# A Link between Cdc42 and Syntaxin Is Involved in Mastoparan-Stimulated Insulin Release†

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ABSTRACT: Mastoparan, a hormone receptor-mimetic peptide isolated from wasp venom, stimulates insulin release from pancreatic $\beta$-cells in a $Ca^{2+}$-independent but GTP-dependent manner. In this report, the role of the Rho family GTP-binding protein Cdc42, in the mastoparan stimulus-secretion pathway, was examined. Overexpression of wild-type Cdc42 in $\beta$HC-9 cells, an insulin-secreting mouse-derived cell line, resulted in a 2-fold increase in mastoparan-stimulated insulin release over vector-transfected $\beta$HC-9 cells. This effect was not seen with secretagogues such as glucose that stimulate secretion via $Ca^{2+}$-dependent pathways. GDP/GTP exchange assay data and studies with pertussis (PTX) toxin suggest that mastoparan may work directly to activate Cdc42 and not via PTX-sensitive heterotrimeric GTP-binding proteins. Using bacterial glutathione *S*-transferase−Cdc42 fusion proteins and co-immunoprecipitation and transient transfection studies, Cdc42 was shown to be an upstream regulator of the exocytotic protein, syntaxin. These results suggest that the GTP-dependent signal underlying the mastoparan effect acts at a "distal site" in stimulus-secretion coupling on one of the SNARE proteins essential for exocytosis. In vitro binding assays, using purified Cdc42 and syntaxin proteins, show that Cdc42 mediates the GTP signal through an indirect association with syntaxin. The H3 domain at the C-terminus of syntaxin, which participates in the formation of the ternary SNARE complex with the core proteins, SNAP-25 and synaptobrevin, is also required for the association with Cdc42. Thus, these studies indicate that Cdc42 could be a putative GTP-binding protein thought to be involved in the mastoparan-stimulated GTP-dependent pathway of insulin release.

Insulin release from the pancreatic $\beta$-cell is under the control of a variety of nutrient, hormone, and neurotransmitter signals that enhance or inhibit insulin release by complex mechanisms (*1*−*3*). Mastoparan, a toxin from wasp venom, can stimulate exocytosis in a number of mammalian cells including pancreatic $\beta$-cells (*4*−*12*). While the mechanism of stimulation of insulin release is unclear, the effect of mastoparan appears to be independent of the requirement for $[Ca^{2+}]_i$ (*7*). Mastoparan stimulates the guanine nucleotide exchange activity of heterotrimeric GTP-binding proteins, such as Gi, Go, Gs, and transducin among others, in a manner similar to that of G-protein-coupled receptors (*13*−*15*) as well as promotes the nucleotide exchange activity of low molecular weight GTP-binding proteins, such as the Rho protein (*16*). In the $\beta$-cell, the secretory effect of mastoparan is enhanced by glucose and α-ketoisocaproic acid (α-KIC), two nutrient insulin secretagogues (*17*). Thus, it appears that mastoparan-stimulated insulin release is due to the activation of a physiological mechanism at a distal step in the stimulation-secretion pathway (*17*). This distinguishes mastoparan as a unique secretagogue in insulin secretion studies. It can be used as a tool in understanding the nature of the direct activation of the exocytotic machinery at this distal site, and additionally, it can be used to determine the series of events involved in GTP-stimulated insulin release.

Insulin is released from the insulin-containing granule through the tightly regulated process of exocytosis. This general process is conserved among species and involves a complex fusion process (*18*−*20*). The basic exocytotic machinery consists of SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) that associate to form the SNARE or core complex that, through a series of protein−protein interactions, is essential for exocytosis (*21*). From genetics and studies with neurotoxins (*20*) and from work in pancreatic $\beta$-cells (*22*), it appears that core complexes form when granules are docked at the membrane. This highly stable complex is made up of two plasma membrane proteins, syntaxin and SNAP-25, and a granule membrane protein, synaptobrevin (also known as VAMP-2) (*18*−*23*). Among the many granule, cytosolic, and membrane proteins that are required at the different stages of exocytosis (*18*−*21*, *23*), recent evidence has indicated that the monomeric Rab proteins and more recently the Rho proteins play an important role in granule trafficking and secretion (*19*, *21*, *23*, *24*).

Although the identity of the G-protein involved in mastoparan-stimulated insulin release from the $\beta$-cell is not

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known, the small monomeric GTP-binding protein, Rab3A, and the alpha subunit of the Gi family of heterotrimeric GTP-binding proteins have been suggested as candidates for mastoparan action (*25*, *26*). Rab3A can stimulate insulin release, in the absence of calcium, by interacting with specific cytosolic proteins that are part of a pre-exocytotic complex (*25*). Through its association and sequestration of the inhibitory proteins REEP-1 and REEP-2, Rab3A promotes secretion (*25*). However, other studies suggest that the effects of mastoparan to stimulate exocytosis are independent of cytosolic proteins and are mediated by the stimulation of Gαi activity in the insulin secretory granule (*26*). Interestingly, the pertussis toxin-sensitive GTP-binding proteins, Gi and Go, are clearly implicated in the inhibition of insulin secretion by physiological agonists such as norepinephrine, somatostatin, and galanin (*3*, *27*).

The family of mammalian Rho GTP-binding proteins, which consists of Cdc42, RhoA−E, Rac1, Rac2, RacE, TC10, and others, are 20−30 kDa proteins that comprise a subset of the Ras superfamily (*28*). Upon activation, Cdc42 associates with specific downstream target proteins to participate in diverse signaling pathways including the regulation of the actin cytoskeleton, protein sorting, membrane trafficking, and cell growth control (*28*−*31*). Cdc42 activity is, in turn, controlled by proteins that regulate its GTP-binding/GTP hydrolytic cycle (*32*, *33*). Identification of putative targets such as the p21-activated kinases (PAKs), IQGAPs, and the Wiscott−Aldrich syndrome proteins (WASPs) has implicated Cdc42 in the stress-induced activation of the c-Jun kinase and p38 and in actin cytoskeletal remodeling (*34*−*39*). However, recent evidence seems to suggest another important role for Cdc42 in both endocytosis and exocytosis (*24*, *40*). In recent years, important information for a functional role for Cdc42 in β-cell stimulus-secretion coupling has been obtained (*41*−*46*). The depletion of GTP by mycophenolic acid resulted in the inhibition of nutrient-induced but not KCl-induced insulin release. Glucose promoted the carboxyl methylation of Cdc42 in intact β-cells, suggesting a physiological role of GTP in the proximal steps of nutrient-induced insulin release (*42*, *43*).

In this study, the involvement of Cdc42 in the mastoparan-stimulated, GTP-dependent pathway of insulin release and the implications at a distal step of stimulus-secretion coupling were examined. A novel connection was found to exist between Cdc42 and syntaxin, a plasma membrane exocytotic protein involved in core complex formation. The association between Cdc42 and syntaxin was promoted by mastoparan. It is proposed that Cdc42 may be involved in the control of mastoparan-stimulated exocytosis.

## EXPERIMENTAL PROCEDURES

*Materials.* Mastoparan, PTX, and glutathione were obtained from Sigma, St. Louis, MO. Mastoparan-17 was purchased from Bachem California Inc., Torrance, CA. IPTG was purchased from GIBCO BRL, New York. GST−syntaxin DNA was kindly provided to us by Dr. Richard H. Scheller. The antibody against syntaxin was purchased from Upstate Biotechnology, Lake Placid, NY. The antiserum against synaptobrevin was a gift from Dr. Reinhard Jahn and was also obtained from Synaptic Systems, Goettingen, Germany. [$^3$H]GDP was purchased from PerkinElmer Life Sciences.

*Cell Culture*. βHC-9 cells (*47*) and the transfected clones from passages 25−35 were used in this study. During the experiment, the cells were maintained at one passage per week and appeared to show a similar response to secretagogues such as mastoparan and depolarizing concentrations of potassium. The response to glucose appeared to decrease at the higher passage numbers (*47*). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 1 mM pyruvate, 15% horse serum, 2.5% fetal bovine serum, 100 units/mL streptomycin, and 100 units/mL penicillin at 37 °C in a 95% air and 5% $CO_2$ atmosphere. The cells were cultured in 75 cm$^2$ flasks for lysate preparations and in 16 mm diameter wells for the insulin secretion assays.

*Stable Transfection of Vector or Cdc42 Constructs*. βHC-9 cells (∼5 × 10$^6$) were cultured on 60 mm diameter tissue culture plates at 37 °C until 80% confluent. Prior to each transfection, 1 μg of DNA of HA-tagged wild-type Cdc42 or the pKH3 vector was diluted in 100 μL of serum-free DMEM, and 10 μL of the lipofectamine reagent (GIBCO BRL) was diluted in 100 μL of serum-free DMEM. The solutions were combined, mixed gently, and incubated at room temperature for 20 min. Serum-free medium (0.8 mL) was added to each sample, mixed, and overlaid onto the βHC-9 cells. The cells were incubated with the transfection mixture for 5 h, and 1 mL of growth medium containing twice the normal concentration of serum was added to the transfection mixture. The cells were split, approximately 24 h after the start of transfection, into two 100 mm plates and grown in neomycin-containing (G418) DMEM. After antibiotic selection, single colonies were transferred onto 24-well plates, grown to confluency, and tested for the expression of Cdc42 using an antibody against the HA tag.

*Transient Transfection of Cdc42 Constructs in a pcDNA3 Vector*. The procedure was carried out as described above with the following exception. Vector alone (pcDNA3), wild-type Cdc42, and (Q61L)Cdc42, the dominant active mutant of Cdc42, were used in the transfection reactions. Forty-eight hours after the reaction, the cells were washed twice with cold PBS and lysed with a 1% NP40-containing lysis buffer (25 mM Hepes, pH 7.4, 1 mM DTT, 15 mM $MgCl_2$, 150 mM NaCl, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.2 mg/mL phenylmethylsulfonic chloride). The supernatants were then immunoprecipitated with the antibody against the myc tag.

*Treatment of βHC-9 Cells*. Flasks (75 cm$^2$) of βHC-9 cells transfected with the vector or overexpressing Cdc42 were washed twice in Krebs−Ringer bicarbonate (KRB) solution composed of 129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $KH_2PO_2$, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.2% bovine serum albumin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 0.1 mM glucose (pH 7.4). Cells were incubated at 37 °C in KRB buffer for 30 min prior to addition of test agents. After the medium was aspirated out, the cells were incubated for an additional 10 or 60 min in 10 mL of fresh KRB buffer ± test agents. The reactions were stopped by removing the buffer and washing the cells with ice-cold phosphate-buffered saline (PBS) solution. After completely aspirating out any leftover solution, the cells were incubated in 700 μL of lysis buffer and rocked at 4 °C for 30 min. The sample was collected in 1.5 mL Eppendorf tubes and centrifuged at 10000*g* for

5 min, and the resulting supernatant was used in experiments as the cell lysate.

*Insulin Secretion under Static Incubation Conditions*. βHC-9 cells were grown in 16 mm diameter wells until confluency at $(0.4-1.6) \times 10^6$ was reached. The cells were washed and incubated for 30 min at 37 °C in 1.5 mL of KRB buffer. After 30 min, the buffer was removed, and 1 mL of fresh KRB containing 15 μM mastoparan or 15 μM mastoparan-17 (KRB buffer alone was used as a control) was added to each well for 60 min at 37 °C. In the experiments with pertussis toxin (PTX), the cells overexpressing Cdc42 were cultured in DMEM for 16−18 h with or without 30 ng/mL PTX prior to treatment with mastoparan. At the end of the incubation period, the medium was collected from the wells and centrifuged briefly to sediment any detached cells. The supernatants were stored at −20 °C until radioimmunoassay. After any leftover KRB buffer was aspirated from the wells, 1 mL of a solution containing 77% ethanol and 1% HCl was added to each well to extract the insulin for determination of the insulin content of the cells. The plates were kept overnight at −20 °C until the assay. The data are expressed as the mean ± SE. Statistical significance was evaluated by Student's *t*-test.

*SDS−PAGE and Immunoblot Analysis*. Samples were resuspended in Laemmli sample buffer [65 mM Tris, 3% SDS, 10% glycerol, bromophenol blue (25 μg/mL), and 5% 2-mercaptoethanol] and boiled for 5 min. The samples were then separated by SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis) and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. The transfer solution contained 20 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. The membrane was then blocked in a buffer (TBST) containing 13.24 mM Tris-HCl, 1.94 mM Tris base, 32.72 mM NaCl, 1.79 mM EDTA, 0.05% Tween 20, and 5% nonfat dried milk for 1 h at 4 °C. Primary antibody incubations were carried out for 1 h at 4 °C. After being washed with TBST buffer at least three times for 5 min each, the membrane was incubated with horseradish peroxidase-conjugated donkey antibodies to mouse IgG (dilution 1:5000) for 1 h at 4 °C. The membrane was washed in TBST buffer, and the immune complexes were visualized by enhanced chemiluminescence (ECL kit).

*Immunoprecipitation Studies*. The reactions were carried out by first incubating the antibody with protein G−agarose beads for 2 h at 4 °C with rocking, followed by three washes with the lysis buffer. The lysate samples prepared from βHC-9 cells were incubated with the antibody-bound protein G−agarose beads for 2 h at 4 °C with rocking. After four washes with the lysis buffer, the beads were resuspended in Laemmli sample buffer and the supernatants subjected to SDS−PAGE.

*Purification of Cdc42 and Syntaxin 1A Constructs*. GST−Cdc42, GST−(T17N)Cdc42, GST−(Q61L)Cdc42 (*38*), and GST−syntaxin 1A (*21*) fusion proteins were expressed in *Escherichia coli* and purified by glutathione−agarose affinity chromatography. A small overnight culture was used to inoculate 1 L of LB (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 mL of 1 N NaOH) media and grown at 37 °C until an $OD_{560}$ of 0.6−0.8 was reached. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactosidase (IPTG). The bacteria were pelleted by centrifugation and the pellets resuspended in a lysis buffer containing 25 mM Hepes, pH 7.4, 1% NP40, 1 mM DTT, 15 mM $MgCl_2$, 150 mM NaCl, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.2 mg/mL phenylmethylsulfonic chloride. The cells were sonicated, and the resulting lysate was cleared of cell debris by centrifugation for 30 min at 18000*g*. The GST fusion proteins were incubated with glutathione−agarose beads for 2 h at 4 °C with rocking. The beads were washed four times with lysis buffer before the proteins were resolved using SDS−PAGE. The gel was stained with 1% Coomassie brilliant blue to estimate the amount of expressed fusion protein.

*Purification of Syntaxin 1A*. Six-His-tagged syntaxin 1A fusion proteins were expressed in *E. coli* and purified by $Ni^{2+}$−agarose affinity chromatography (Qiagen). The cells were lysed in 40 mM Tris-HCl, 250 mM NaCl, and 5 mM imidazole, pH 7.9. The lysed cells were incubated with $Ni^{2+}$−agarose beads for 2 h at 4 °C with rocking. The bound proteins were washed in buffer supplemented with 40 mM imidazole and eluted using 250 mM imidizole. The sample was then dialyzed in lysis buffer containing 20% glycerol and stored at −20 °C.

*GTP/GDP Exchange Assays*. GTP/GDP exchange assays were performed at room temperature as previously described (*48*). The loading of [$^3$H]GDP to 2 μM GST−Cdc42 was carried out in a buffer containing 10 mM Hepes, 100 mM NaCl, 7.5 mM EDTA, pH 7.5, and [$^3$H]GDP for 30 min at room temperature followed by incubation with 20 mM $MgCl_2$ for 15 min. GTP/GDP exchange was determined in the presence of 20 mM EDTA or 30 μM mastoparan for the indicated times in a buffer containing 10 mM Hepes, 5 mM $MgCl_2$, 0.1 M NaCl, pH 7.5, 500 μM DTT, and 100 μM GTPγS. The reactions were stopped at the indicated times by dilution (20 μL aliquots) in ice-cold buffer (20 mM Tris base, 100 mM NaCl, and 20 mM $MgCl_2$, pH 7.5), and the radioactivity of protein-bound nucleotide bound to nitrocellulose filters was determined.

## RESULTS

*Identification of Cdc42-Expressing βHC-9 Cells*. To characterize the role of Cdc42 in the insulin secretion pathway, we used stable transfection of epitope-tagged wild-type Cdc42 in βHC-9 cells. Single colonies of G418-selected cells were grown to confluency and tested for expression of Cdc42. Antibodies against the hemagglutinin (HA) tag were used to distinguish between overexpressed and endogeneous Cdc42. A clone that showed an increase in Cdc42 expression was then used as our system of study (Figure 1, lane 5).

*Insulin Release from Cdc42-Expressing βHC-9 Cells.* The Cdc42-overexpressing βHC-9 stable line was first tested for its response to different secretagogues using vector-expressing cells as the control. Once it was determined that the control vector-transfected cells responded to stimuli in a manner similar to that observed with normal nontransfected βHC-9 cells (data not shown), we compared the secretion profile of cells overexpressing Cdc42 to that observed with the vector-expressing cells. As shown in Figure 2, insulin secretion was enhanced almost 2-fold by mastoparan treatment of cells overexpressing Cdc42 compared to similarly treated vector-transfected cells. This effect was not seen with secretagogues such as glucose that stimulate secretion via $Ca^{2+}$-dependent pathways. These results indicate that Cdc42 plays a regulatory role in the mastoparan-stimulated release

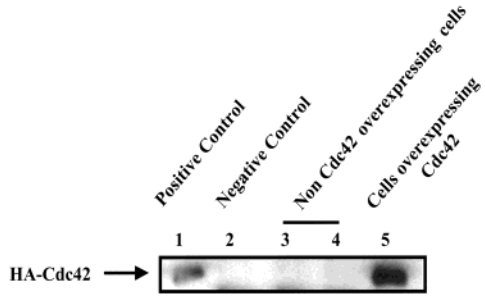


FIGURE 1: Stable transfection of vector or Cdc42 constructs. Cell lysates were prepared from $\beta$HC-9 cells that were stably transfected with wild-type Cdc42 with a hemagglutinin triple tag. Western blot analysis was carried out using the antibody against the HA tag. Lysate from Cos-7 cells overexpressing Cdc42 was used as a positive control (lane 1), lysate from nontransfected $\beta$HC-9 cells was used as the negative control (lane 2) and lysates representing three of the single colonies that were transfected with wild-type Cdc42 were used (lanes 3−5). Cells overexpressing Cdc42 (lane 5) were then used in subsequent experiments. Results are representative of four independent experiments with a similar pattern of expression.

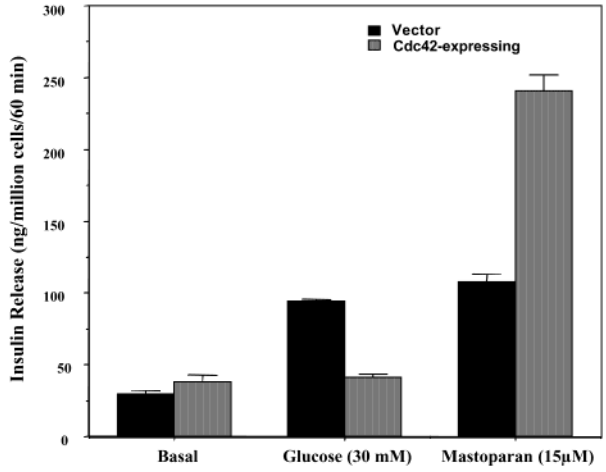


FIGURE 2: Insulin release from $\beta$HC-9 cells expressing Cdc42. Cells grown in 16 mm diameter wells were treated with 15 $\mu$M mastoparan in KRB buffer for 60 min, with KRB buffer alone serving as the control. The samples were collected and insulin release was measured by RIA. The graph denotes absolute secretion rates expressed as nanograms per million cells per 60 min measured under the mentioned conditions. Values are the mean ± SE for each condition. The number of wells ($n$) used for each condition was 8. Statistical significance is $P < 0.05$ vs basal for glucose and mastoparan in vector and Cdc42-expressing cells.

of insulin from $\beta$HC-9 cells. In keeping with previous work (*42*−*46*), it does not preclude a role for Cdc42 or Rho proteins in nutrient-stimulated secretion through other more proximal pathways.

*Immunoprecipitation Studies*. As a first step toward understanding the mechanism underlying the action of Cdc42 in the mastoparan stimulation pathway, we set out to identify the protein(s) involved in the exocytotic process that might be targeted by Cdc42. As syntaxin is the primary coordinator of exocytosis, different techniques were employed to detect possible interactions with Cdc42. First, co-immunoprecipitation reactions were performed on lysates prepared from $\beta$HC-9 cells overexpressing Cdc42, using an antibody against the epitope tag attached to Cdc42. This was followed by western blotting of the precipitated proteins with anti-syntaxin antibodies. As observed in Figure 3A, the anti-HA antibody can weakly but specifically coprecipitate a 35 kDa protein (lane 4 versus lanes 2 and 3) corresponding to endogeneous syntaxin (lane 1).

*Transient Transfection Studies*. In transient transfection experiments, a myc-tagged pcDNA3 vector containing the cDNA of either wild-type Cdc42 or the dominant active mutant (Q61L)Cdc42 was introduced into $\beta$HC-9 cells. Transfection of pcDNA3 vector alone was used as a control (Figure 3B, lane 1). Forty-eight hours after the transfection reactions, cell lysates were prepared and subjected to immunoprecipitation with the anti-myc antibody. The results in Figure 3B indicate that the activated (Q61L)Cdc42 mutant is more effective than wild-type Cdc42 in co-immunoprecipitating syntaxin (lane 3 versus lane 2).

*Affinity Precipitation Studies*. In vitro binding studies were next used to determine the ability of *E. coli*-expressed GST−Cdc42 fusion proteins to bind to endogeneous syntaxin. Cell lysates prepared from $\beta$HC-9 cells overexpressing Cdc42 were incubated with the following GST−Cdc42 fusion proteins: wild-type Cdc42, the dominant negative mutant (T17N)Cdc42, and the dominant active mutant (Q61L)-Cdc42. GST alone was used as a control. As seen in Figure 3C, syntaxin can be affinity precipitated by the activated form of Cdc42 (lane 4). However, wild-type Cdc42 is not as effective in precipitating syntaxin (lane 2). Some association was also observed with GST alone (lane 1) and the dominant negative mutant of Cdc42 (lane 3). When tested under more stringent conditions using 300 mM NaCl, nonspecific binding was reduced (lanes 5 and 7), but the association between syntaxin and both wild-type Cdc42 and the (Q61L)Cdc42 mutant persisted (lanes 6 and 8).

*Effect of Mastoparan on the Association between Syntaxin and Cdc42*. On the basis of these data, obtained using three different techniques, it was concluded that a complex was shown to exist between syntaxin and Cdc42. Colocalization of syntaxin and Cdc42 was also observed through immunofluorescence studies (data not shown). Although syntaxin associates with wild-type Cdc42, it interacts more strongly with either the dominant active mutant of Cdc42 or the GTP-bound form of Cdc42, compared to the GDP-bound form of the protein. This would imply that syntaxin is acting downstream of activated Cdc42. Upon activation, Cdc42 can regulate various pathways through an interaction with different effector molecules. Hence, the effect on the interaction between Cdc42 and syntaxin by mastoparan stimulation of insulin secretion was then examined. In vitro binding studies were carried out in lysates prepared from $\beta$HC-9 cells overexpressing Cdc42 that had been treated with mastoparan for 60 min (Figure 4). These lysates were incubated with the three different GST−Cdc42 fusion proteins and precipitated with glutathione−agarose beads. The upper bands represent the nonspecific binding of GST fusion proteins. As seen in Figure 4 (lane 3), mastoparan promoted the association between syntaxin and wild-type Cdc42 in a manner similar to that observed with syntaxin and the activated mutant under basal conditions (lane 6). Mastoparan, however, did not cause syntaxin to associate with the dominant negative mutant (T17N)Cdc42 (lane 5). It was also observed that the association of syntaxin was specific to Cdc42, as indicated by its failure to bind other Rho family members, RhoA and Rac1 (data not shown). Thus, the effect of Cdc42 on mastoparan-stimulated insulin

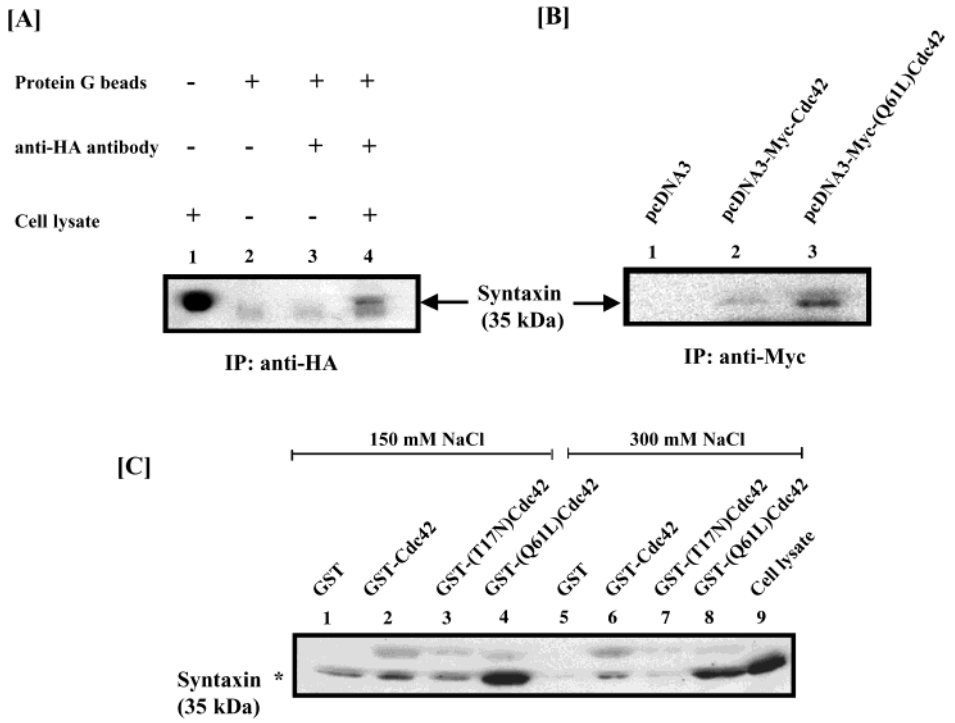


FIGURE 3: Syntaxin associates with Cdc42. In [A], co-immunoprecipitation studies were carried out with the antibody against the HA tag in lysates prepared from $\beta$HC-9 cells overexpressing Cdc42 (lane 4). The protein G−agarose beads or HA antibody-bound beads were used as controls (lanes 2 and 3, respectively). Cell lysate was used as a size marker for syntaxin (lane 1). The data are representative of three independent experiments with similar results ($P < 0.2$ for the co-immunoprecipitation reactions with cell lysates compared to the control). In [B], transient transfection of $\beta$HC-9 cells was carried out using a myc-tagged pcDNA3 vector containing either the wild-type Cdc42 cDNA (lane 2) or the dominant active Cdc42 cDNA (lane 3). Vector alone was used in lane 1. Cell lysates were prepared 48 h after the transfection. The co-immunoprecipitation reactions were carried out with an anti-myc antibody, and the blots were probed for syntaxin. Results are representative of three independent experiments [$P < 0.05$ for wild-type Cdc42 and $P < 0.06$ for (Q61L)Cdc42 compared to the control]. In [C], cell lysates prepared from $\beta$HC-9 cells were incubated with different GST−Cdc42 fusion proteins and then subjected to western blot analysis and probed for syntaxin. Wild-type Cdc42 (lanes 2 and 6), the dominant negative mutant (T17N)Cdc42 (lanes 3 and 7), and the dominant active mutant (Q61L)Cdc42 (lanes 4 and 8) were used in the experiment with GST alone as the control (lanes 1 and 5). In lanes 1−4, the washes were carried out in lysis buffer supplemented with 150 mM NaCl; in lanes 5−8, samples were washed with buffer containing 300 mM NaCl. Data are repesentative of three independent experiments with similar results.

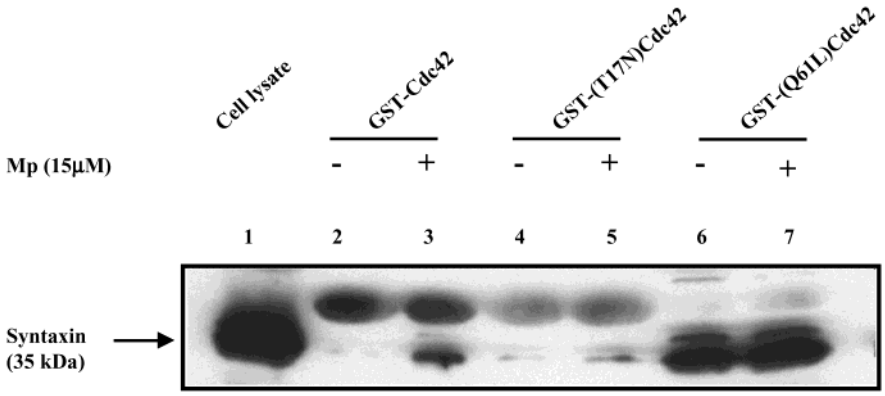


FIGURE 4: Mastoparan promotes the association between wild-type Cdc42 and syntaxin. Cell lysates were prepared from control and 15 $\mu$M mastoparan-treated $\beta$HC-9 cells and incubated with different GST−Cdc42 fusion proteins followed by western blot analysis using the anti-syntaxin antibody. Wild-type Cdc42 (lanes 2 and 3), the dominant negative mutant (T17N)Cdc42 (lanes 4 and 5), and the dominant active mutant (Q61L)Cdc42 (lanes 6 and 7) were used in the binding assay. The upper bands represent the nonspecific binding of GST fusion proteins. Data are repesentative of four independent experiments with similar results.

secretion is not the result of a sequential activation of signaling pathways by the Rho proteins. The binding assays were repeated using mastoparan-17, an inactive analogue of mastoparan (*14*). Mastoparan-17 does not stimulate insulin secretion (Figure 5A), and it did not promote the binding of syntaxin and Cdc42 (Figure 5B, lanes 3 and 9). Therefore, only the active form of mastoparan facilitates the association of syntaxin with Cdc42.

*Syntaxin Requires Cytosolic Protein(s) To Mediate Its Association with Cdc42.* The next series of experiments was performed to determine whether the interaction between syntaxin and Cdc42 was direct or whether other factors were necessary for this association. An in vitro system was used to test the binding of purified *E. coli*-expressed His-tagged syntaxin to GST−Cdc42 proteins. Wild-type Cdc42, dominant negative Cdc42, and activated Cdc42, expressed as GST

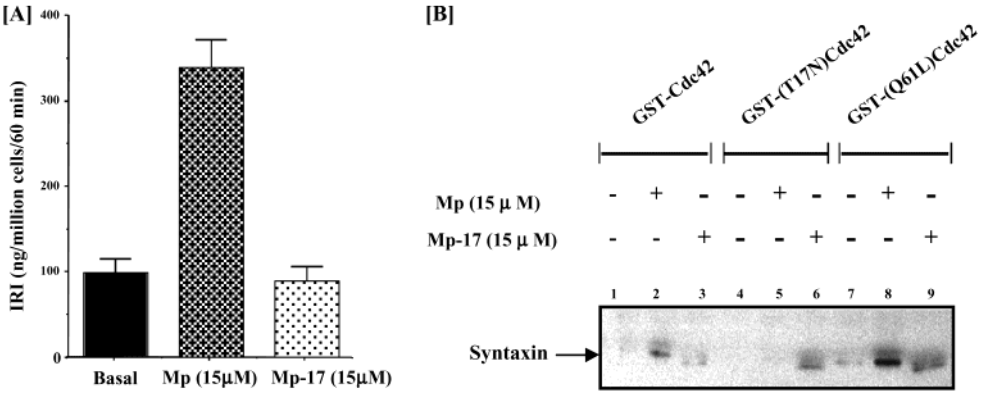


FIGURE 5: Mastoparan-17 does not promote the association between wild-type Cdc42 and syntaxin. In [A], $\beta$HC-9 cells expressing Cdc42 were grown in 16 mm diameter wells and preincubated with KRB buffer containing 0.1 mM glucose for 30 min. The buffer was then replaced with KRB buffer containing 15 $\mu$M mastoparan or mastoparan-17, an inactive analogue of mastoparan, with KRB buffer serving as the control. This treatment was done for 60 min, after which time the samples were collected and insulin release was measured by RIA. The graph denotes absolute secretion rates expressed as nanograms per million cells per 60 min measured under the mentioned conditions. Values are the mean ± SE for each condition. The number of wells ($n$) used for each condition was 8. Insulin release was statistically significant in the cells overexpressing Cdc42 ($P < 0.01$ compared under mastoparan conditions and $P < 0.05$ compared under mastoparan-17 conditions). In [B], cell lysates were prepared from $\beta$HC-9 cells overexpressing Cdc42 that had been treated with 15 $\mu$M mastoparan or 15 $\mu$M mastoparan-17 for 10 min, with KRB buffer alone serving as the control. Equal amounts of the treated lysates were incubated with different GST−Cdc42 fusion proteins followed by western blot analysis with the anti-syntaxin antibody. Wild-type Cdc42 (lanes 1−3), the dominant negative mutant (T17N)Cdc42 (lanes 4−6), and the dominant active mutant (Q61L)Cdc42 (lanes 7−9) were used in the binding assay. Data are repesentative of three separate experiments with similar results.

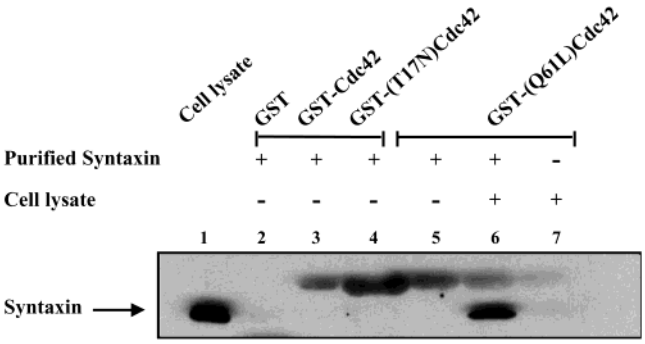


FIGURE 6: Studying the binding of purified syntaxin with GST-bound mutants of Cdc42. Six-His-tagged syntaxin was expressed in *E. coli*, and protein expression was induced with IPTG. The cells were lysed in 40 mM Tris-HCl, 250 mM NaCl, and 5 mM imidazole, pH 7.9. The lysed cells were incubated with $Ni^{2+}$ beads for 2 h at 4 °C with rocking. The bound proteins were washed with 40 mM imidazole containing lysis buffer and eluted using 250 mM imidizole. The elutant was dialyzed overnight in lysis buffer containing 20% glycerol. Purified syntaxin was incubated with GST and GST−Cdc42 fusion proteins (lanes 3−6). In lanes 6 and 7, 10 $\mu$g of $\beta$HC-9 cell lysate was added to the incubation reactions. The upper bands represent the nonspecific binding of GST fusion proteins. Data are repesentative of three independent experiments with similar results.

fusion proteins and bound to glutathione−agarose beads, were each incubated with purified syntaxin protein. The precipitated proteins were separated by SDS−PAGE and immunoblotted with the anti-syntaxin antibody. As shown in Figure 6, syntaxin was incapable of associating with GST (lane 2), wild-type Cdc42 (lane 3), and the (T17N)Cdc42 mutant (lane 4) as well as with activated Cdc42 (lane 5). However, the addition of 10 $\mu$g of cell lysate prepared from untreated $\beta$HC-9 cells promoted the association of the recombinant syntaxin with activated Cdc42 (lane 6). This finding implies that syntaxin cannot bind directly to Cdc42 and that the association between these two proteins requires an additional detergent-soluble factor(s) that is present in the $\beta$HC-9 cell.

*The H3 Domain of Syntaxin Is Required for Its Association with Cdc42*. Syntaxin participates in various stages of the exocytotic process by associating with multiple binding partners and appears to be the major coordinator of exocytosis (*49*−*56*). Through a series of deletion mutants, the regions on syntaxin required for binding to munc-18, synaptobrevin, SNAP-25, and α-SNAP have been determined (*49*, *57*). Among the many isoforms of syntaxin that are present in the $\beta$-cell, only syntaxin 1A is localized to the plasma membrane and participates in the exocytosis of insulin (*58*). To determine the region of syntaxin required for its association with Cdc42, full-length (syntaxin 1A-1, amino acids 4−288) and a C-terminal deletion mutant of syntaxin (syntaxin 1A-6, amino acids 4−194), which lacks the H3 domain (194−288) required for binding the other SNARE proteins, were tested in in vitro binding studies. Full-length GST-syntaxin, but not the mutant lacking the H3 domain, was able to bind strongly to endogeneous Cdc42, synaptobrevin, and munc-18 (Figure 7, lane 3). The truncated syntaxin mutant, however, still interacted weakly with munc-18 (lane 4), which requires both the H3 domain and N-terminal sequences of syntaxin for association (*49*, *57*). As observed from the crystal structure (2.4 Å) of the SNARE complex, the H3 domain of syntaxin participates in the formation of the tight four-helix bundle along with the N- and C-terminal domains of SNAP-25 and a C-terminal region of synaptobrevin, contributing to the stability of the SNARE complex (*59*, *60*). Our studies indicate that the same region, the H3 domain at the carboxy terminus of syntaxin that spans residues 194−288, is required for the association with Cdc42.

*Determining the Mechanism of Activation of Cdc42 by Mastoparan*. We next determined if the ability of mastoparan to activate Cdc42 and promote insulin release was the result of its action as a guanine nucleotide exchange factor (GEF). GST−Cdc42 was expressed in *E. coli* and purified by glutathione−agarose affinity chromatography. The ability of mastoparan to stimulate the guanine nucleotide exchange activity of Cdc42 was determined by measuring the rate of

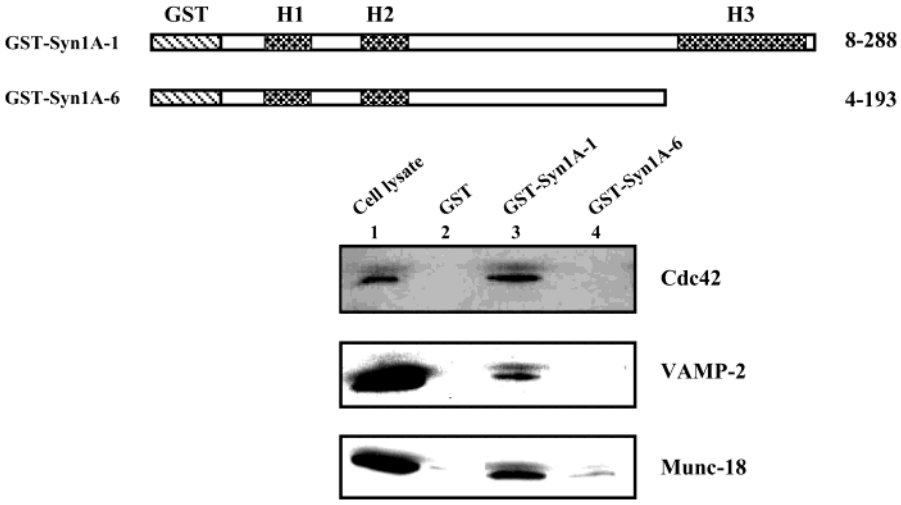


FIGURE 7: Identifying the region on syntaxin required for the association with Cdc42. GST, GST−syntaxin 1A-1, and GST−syntaxin 1A-6, represented in lanes 2, 3, and 4, respectively, were expressed in *E. coli* and purified using glutathione−agarose beads. Cell lysate was used as the positive control (lane 1). The protein-bound beads were incubated with $\beta$HC-9 cell lysates for 2 h at 4 °C with rocking. The samples were loaded on SDS−polyacrylamide gels and immunoblotted for Cdc42, VAMP-2, and munc-18 following transfer onto a PVDF membrane. Data are repesentative of three experiments for Cdc42 and two experiments for VAMP-2 and Munc-18.

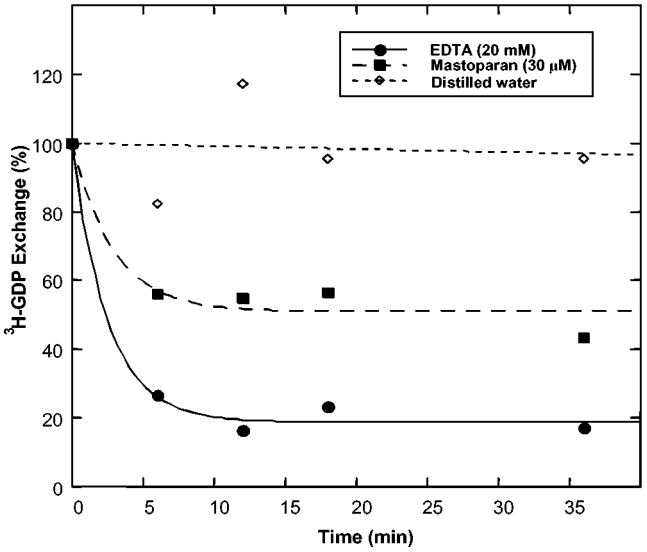


FIGURE 8: Stimulation of guanine nucleotide exchange activity of Cdc42 by mastoparan. Under GTP/GDP exchange assay conditions (with 1 mM free GTP), the time course of [$^3$H]GDP release from 2 $\mu$M GST−Cdc42 stimulated by 20 mM EDTA, 30 $\mu$M mastoparan, or distilled water was determined. Data are representative of six to seven independent experiments with similar results.

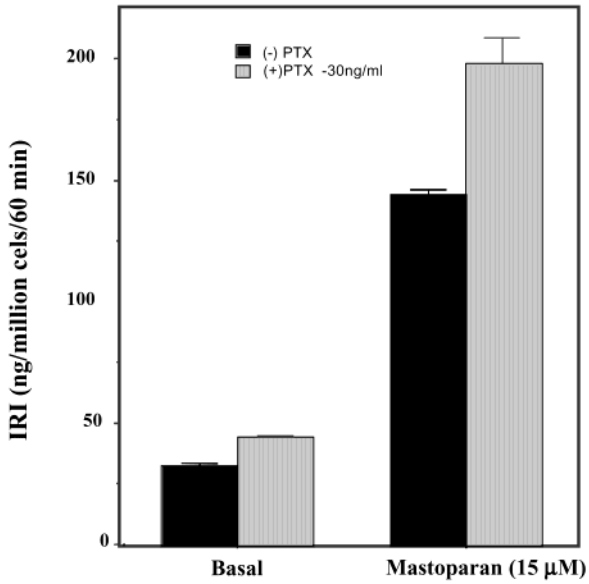


FIGURE 9: Effect of pertussis toxin on mastoparan-stimulated insulin release. $\beta$HC-9 cells expressing Cdc42 were grown in 16 mm diameter wells and pretreated with 30 ng/mL pertussis toxin (PTX) in DMEM for 16−18 h prior to exposure to mastoparan. This treatment was carried out for 60 min following incubation with KRB buffer containing 0.1 mM glucose for 30 min. The samples were collected, and insulin release was measured by RIA. The graph denotes absolute secretion rates expressed as nanograms per million cells per 60 min measured under the mentioned conditions. Values are the mean ± SE for each condition. The number of wells ($n$) used for each condition was 8. Insulin release was statistically significant in the cells expressing Cdc42 ($P < 0.01$ compared under mastoparan conditions and $P < 0.05$ compared under mastoparan + PTX conditions).

dissociation of [$^3$H]GDP in the presence of 30 $\mu$M mastoparan, with 20 mM EDTA and distilled water as the positive and negative controls, respectively (Figure 8). It was observed that mastoparan, like EDTA, caused the dissociation of bound [$^3$H]GDP. This effect was not observed with the inactive peptide, mastoparan-17 (data not shown). Thus, it appears that mastoparan is acting as an exchange factor by promoting GTP/GDP exchange on Cdc42 as suggested previously by Koch et al. with the Rho protein (*16*). On the other hand, mastoparan did not affect GTP hydrolysis (data not shown), implying that it does not activate Cdc42 by inhibiting a GTPase activity. The effect of PTX pretreatment on mastoparan-stimulated insulin release was also examined. As observed in Figure 9, PTX enhances the mastoparan stimulation of insulin release in cells overexpressing Cdc42 as it does for glucose and other stimuli. There was no interference with the stimulation by mastoparan, confirming a previous report from our laboratory (*7*). Therefore, the mode of regulation of Cdc42 by mastoparan appears to involve a direct activation of Cdc42 and does not require a prior activation of PTX-sensitive heterotrimeric G-proteins.

## DISCUSSION

Mastoparan, an amphiphilic tetradecapeptide, inserts itself into the phospholipid bilayer of the plasma membrane and

displays four positive charges near the inner surface of the membrane (*13*−*15*). This structurally mimics the positively charged loops of hormone receptors that associate with the α-subunit of heterotrimeric GTP-binding proteins (*13*). By acting as a structural analogue of the regulatory domain of G-protein-coupled receptors, mastoparan can mimic the action of these receptors and initiate signaling pathways by stimulating the guanine nucleotide exchange activity of heterotrimeric G-proteins, prominent among which are Gi and Go (*13*). Hence, mastoparan can be used to study pathways that are physiologically regulated by the activation of G-protein-coupled receptors. Additionally, there is a single report that mastoparan can activate the low molecular weight Rho protein (*16*). One of the primary consequences of mastoparan action in pancreatic β-cells is the stimulation of exocytosis (*4*−*12*). Unlike other stimulants of insulin release from the β-cell, mastoparan has a unique mode of action in that neither the activation of protein kinases A and C nor the involvement of phospholipase $A_2$ is required (*7*). While mastoparan promotes an increase in intracellular [$Ca^{2+}$], resulting from an inhibition of $K_{ATP}$ channel activity (*61*), the ability of this peptide to stimulate insulin release from the β-cell remains unaltered even under stringent $Ca^{2+}$-free conditions (*7*). Thus, it has been concluded that mastoparan acts at a distal site, beyond the requirement for $[Ca^{2+}]_i$, to stimulate insulin secretion (*6*). While $Ca^{2+}$-independent exocytosis can occur in the β-cell, it is extremely sensitive to GTP depletion (*6*, *41*), and neither the nature of this sensitivity nor the relationship of GTP to the mechanisms of exocytosis is known.

Several studies have attempted to identify the putative GTP-binding protein that may be responsible for mastoparan-stimulated insulin release (*25*, *26*). With the use of *Clostridium difficile* toxins A and B, a role for Rho proteins in nutrient- and calcium-stimulated insulin release has been reported (*42*−*46*). Glucose and GTPγS can promote the carboxyl methylation of Cdc42 in normal human and rat islets and in pure β-cells, indicating an effect in relatively proximal steps of stimulus-secretion coupling (*42*, *43*). Cdc42 and other Rho GTP-binding proteins have been primarily implicated in the reorganization of the actin cytoskeleton (*34*−*39*), with recent evidence of an involvement of Cdc42 both in vesicular transport and in endocytosis (*24*, *31*, *40*). It was observed that a direct association of activated Cdc42 with the γ-COP subunit of the COP1 complex resulted in the stimulation of viral protein VSV-G trafficking activity (*24*). Cdc42 has also been implicated in the sorting of EGF receptors to endosomes through its interaction with ACK2 (for activated Cdc42-associated tyrosine kinases) (*62*). These actions of Cdc42 in intracellular trafficking events, together with the documented effect in β-cells, suggest that Cdc42 could play a role in GTP-stimulated insulin exocytosis.

The techniques of molecular biology and biochemistry were applied to a physiological system, the mouse-derived βHC-9 cells (*47*), to study the role of Cdc42 in mastoparan-stimulated insulin release. A specific although indirect association between the plasma membrane protein syntaxin and Cdc42 was observed, and mastoparan promoted the association between syntaxin and the GDP-bound form of Cdc42.

To understand the nature of the activation by mastoparan that causes wild-type Cdc42 to associate with syntaxin, the ability of the peptide to promote GDP/GTP exchange on purified Cdc42 was measured. Our data indicate that mastoparan stimulates the exchange of labeled GDP for cold GTP and appears to corroborate previous data by Koch et al. (*16*) with purified Rho protein. While mastoparan-stimulated insulin release in Wistar rat islets (*5*, *44*), as well as in clonal beta cells such as βTC-3 (*26*), seems to involve a pertussis toxin- (PTX-) sensitive mechanism, the effect of PTX on insulin secretion is selective and was not observed in RINm5F cells (*7*, *8*). In the present studies, PTX did not inhibit the mastoparan-stimulated insulin release from cells overexpressing Cdc42. However, this does not eliminate the possible involvement of non-PTX-sensitive trimeric G-proteins in the transmission of signals to downstream effector molecules via an indirect activation of Cdc42 (*63*−*65*). It was reported that mastoparan stimulation of the c-Jun N-terminal kinase (JNK) activity was promoted by constitutively activated mutants of Gαi1, Gαi2, and Gαi3 and inhibited by dominant negative mutants of Rho and Cdc42, implying that both heterotrimeric and monomeric G-proteins participate in this pathway (*63*). Other studies have demonstrated that βγ subunits of heterotrimeric G-proteins, in particular, can associate with proteins that contain a pleckstrin homology (PH) domain (*64*, *65*). Guanine nucleotide exchange factors (GEFs) such as Dbl (*32*), which are regulators of Rho proteins, have conserved DH (dbl homology) and PH domains (*32*), both of which are necessary for the activation of Rho proteins. Thus, it is possible that either the α or βγ subunits bind and activate GEFs specific for Cdc42, which in turn leads to Cdc42 activation.

While mastoparan promotes the association of wild-type Cdc42 with syntaxin, this effect is not seen with mastoparan-17, which does not activate G-proteins nor stimulate insulin secretion. These results could suggest that mastoparan treatment results in the removal of an inhibitory effect from endogeneous syntaxin to allow it to associate better with intermediary proteins that interact with wild-type Cdc42. Alternatively, mastoparan could be promoting an activation of proteins that facilitate the association of syntaxin with Cdc42. We addressed this possibility by examining the binding of endogeneous Cdc42 to GST−syntaxin. Under these conditions, both basal and mastoparan-treated lysates reveal an equal affinity of GST−syntaxin for Cdc42 (data not shown). Thus, it appears that purified syntaxin does not bind directly to Cdc42 but instead requires the activation of a cellular protein to promote this interaction. To determine the identity of this (these) protein(s), the site of action of the mastoparan effect would have to be determined. If the Cdc42−syntaxin interaction is participating in vesicular trafficking, then a possible candidate mediating this association could be γ-COP, a subunit of the coatomer complex (*24*). Interestingly, we have been able to affinity-purify endogeneous γ-COP with GST−syntaxin (data not shown). Further studies would determine the functional significance of this association in response to mastoparan.

In summary, the effect of mastoparan to stimulate insulin secretion via Cdc42 is specific to the β-cell and indicates a novel role for this Rho family GTP-binding protein. We have also determined that the activation of Cdc42 by mastoparan requires an indirect association with the SNARE protein syntaxin.

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