Syntaxin 4 and Synip (Syntaxin 4 Interacting Protein) Regulate Insulin Secretion in the Pancreatic β HC-9 Cell*

Received for publication, May 15, 2003, and in revised form, June 25, 2003
Published, JBC Papers in Press, July 9, 2003, DOI 10.1074/jbc.M305114200

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Although syntaxin 1 is generally thought to function as the primary target-N-ethylmaleimide-sensitive factor attachment protein receptor required for pancreatic β cell insulin secretion, we have observed that overexpression of a dominant-interfering syntaxin 4 mutant (syntaxin 4/ΔTM) attenuated glucose-stimulated insulin secretion in βHC-9 cells. Furthermore, these cells express the selective syntaxin 4-binding protein Synip (syntaxin 4 interacting protein), and Synip was specifically co-immunoprecipitated with syntaxin 4 but not syntaxin 1. Overexpression of the full-length Synip protein (Synip/wild type) inhibited VAMP2 association with syntaxin 4 and decreased glucose-stimulated insulin secretion. This did not occur with a Synip mutant (Synip/ΔEF) that was incapable of binding syntaxin 4. Consistent with a functional role of syntaxin 4 in this process, expression of syntaxin 4/ΔTM also inhibited glucose-stimulated insulin secretion. Furthermore, analysis of first and second phase insulin secretion demonstrated that syntaxin 4/ΔTM mainly suppressed the second phase of insulin secretion. In contrast, overexpression of Synip resulted in an inhibition of both the first and second phase of glucose-stimulated insulin secretion. These data demonstrate that syntaxin 4 plays a functional role on insulin release and granule fusion in β cells and that this process is regulated by the syntaxin 4-specific binding protein Synip.

It is well established that glucose-stimulated insulin secretion occurs through a complex metabolic network in which the increased flux of glucose in pancreatic β cells elevates the intracellular ATP/ADP ratio (1–5). The increased ATP/ADP ratio results in the closure of the ATP-sensitive potassium channel (K<sub>ATP</sub> channels) and subsequent cellular depolarization (6, 7). In turn, depolarization causes the activation of the L-type voltage-dependent calcium channels that result in the influx of extracellular calcium. This increased intracellular calcium then serves as a trigger for the initial fusion of insulin-containing dense core granules with the plasma membrane (8, 9).

Although the calcium trigger mechanism, trafficking, and fusion of insulin granules have not been fully elucidated, recent studies have suggested that the majority of granules that initially fuse with the plasma membrane are localized beneath the plasma membrane in a bound ( docked) state (10–12). This pool of insulin granules has been referred as the readily releasable pool, and it appears to account for the first phase of stimulus-mediated insulin secretion (13). This initial release process requires the functional interactions of the t-SNARE<sup>1</sup> proteins, syntaxin 1 and SNAP25, with the v-SNARE protein, VAMP2. For example, the use of SNARE mutants or various SNARE protein-specific proteolytic toxins prevent regulated insulin secretion (14–17). However, since these endotoxins do not proteolyze complexed SNARE proteins, it is thought that the plasma membrane-bound ( docked) insulin granules first undergo a priming step ( core SNARE complex formation) followed by SNARE-mediated fusion.

During our investigation of SNARE protein complexes, we observed that the regulated insulin-secreting pancreatic β cell line, βHC-9, not only expressed syntaxin 4 but also expressed the specific syntaxin 4-interacting protein, Synip. In this study, we demonstrate that syntaxin 4 also plays a role in glucose-stimulated insulin secretion and that this process may be negatively regulated by the syntaxin 4-interacting protein Synip.

EXPERIMENTAL PROCEDURES

Materials—The FLAG M2 monoclonal antibody was obtained from Sigma, and syntaxin 4 sheep polyclonal and Synip rabbit polyclonal antibodies were prepared and affinity-purified as described previously (18, 19). ECL plus Western blotting detection system and [α<sup>32</sup>P]dCTP were purchased from Amersham Biosciences. The anti-sheep and anti-rabbit IgG-horseradish peroxidase were obtained from Pierce. The VAMP2 antibody was purchased from Calbiochem, and all restriction enzymes, cell culture media, and reagents were from Invitrogen.

Cell Culture—βHC-9 cells were kindly provided by Dr. Douglas Hanahan (University of California at San Francisco) and were maintained in Dulbecco’s modified Eagle’s medium containing high glucose, 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 20 mM glutamine, under a humidified condition of 95% air and 5% CO<sub>2</sub> at 37 °C.

Ectoproduction of βHC-9 Cells—To obtain a high degree of transfection efficiency necessary for insulin secretion assay, immunoprecipitation, and Western blotting of whole cell extracts, βHC-9 cells were electroporated with a total of 300 µg of plasmid DNA at 950 microfarads and 0.2 kV as described previously (20). Within this condition, ~70% of the cells were functionally transfected as determined by in situ staining for the expression of β-galactosidase activity. Forty-eight h following transfection, the cells were washed twice in Krebs-Ringer bicarbonate buffer composed of 129 mM NaCl, 5 mM NaHCO<sub>3</sub>, 4.8 mM KCl, 1.2 mM

<sup>1</sup> The abbreviations used are: SNARE, N-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, target-SNARE; v-SNARE, vesicle-associated-SNARE; VAMP2, vesicle-associated membrane protein 2; RT, reverse transcriptase; SNAP25, synaptosome-associated proteins of 25 kDa; WT, wild type; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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probe was hybridized to a Northern blot containing 2 was purified with a QIAquick nucleotide removal kit (Qiagen). The period prior to the test periods with 30 mM glucose. Samples were secretion.

we designed 5 and the lower primer de- designing an upstream primer containing an Eco RI site for just up-

TCATGAGTGATGGTACAGCTTCTGCC-3, and the lower primer de-

GAAT-5TCGGGTCTGTTAGCTC-3 — Synip and VAMP2 antibodies (Pierce). Specific signals were visualized, scanned, and ana-

lyzed by Molecular Imager FX (Bio-Rad).

The extracted and purified RT-PCR products were applied on a PRISM agarose gel, and a specific single band was extracted from agarose gel. The extracted and purified RT-PCR products were applied on a PRISM model 310 autosampler (PE Applied Biosystems, Tokyo, Japan) for analysis of sequence.

Western Blots—Samples were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with monoclonal or polyclonal specific antibody as indicated in the figures and figure legends. The primary monoclonal and polyclonal antibodies (except for PY20-horseradish peroxidase) for immunoblots were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Pierce). Specific signals were visualized, scanned, and analyzed by Molecular Imager FX (Bio-Rad).

**Synip**DEFS—Deletion of the EF-hand motif of Synip was prepared by designing an upstream primer containing an EcoRI site for just upstream of ATG. In this case, upstream primer design was 5′-GGAAT TCATGAGTGATGGTACAGCTTCTGCC-3′, and the lower primer design was 5′-CTTAAGCGCAAGAAGGATTTAG-3′. To amplify the Synip region, the EcoRI site was chosen immediately upstream of the stop codon. Using 5′-GGAGATTTCCGAGTCCGATG-3′ oligonucleotide DNA as the upper primer and 5′-TCCTAGATAGGAGTCCGATG-3′ oligonucleotide DNA as the lower primer, we inserted the FLAG epitope tag before the stop codon and the XhoI site after the stop codon. Thereafter, pcDNA3 vector was digested with EcoRI and XhoI, and triple ligation was attempted with amplified two of Synip PCR products.

**Insulin Release under Static Incubation Conditions**—Insulin release was determined as described previously with minor modifications (8). Following electroration and stimulation, the cell medium was collected for analysis by an insulin radioimmunoassay. A total amount of cell protein was determined by extraction of cells with a Nonidet P-40 lysis buffer and used to normalize for the amount of insulin release (18). All data are expressed as mean ± S.D. Data were evaluated for statistical significance by analysis of variance and paired Student’s t test when appropriate.

**Insulin Secretion under Perfusion Conditions**—The perfusion system was performed as described elsewhere with modifications (13). In brief, an equal number of electroporated βHC-9 cells were grown on glass coverslips in each experiment; coverslips were then placed in 25-mm Swinnex chambers (Millipore, Bedford, MA) with the cells fac-

ing the inside of the chamber. The cells were perfused with Krebs-Ringer buffer and 0.1 mM glucose at 37 °C for a 60-min equilibration period prior to the test periods with 30 mM glucose. Samples were collected at 1- and 2-min intervals for the measurement of insulin secretion.

**Statistical Analysis**—All data expressed as means ± S.E. Data were evaluated for statistical significance by analysis of variance or t test.

RESULTS

**Synip Expression in βHC-9 Cells**—Previously, we reported that Synip is expressed in muscle and adipose tissue (18). In Northern blot analysis of RNA from differentiated 3T3L1 adipocytes and βHC-9 cells, the RNA was separated by agarose gel electrophoresis and subjected to Northern blot analysis using a Synip cDNA probe as described under “Experimental Procedures.” As shown in B, whole cell extracts (lysates) were prepared from 3T3L1 adipocytes and βHC-9 cells as described under “Experimental Procedures.” The proteins were separated by SDS-PAGE and subjected to immunoblottings (IB) with a Synip, syntaxin 4, and VAMP2 antibodies. These are representative experiments independently performed three times.

**Fig. 1. Synip mRNA and protein are expressed in the βHC-9 pancreatic beta cell line.** As shown in A, total RNA was extracted from 3T3L1 adipocytes and βHC-9 cells. The RNA was separated by agarose gel electrophoresis and subjected to Northern blot analysis using a Synip cDNA probe as described under “Experimental Procedures.” As shown in B, whole cell extracts (lysates) were prepared from 3T3L1 adipocytes and βHC-9 cells as described under “Experimental Procedures.” The proteins were separated by SDS-PAGE and subjected to immunoblottings (IB) with a Synip, syntaxin 4, and VAMP2 antibodies. These are representative experiments independently performed three times.
As shown in Fig. 2A (panels 2 and 3), 48 h following electroporation with the LacZ cDNA, ~80% of the cells stained blue for the presence of β-galactosidase activity. Importantly, the electroporated cells are not morphologically different from the non-electroporated cells (Fig. 2A, panel 1). The electroporation of the βHC-9 per se also had no detrimental effect on the ability of these cells to display regulated insulin secretion (see Figs. 5 and 6). Furthermore, expression of a FLAG epitope-tagged Synip demonstrated the co-localization of Synip with endogenous syntaxin 4 by immunofluorescent microscopy (Fig. 2B, panels 1–3).

**Glucose Stimulation Regulates the Binding Interactions among Syntaxin 4, VAMP2, and Synip**—To assess the interaction of syntaxin 4 with Synip and Synip, the βHC-9 cells were either left unstimulated or incubated with 30 mM glucose for 10 and 30 min (Fig. 3A). In the basal state (zero time), immunoprecipitation of syntaxin 4 resulted in the co-immunoprecipitation of Synip and VAMP2 (Fig. 3A, lane 1). Glucose stimulation for 10 min resulted in an increase in the amounts of Synip and VAMP2 co-immunoprecipitated with syntaxin 4 (Fig. 3A, lane 2). However, the amounts of Synip and VAMP2 co-immunoprecipitated with syntaxin 4 were reduced following 30 min of glucose stimulation (Fig. 3A, lane 3). The statistical evaluations of Synip and VAMP2 band intensity changes are represented in Fig. 3B. This change associated with syntaxin 4 occurred without any significant effect to the total amount of Synip, syntaxin 4, or VAMP2 protein content in the βHC-9 cells (Fig. 3A, lanes 4–6).

In contrast to glucose-stimulated biphasic interaction of VAMP2 with syntaxin 4 in control cells, overexpression of Synip completely prevented any detectable co-immunoprecipitation of VAMP2 with syntaxin 4 (Fig. 4A, lanes 1–3). Furthermore, due to the higher level of Synip expression, glucose was no longer able to induce either an association or a dissociation of Synip with syntaxin 4, suggesting that the majority of functionally available syntaxin 4 is now bound to Synip. Again, glucose stimulation had no significant effect on the amount of expressed Synip or the endogenous syntaxin 4 and VAMP2 proteins (Fig. 4A, lanes 4–6). As a control, we also expressed a deletion mutant of Synip (SynipΔEF) that was unable to significantly bind to syntaxin 4 (Fig. 4B, lane 1). Expression of this mutant had no significant effect on the basal state association of syntaxin 4 with VAMP2 and did not prevent the glucose-stimulated association and subsequent dissociation of VAMP2 from syntaxin 4 (Fig. 4B, lanes 2 and 3). The control total cell extract blots for the constitutive expression of SynipΔEF, syntaxin 4, and VAMP2 are shown in Fig. 4B (lanes 4–6).

**Synip-Syntaxin 4 Interactions Regulate Glucose-stimulated Insulin Secretion**—It is well established that the syntaxin 1-SNAP25 complex functions as the primary t-SNARE for the first phase of glucose-stimulated insulin secretion (13, 21). However, the potential contribution of syntaxin 4 in this process has not been carefully evaluated. Therefore, to examine the potential physiological role of syntaxin 4 and Synip on insulin secretion, we initially expressed the syntaxin 4ΔTM. As typi-
cally observed in β cell lines, glucose stimulation for 60 min resulted in an approximate 2.0-fold increase of insulin release (Fig. 5, bars 1 and 2). Similarly, glucose stimulation resulted in a 2.2-fold increase of insulin release from empty vector transfected cells (Fig. 5, bars 3 and 4). Although expression of syntaxin 4/ΔTM had no effect on basal insulin release, there was a statistically significant reduction in glucose-stimulated insulin secretion (Fig. 5, bars 5 and 6). Furthermore, expression of Synip/WT inhibited glucose-stimulated insulin secretion, whereas expression of Synip/ΔEF had no effect (Fig. 5, bars 7–10). As a control for syntaxin 4 and Synip specificity, we also expressed Synip/WT and examined its binding to syntaxin 1. As reported previously (18), the immunoprecipitation of Synip/WT resulted in the co-precipitation of syntaxin 4 but not syntaxin 1 (data not shown). Together, these data suggest that syntaxin 4 and Synip may both play functional roles in glucose-stimulated insulin secretion.

**Synip and Syntaxin 4 Have Different Effects on First and Second Phase Insulin Secretion**—Although Synip displays high specificity for syntaxin 4, syntaxin 4 is more promiscuous, and in particular, syntaxin 4/ΔTM can also directly interact with VAMP2 (19). Thus, the mode of action of Synip and syntaxin 4/ΔTM to inhibit glucose-stimulated insulin secretion may result from different mechanisms. To address this issue, we next examined the effect of Synip and syntaxin 4/ΔTM on first and second phase insulin secretion (Fig. 6A). Empty vector transfected cells displayed a rapid first phase of glucose-stimulated insulin secretion that peaked at 3 min and declined over the next 4–5 min. At this time, the second phase was readily apparent with a relative constant rate of insulin secretion over...
the next 30 min. In contrast, in the case of cells expressing syntaxin 4/H9004, there was no significant effect on first phase insulin secretion, but there was a small reduction in second phase secretion (Fig. 6, B and C). However, overexpression of Synip markedly reduced both the first phase as well as the second phase of glucose-stimulated insulin secretion (Fig. 6, A–C). Based upon the known binding interactions, these data suggest that syntaxin 4/HTM inhibits second phase secretion by binding to VAMP2, which is associated with pre-docked insulin granules. In contrast, Synip inhibits first and second phase secretion by preventing the initial docking of insulin granules as well as the recruitment of new insulin granules. Thus, Synip appears to regulate first phase insulin secretion as well as the recruitment of new insulin granules through the modulation of syntaxin 4 function.

DISCUSSION

Insulin secretion is a complex regulatory process that can occur through distinct signaling mechanisms and multiple insulin granule pools (21, 22). In the basal unstimulated state, there is a slow constitutive release of insulin that is thought to result from the trafficking and fusion of insulin from the trans-Golgi network and/or immature secretory granules to the plasma membrane (23). However, the majority of insulin is stored in dense core granules within the cell interior and juxtaposed to the plasma membrane (24). Functionally, glucose stimulation results in a relatively rapid initial release of insulin (first phase) followed by a second slower and more prolonged release of insulin (second phase). It is generally accepted that the first phase of insulin release results from the fusion of plasma membrane pre-docked insulin granules, termed the readily releasable pool of insulin granules.

It is also well established that vesicle fusion first requires the association of granules with the plasma membrane in a process termed tethering (25). The tethering process is apparently SNARE-independent but is then converted to a primed or readily releasable state that results from SNARE complex formation (26). Through an unknown mechanism that is calcium-dependent, fusion readily ensues, probably through the formation of SNAREpin complex (27, 28). In the case of insulin granule fusion, the t-SNARE complex syntaxin 1-SNAP25 directly interacts with the insulin granule v-SNARE protein VAMP2 (29). This conclusion is based upon the expression of several SNARE protein mutants and the use of various endotoxins that are specific proteases for these proteins (14–17). Although there may be several types of fusion-competent insulin granules, these data suggest that the syntaxin 1-SNAP25-VAMP2 complex plays a predominant role in first phase insulin secretion and is probably responsible for the fusion of the readily releasable pool.

Subsequent to the initial single granule fusion event, the
SNARE complex must undergo dissociation to recycle and separate the t-SNARE proteins (syntaxin 1-SNAP25) from the v-SNARE protein (VAMP2). In this regard, syntaxin 1 was found to co-immunoprecipitate with VAMP2 prior to glucose stimulation consistent with this complex being responsible for the high affinity association of the releasable pool of insulin granules with the plasma membrane (13, 21). Moreover, the interaction of syntaxin 1 with VAMP2 was disrupted following glucose stimulation. This latter process appears to be necessary for the recycling of the SNARE proteins to allow for subsequent rounds of insulin granule docking and fusion. The data presented in this manuscript are generally consistent with this model, but our data reveal several important details.

First, syntaxin 4 was reported previously not to be involved in the regulation of insulin secretion based upon the overexpression of the full-length syntaxin 4 protein (14, 30, 31). However, the full-length syntaxin 4 protein does not function as a dominant-interfering mutant in other cell systems, and currently, there are no specific endotoxins for the syntaxin 4 isoform. Since β cells also express syntaxin 4 that can interact with VAMP2, it is not unreasonable to expect that syntaxin 4 may play a role, albeit different from that of syntaxin 1. Indeed, we found that expression of a dominant-interfering syntaxin 4 mutant partially inhibited glucose-stimulated insulin secretion. Second, similar to syntaxin 1, we have observed that the plasma membrane t-SNARE protein syntaxin 4 is also associated with VAMP2 in the basal state. Following glucose stimulation, there was a transient increase in the amount of VAMP2 bound to syntaxin 4 followed by a marked decrease. This dynamic change in the association of VAMP2 with syntaxin 4 occurred independent in any change in either syntaxin 4 or VAMP2 protein levels.

Based upon the time frame of these events, we hypothesize that insulin secretory granules utilize the syntaxin 1-SNAP25 complex for the initial membrane fusion and release of insulin. Subsequently, additional granules utilize VAMP2 to associate with syntaxin 4. This accounts for the initial increase in the amount of VAMP2 that co-immunoprecipitates with syntaxin 4. As fusion ensues, the trans-SNARE complexes become cis-SNARE complexes and undergo dissociation and recycling. This then accounts for the decrease in the interaction between VAMP2 and syntaxin 4 at later times. This time frame is also consistent with the function of syntaxin 4 in the second phase of insulin secretion, whereas the syntaxin 1-VAMP2 complex probably functions in the priming and initiation of the first phase insulin secretion. The observation that overexpression of a dominant-interfering syntaxin 4 mutant (syntaxin 4/ΔTM) inhibited second phase but not first phase insulin secretion is consistent with this model.

We also found that the syntaxin 4-specific binding protein Synip is highly expressed in βHC-9 cells. Previously, we reported that Synip binding to syntaxin 4 competes for VAMP2 binding and is therefore mutually exclusive (18). As expected, overexpression of Synip completely prevented the association of VAMP2 with syntaxin 4 before and after glucose stimulation and inhibited glucose-stimulated insulin secretion. This effect was unrelated to syntaxin 1 function as Synip did not interact with this t-SNARE isoform. Importantly, overexpression of Synip inhibited second phase insulin secretion consistently with a role for syntaxin 4 in this process. Surprisingly, however, although Synip does not interact with syntaxin 1, overexpression of Synip also reduced first phase insulin secretion.

How can Synip inhibit both first and second phase insulin secretion if Synip is a specific syntaxin 4-binding protein? The most likely elucidation is that these are not static complexes but are undergoing successive rounds of docking, fusion, dissociation, and reassociation/docking. Initially, the insulin granules are probably pre-docked mainly through the syntaxin 1-VAMP2 and partially through syntaxin 4-VAMP2 complex.
These complexes may not necessarily be independent as multiple SNARE complexes are required for docking and fusion of a single granule (32). Thus, the functional complex may be composed of both syntaxin 1-VAMP2 and syntaxin 4-VAMP2 SNAREpins. Following fusion of this readily releasable pool, these core complexes undergo dissociation, allowing for the recycling of the VAMP2 protein. Although this cycle is continuous as part of the first phase of insulin secretion, newly recruited insulin granules or an alternative pre-existing secondary pool of insulin granules become associated through the syntaxin 4-VAMP2 complex. This secondary pool then fuses with the plasma membrane with the subsequent dissociation of VAMP2. When Synip is overexpressed, this effectively also prevents the reassociation of VAMP2 with syntaxin 4, thereby blocking subsequent rounds of granule docking and fusion. Since first phase secretion is also inhibited after Synip overexpression, these data suggested that the next round of predocking is also dependent on syntaxin 4. Although this model is consistent with the current data, it also implies that Synip must undergo continuous cycling on and off from syntaxin 4. Current studies are now necessary to determine the dynamic reciprocal relationship between VAMP2 and Synip interaction with syntaxin 4.

In summary, our data demonstrate that in addition to syntaxin 1, syntaxin 4 also plays a role in the glucose stimulation of insulin secretion. In the basal state, syntaxin 4 is associated with the insulin granule v-SNARE protein, VAMP2. VAMP2 undergoes a glucose-induced dissociation from syntaxin 4. Synip seems to regulate syntaxin 4 readily releasable pool size and affect insulin secretion since overexpression of Synip prevents the association of syntaxin 4 with VAMP2 and inhibits glucose-stimulated insulin secretion.

Acknowledgments—We thank Dr. Koushi Hashimoto (Gunma University Graduate School of Medicine, Maebashi, Japan) and Dr. Mitsuhisa Komatsu (Shinshu University School of Medicine, Matsumoto, Japan) for excellent technical advice.

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J. Biol. Chem. 2003, 278:36718-36725.
doi: 10.1074/jbc.M305114200 originally published online July 9, 2003

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