Overexpression of Rab3D Enhances Regulated Amylase Secretion from Pancreatic Acini of Transgenic Mice

Hirohide Ohnishi,* Linda C. Samuelson,* David I. Yule,* Stephen A. Ernst,‡ and John A. Williams*§

**Department of Physiology, ‡Department of Anatomy and Cell Biology, and §Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109*

Abstract

Rab3D, a member of the ras-related GTP-binding protein Rab family, is localized to secretory granules of various exocrine tissues such as acinar cells of the pancreas, chief cells of the stomach, and parotid and lacrimal secretory cells. To elucidate the function of Rab3D in exocytosis, we have generated transgenic mice that over-express Rab3D specifically in pancreatic acinar cells. Hemagglutinin-tagged Rab3D was localized to zymogen granules by immunohistochemistry, and was shown to be present on zymogen granule membranes by Western blotting; both results are similar to previous studies of endogenous Rab3D. Secretion measurements in isolated acinar preparations showed that overexpression of Rab3D enhanced amylase release. Amylase secretion from intact acini of transgenic mice 5 min after 10 pM cholecystokinin octapeptide (CCK) stimulation was enhanced by 160% of control. In streptolysin-O–permeabilized acini of transgenic mice, amylase secretion induced by 100 μM GTP-γ-S was enhanced by 150%, and 10 μM Ca^{2+}-stimulated amylase secretion was augmented by 206% of that of the control. To further elucidate Rab3D involvement in stimulus–secretion coupling, we examined the effect of CCK on the rate of GTP binding to Rab3D. Stimulation of permeabilized acini with 10 pM CCK increased the incorporation of radiolabeled GTP into HA-tagged Rab3D. These results indicate that overexpression of Rab3D enhances secretagogue-stimulated amylase secretion through both calcium and GTP pathways. We conclude that Rab3D protein on zymogen granules plays a stimulatory role in regulated amylase secretion from pancreatic acini. (*J. Clin. Invest.* 1997. 100: 3044–3052.) Key words: pancreatic zymogen granules • regulated exocytosis • GTP-binding protein • permeabilized acini • hemagglutinin tag

Introduction

Regulated exocytosis involves highly controlled targeting, docking, and fusion of secretory vesicles to the plasma membrane. Recent evidence has shown that secretory granule proteins play key roles at each step of exocytosis (1, 2). Studies using cell permeabilization and nonhydrolyzable GTP analogues such as GTP-γ-S have indicated that G proteins called G_E act at a late step to facilitate exocytosis (3). Rab proteins, members of the ras-related small G-protein family, are well known to participate in various steps of intracellular vesicle-trafficking, including exocytosis (4–7), and the Rab3 subfamily proteins are candidates for G_E. Rab3 proteins have been localized on the secretory granules of neuroendocrine cells. Rab3A and C, present on secretory vesicles of neurons and chromaffin cells, act to inhibit exocytosis (8–10). In contrast, Rab3B was demonstrated to play a stimulatory role in secretion in anterior pituitary and PC12 cells (11, 12), suggesting that Rab3 isoforms may function in different aspects of exocytosis. Recently, a fourth isoform of Rab3, Rab3D, was cloned from adipocytes and assumed to be localized to glucose transporter–containing vesicles (13). We and others have reported that Rab3D is localized to secretory granules of various exocrine secretory cells such as acinar cells of the exocrine pancreas, parotid and lacrimal glands, and chief cells of the stomach (14–17). Although this localization implies that Rab3D may be involved in regulated exocytosis, it has not yet been demonstrated directly. Using permeabilized acini, it was previously shown that a synthetic peptide called Rab3AL, corresponding to the effector domain of Rab3A, stimulated amylase secretion in pancreatic acini (18). The isoform of Rab3 proteins that exists in pancreatic acini, however, is Rab3D (14, 15), not Rab3A (19). In addition, recent evidence has cast doubt on the specificity of the Rab3AL peptide, as Rab3AL peptide appears to act similarly to mastoparan, which activates heterotrimeric G-proteins (20). Thus, the role of Rab3D in exocytosis is still an open question.

In this work, we have generated transgenic mice that overexpress Rab3D specifically in pancreatic acinar cells to study the function of Rab3D in pancreatic exocytosis. We found that regulated amylase secretion was augmented in both intact and streptolysin-O–permeabilized acini from the transgenic mice. We also examined the effect of cholecystokinin (CCK)[1] on GTP incorporation into Rab3D. Since Rab proteins are assumed to be activated by GDP/GTP exchange (21), we have to address the possibility that CCK can stimulate pancreatic exocytosis in part by regulating the GTP-binding rate of Rab3D. We found that CCK enhanced GTP incorporation into hemagglutinin (HA)-tagged Rab3D. These results suggest that Rab3D protein is involved in the stimulus–secretion coupling pathway, and plays a stimulatory role in regulated exocytosis.

Methods

Reagents. All restriction enzymes and DNA-modifying enzymes were purchased from Promega Corp. (Madison, WI); Taq DNA poly-

Address correspondence to John A. Williams, M.D., Ph.D., Department of Physiology, University of Michigan Medical School, 7744 Medical Science II, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0622. Phone: 313-764-4376; FAX: 313-936-8813; E-mail: jawillms@umich.edu

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1. *Abbreviations used in this paper:* CCK, cholecystokinin; HA, hemagglutinin; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

merase was from Perkin Elmer Corp. (Foster City, CA); streptolysin-O was from Murex (Dartford, England); chromatographically purified collagenase was from Worthington Biochem. Corp. (Freehold, NJ); and protein G agarose and SuperSignal Ultra were from Pierce (Rockford, IL). The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): Mg-ATP, GTP, GTP-γ-S, and soybean trypsin inhibitor. [α-^{32}P]GTP (3,000 Ci/mmole) was purchased from DuPont-NEN (Boston, MA). Cholecystokinin octapeptide (CCK) was a gift from the Squibb Institute (Princeton, NJ). Anti-HA monoclonal antibody was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Anti-Rab3D antisera was a gift from Dr. Mark McNiven (Mayo Clinic, Rochester, MN). Peroxidase-coupled goat anti–rabbit and anti–mouse IgG and ECL reagent were purchased from Amersham Corp. (Arlington Heights, IL), and FITC-labeled goat anti–mouse IgG was purchased from Sigma.

Transgenic mice. HA-tagged Rab3D under the control of the rat elastase I promoter was constructed as follows. HA-tagged Rab3D cDNA was generated by PCR using mouse Rab3D cDNA, a gift from Dr. Harvey Lodish (Whitehead Institute, Boston, MA), as a template. PCR primers designed to incorporate the published HA sequence (21, 22) at the amino terminal of Rab3D had the following sequence: sense, 5′ CGC GGATCC-ACTGAGATGTACCCATACA-TGTTCCGGATTACGCTAGCCTCGCATCCGCTAGTGAGCC-CCCT 3′; antisense, 5′ CCGGAATTCCTAACAGCT-GCAGCT-GCTCGG 3′. BamHI and EcoRI sites used for cloning the amplified product into pcDNA3 (Invitrogen Corp., San Diego, CA) are underlined. A 510-bp HindIII–BamHI rat elastase I enhancer/ promoter fragment (a gift from Dr. Raymond MacDonald, University of Texas, Southwestern Medical School) was cloned upstream of the HA-Rab3D cDNA. The integrity of the transgene construct was confirmed by DNA sequencing. A 2.2-kb Hind III–Sma I fragment containing the rat elastase I enhancer/promoter, HA-tagged Rab3D and bovine growth hormone poly A site originally present in pcDNA3 was excised from the plasmid vector and purified after agarose gel electrophoresis. The purified DNA fragment was microinjected into F2 hybrid eggs from (C57BL/6J × SJL/J) F1 parents (23). Eggs were transferred to day 0.5 postcoitum pseudopregnant CD-1 females. Four founder transgenic mice were identified and mated to C67BL/6J mice to establish three independent transgenic lines. Transgenic mice were prepared by the Transgenic Animal Model Core of the University of Michigan Biomedical Core Research Facilities. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals.

Identification of transgenic individuals by PCR of genomic DNA. Transgenic individuals were identified by PCR of genomic DNA isolated from tail biopsies (24). The primers used were as follows: sense, 5′CGCGGATCCACTGAGATGTACC-CATACGATGTTCCGGA-TTACGCTAGCCTC 3′; antisense, 5′ ACCCGTTCGT-CTTCCAG-GTCACACTTGT 3′; the product size was 463 bp. Since the antisense primer corresponds to the HA-tag sequence of the transgene, PCR with this primer pair detects the transgene but not the endogenous Rab3D gene. PCR reactions were run for 35 cycles of 94°C denaturing, 65°C annealing, and 72° extending temperatures. Mouse β-globin primers (sense, 5′ CCAATCTGCTCACACAGGATAGA-GAGGGCAGG 3′; antisense, CCTTGAGGCTGTCCAAGTGAT-TCAGGCCATCG 3′) were used to amplify a 494-bp fragment as an internal control for DNA quality. The PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

Western blotting. Tissue samples, including pancreas, brain, liver, kidney, and spleen, were homogenized with a Polytron homogenizer in homogenation buffer containing 5 mM Mops (pH 7.0), 250 mM sucrose, 0.1 mM PMSF, and 0.1 mM $MgSO_4$, and centrifuged at 500 *g* for 10 min at 4°C. A lysate of COS-7 cells transiently transfected with HA-tagged Rab3D under the control of the CMV promoter was prepared as described previously (14). Electrophoresis was performed as described by Laemmli (25). Tissue supernatants or lysates were loaded onto 10% SDS-polyacrylamide electrophoresis gels and run at 200 V. After gel electrophoresis, proteins were transferred to nitrocellulose membranes at 30 V overnight. Western blotting was carried out as described previously (26), but using 10% dry milk as a blocker and either ECL or SuperSignal Ultra chemiluminescence reagents to visualize the secondary antibody (26). The intensity of the bands was quantitated by densitometry with an Agfa Arcus II Scanner and Molecular Analyst software (Bio-Rad Laboratories, Richmond, CA).

Immunofluorescence. Fixation and evaluation of immunofluorescence was carried out similar to previous studies using anti-Rab3D serum (14) except that tissue was fixed in 2% formaldehyde and the primary antibody was ascites fluid containing a monoclonal Ab directed to the HA epitope used at a dilution of 1:25–1:200. Specimens were photographed using a 63× oil immersion lens on an Aristoplan microscope (Leitz), digitized, and processed using Photoshop 3.0 software (Adobe, Mountain View, CA).

Preparation of mouse acinar zymogen granules and zymogen granule membranes. Purified zymogen granules were prepared by Percoll gradient separation as previously described (14, 26). Granule membranes were isolated by ultracentrifugation after lysis with nigericin (26). Protein content was determined with the Bio-Rad protein assay kit using BSA as standard.

Preparation of pancreatic acini and amylase release from intact and permeabilized acini. Acini were prepared by collagenase digestion as previously described (27). For intact acinar experiments, isolated acini were suspended in incubation buffer consisting of 10 mM

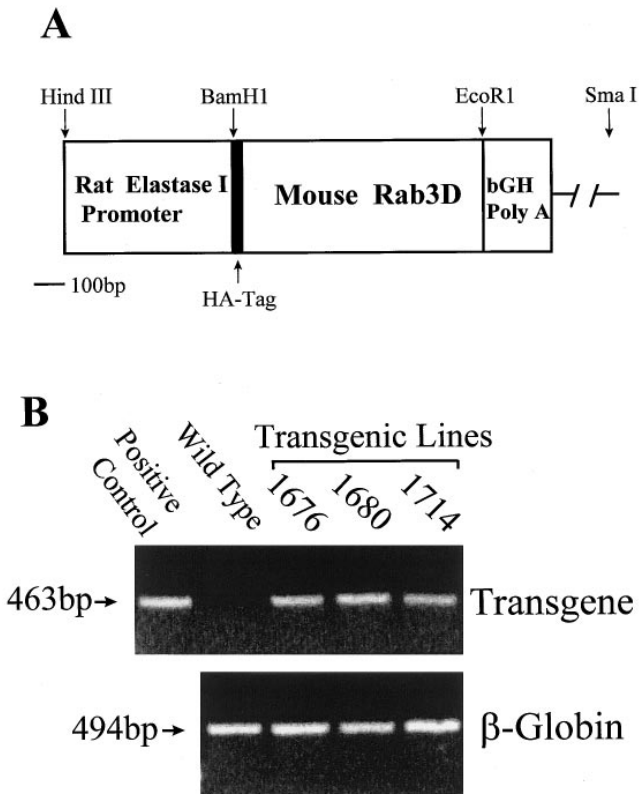

Figure 1. Production of HA-Rab3D transgenic mice. (*A*) HA-tagged mouse Rab3D transgene construct. The transcription initiation site and start codon are just upstream of the HA-tag sequence. The rat elastase I promoter/enhancer has been previously described to direct expression to pancreatic acinar cells (33). After the Rab3D coding region is 1061 bp of pcDNA3 sequence starting with 231 bp coding for the bovine growth hormone polyadenylation signal. (*B*) PCR products from genomic DNA of three transgenic lines and a wild-type control are shown (*top*). The transgene plasmid was used as a template for the positive control. β-globin positive control PCR is shown (*bottom*).

Hepes (pH 7.4), 127 mM NaCl, 4.7 mM KCl, 0.6 mM $MgCl_2$, 1.3 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 2.0 mg/ml glucose, Eagle's MEM amino acid supplement, 2 mM L-glutamine, 1% BSA, and 0.01% soybean trypsin inhibitor, and preincubated at 37°C for 30 min. After preincubation, acini were centrifugated at a low speed, resuspended in fresh incubation buffer, and incubated at 37°C in the presence or absence of 10 pM CCK. For permeabilized acinar experiments, isolated acini were suspended in permeabilization buffer consisting of 20 mM Pipes (pH 7.0), 140 mM potassium glutamate, 0.91 mM $MgCl_2$, 5 mM EGTA, 1 mg/ml BSA, 0.1 mg soybean trypsin inhibitor, 1 mM ATP, and 0.5 IU/ml streptolysin-O, and aliquoted into 200-μl samples. Amylase release was initiated by adding 200 μl permeabilization buffer supplemented with $CaCl_2$ to give a final concentration of 1 mM free Mg^{2+} and specified concentrations of free Ca^{2+} that were calculated using a computer program as described previously (28). Amylase released into the supernatant during the incubation was quantified using a Phadebas Amylase Test (Pharmacia Diagnostics, Columbus, OH) and expressed as a percent of total amylase in the acini at the beginning of the incubation.

Measurement of GTP binding to HA-tagged Rab3D. GTP binding to HA-tagged Rab3D was determined by the modified method of Buday and Downward (29). Pancreatic acini prepared from transgenic mice were suspended in permeabilization buffer described above supplemented with 100 nM Ca^{2+}, and aliquoted into 1-ml samples. 10 μCi [α-^{32}P]GTP was added with or without 10 pM CCK, followed by incubation at 30°C for 0, 5, 10, and 15 min. The acini were then washed with ice-cold PBS buffer, resuspended in immunoprecipitation buffer containing 25 mM Tris-HCl (pH 7.5), 3 mM benzamidine, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 mM PMSF, and 0.1 mM unlabeled GTP, and sonicated for 10 s. The insoluble fraction was removed by centrifugation in a microcentrifuge at 4°C for 15 min. HA-tagged Rab3D was immunoprecipitated from the supernatant (2 mg protein) by incubating with 4 μg anti-HA-tag monoclonal antibody at 4°C for 2 h followed by incubation with protein G-agarose beads for 1 h. The HA-tagged Rab3D-bound beads were washed with the immunoprecipitation buffer, and the incorporation of radioactive [α-^{32}P]GTP into HA-tagged Rab3D was measured by liquid scintillation.

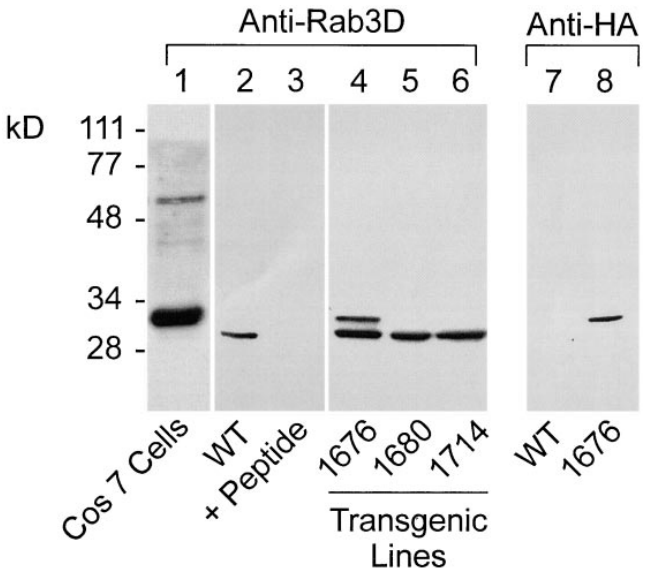

Figure 2. Western blotting of HA-tagged Rab3D in the pancreas of transgenic lines using anti-Rab3D (lanes *1–6*) and anti–HA tag (lanes *7* and *8*) antibodies. HA-tagged Rab3D transiently transfected in COS-7 cells (lane *1*) were 3 kD higher than that of endogenous Rab3D (lane *2*). The band visualized by anti-Rab3D was abolished when the antisera was preincubated with the peptide used for immunization (lane *3*). Of the three transgenic lines, line 1676 expressed sufficient HA-tagged Rab3D protein to be detected easily (lane *4*). Anti-HA-tag monoclonal antibody confirmed the expression of HA-tagged Rab3D in the pancreas of line 1676 (lane *8*), but showed no labeling in wild-type mouse pancreas (lane *7*). The same amount of each pancreatic homogenate (30 μg protein) was applied to lanes *2–7*. To lane *1*, 10 μg protein of a lysate from COS-7 cells transfected with a HA-tag Rab3D expression construct was applied. Molecular mass markers are indicated on the *left.* Primary anti-Rab3D sera was used at a dilution of 1:1,000, anti-HA-tag monoclonal at 2 μg/ml, HRP tagged goat anti-rabbit Ab at 1:10,000 and HRP-tagged anti–mouse at 1:50,000. Chemiluminesce was generated with SuperSignal Ultra.

Results

Transgenic mouse lines. A 2.2-kb mouse Rab3D transgene under the control of the rat elastase I promoter was microinjected into fertilized eggs to produce transgenic mice (Fig. 1 *A*). We designed the transgene to incorporate the HA tag on the amino terminal of Rab3D to distinguish it from endogenous Rab3D. HA tags at the amino termini of various Rab proteins have been previously shown not to affect Rab function (9, 22, 30, 31). Three transgenic lines containing the HA-tagged Rab3D transgene were established (Fig. 1 *B*). Of the three lines, line 1676 expressed sufficient amounts of HA-Rab3D protein in the pancreas to be detected easily in Western blotting (Fig. 2). HA-Rab3D levels in 1676 were comparable to endogenous Rab3D levels, and line 1676 was used for all subsequent experiments. HA-tagged Rab3D expressed in transgenic mice was 3 kD higher than endogenous Rab3D in SDS-PAGE gels as previously described for HA-tagged Rab3D transfected in COS-7 cells (14). Thus, HA-tagged Rab3D was easily distinguished from endogenous Rab3D in the Western blotting with anti-Rab3D antibody or anti-HA antibody. The specificity of the anti-Rab3D antibody is shown

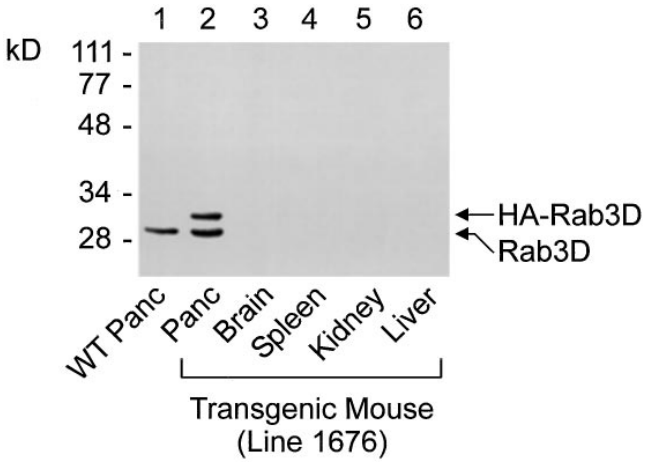

Figure 3. Western blotting of various tissues of the transgenic mouse using anti-Rab3D antiserum. Endogenous Rab3D of 28 kD was recognized in wild-type mouse pancreas (lane *1*). Both endogenous Rab3D (28 kD) and the HA-tagged Rab3D of 31 kD were detected in transgenic mouse pancreas (lane *2*). In brain (lane *3*), spleen (lane *4*), kidney (lane *5*), or liver (lane *6*) of the transgenic mouse, no appreciable labeling was observed. 30 μg protein of homogenate of each tissue was applied to each lane; primarily anti-Rab3D sera was used at a dilution of 1:1,000, secondary antibody at a dilution of 1:10,000, and chemiluminescence was generated with SuperSignal Ultra.

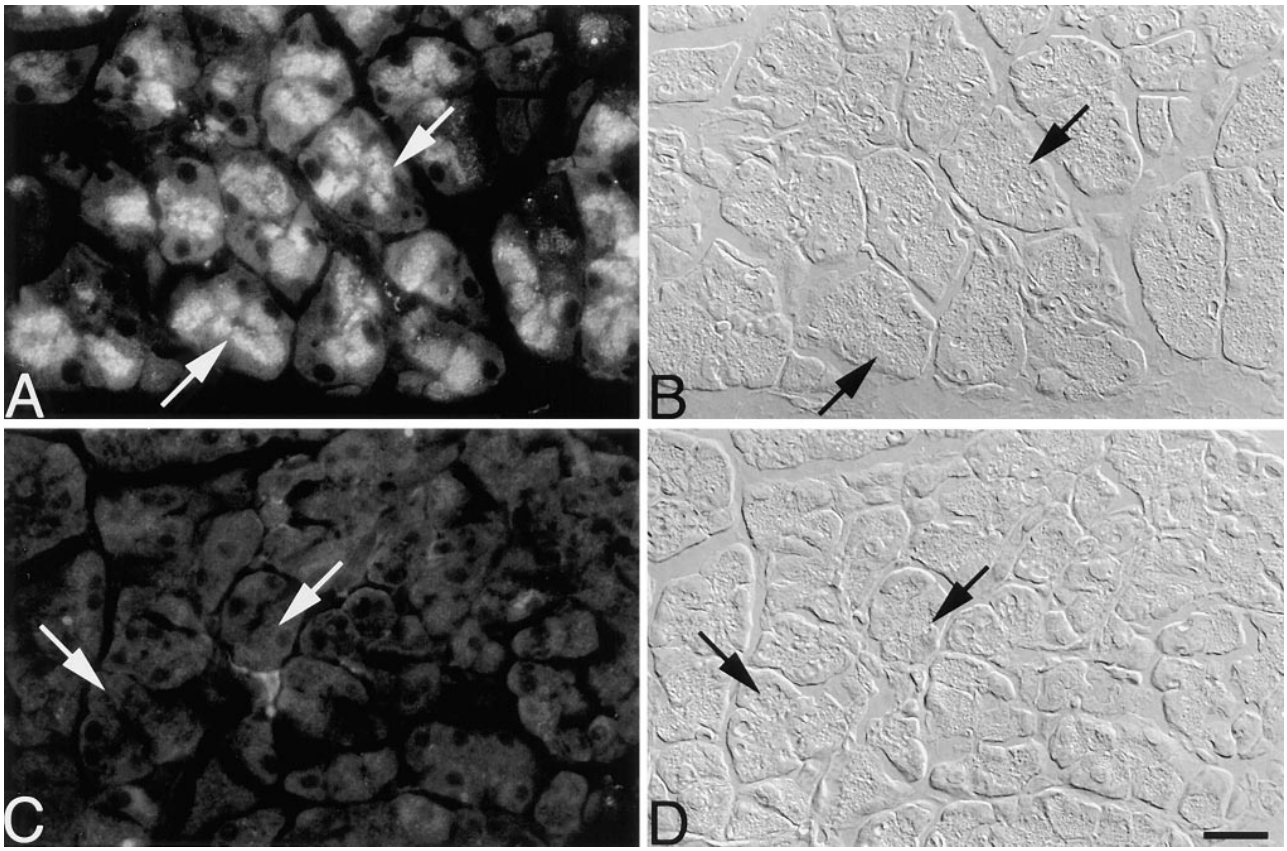

Figure 4. Immunofluorescence localization of HA-tagged Rab3D in transgenic and control pancreas using an anti-HA-tag antibody. (*A*) Immunofluorescence in transgenic pancreas. (*B*) Corresponding Nomarski image. (*C*) Immunofluorescence in control pancreas. (*D*) Corresponding Nomarski image. *Arrows* indicate position of zymogen granules. Nonspecific staining of basolateral membranes or extracellular structures was occasionally seen in both transgenic and control pancreas. Primary antibody dilution, 1:50 (8 μg/ml). Calibration bar, 20 μm.

by competition with the synthetic peptide used to generate the antibody (Fig. 2, lane *3*). The specificity of the anti-HA tag is shown by the absence of signal in pancreas from wild-type mice. At longer exposures much weaker bands corresponding in mass to mouse antibody were observed in all tissues of wild-type and transgenic mice when the goat anti–mouse secondary antibody was used (not shown). In transgenic line 1676 the volume density of the HA-tagged Rab3D band was 78.3±8.8% of the native Rab3D band ($n = 3$), while the native Rab3D band was 119±12% of a wild-type control run on the same gel. Thus, total Rab3D is doubled in transgenic line 1676.

Localization of HA-tagged Rab3D in transgenic mice. To confirm that HA-tagged Rab3D is specifically targeted to the pancreas by the rat elastase I promoter, we carried out Western blotting of various tissue homogenates of the transgenic mice using an anti-Rab3D antibody. Both HA-tagged Rab3D and endogenous Rab3D were observed in the transgenic pancreatic homogenates, however, no labeling of HA-tagged Rab3D was detected in other tissues from the transgenic mouse, such as brain, liver, kidney, and spleen (Fig. 3). Thus, HA-tagged Rab3D appeared to be expressed specifically in the pancreas. To further elucidate the cellular and subcellular localization of HA-tagged Rab3D in pancreas, we carried out immunohistochemistry of pancreas using an anti-HA-tag antibody. Although the intensity of expression was variable, HA-tagged Rab3D was localized to zymogen granules of pancreatic acinar cells of transgenic mice (Fig. 4, *A* and *B*). No appreciable staining was observed in the pancreas of wild-type mice (Fig. 4, *C* and *D*) or over ducts, islets, or blood vessels of transgenic mouse pancreas. When anti–HA tag antibody was preincubated with the HA peptide used to generate the antibody, granular staining in the apical region of the pancreatic

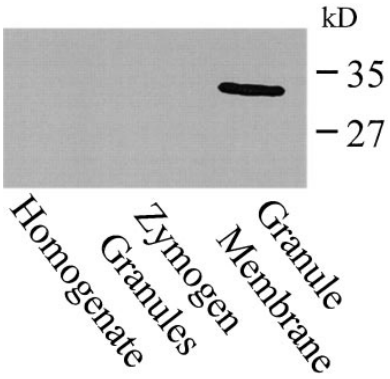

Figure 5. Western blotting of pancreatic fractions of the transgenic mouse using anti-HA-tag monoclonal antibody. 10 μg protein of pancreatic homogenate, zymogen granules, and zymogen granule membranes of the transgenic mice were applied to each lane. Anti–HA tag antibody demonstrated that expressed HA-tagged Rab3D was highly concentrated on pancreatic zymogen granule membranes of the transgenic mouse. Primary anti-HA antibody was used at 4 μg/ml, and chemiluminescence was generated with ECL reagent.

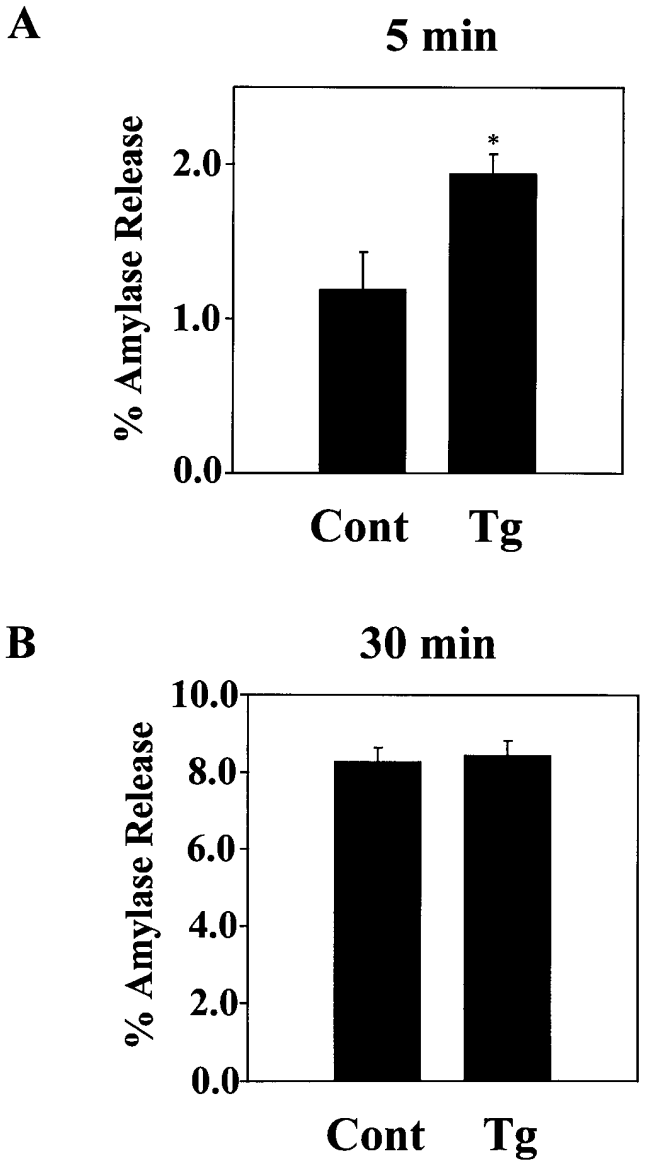

Figure 6. CCK-stimulated amylase release from intact pancreatic acini of transgenic and wild-type mice. Enzymatically isolated acini of control or transgenic mice were incubated for 5 min (panel *A*) or 30 min (panel *B*) with 10 pM CCK. Values are the mean±SE of four independent experiments, each with duplicated determinations. Basal amylase secretion at 5 min from both transgenic and control mice were below the sensitivity of the assay. Basal amylase secretion at 30 min for acini from transgenic and control mice was similar, and was subtracted from each CCK-stimulated amylase release at 30 min, respectively. Tg, transgenic mice; cont, control mice. (*$P < 0.02$ by Student's *t* test).

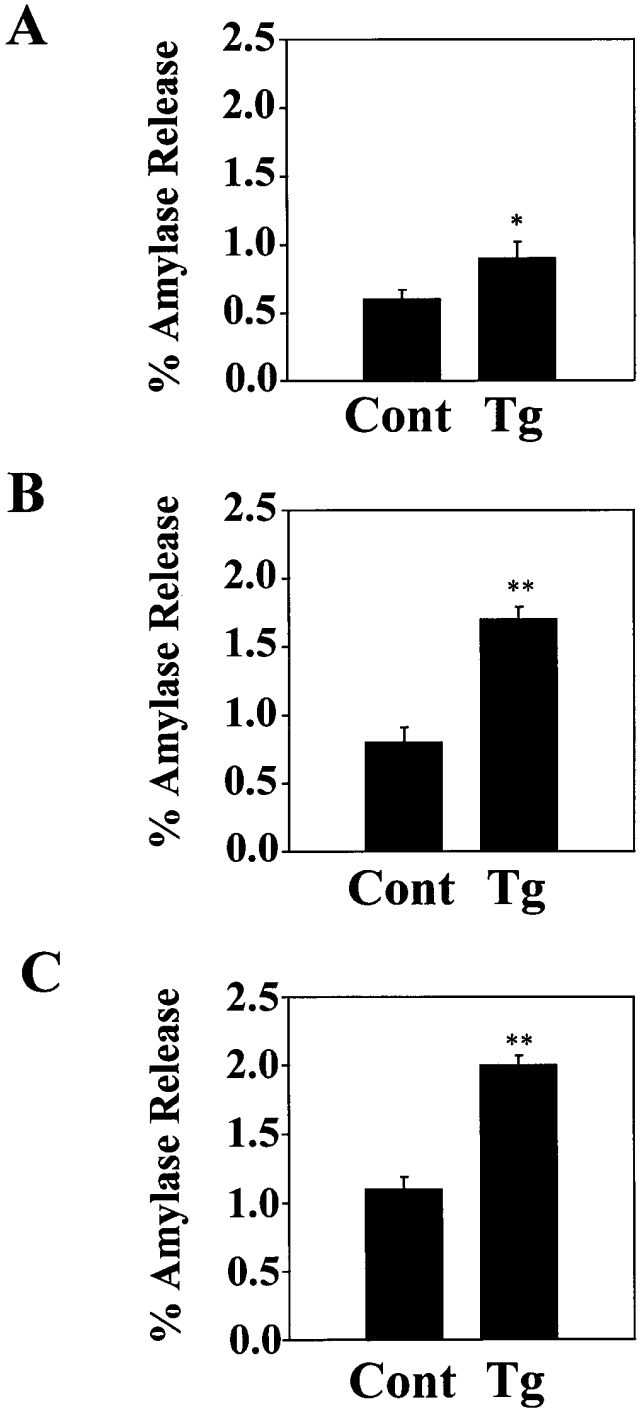

Figure 7. GTP-γ-S- and/or calcium-triggered amylase release from streptolysin-O–permeabilized acini of transgenic and control mice. Permeabilized acini of transgenic or control mice were incubated for 5 min with 100 μM GTP-γ-S and 100 nM Ca^{2+} (*A*), or with 10 μM Ca^{2+} (*B*), or with 100 μM GTP-γ-S and 10 μM Ca^{2+} (*C*). Values are the mean±SE of five independent experiments, each with duplicated determinations. Basal amylase secretion measured in the presence of 100 nM Ca^{2+} was similar in control and transgenic mouse acini, and was subtracted from each stimulated amylase release. Tg, transgenic mice; cont, control mice. *$P < 0.05$, **$P < 0.0005$ by Student's *t* test.

acinar cells of the transgenic mice was abolished (data not shown). To confirm that HA-tagged Rab3D was present on zymogen granule membranes of pancreatic acinar cells, we performed Western blots of zymogen granule membranes purified from pancreas of transgenic mice. Using less protein and the original ECL reagent, a 31-kD band was observed on granule membranes with anti-HA-tag antibody, while homogenates and intact granules did not show a signal (Fig. 5). This result is consistent with our earlier results on native Rab3D (14),

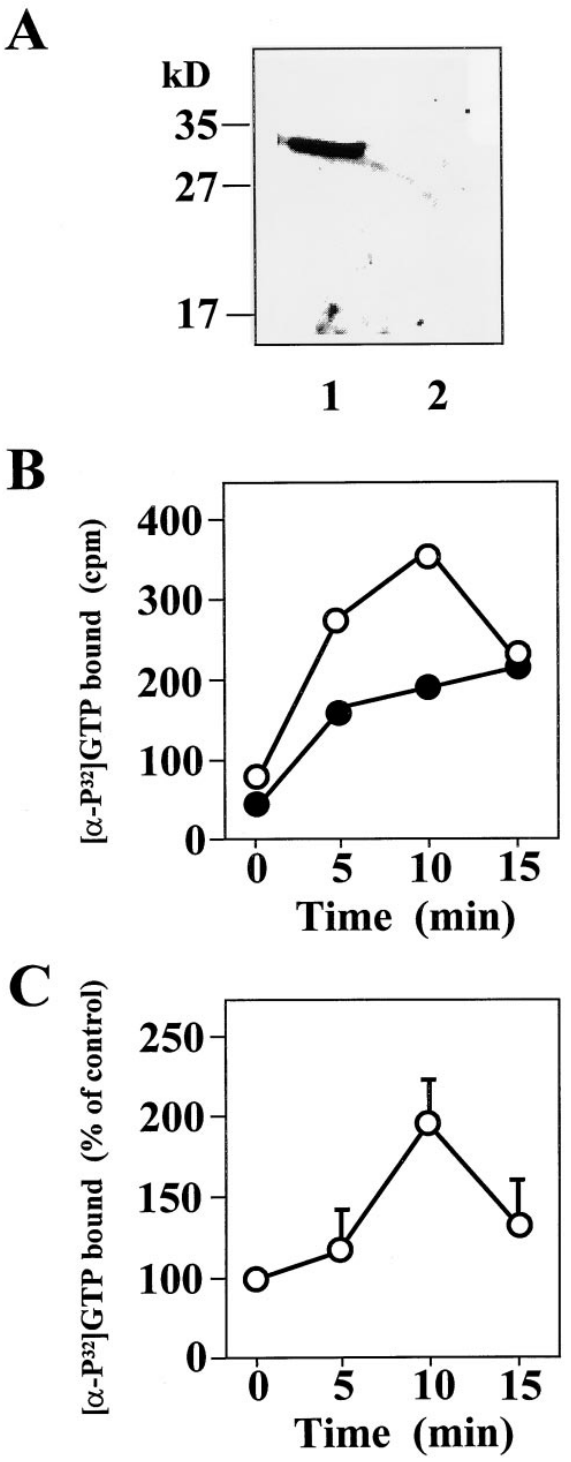

Figure 8. Effect of CCK on GTP incorporation into HA-tagged Rab3D. (*A*) To determine the incorporation of [α-^{32}P]GTP into HA-tagged Rab3D, HA-tagged Rab3D was immunoprecipitated using anti-HA-tag monoclonal antibody and visualized by Western blotting using anti-Rab3D antisera (lane *1*). Control was performed with pancreatic acini of a wild-type mouse (lane *2*). (*B* and *C*) Acini of transgenic mice were permeabilized with streptolysin-O and stimulated with 10 pM CCK for various times in the presence of [α-^{32}P]GTP. HA-tagged Rab3D was then immunoprecipitated, and bound radioactivity was determined by liquid scintillation count. Control experiments were performed but in the absence of CCK. (*B*) A representative experiment showing specific cpm bound. Nonspecific cpm bound determined by the same method without pancreatic lysate was subtructed from all data. (*open circles*, CCK-stimulated; *closed circles*, control.) (*C*) means±SE obtained from three independent experiments in which bound nucleotide is expressed as % of control.

and is explained by the fact that the granule membrane makes up only 1% of the granule volume. These data using immunocytochemistry and Western blotting show that, similar to endogenous Rab3D, HA-tagged Rab3D is targeted to pancreatic zymogen granule membranes.

Effect of overexpression of Rab3D on CCK-stimulated amylase release from intact acini. To study the function of Rab3D in regulated exocytosis, we compared CCK-stimulated amylase release from isolated pancreatic acini of transgenic and control mice. Mice from the same litter as the transgenic mice, but not carrying the transgene, were used as controls. We used 10 pM CCK for stimulation as this concentration induced the maximum amylase secretion from pancreatic acini of both transgenic and control mice (data not shown). As shown in Fig. 6 *A*, amylase secretion was enhanced in transgenic mice by 60% compared to controls 5 min after stimulation. However, amylase release at 30 min of stimulation was similar to that of the controls. These data suggest that overexpressed Rab3D protein augments the early phase of amylase release.

Effect of Rab3D overexpression on calcium and/or GTP-stimulated amylase release from streptolysin-O–permeabilized acini. To further characterize the stimulatory effect of overexpressed Rab3D in regulated amylase secretion, we measured amylase secretion from streptolysin-O–permeabilized acini. When stimulated with 100 μM GTP-γ-S, amylase release from permeabilized acini of transgenic mice was increased by 50% compared to control (Fig. 7 *A*). Moreover, amylase secretion induced by 10 μM Ca^{2+} from permeabilized acini was 106% greater compared to acini from control pancreas (Fig. 7 *B*). Amylase release triggered by the combination of 100 μM GTP-γ-S and 10 μM Ca^{2+} was also augmented in transgenic mice (Fig. 7 *C*). These data suggest that overexpression of Rab3D enhanced both calcium- and GTP-regulated amylase secretion from pancreatic acini.

Effect of CCK on GTP binding to HA-tagged Rab3D. To further elucidate the involvement of Rab3D in stimulus–secretion coupling, and to investigate whether secretagogues regulate the activity of Rab3D, we examined the effect of CCK on the rate of GTP binding to HA-tagged Rab3D. To isolate HA-tagged Rab3D from the pancreas of transgenic mice, we performed immunoprecipitation using anti-HA monoclonal antibody (Fig. 8 *A*). To introduce labeled GTP, isolated pancreatic acini were permeabilized with streptolysin-O in the presence of [α-^{32}P]GTP. After incubation for indicated periods with or without 10 pM CCK, HA-tagged Rab3D was immunoprecipitated, and the associated radioactivity was determined. As shown in Fig. 8, *B* and *C*, 10 pM CCK significantly increased the incorporation of GTP into HA-tagged Rab3D with the maximal effect at 10 min compared with control. These data suggest that CCK regulates Rab3D by changing its GTP-binding rate, and that Rab3D is involved in the pathway of stimulus–secretion coupling.

Stability of HA-Rab3D in acinar cells. Because the decrease in GTP labeling at the latter time points could reflect protein

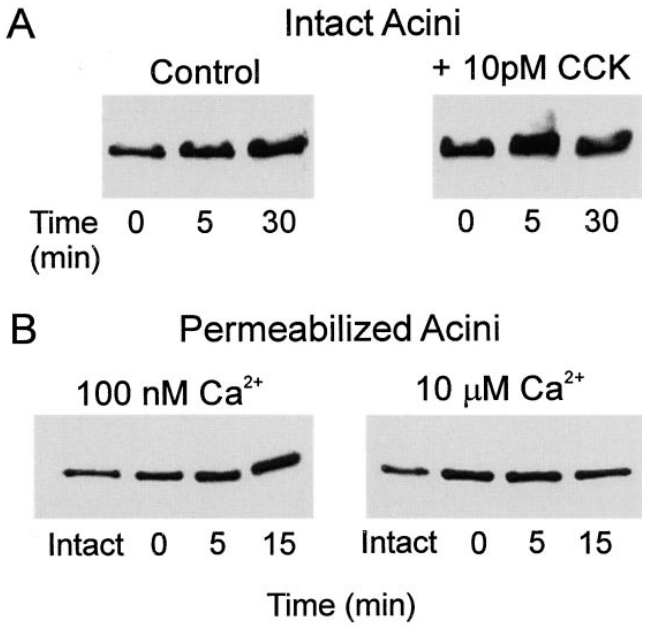

Figure 9. Effect of stimulation on amount of transgenic HA-Rab3D in pancreatic acini. Mouse acini from transgenic mice were incubated intact with 10 pM CCK similar to studies of amylase release shown in Fig. 6 *A* or permeabilized with streptolysin O and incubated with low or high Ca^{2+} as in Figs. 7 and 8 *B*. At times specified, acini were centrifuged, lysed, and prepared for Western blotting using 20 μg of acinar protein per lane. Blots were probed with anti-HA monoclonal antibody similar to Fig. 2.

degradation, we evaluated the amount of HA-Rab3D after stimulation. Neither stimulation of intact cells by CCK or permeabilized cells with Ca^{2+} reduced the amount of HA-Rab3D (Fig. 9). Interestingly, permeabilization increased the Rab3D signal per unit protein over that determined in intact cells, consistent with Rab3D being attached to membranes in cells. When the blotting membranes were stripped and reprobed with anti-Rab3D, similar changes were seen (data not shown).

Discussion

Although Rab3D has been localized to secretory granules of various exocrine tissues including pancreas, a functional role had not been previously demonstrated. This work was conducted to determine if Rab3D had a functional role, either stimulatory or inhibitory, in regulated exocytosis by using transgenic mice. We observed enhanced CCK-stimulated amylase secretion from intact acini of transgenic mice overexpressing Rab3D. Both GTP-γ-S- and Ca^{2+}-triggered amylase release from streptolysin-O–permeabilized acini was augmented in these transgenic mice. Furthermore, we demonstrated that CCK potentiated GTP incorporation into Rab3D. These results suggest that Rab3D is involved in stimulus–secretion coupling, and plays a stimulatory role in regulated exocytosis of pancreatic acini.

Exocrine pancreas, especially chemically permeabilized pancreatic acini, have been widely used for studying the regulation of exocytosis by guanyl nucleotides, intracellular calcium, and other intracellular signal mediators (28, 32). However, this system has some limitations since permeabilized pancreatic acini run down too rapidly to permit functional remodeling after application of antibodies or exogenous proteins (33). Thus, we have generated transgenic mice to analyze the function of Rab3D in regulated exocytosis. To our knowledge, this is the first attempt to use transgenic mice for functionally studying exocytosis of the exocrine pancreas. We designed the transgene to incorporate the HA tag at the amino terminal of Rab3D to distinguish it from endogenous Rab3D. The HA epitope has been used to tag various Rab proteins in transfected cell lines without affecting Rab protein function (9, 22, 30, 31). Thus, it is reasonable to interpret that the stimulatory effect of expressing HA-tagged Rab3D on amylase secretion described in our studies represents the function of Rab3D and not the HA tag.

The rat elastase I promoter is a powerful tool to overexpress proteins selectively in pancreatic acini of transgenic mice (34). We carried out immunohistochemistry and Western blotting to confirm that HA-tagged Rab3D is expressed in pancreatic acini and targeted to zymogen granules of transgenic mice. These data indicate that the transgenic mouse line we have chosen is useful for analyzing Rab3D function.

The process of exocytosis occurs in two phases. The first step of docking and fusion of primed secretory vesicles to plasma membranes is completed in the first 5 min. The second step involves the sequential release of vesicles from a reserve pool (35). According to this scheme, our finding that CCK-stimulated amylase secretion from intact acini in transgenic mice was enhanced during 5-min, but not 30-min incubation, suggests that Rab3D participates in the first phase of pancreatic acinar exocytosis, such as priming zymogen granules for docking, or fusion to plasma membranes. Another interpretation of the insignificant enhancement of amylase release at 30 min is that other proteins essential for the stimulatory effect of Rab3D were not expressed at high enough levels in pancreatic acini of transgenic mice for Rab3D to maintain enhanced amylase secretion for 30 min. In a recent developmental study, Rab3D was found to localize to secretory granules after birth concurrent with the onset of regulated exocytosis, while Rab GDI was expressed both before and after birth (36). Further studies are clearly needed to identify the protein(s) that interact with Rab3D in exocrine pancreas.

By using permeabilized acini of transgenic mice, we have shown that both GTP- and calcium-triggered amylase secretion were potentiated in Rab3D transgenic mice. Our results suggest that the G_E activity associated with exocytosis of zymogen granules is likely to include Rab3D. One mechanism by which Rab3D might enhance calcium-regulated exocytosis is by association with a calcium-binding protein, similar to the role of Rabphilin-A for Rab3A (37, 38). Alternatively, Søgaard et al. recently reported that a Rab protein is required for the assembly of soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complexes in transport vesicle docking in yeast (39). Since SNARE complexes are known to participate in exocytosis of pancreatic acini (40, 41), an interaction of Rab3D and SNARE complexes could explain calcium-regulated amylase release enhancement.

The activity of Rab proteins is believed to be controlled by GTP/GDP cycling (42). Rab proteins require GTP hydrolysis for participation in vesicle trafficking and fusion, and are removed from acceptor membranes by GDP dissociation inhibi-

tor proteins. Stahl et al. recently reported a relationship between regulated exocytosis and the GDP/GTP status of Rab3A (43). They reported that stimulation of synaptic vesicle exocytosis by α-latrotoxin, the active component of black widow spider venom, increased the GDP/GTP ratio of Rab3A, and therefore concluded that cleavage of Rab3A-bound GTP is a crucial step in regulated exocytosis of synaptic vesicles. However, the effect of stimulation of exocytosis on GTP incorporation into Rab3 proteins and the mechanism of secretagogue regulation of Rab3 protein function are still unclear. Our finding that overexpression of Rab3D enhanced CCK-stimulated amylase secretion and CCK-augmented GTP incorporation into HA-tagged Rab3D, suggested that CCK regulates exocytosis of pancreatic acini in part by promoting GTP binding to Rab3D. Since a multistep mechanism may regulate proteins by GDP/GTP cycling as described for Rab5 by Ullrich et al. (21), further studies are needed to identify and characterize possible GDP/GTP exchange factor(s) of Rab3D and GAPs that might modify guanine nucleotide binding.

The mechanism by which CCK enhances GTP binding to HA-tagged Rab3D is unknown. The fact that the CCK effect was maximal at 10 min probably reflects the time required for permeabilization and cell penetration, with the actual event occurring much quicker. In support of this interpretation, GTP binding to Ras in pancreatic acini by a similar technique was maximal at 10 min, whereas downstream events such as MEK and MAP kinase activation could be observed by 1–2.5 min (44). CCK is believed to induce secretion by increasing Ca^{2+} and activating protein kinase C. In the present studies Ca^{2+} was buffered. Adding phorbol ester, which increases binding of GTP to Ras (44), had no effect (data not shown).

In summary, we have demonstrated using transgenic mice that epitope-tagged Rab3D can be targeted to pancreatic zymogen granules, increasing the amount of Rab3D present. Although the observed effects of elevated Rab3D on secretory events was modest, they were seen consistently in two different aspects of regulated secretion: CCK-stimulated amylase release in intact acini, and Ca^{2+}-stimulated amylase release in permeabilized acini. In addition, CCK was shown to stimulate GTP binding to Rab3D. While the modest increase in secretion may reflect the limited increase in Rab3D on the granule, it may also reflect the presence of a limiting component downstream in the sequence of events leading to exocytosis. Studies with other mouse lines further overexpressing normal Rab3D or expressing mutant Rab molecules affecting the GTPase cycle are necessary to further understand regulation of secretory vesicle exocytosis by Rab3D, and possible other G-proteins on secretory vesicles.

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