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Involvement of the Rab27 Binding Protein Slac2c/MyRIP in Insulin Exocytosis

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Rab27a is a GTPase associated with insulin-containing secretory granules of pancreatic β-cells. Selective reduction of Rab27a expression by RNA interference did not alter granule distribution and basal secretion but impaired exocytosis triggered by insulin secretagogues. Screening for potential effectors of the GTPase revealed that the Rab27a-binding protein Slac2c/MyRIP is associated with secretory granules of β-cells. Attenuation of Slac2c/MyRIP expression by RNA interference did not modify basal secretion but severely impaired hormone release in response to secretagogues. Although β-cells express Myosin-Va, a potential partner of Slac2c/MyRIP, no functional link between the two proteins could be demonstrated. In fact, overexpression of the Myosin-Va binding domain of Slac2c/MyRIP did not affect granule localization and hormone exocytosis. In contrast, overexpression of the actin-binding domain of Slac2c/MyRIP led to a potent inhibition of exocytosis without detectable alteration in granule distribution. This effect was prevented by point mutations that abolish actin binding. Taken together our data suggest that Rab27a and Slac2c/MyRIP are part of a complex mediating the interaction of secretory granules with cortical actin cytoskeleton and participate to the regulation of the final steps of insulin exocytosis.

INTRODUCTION

Insulin release from pancreatic β-cells plays an essential role in the achievement of blood glucose homeostasis and defects in the regulation of this process lead to profound metabolic disorders. The precise mechanism that allows the fine-tuning of insulin exocytosis is still not well understood. However, some components of the secretory machinery of pancreatic β-cells have been identified. These components include the SNAREs VAMP-2, SNAP25, and syntaxin-1a and different Rab GTPases (Lang, 1999; Easom, 2000). Rab GTPases constitute a large family of proteins (more than 60 members in humans) that govern intracellular vesicular trafficking in eukaryotic cells (Martinez and Goud, 1998; Zerial and McBride, 2001). Although the exact role of most Rabs remains to be elucidated, the members of this family are generally believed to control one or more steps in the secretory or in the endocytic pathway (Martinez and Goud, 1998; Zerial and McBride, 2001). In a variety of cell systems, Rab3 and Rab27 are associated with secretory vesicles and are involved in the regulation of exocytosis. The secretory granules of pancreatic β-cells are endowed with different isoforms of Rab3 (Rab3a-d) and Rab27 (Rab27a-b; Regazzi *et al.,* 1996; Iezzi *et al.,* 1999; Yi *et al.,* 2002, Zhao *et al.,* 2002). Overexpression of constitutively active mutants of Rab3a-d leads to a decrease in insulin release in response to secretagogues (Iezzi *et al.,* 1999). In contrast, overexpression of the active form of Rab27a improves the secretory response of pancreatic β-cells (Yi *et al.,* 2002), suggesting that the two GTPases may have opposite effects on insulin exocytosis.

Rab GTPases exert their action on vesicular transport by interacting with one or more downstream effectors. Pancreatic β-cells express several putative Rab3 targets susceptible to mediate the effect(s) of the GTPase on insulin exocytosis, including RIM, Noc2, and calmodulin (Kotake *et al.,* 1997; Coppola *et al.,* 1999; Iezzi *et al.,* 2000; Ozaki *et al.,* 2000). The mechanism of action of Rab27 in endocrine cells has been investigated only very recently and is still incompletely understood. Synaptotagmin-like proteins (Slp) and the related Slac2 proteins (Slps lacking C2 domains) are characterized by the presence of a unique amino terminal domain that confers the capacity to bind Rab27 (Kuroda *et al.,* 2002a, 2002b). One member of this protein family, Slp4/Granuphilin is localized on secretory granules of pancreatic β-cells and its overexpression causes a profound inhibition of insulin secretion (Wang *et al.,* 1999; Coppola *et al.,* 2002; Torii *et al.,* 2002). This effect is prevented by point mutations that impair binding of Rab27 (Torii *et al.,* 2002), suggesting that Slp4/Granuphilin may mediate at least part of the functions of this GTPase. However, Slp4/Granuphilin is unlikely to be the only downstream effector of Rab27. In fact, the active form of Rab27 and Slp4/Granuphilin have opposite effects on insulin exocytosis.

In view of these observations, we tested whether pancreatic β-cells express other members of the Slp/Slac2 family. We found that insulin-secreting cells contain two other

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Figure 1. Rab27a expression can be selectively decreased by RNA interference. (A) INS-1E cells were transiently transfected with GFP-tagged Rab27a or with GFP-tagged Rab3a. Silencing of the genes was determined by cotransfecting the cells either with an empty pSUPER vector (control) or with a pSUPER vector containing a sequence that directs the synthesis rat Rab27a-specific siRNAs (RNAi). The cells were homogenized and analyzed by Western blotting using an anti-GFP antibody. The figure shows the results of three independent experiments. (B) INS-1E cells were transiently cotransfected with GFP and with the Rab27a silencer. After three days the cells were fixed and analyzed by immunofluorescence using an antibody against Rab27a. Top: GFP fluorescence. Bottom: expression of endogenous Rab27a. The arrow indicates the position of a GFP-positive cell expressing the silencer.

Rab27 binding proteins and that at least one of them, Slac2c/Myrip is involved in the regulation of insulin release.

MATERIALS AND METHODS

The antibodies against Rab27a and Myosin-Va (clone LF18) were purchased from Transduction Laboratories (Lexington, KY) and Sigma (St. Louis, MO), respectively. The antibody against insulin was from Linco Research (St. Charles, MO). The production of the antibodies against Slac2c/MyRIP and Myosin VIIa as well as the generation of the antibodies against Slp1, Slp2a, Slp3a, Slp5, and Slac2a/melanophilin have been described previously (El-Amraoui *et al.*, 2002; Kuroda *et al.*, 2002a, 2002b; Fukuda *et al.*, 2002). Fluorescently labeled secondary antibodies and Oregon green–coupled phalloidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). The generation of plasmids encoding Slac2c/MyRIP constructs has been reported elsewhere (El-Amraoui *et al.*, 2002). The pSUPER plasmid (Brummelkamp *et al.*, 2002) was kindly provided by Dr. Agami, Netherlands Cancer Institute, Amsterdam. Rat Rab27a was cloned from an INS-1E expression library. The plasmids encoding GFP-tagged rat Rab27a and human Rab3a were obtained by inserting the coding sequence of the two GTPases in frame with GFP in pcDNA3.

Preparation of Vectors for Rab27a and Slac2c/MyRIP Silencing

Mammalian expression vectors directing the synthesis of small interfering RNAs (siRNAs) targeted against Rab27a or Slac2c/MyRIP were prepared according to the method of Brummelkamp *et al.* (2002). Two cDNA fragments encoding a 19-nucleotide sequence derived from the target transcript and separated from its reverse 19-nucleotide complement by a short spacer were synthesized by MWG Biotech Company (Ebersberg, Germany). The two cDNA fragments were annealed and cloned in front of the H1-RNA promoter in the pSUPER vector (Brummelkamp *et al.*, 2002). The Rab27a silencer was generated using the sequence corresponding to nucleotides 112–130 of rat Rab27a cDNA; the Slac2c/MyRIP silencers were constructed using the nucleotide sequences 1932–1950 (sequence 1) and 2339–2357 (sequence 2) of human Slac2c/MyRIP. These two sequences were chosen because of their full match with the nucleotide sequence of rat Slac2c/MyRIP. The specificity of each sequence was verified by BLAST search against the gene data bank. To prepare mammalian expression vectors encoding both siRNAs and human growth hormone (hGH) a cassette containing the H1-RNA promoter and the silencing sequence was amplified by PCR and subcloned within the *Hin*dIII and *Xba*I sites of pXGH5 (Nichols Institute, San Juan Capistrano, CA).

Cell Culture and Transfection

The rat pancreatic β-cell line INS-1E was cultured in RPMI 1640 medium supplemented with 50 μM β-mercaptoethanol, 100 μM sodium pyruvate, and 5% fetal calf serum as described (Asfari *et al.*, 1992). Transient transfection was performed using the Effectene reagent (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. In all the experiments the DNA/Effectene ratio was 1/25.

Immunohistochemistry

Immunohistochemistry was performed on a 2-week-old mouse. The pancreas was isolated after fixation by perfusion with 4% paraformaldehyde in PBS. Twenty-micrometer thick cryosections were incubated overnight at 4°C with primary antibodies. Immunolabeled proteins were visualized by incubating the slices for 1 h at room temperature with fluorescently labeled secondary antibodies. Images were obtained by confocal microscopy (Leica, model TCS NT; Lasertechnik, Heidelberg, Germany).

Immunocytochemistry

INS-1E cells were cultured on glass coverslips coated with 20 μg/ml laminin and 0.2 mg/ml poly-L-lysine. The day of the experiment the cells were fixed in 4% paraformaldehyde and incubated for 2 h at room temperature with the first antibody diluted in buffer A (PBS, pH 7.5, supplemented with 0.1% goat serum [vol/vol], 0.3% Triton-X-100 [vol/vol] and 20 mg/ml bovine serum albumin). The coverslips were rinsed with PBS and incubated for 30 min at room temperature with the secondary antibody diluted in buffer A. Filamen-

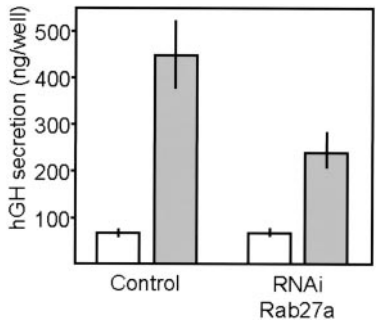

Figure 2. Effect of Rab27a knock down on exocytosis. INS-1E cells were transiently transfected with a plasmid encoding hGH and with either the empty vector (control) or a with the vector containing a sequence that directs the synthesis of Rab27a-specific siRNAs (RNAi). Three days later the cells were incubated under basal (□) or stimulatory (■) conditions. The amount of hGH released during the incubation period was determined by ELISA.

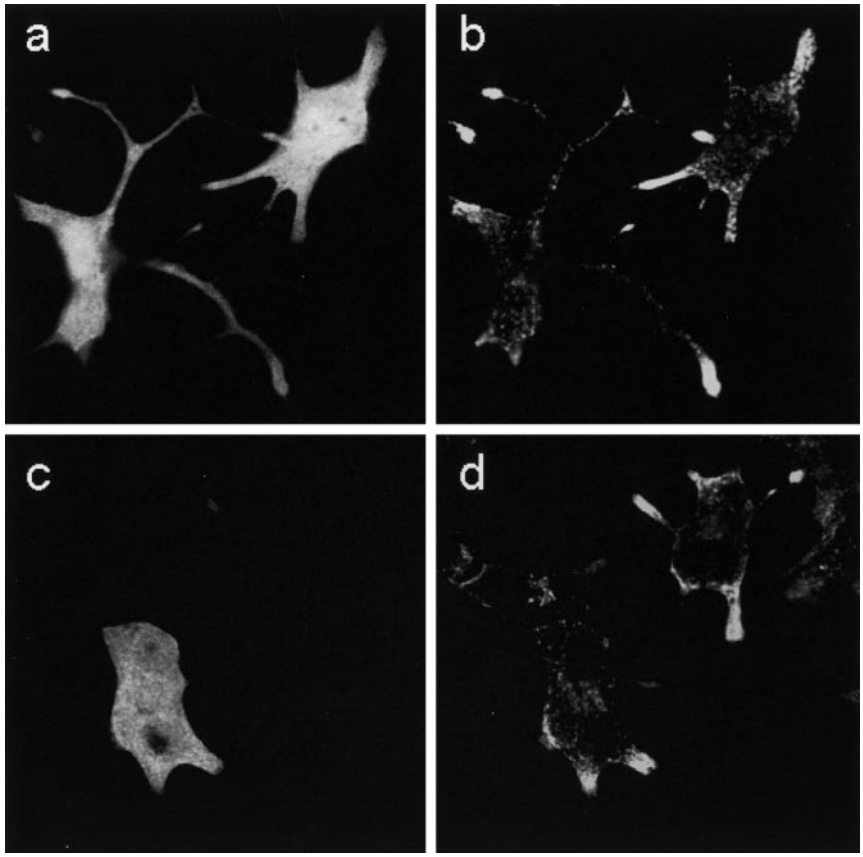

Figure 3. A decrease in Rab27a function does not affect the distribution of insulin-containing granules. INS-1E cells were transiently transfected with the Rab27a silencer together with GFP (a and b) or with GFP-tagged Rab27a T23N (c and d). Three days later they were fixed and analyzed by confocal microscopy. Transfected cells were identified by the GFP fluorescence (a and c). The distribution of secretory granules was visualized using an antibody against insulin (b and d).

tous actin was visualized with Oregon green–coupled phalloidin. The coverslips were washed and mounted for confocal microscopy.

Secretion

INS-1E cells were transiently cotransfected with a plasmid encoding hGH and with plasmids encoding either the RNAi silencers or the Slac2c/MyRIP expression constructs. In some experiments the cells were transfected with a single plasmid directing the synthesis of both siRNAs and human growth hormone. Three days later, the cells were preincubated for 30 min in 20 mM HEPES, pH 7.4, 128 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.7 mM $CaCl_2$. The medium was then aspirated, and the cells were incubated for 45 min at 37°C either in the same buffer (basal) or in a buffer containing 20 mM HEPES, pH 7.4, 53 mM NaCl, 80 mM KCl, 1 mM $MgCl_2$, 2.7 mM $CaCl_2$, 20 mM glucose, 1 μM Forskolin, and 1 mM IBMX (stimulated). Exocytosis from transfected cells was assessed by measuring by ELISA the amount of hGH released in the medium during the incubation period (Roche, Rotkreuz, Switzerland).

Immunoblotting

For Western blotting, transfected cells grown in 24-well plates were washed twice in ice-cold PBS and harvested. Cells from one well were solubilized in 200 μl SDS sample buffer and boiled 10 min. Equal amounts of protein were separated on polyacrylamide gels and transferred to nitrocellulose membranes. Immunostaining with specific antibodies and horseradish peroxidase–conjugated secondary antibodies diluted 1:2000 was carried out using the ECL plus technique (Amersham Pharmacia Biotechnology, Piscataway, NJ).

Actin Binding Assay

An actin-binding-defective Slac2c mutant carrying a RA (R774A/R777A/R778A/R779A) substitution was produced by two-step PCR techniques using the following mutagenic oligonucleotides as described previously (Fukuda *et al.,* 1995; Kuroda *et al.,* 2003): 5′-CCGCGGCTGCTGTGAGAGCCACACACGTG-3′ (RA primer 1) and 5′-CCGCGGACCAGAAGCAAAGGAGCCAG-3′ (RA primer 2). The mutant Slac2c fragment was subcloned into the pEF-T7 tag vector (named pEF-T7-Slac2c-ABD (RA)) and verified by DNA sequencing. Actin-binding assay was performed as described previously (Fukuda and Kuroda, 2002).

RESULTS

Rab27a is associated with insulin-containing secretory granules of pancreatic β-cells (Yi *et al.,* 2002). To directly investigate the role of Rab27a in insulin exocytosis, we took

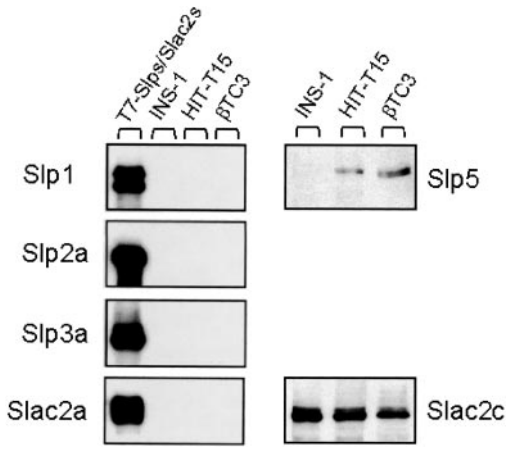

Figure 4. Screening for members of the Slp family expressed in insulin-secreting cell lines. Total homogenates of INS-1E, HIT-T15, and βTC3 were analyzed by Western blotting using antibodies against Slp1, Slp2a, Slp3a, Slp5, Slac2a, and Slac2c. When no detectable signal was obtained in insulin-secreting cells, homogenates of COS cells transfected with the appropriate T7-tagged Slps were included as a positive control.

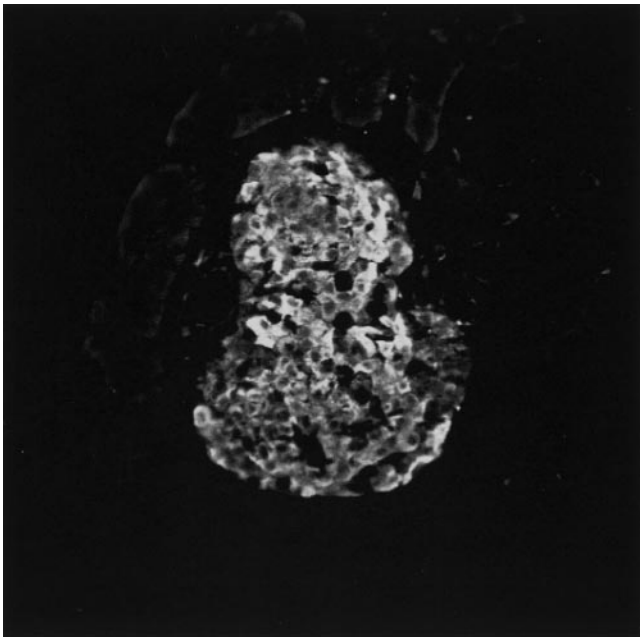

Figure 5. Slac2c/MyRIP is expressed in pancreatic islets. Mouse pancreatic sections were incubated with a polyclonal antibody directed against Slac2c/MyRIP. The presence of immunocomplexes was revealed using a Cy3-labeled anti-rabbit antibody. The image was obtained by confocal microscopy.

advantage of a recently described phenomenon called RNA interference (RNAi; Hammond *et al.,* 2001; Tuschl, 2001). RNAi is an evolutionarily conserved process, initially discovered in invertebrates, that is initiated by double-stranded RNAs. RNAi leads to sequence-specific degradation of the mRNA target resulting in effective silencing of the gene. To this purpose we generated a plasmid that directs the synthesis of siRNAs targeted against the sequence of the Rab27a GTPase. Transient expression of the Rab27a silencer in the insulin-secreting cell line INS-1E selectively reduced the production of GFP-Rab27a but did not affect the expression of GFP-Rab3a (Figure 1A) or of other more distantly related proteins such as GFP-Rab8 (unpublished data). Densitometric scanning of the films from three independent experiments revealed that the expression of GFP-Rab27a was decreased by 40 ± 15%, whereas the expression of GFP-Rab3a was not significantly affected (94 ± 11% of the level found in control cells). To verify the silencing effect on the endogenous protein, INS-1E cells were transiently cotransfected with the Rab27a silencer and with GFP. Three days later the cells were fixed and analyzed by immunofluorescence. Efficiently transfected cells (usually ~30%) identified by the expression of GFP, were found to contain lower levels of Rab27a (Figure 1B), confirming silencing also of the endogenous protein. To assess the involvement of Rab27a in β-cell exocytosis, INS-1E cells were transiently transfected with the Rab27a silencer together with a plasmid encoding human growth hormone (hGH). hGH is targeted to insulin-containing secretory granules and can be used to monitor exocytosis in the subpopulation of cells that are transiently transfected (Coppola *et al.,* 1999; Iezzi *et al.,* 2000). Selective knock down of Rab27a by RNAi did not affect basal secretion but resulted in a decrease in the amount of hormone released under stimulatory conditions (Figure 2). Experiments using a single plasmid engineered to express both hGH and the siRNAs against Rab27a gave similar results (unpublished data). These findings are consistent with the proposed positive role of Rab27a in insulin secretion (Yi *et al.,* 2002).

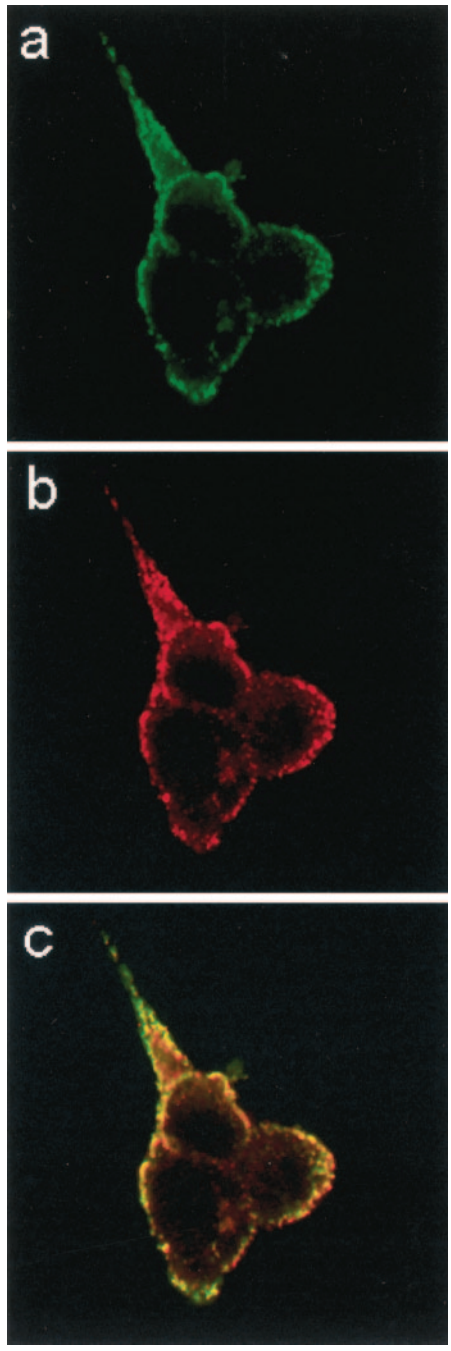

Figure 6. Subcellular localization of Slac2c/MyRIP. INS-1E cells grown on glass coverslips were fixed with paraformaldehyde and labeled with a guinea pig antibody against insulin and a rabbit antibody against Slac2c/MyRIP. Images were obtained by confocal microscopy after incubation of the coverslips with an Oregon-green–coupled anti–guinea pig antibody and a Cy3-coupled anti-rabbit antibody. (a) The localization of insulin; (b) the position of Slac2c/MyRIP; (c) the superposition of the green and red channels.

In melanocytes lacking Rab27a or expressing dominant negative Rab27a mutants, melanosomes are not correctly

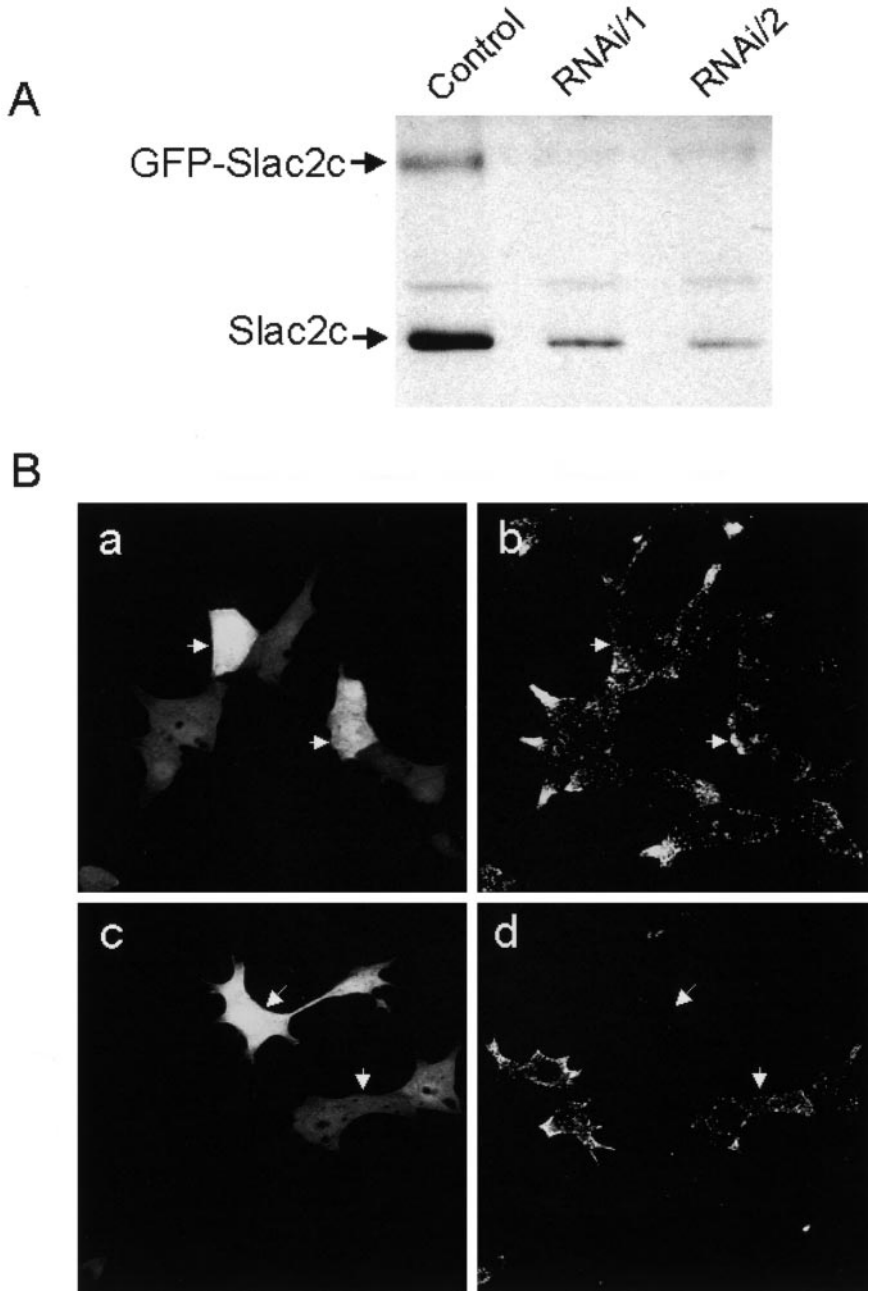

Figure 7. Slac2c/MyRIP expression can be selectively decreased by RNA interference. (A) INS-1E cells were transiently transfected with GFP-tagged Slac2c. Silencing of the genes was determined by cotransfecting the cells either with an empty vector (control) or with vectors containing sequences that direct the synthesis Slac2c/MyRIP-specific siRNAs (RNAi/1 and RNAi/2). Three days later the cells were homogenized and equal amounts of proteins were analyzed by Western blotting using an anti-Slac2c antibody. The positions of endogenous Slac2c and of its transfected GFP-tagged counterpart are indicated by the arrows. (B) INS-1E cells were transiently cotransfected with GFP and with an empty vector (a and b) or with a vector containing the RNAi/2 silencer sequence (c and d). After 3 d the cells were fixed and analyzed by immunofluorescence using the antibody against Slac2c/MyRIP. The arrows indicate the position of transiently transfected cells expressing the silencer.

positioned in dendritic tips and remain clustered in the cell center (Hume *et al.*, 2001; Wu *et al.*, 2002). The morphology of INS-1E cells is often characterized by the presence of dendritic-like protrusions in which insulin-containing secretory granules accumulate. In cells transfected with the Rab27a silencer or with a plasmid encoding a dominant negative mutant of Rab27a (Rab27T23N), the distribution of insulin-containing secretory granules was not altered (Figure 3). This indicates that in pancreatic β-cells Rab27a is not essential for the delivery or the retention of secretory granules to the cell periphery but is rather involved in the final steps of insulin exocytosis.

Slps and their homologues lacking C2 domains (Slac2) are Rab27-binding proteins involved in vesicular trafficking (Fukuda and Mikoshiba, 2001; Fukuda *et al.*, 2001). One member of this protein family, Slp4/Granuphilin, is expressed in pancreatic β-cells and controls insulin exocytosis (Coppola *et al.*, 2002; Torii *et al.*, 2002). However, Slp4 appears to inhibit rather than favor exocytosis, suggesting that other Rab27 binding proteins may also participate in the regulation of insulin release. In attempt to identify additional Rab27a partners, we searched for other Slps and Slac2 proteins present in homogenates of the insulin-secreting cell lines, INS-1E, HIT-T15 and βTC3. Slp1, Slp2a, Slp3a, and Slac2a/melanophilin were not detectable in the cell lines tested (Figure 4). Slp5 was readily visualized in HIT-T15 and βTC3 homogenates but was poorly detectable in INS-1E cells. In contrast, Slac2c/MyRIP was expressed at high levels in each of the cell lines tested. In view of these findings we decided to investigate the role of this protein in more detail.

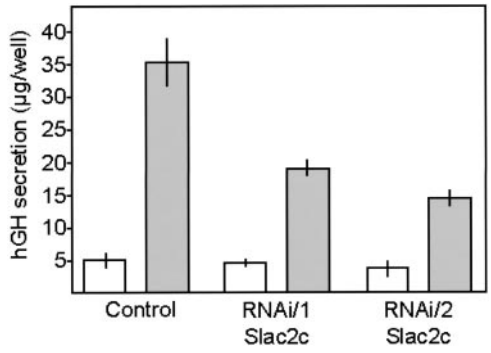

Figure 8. Effect of Slac2c/MyRIP knock down on INS-1E exocytosis. INS-1E cells were transiently transfected with plasmids that encode hGH and contain sequences under the control of an H1-promoter that direct the synthesis Slac2c/MyRIP-specific siRNAs. A vector encoding hGH but devoid of RNAi-inducing sequences was used as a control. Three days later the cells were incubated under basal (□) or stimulatory (■) conditions. The amount of hGH released during the incubation period was determined by ELISA.

To verify the presence of Slac2c/MyRIP in primary β-cells we prepared mouse pancreatic sections and analyzed them by immunohistochemistry. As shown in Figure 5, pancreatic islets were readily stained with the antibody directed against Slac2c/MyRIP. In contrast, the other pancreatic structures, including the exocrine portion of the gland and blood vessels, were only barely stained. To determine the subcellular distribution of Slac2c/MyRIP, INS-1E cells were analyzed by confocal microscopy. Double labeling experiments demonstrated that Slac2c/MyRIP colocalizes with insulin-containing secretory granules (Figure 6). Similar results were obtained after confocal analysis of cultured primary β-cells (unpublished data).

The association of Slac2c/MyRIP with insulin-containing granules prompted us to investigate the involvement of Slac2c/MyRIP in the secretory process of pancreatic β-cells. To this end, we transiently cotransfected INS-1E cells with GFP-Slac2c/MyRIP and with plasmids that direct the synthesis of siRNAs targeted against two different sequences of Slac2c/MyRIP. As shown in Figure 7A, after 3 d in culture the silencers nearly abolished the expression of GFP-Slac2c/MyRIP. Despite the fact that only a fraction of the cells are transfected with the silencers, reduction of endogenous Slac2c/MyRIP expression was also detectable. The efficacy of the silencers on the endogenous protein was also confirmed by immunofluorescence (Figure 7B). A decrease in Slac2c/MyRIP expression did not alter basal secretion but hormone release in the presence of secretagogues was significantly reduced with both sequences (Figure 8).

Slac2c/MyRIP can interact with Myosin-Va, Myosin-VIIa, and actin (El-Amraoui *et al.*, 2002; Fukuda and Kuroda, 2002). The presence of unconventional Myosins in insulin-secreting cells is poorly documented. To identify potential Slac2c/MyRIP partners in insulin-secreting cells, we investigated the expression of Myosin-Va and Myosin-VIIa in mouse pancreatic sections. Pancreatic islet cells were found to express Myosin-Va but not Myosin-VIIa (Figure 9). None of the myosins was detected in the exocrine portion of the

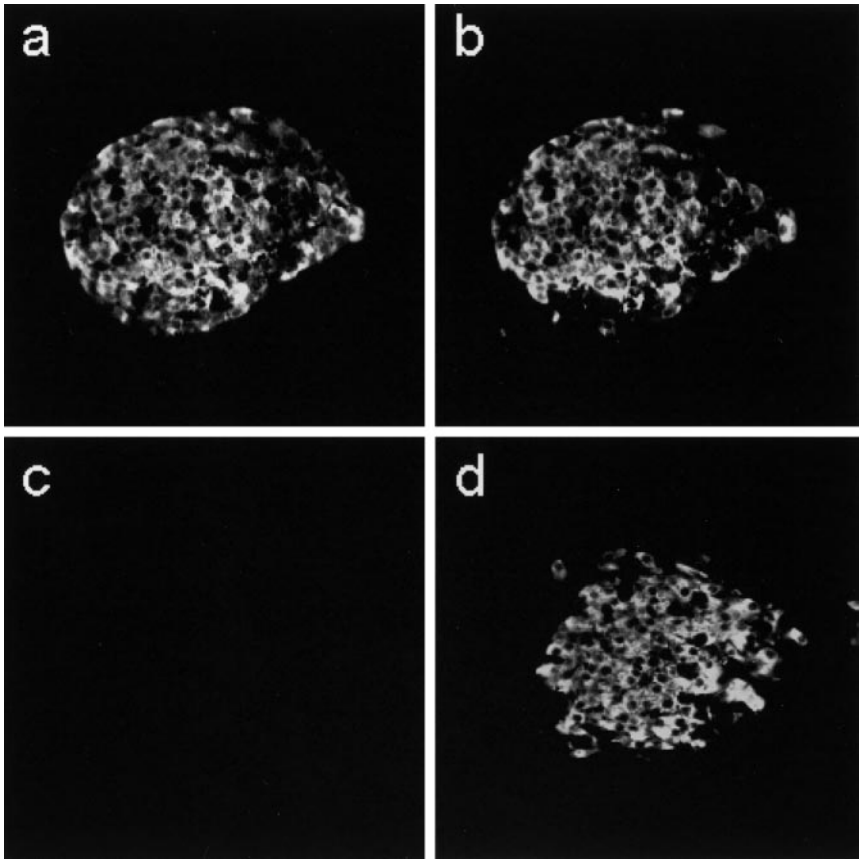

Figure 9. Expression of unconventional myosins in pancreatic islets. Mouse pancreatic sections were incubated with antibodies directed against Myosin Va (a) or Myosin VIIa (c) and with an antibody against insulin (b and d). Images were obtained by confocal microscopy.

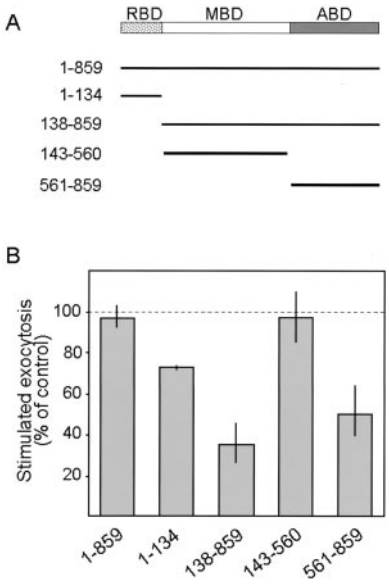

Figure 10. Effect of Slac2c/MyRIP constructs on INS-1E exocytosis. (A) Schematic representation of Slac2c/MyRIP and of the deletion mutants used in this study. The numbers on the left indicate the amino acids included in each construct. RBD, Rab-binding domain; MBD, Myosin-binding domain; ABD, actin-binding domain. (B) The different Slac2c/MyRIP constructs were transiently cotransfected in INS-1E cells together with a plasmid encoding hGH. Three days later the cells were incubated under basal or stimulatory conditions. The amount of hGH released under each condition was determined by ELISA. None of the constructs affected basal hGH secretion. The figure shows the secretory response of the cells under stimulatory conditions. The results correspond to the mean ± SEM of three independent experiments performed in triplicates. The amount of hormone released under stimulatory conditions from cells cotransfected with the hGH plasmid and an empty vector was set to 100%.

gland. These results were confirmed by Western blotting (unpublished data).

To clarify the mode of action of Slac2c/MyRIP, we transiently transfected INS-1E cells with the full-length protein or with deletion mutants corresponding to its putative functional domains (Figure 10A). All the constructs were expressed to comparable levels and none of the constructs altered basal hGH release (unpublished data). Exocytosis triggered by insulin secretagogues was not affected in cells overexpressing full-length Slac2c/MyRIP or its putative Myosin-Va–binding domain (Figure 10B). In contrast, transfection of the Rab27 binding domain led to a small but significant decrease in stimulated hGH release. Because the Rab27 binding domain colocalizes with insulin-containing granules (Figure 11), this effect is most likely due to a competition with endogenous Rab27 partners. The strongest alteration in INS-1E exocytosis was observed upon expression of the two protein constructs including the actin-binding domain. In this case, hGH secretion was inhibited by more than 50% (Figure 10B). None of the constructs tested modified the distribution of insulin-containing granules (Figure 11), indicating that the inhibition observed in the presence of the Rab27-binding domain or of the actin-binding domain is not caused by a defect in the transport or in the retention of secretory vesicles to the cell periphery. When expressed in INS-1E cells, the Myosin-Va–binding domain and the actin-binding domain did not colocalize with insulin-containing granules (Figure 11). The Myosin-Va–binding domain was homogenously distributed throughout the cell cytoplasm (Figure 12). In contrast, the actin-binding domain was concentrated at the cell periphery and partially colocalized with cortical actin filaments. The actin-binding domain of Slac2c/MyRIP contains a polyarginine sequence that is homologous to the polybasic motif required for the interaction of Slac2a/melanophilin with actin (Kuroda *et al.*, 2003). We found that replacement of the polyarginine sequence of Slac2c/MyRIP with alanines abolished the capacity of the C-terminal fragment to interact with actin and prevented the inhibitory effect on INS-1E exocytosis (Figure 13).

DISCUSSION

Rab27a is associated with secretory organelles of melanocytes, cells of hematopoietic origin and endocrine cells (Chen *et al.*, 1997). In melanocytes loss of function mutations in the Rab27a gene lead to a defect in melanosome distribution (Hume *et al.*, 2001; Wu *et al.*, 2002) and in cytotoxic T lymphocytes to impairment in granule exocytosis (Stinchcombe *et al.*, 2001; Haddad *et al.*, 2001). The mechanism of action of Rab27a in melanocytes has been intensively investigated and is now relatively well understood. The activation of Rab27a determines the binding of Slac2a/melanophilin to melanosomes (Fukuda *et al.*, 2002; Wu *et al.*, 2002). This causes the recruitment of the actin-based motor protein Myosin-Va to the organelle surface and allows peripheral retention of melanosomes within the cortical actin network (Wu *et al.*, 2002). Absence of any of the components of the Rab27a/Slac2a/Myosin-Va complex results in the accumulation of melanosomes in the perinuclear area. In cytotoxic T lymphocytes the mode of action of Rab27a is less clear and is apparently not linked to Myosin-Va and Slac2a, indicating that the function of the GTPase may be linked to the existence of cell specific targets.

Rab27a is expressed at high levels in pancreatic β-cells and is localized on insulin-containing secretory granules (Yi *et al.*, 2002). Here we took advantage of the RNAi process to elucidate the role of Rab27a on insulin exocytosis. Recently, several groups reported strategies based on transcription of short hairpin RNAs that allow the use of RNAi in mammalian cells (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002). In this study we demonstrate that RNAi can be initiated also in insulin-secreting cells and can be efficiently used to investigate β-cell function. Silencing of Rab27a gene in INS-1E cells led to a decrease in the capacity of insulin secretagogues to trigger exocytosis. Pancreatic β-cells express low levels of Rab27b (Zhao *et al.*, 2002), an isoform that can replace Rab27a in other cell systems (Barral *et al.*, 2002). The sequence selected for Rab27a silencing is specific for rat Rab27a and differs by three nucleotides from the sequence of rat Rab27b. Because of the known specificity of the RNAi process, it is very unlikely that the Rab27a silencer affects the expression of Rab27b. It is therefore possible that the effect of the Rab27a silencer on insulin exocytosis is attenuated by the presence of Rab27b. Despite the decrease in secretagogue-induced release, basal secretion was not altered and the distribution of secretory granules was unchanged. This indicates that the inhibition of exocytosis is most probably caused by impairment in late secretory pathway events rather than defects in the transport of secretory vesicles to the cell periphery. Thus, the effect of Rab27a silencing in pancreatic β-cells is clearly different from the phenotype displayed by melanocytes lacking Rab27a, Slac2a/Melanophilin or Myosin-Va (Hume *et al.*, 2001; Wu *et al.*, 2002). INS-1E cells the decrease in Rab27a expression results in a

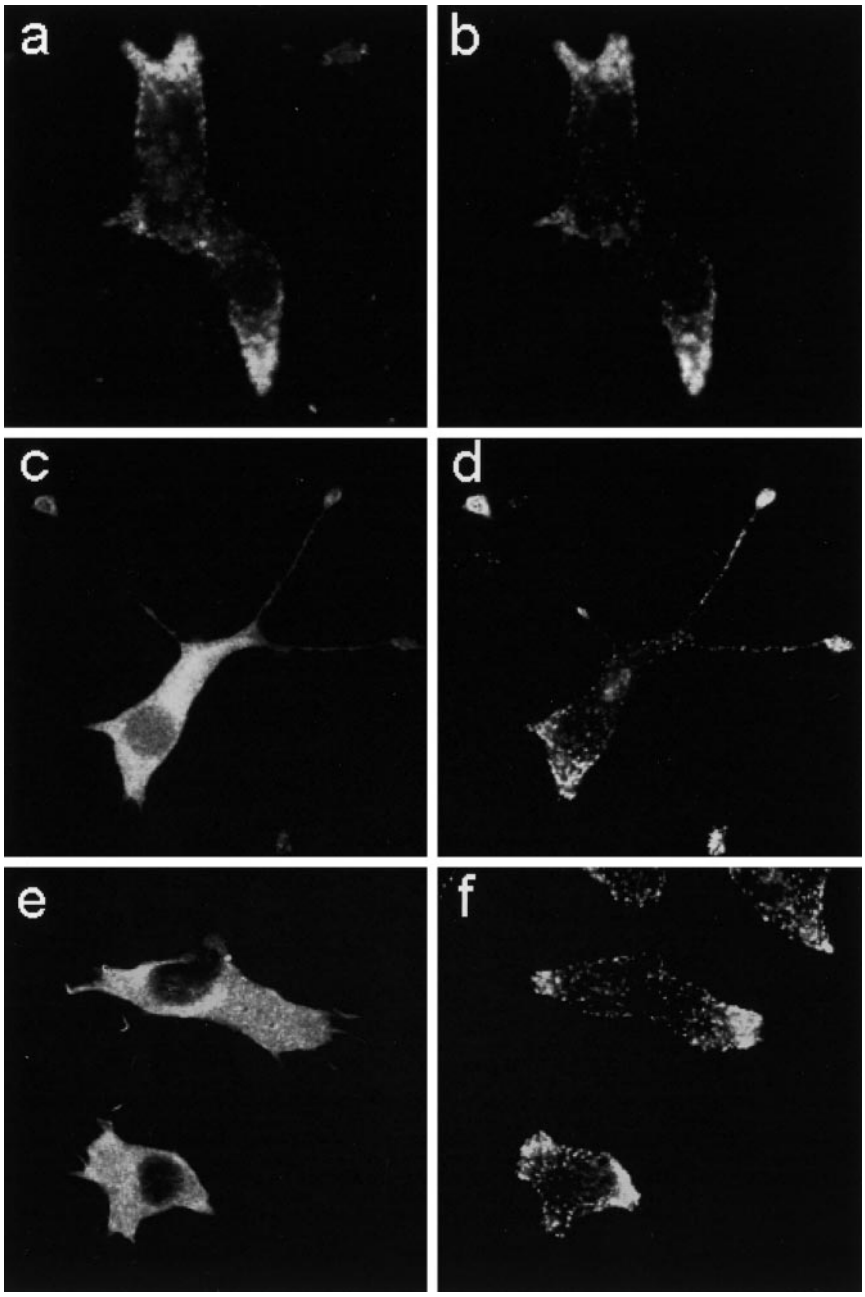

Figure 11. Subcellular distribution of Slac2c/MyRIP constructs in INS-1E cells. INS-1E cells were transiently transfected with myc-tagged constructs encoding the Rab-binding domain (a and b), the Myosin-binding domain (c and d) or the actin-binding domain (e and f). Two days later the cells were fixed, double stained with antibodies directed against the myc tag (a, c, and e) and against insulin (b, d, and f), and analyzed by confocal microscopy.

defect reminiscent to that observed in cytotoxic T lymphocytes lacking the GTPase (Haddad *et al.,* 2001; Stinchcombe *et al.,* 2001).

Pancreatic β-cells express at least three potential partners for Rab27a: Slp4/Granuphilin, Slp5, and Slac2c/MyRIP. Overexpression of Slp4/Granuphilin in pancreatic β-cells results in a profound inhibition of insulin secretion (Coppola *et al.,* 2002; Torii *et al.,* 2002), suggesting that the protein functions as a negative modulator of exocytosis. In agreement with this hypothesis a decrease in Slp4/Granuphilin expression is associated with enhancement in the secretory response of pancreatic β-cells (Waselle, Coppola, and Regazzi, unpublished observations). Thus, Rab27a and Slp4/Granuphilin have opposite effects on exocytosis. This apparent discrepancy may be at least in part explained by the capacity of Slp4/Granuphilin to interact with Rab3a, another GTPase associated with insulin-containing granules (Coppola *et al.,* 2002). Alternatively, fine-tuning of insulin release may result from a balance between the activation of multiple effectors with opposing actions on exocytosis. Slac2c/MyRIP silencing is associated with a decrease in hormone release elicited by secretagogues without appreciable alterations in granule distribution and basal secretion. This effect is identical to that observed after Rab27a silencing, indicating that the two proteins may indeed be functionally linked. In attempt to clarify the mechanism of action of Slac2c/MyRIP, we performed experiments with several deletion mutants. In vitro Slac2c/MyRIP interacts with two unconventional myosins: Myosin-Va and Myosin-VIIa. Myosin-Va is expressed in pancreatic β-cells and could repre-

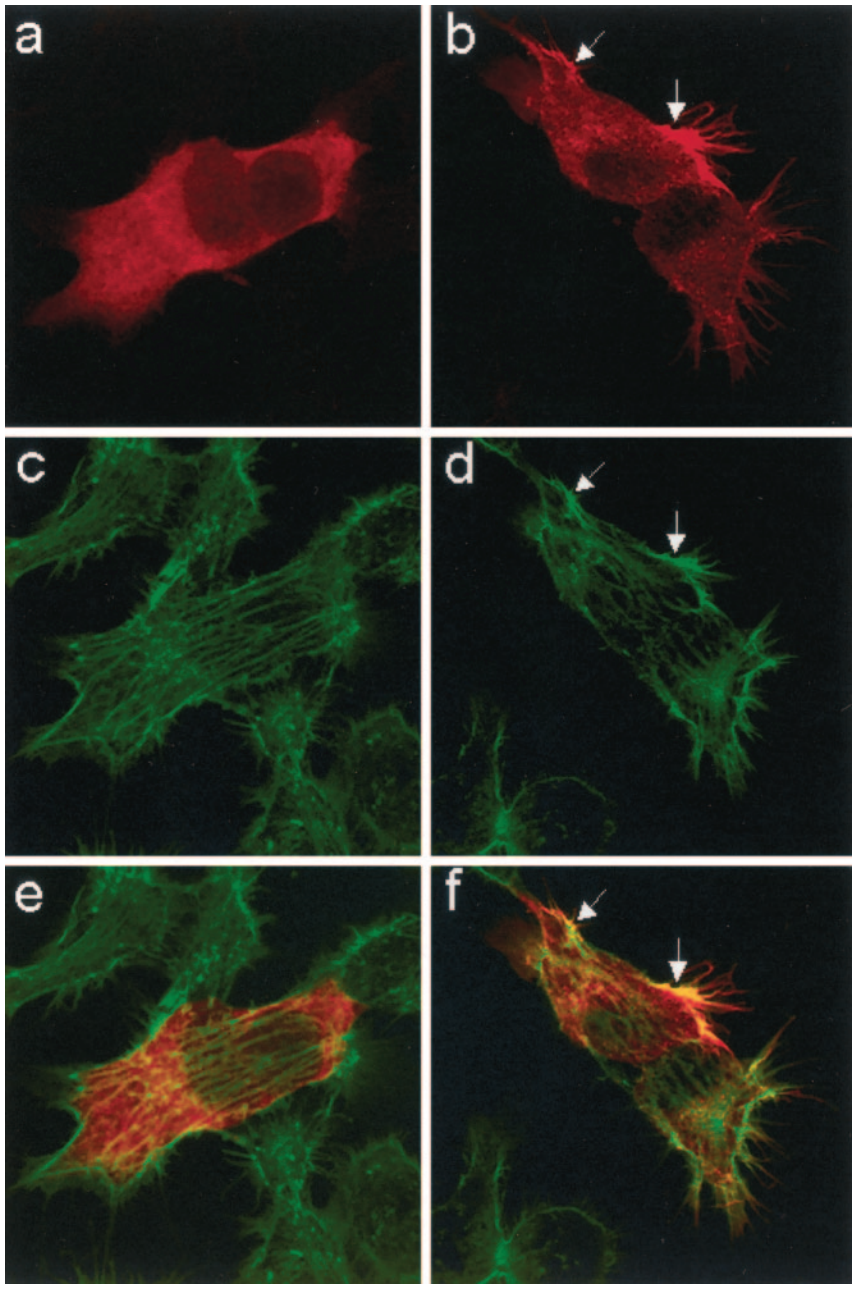

Figure 12. The C-terminal domain of Slac2c/MyRIP colocalizes with cortical actin cytoskeleton. INS-1E cells were transiently transfected with the Myosin-binding domain (a, c, and e) or the actin-binding domain (b, d, and f) of Slac2c/MyRIP. Two days later the cells were fixed and stained with an antimyc antibody (a and b) and with fluorescently labeled phalloidin (c and d). Images were taken using confocal microscopy. Panels e and f correspond to the overlay of the antimyc and phalloidin signal. The arrows indicate regions where the C-terminus of Slac2c/MyRIP and actin filaments colocalize.

sent a potential partner for Slac2c/MyRIP. However, overexpression of the Myosin-Va–binding domain of Slac2c/MyRIP did not affect granule distribution and was without effect on hormone secretion. Thus, although our data do not exclude a link between Slac2c/MyRIP and Myosin-Va, at present a functional interaction between the two proteins remains to be demonstrated. Overexpression of the C-terminal domain of Slac2c/MyRIP reduced exocytosis of INS-1E cells. The C-terminal Slac2c/MyRIP fragment was concentrated at the cell periphery and appeared to colocalize with the cortical actin network. Point mutations that abolish actin binding prevent the effect on exocytosis, indicating that the interaction with the actin cytoskeleton is probably essential for Slac2c/MyRIP function. The inhibitory effect of the C-terminus of Slac2c on exocytosis is unlikely to be due to actin sequestration. In fact, the actin sequestering compound latrunculin B was recently found to have a stimulatory effect on insulin exocytosis (Thurmond *et al.*, 2003). Our findings would be consistent with a model in which Slac2c/MyRIP is part of a complex bridging secretory vesicles to the cortical actin network. Dynamic association of insulin-containing granules with actin cytoskeleton is thought to be required for pancreatic β-cell exocytosis. Therefore, the decrease in hormone release in cells transfected with the C-terminal domain of Slac2c/MyRIP is likely to be explained by perturbations in the interaction of secretory vesicles with cortical actin network. Future experiments, involving detailed structure/function studies will have to elucidate the precise role played by Slac2c/MyRIP in the regulation of insulin exocytosis.

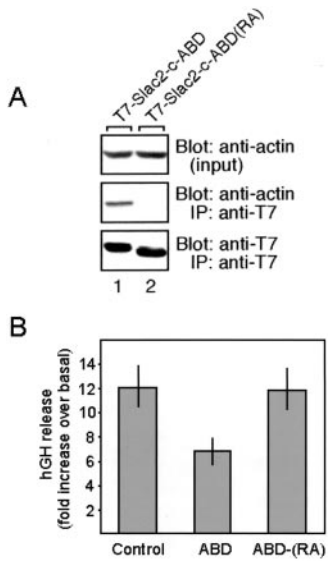

Figure 13. The actin binding activity of the C-terminus of Slac2c is required for the control of exocytosis. (A) The Slac2c-ABD(RA) mutant lacks actin-binding activity. T7-Slac2c-ABD and the Slac2c-ABD(RA) mutant were expressed in COS-7 cells and immunoprecipitated with anti-T7 tag antibody-conjugated agarose. Coimmunoprecipitated actin was first detected by antiactin antibody (middle panel) and the immunoprecipitated T7-Slac2c-ABD proteins were then visualized with anti-T7 tag antibody (bottom panel). The top panel shows total expressed proteins (1/80 volume; input) used for immunoprecipitation. (B) INS1-E cells were transiently cotransfected with a plasmid encoding hGH and with T7-Slac2c-ABD, Slac2c-ABD(RA) or with an empty vector (control). Three days later the cells were incubated under basal or under stimulatory conditions. The results show the ratio between the amount of hGH released under basal and stimulatory conditions.

In conclusion, this study has uncovered the involvement of Slac2c/MyRIP in the regulation of pancreatic β-cell exocytosis. At a first glance, the mode action of Slac2c/MyRIP in insulin-secreting cells appears to be different from that of Slac2a/Melanophilin in melanocytes. However, both members of the Slac2 family are part of complexes that mediate the interaction of secretory vesicles with the actin cytoskeleton. Thus, the capacity to bridge secretory vesicles to cytoskeletal elements may be a common property of the members of the Slac2 family.

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