The putative Rab3 effector RIM (Rab3-interacting molecule) was detected by Northern blotting, RT-PCR and Western blotting in native pancreatic beta-cells as well as in the derived cell lines INS-1E and HIT-T15. RIM was localized on the plasma membrane of INS-1E cells and beta-cells. An involvement of RIM in insulin exocytosis was indicated by transfection experiments of INS-1E cells with the Rab3 binding domain of RIM. This domain enhanced glucose-stimulated secretion in intact cells and Ca(2+)-stimulated exocytosis in permeabilized cells. Co-expression of Rab3A reversed the effect of RIM on exocytosis. These results suggest an implication of RIM in the control of insulin secretion.
The Rab3-interacting molecule RIM is expressed in pancreatic β-cells and is implicated in insulin exocytosis

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1. Introduction

In the pancreatic β-cell, insulin is stored in large dense core vesicles (secretory granules) and is released by exocytosis in a Ca²⁺-dependent manner [1]. This process shares many characteristics with neurotransmitter release [2,3]. In this context, monomeric Rab GTPases constitute a large protein family implicated in the vectorial movement of vesicles in the secretory pathway [4,5]. They act as molecular switches that flip between two conformational states, the active GTP-bound form and the inactive GDP-bound form [4-6]. In particular, Rab3 seems to control exocytosis, thus Rab3A deficient mice exhibit enhanced neurotransmitter release [7]. Not only Rab3A, but also Rab3B, -C and -D are expressed in insulin-secreting cells and in part associated with the secretory granules [8,9]. The reversible association with the membrane of secretory vesicles, as well as guanine nucleotide binding and the GTPase activity of Rab3, are under the control of several regulatory proteins and effectors such as rabphilin-3A [10] and Rab3-interacting molecule RIM [11]. Using a yeast two-hybrid selection, RIM was identified from a rat brain cDNA library. RIM is composed of an N-terminal Rab3A binding domain mediating the attachment to plasma membrane proteins [11]. Two C-terminal atypical C2 domains are separated by alternatively spliced sequences [11]. In rabphilin-3A two typical C-terminal C2 domains are present, which bind phospholipid in a Ca²⁺-dependent manner [10]. There is no conclusive evidence that rabphilin-3A is expressed in primary β-cells and furthermore rabphilin-3A knock-out mice display a normal phenotype [13]. However the implication of RIM in regulated exocytosis is suggested by two observations: (i) RIM is restricted to the active zone in the presynaptic membrane, where vesicles dock, and (ii) overexpression of the Rab3A binding domain of RIM in PC12 cells enhances regulated exocytosis in a Rab3-dependent manner [11]. Based on Northern blotting of various tissues, RIM was proposed to be expressed exclusively in brain [11]. Here we demonstrate the expression of RIM and its function in pancreatic β-cells and derived cell lines.

2. Materials and methods

2.1. Materials

The plasmids encoding the full-length RIM (pcMV-RIM) and the GST-RIM fusion protein (pGEX-RIM-52) were generously provided by Dr. T.C. Südhof (Southwestern Medical Center at Dallas, University of Texas). The cDNA coding for human growth hormone was obtained from Dr. J. Lang and Dr. H. Zhang (Department of Clinical Biochemistry, University of Geneva). The mouse monoclonal antibody specific for human c-myc (9E10) was produced from myeloma SP2/O and the supernatant from the hybridoma was used for experiments.

2.2. Pancreatic islets

Pancreatic islets were obtained as described [14]. Islet cells were then separated into two populations, non-β- and β-cells, by fluorescence-activated cell sorter (FACS) as described [14] and the latter were used for RT-PCR experiments. Alternatively, following the Ficoll gradient, islet cells were cultured on poly-ornithine-coated glass coverslips and used for immunofluorescence experiments as reported [14]. Human pancreatic islets were isolated by collagenase digestion and cultured free floating in CMRL-1066 medium with 10% FCS prior to the experiments [15].

2.3. Generation of the soluble N-terminal portion of RIM

The nucleotides coding for the residues 1–399 of RIM were inserted in a myc-tagged expression vector derived from pcDNA3 (Invitrogen, San Diego, CA, USA) by the following procedure. First, nucleotides corresponding to the residues 1–10 were inserted in the myc-tagged pcDNA3, then the fragment coding for residues 11–398 (excised from pGEX-RIM-52) was added, and finally the residue 399 was generated by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene, Basel, Switzerland). The obtained construct was confirmed by DNA sequencing of the plasmid.

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Abbreviations: FACS, fluorescence-activated cell sorter; hGH, human growth hormone; KRB, Krebs-Ringer bicarbonate buffer; PMSF, phenylmethylsulfonylfluoride; RIM, Rab3-interacting molecule; RT-PCR, reverse transcription-polymerase chain reaction
2.4. Northern blotting and RT-PCR

For Northern blotting total RNA was extracted from whole rat brain and insulin-secreting cell lines cultured in 10-cm dishes by the guanidinium thiocyanate/phenol/chloroform method [16]. Poly A+ mRNA was purified from 250 μg of total RNA (Oligotex mRNA, Qiagen AG, Basel, Switzerland) detergent with glyoxal and di-methylsulphoxide and separated on 1% agarose gels. Resolved RNA was blotted to nylon membrane by vacuum transfer (VacuGene XL, Pharmacia, Uppsala, Sweden), followed by UV cross-linking. The membrane was hybridized to 5′-labelled cDNA probe derived from the full-length RIM cDNA (pCMV-RIM), using the random priming technique (Boehringer, Mannheim, Germany).

For RT-PCR total RNA was extracted from rat kidney, rat brain, primary β-cells purified by FACS and cell lines by the use of Trizol (Gibco Life Sciences, Basel, Switzerland). Reverse transcription was done with an input of 1 μg total RNA using AMV reverse transcriptase (Promega, Catalys AG, Wallisellen, Switzerland). The primers used for reverse transcription and amplification of rat kidney, brain, islet β-cells and INS-1E cDNAs were: 5′-GGTACGAGTTCCCTCTCTTTG-3′ (sense strand, position 2031-2050 in reference [16]) and 5′-TTACTATAGCCGGATGCAGGG-3′ (antisense strand, positions 5059-5079). The following primers were used for amplification of HIT-T15 cDNA: 5′-ACGTGCAAGATCTCGTGAAGCA-3′ (sense strand, positions 5059-5079) and 5′-ACGTGCAAGATCTCGTGAAGCA-3′ (antisense strand, positions 2399-2416). The PCR program for cDNA products of rat origin involved an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and elongation at 68°C for 2 min. For HIT-T15 cDNA product, the following PCR program was carried out: initial denaturation cycle at 94°C for 4 min, followed by 40 cycles at 92°C, 58°C and 72°C each for 30 s. A final elongation step of 7 min at 68°C (on rat cDNAs) and 2 min at 72°C (on HIT-T15 cDNA), allowed to extend truncated product to full-length. PCR reactions were performed using TDI Taq polymerase (Promega, Wallisellen, Switzerland). Catalys AG, Wallisellen, Switzerland. The RIM cDNA with 1 μM of each primer. Control experiments demonstrated that amplification products were absent after reactions omitting reverse transcrip'tase.

2.5. Production, purification and characterization of the RIM antibody

Approximately 500 μg of GST-RIM fusion protein encoded by pGEX-RIM-52 was injected into a rabbit (Eurogentech, Seraing, Belgium). The immune response of the rabbit was tested by Western blotting demonstrating that the serum reacted with the GST-RIM protein whereas the pre-immune did not; similarly, immunofluorescence using pre-immune serum showed no specific plasma membrane labelling. Affinity purification of the RIM antibody was performed by incubating 1 ml of serum sample with 250 μg of protein A Sepharose CL-4B (Sigma). After washing with PBS and washed five times in 0.1 M glycine buffer pH 2.5 and desalted using centrifugal filters by preparative Western transfer; these immobilized antibodies were affinity-purified by binding GST and GST-RIM fusion proteins to the membrane as described [17]. For cotransfection experiments, INS-1E cells were seeded on poly-ornithine-coated 24-well plates (2 x 10⁶ cells per well) and transfected 3 days later. To this end, cells were washed with Mg⁺² and Ca⁺²-free PBS. 8 μl of Lipofectamine (Life Technologies, Basel, Switzerland) was complexed for 30 min with 2 μg of pcDNA3-bGH encoding human growth hormone (hGH), and 2 μg of the mammalian expression plasmids (residues 1-399 of RIM and Rab3A Q81L mutant cDNAs, inserted in the N-terminal myc-tagged pcDNA3) or the control vector myc-tagged pcDNA3 in 400 μl RPMI 1640 medium containing 10% FCS, and other additions as described for each cell line [19,20]. For cotransfection experiments, INS-1E cells were seeded on poly-ornithine-coated 24-well plates (2 x 10⁶ cells per well) and transfected 3 days later. To this end, cells were washed with Mg⁺² and Ca⁺²-free PBS. 8 μl of Lipofectamine (Life Technologies, Basel, Switzerland) was complexed for 30 min with 2 μg of pcDNA3-bGH encoding human growth hormone (hGH), and 2 μg of the mammalian expression plasmids (residues 1-399 of RIM and Rab3A Q81L mutant cDNAs, inserted in the N-terminal myc-tagged pcDNA3 or the control vector myc-tagged pcDNA3 in 400 μl RPMI 1640 medium containing 10% FCS, and other additions as described for each cell line [19,20]. The cytosolic localization of the RIM fragment in transfected INS-1E cells was analyzed as described [9].

2.6. Preparation of homogenates, GST-Rab3A Q81L binding assay and Western blotting

Human and rat pancreatic islets, human and rat brain, rat kidney, INS-1E, HIT-T15, BHK, COS and A431 cells (source of BHK, COS and A431 cells: ATCC, Rockville, MD, USA) were washed twice with ice-cold PBS and incubated for 10 min at 4°C in homogenization buffer (HB): 0.5% Triton X-100, 1 mM EDTA, 0.1 M NaCl, 0.1 g 1 M PMSF and 50 mM HEPES-NaOH, pH 7.4, and then disrupted by sonication (3 x 1 s). The homogenates were centrifuged for 30 min at 4°C at 14000 x g and the supernatants were used in the binding experiment for Western blotting.

To perform the binding assay, glutathione Sepharose beads were incubated with the GST-Rab3A Q81L fusion protein for 1 h at 4°C and washed five times in PBS, 5 mM EDTA and 0.5% Triton X-100. The protein-coupled Sepharose beads were then incubated with ~1 mg of rat brain or kidney homogenates (prepared in HB), in the presence of 100 μM GTP·Sα at 4°C overnight. After incubation the samples were washed three times in 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 6 mM MgCl₂ and 1 mM DTT. The proteins remaining associated to the beads were analyzed by Western blotting as previously described [18].

2.7. Immunofluorescence microscopy

To analyze the subcellular distribution of the endogenous RIM, INS-1E and primary β-cells subconfluent monolayers were rinsed in PBS, fixed and permeabilized for 1 min in cold ethanol. The cells were washed in PBS and were then incubated with the affinity-purified RIM antibody overnight at 4°C. After rinsing with PBS, the cells were exposed to a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody. The coverslips were analyzed by confocal microscopy (Leica Lasertechnick, Heidelberg, Germany, model TCS NT). Images were taken with a 40 × objective.

3. Results and discussion

3.1. Expression of RIM in insulin-secreting cells

Fig. 1A illustrates the expression of RIM poly A⁺ RNA in insulin-secreting hamster HIT-T15 cells and in rat insulinoma INS-1E cells. Poly A⁺ RNA from rat brain was used as a positive control. Two principal bands were observed corresponding to approximately 8 kb and 12 kb in all three preparations. These RNA species were not detectable when total RNA was examined. Poly A⁺ RNA extracts of rat kidney, hamster BHK and human A431 epidermoid cells [21] did not reveal positive bands in Northern blotting under the conditions used in Fig. 1A (data not shown). To date, there is no published information on the RIM transcript. The expected size of the coding mRNA for the published RIM sequence of 1553 amino acids [11] is 4659 bp. The observed bands of ~8 kb and ~12 kb suggest that these species contain 3'- or 5'- untranslated regions (UTR). The two bands could represent the two major alternatively spliced variants of the up to 18 proposed forms [11]. It is possible that several splicing variants of similar size comigrate in the two bands, in particular in the broader ~8 kb.

Rat pancreatic islets contain at least 30% other cell types...
and therefore RT-PCR was performed on β-cells purified by FACS (Fig. 1B). RT-PCR was also performed on RNA from INS-1E cells and rat brain. The DNA sequencing of these three amplification products confirmed the expression of RIM. The database contains a partial coding sequence of human brain mRNA. The encoded protein (Database accession number AB002338; reference [22]) is 59% homologous to the published rat brain RIM and is likely to correspond to a human RIM splicing form. Indeed, we could amplify one region from hamster HIT-T15 RNA conserved between rat and human. The amplification of HIT-T15 cDNA using PCR primers designed for regions specific for the human or the rat sequence did not yield positive signals (data not shown).

To investigate the expression of the RIM protein, we used the rabbit polyclonal antibody against RIM. The specificity of the antibody was tested by several methods (see Section 2) including its reactivity with proteins capable of binding Rab3A in the GTP-bound form. Fig. 2A shows that the antibody reacts with a rat brain protein of ~170 kDa complexed to Rab3A-GTP. This protein was absent from rat kidney extract used as control.

Western blotting (Fig. 2B) revealed that rat brain, rat islets, and INS-1E cells express similar quantities of a protein migrating at ~170 kDa. This molecular weight fits well with the published 1553 amino acids of the RIM alternatively spliced variant [11]. The weak expression of the ~170 kDa band in HIT-T15 cells cannot be quantitatively compared before the RIM sequence in hamster becomes available. The nature of the minor bands of lower molecular weight (between 100 and 170 kDa) observed in Fig. 2B remains to be established. It may be speculated that these proteins could represent several spliced RIM variants of similar size or perhaps degradation products. The immunoreactivity of ~170 kDa was also present in human brain (Fig. 2C). This band was not detected in human islets. However the islets contained a major band migrating with an ~160 kDa as well as other proteins of lower molecular weight. The complete sequence of the human RIM being unknown, the identity of these bands remains only a speculation. Nonetheless, as aforementioned these proteins may correspond to different RIM isoforms produced by alternative splicing. Control extracts from rat kidney and hamster BHK cells did not react with the antibody. Likewise no positive bands were observed in monkey COS cells and in human A431 epidermoid cells (data not shown). Taken together, these results demonstrate the presence of RIM in insulin-secreting cells.
3.2. Subcellular distribution of RIM

The cellular localization of RIM was examined by immunofluorescence confocal microscopy. Fig. 3A shows that RIM is mainly localized on the plasma membrane of rat islet β-cells and the staining is found in patches. A similar distribution of the protein is observed in INS-1E cells (Fig. 3C). Co-staining with an anti-insulin antibody revealed that RIM is also expressed in islet endocrine cells not positive for insulin (data not shown). By contrast, there was no plasma membrane reactivity in rat hepatocytes and smooth muscle cells (data not shown).

Fig. 3. Subcellular localization of RIM by immunofluorescence confocal microscopy. Pancreatic β-cells (A) and INS-1E cells (C) were stained with the antibody against RIM and revealed using fluorescein-conjugated secondary antibody. The same cells were visualized on phase contrast (B and D).

3.3. Role of soluble RIM in insulin exocytosis

The plasma membrane localization of RIM in insulin-secreting cells is compatible with a possible role in exocytosis. To investigate the RIM function, the soluble Rab3A binding N-terminal region of RIM was transiently overexpressed in INS-1E cells. The cytosolic localization of the overexpressed RIM fragment is shown in Fig. 4A using an anti-myc antibody.

The cDNAs encoding for the proteins under study were cotransfected with human growth hormone (hGH) which serves as a marker for exocytosis from the subpopulation of

Fig. 4. Immunolabelling of transfected soluble RIM fragment in INS-1E cells. INS-1E cells transiently transfected with the myc-tagged N-terminal region of RIM were analyzed by confocal microscopy after immunofluorescence with an antibody against the myc epitope (revealed using fluorescein-conjugated antibodies) (A). The same cells were visualized on phase contrast (B).

Fig. 5. Effect of Rab3A Q81L and the N-terminal domain of RIM on exocytosis in intact and α-toxin permeabilized INS-1E cells. A: INS-1E cells were transiently cotransfected with a plasmid encoding human growth hormone (hGH) as a reporter for secretion, and with respectively the control vector pcDNA3 (control), the mutant Rab3A Q81L (Q81L) or the N-terminal region of RIM (residues 1–399) (RIM). The latter cDNA was also cotransfected with Rab3A Q81L (Q81L+RIM). After 3 days, the cells were incubated in the presence of 2.8 mM glucose or with 12.8 mM glucose for 30 min. The cells were then collected and the expression of the exogenous proteins was analyzed by Western blotting using an antibody against the myc epitope (c-myc). One representative experiment is shown at the top. The amount of hGH secreted by the cells in response to the stimuli was measured by ELISA. The basal secretion rates ranged from 16 to 27 pg/well. The hGH cellular content rates ranged from 484 to 502 pg/well. The lower panel shows the mean ± S.E.M. of three independent experiments performed in duplicate.

B: Three days after transfection the cells were permeabilized with K+ toxin for 10 min and subsequently exposed to 0.1 mM cAMP or 10 mM Ca2+ during 10 min. The basal secretion rates ranged from 32 to 41 pg/well. The results represent the mean ± S.E.M. of two independent experiments performed in triplicate.
transfected cells [9,23]. Fig. 5A shows that an increase in the glucose concentration from 2.8 to 12.8 mM, caused a 4.7-fold increase in hGH release. As reported previously for transiently transfected HIT-T15 cells [8,9], overexpression of the GTPase deficient mutant (Rab3A Q81L) inhibited the stimulated secretion by 41%. Transfection of the soluble N-terminal domain of RIM enhanced stimulated exocytosis by 75%, while basal secretion was unaffected (Fig. 5A, legend). The combined expression of Rab3A Q81L and RIM completely neutralized the effect of RIM on exocytosis (100% of control). The observed changes in the secretory responses are not due to varying degrees of overexpression of RIM or Rab3A Q81L as shown by Western blotting using an anti-myc antibody (Fig. 5A, upper panel), or to variations in the hGH content (Fig. 5A, legend). The results obtained with the GTPase deficient mutant Rab3A Q81L indicate an in vivo interaction between RIM and the GTP-bound form of Rab3A as suggested previously [11].

To investigate whether RIM acts by increasing the Ca\textsuperscript{2+} signalling pathway or by directly enhancing the exocytotic process, we performed experiments in \(\alpha\)-toxin permeabilized cells, to bypass the function of ion channels and other plasma membrane events. Ca\textsuperscript{2+} (10 \(\mu\)M) stimulated exocytosis 1.9-fold (Fig. 5B). Rab3A Q81L inhibited the release by 35% whereas RIM augmented the secretion by 78%. Again, the stimulant action of RIM was reversed by the Rab3A mutant (100% of control). These data clearly show that RIM acts directly on the exocytotic process without affecting upstream signalling. The results obtained with the soluble domain of RIM both in intact and in permeabilized cells, strongly suggest that the overexpressed fragment increases exocytosis by interfering with the normal function of Rab3.

3.4. Conclusions

Our results demonstrate that RIM is not only neuron specific but is also expressed in native pancreatic \(\beta\)-cells and derived cell lines. The protein is localized at the plasma membrane of the endocrine cell types. The observed effects of RIM overexpression on exocytosis further substantiate the model proposed for neuro-exocytosis [11]. Hence, the insulin-containing secretory granule would be clamped by the interaction of Rab3-GTP on its surface with RIM at the plasma membrane close to L-type Ca\textsuperscript{2+} channels [24,25]. Recently, functional interaction between the exocytotic machinery and this type of voltage-sensitive Ca\textsuperscript{2+} channel was reported [25]. Following membrane depolarization by glucose and other secretagogues, the Ca\textsuperscript{2+} concentration increases. The subsequent binding of Ca\textsuperscript{2+} to calmodulin dissociates Rab3 from the secretory granule [26] as demonstrated for PC12 cells and HIT-T15 cells recently [23]. This allows exocytosis to proceed at the site determined by the localization of RIM.

Acknowledgements: We should like to thank Dr. J. Lou, Dr. J. Oberholzer and Dr. P. Morel (Department of Surgery, University of Geneva) for providing human islets and A. Valeva (Institute of Medical Microbiology, University of Mainz) for \(\alpha\)-toxin. We are grateful to Ms. J. Bassi and to Ms D. Harry for isolation and FACS sorting of rat islet cells. We should thank to Dr. H. Wang, Dr. P. Maechler and Dr. U. Vischer for stimulating discussions. This work was supported by the Swiss National Science Foundation Grants 32-49755.96 to C.B.W., 31-50640.97 to R.R. and by a Grant to C.B.W. from the Human Frontier Science Program (Strassbourg, France) RG0197/1998-M.

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