, consistent with dynamin II-regulated hormone secretion. Furthermore, overexpression of the wild type or dynamin II mutant regulates de novo production of secretory vesicles from purified Golgi apparatus and semi-permeabilized cells. Thus, dynamin II may be a major form of the dynamin family playing an obligatory role in secretory vesicle biogenesis at the Golgi apparatus during hormone secretion from neuroendocrine cells, consistent with the recent findings that dynamin II regulates post-Golgi transport of a plasma-membrane protein (29).

In addition, structure-function analysis suggests a crucial role for the PH domain in targeting dynamin II to the Golgi. The finding that overexpressed dynamin II PH domain induces dissociation of endogenous dynamin II from the Golgi and inhibition of hormone secretion strongly suggests that the PH domain of dynamin II is involved in interacting with Golgi membrane docking molecule(s) of dynamin II. Given that PH domains from diverse proteins are capable of binding to the phosphate groups of acidic phospholipids (such as phosphatidylinositol (4,5)-bisphosphate) (30), it is possible that dynamin II interacts with these lipid molecules but is specifically targeted at the Golgi apparatus by protein-protein interactions involving its PH domain. In an attempt to search for dynamin II interactive proteins, we detected no interaction between dynamin II and γ-adaptin by immunoprecipitation, although previous studies have shown that dynamin I interacts with α-adaptin (31) and dynamin II mediates clathrin-coated vesicle budding from the TGN (23). In contrast, the interaction between dynamin II and the βγ subunit of G-proteins via the dynamin II PH domain is specific, since the small GTP-binding protein Rab6 does not coimmunoprecipitate with G-protein β subunit, nor does dynamin II with β-COP (a Golgi coatamer protein also possessing a WD40 repeat). Previous studies have also shown that purified βγ subunits bind to purified dynamin (32) and stimulate secretory vesicle formation (33).

In addition to the PH domain, our data also support a role for the SH3-binding domain in dynamin II-mediated secretory vesicle production at the Golgi apparatus. Overexpression of dynamin II lacking the SH3-binding domain not only induces dislocation of endogenous dynamin II from the Golgi but also produces inhibition of hormone secretion. The translocation of endogenous dynamin II from the Golgi to the plasma membrane suggests an involvement of the SH3-binding domain in dynamin II association with secretory vesicle membranes (Fig. 10). Thus, uncoupling of the SH3-binding domain renders dynamin II ineffective in mediating secretory vesicle biogenesis, an effect paralleling previous finding for dynamin I that dis-
ruption of the SH3-binding domain impairs synaptic vesicle recycling (34).

It is also noteworthy that the SH3-binding domain deletion mutant is more potent than the PH domain mutant in causing inhibition of hormone release (Fig. 7A) and stimulation of receptor-mediated endocytosis (Fig. 7B). This difference in potency may reflect a difference between the mutants in competing with endogenous dynamin II at the Golgi. Since the region immediately C-terminal to the PH domain of β-adrenergic receptor kinase is also involved in binding to the βγ subunit (35), it is possible that other regions outside the PH domain of dynamin II are also involved in targeting the protein to the Golgi, and thus that the SH3-binding domain deletion mutant is more effective than the PH domain-only mutant in causing translocation. Once on the plasma membrane, the misallocated dynamin II is capable of mediating clathrin-dependent endocytosis, and that its physiological role in the secretory pathway is determined by its localization to the Golgi.

Collectively, these data show that dynamin II GTPase is a dominant regulator of both constitutive and regulated hormone secretions from neuroendocrine cells, operating as a polymer at the Golgi controlling secretory vesicle biogenesis. During this process, dynamin II interacts with βγ subunit of G-proteins via a region containing the PH domain; disruption of the interaction causes translocation of dynamin II from the Golgi to plasma membrane. The parallel increases and decreases in dynamin II expression with constitutive and regulated hormone secretion are consistent with expressed levels of dynamin II playing an important physiological role in regulating the capacity of cell secretion in response to environmental stimuli. Molecular targeting to particular dynamin isoforms may therefore provide a potential therapeutic means for controlling Golgi protein trafficking and hormone secretion under particular circumstances (36).

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Dynamin II and Hormone Secretion

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