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Reference

IEZZI-BAKHTIARI, Mariella, REGAZZI, Romano, WOLLHEIM, Claes. The Rab3-interacting molecule RIM is expressed in pancreatic beta-cells and is implicated in insulin exocytosis. FEBS Letters, 2000, vol. 474, no. 1, p. 66-70

PMID : 10828453
DOI : 10.1016/s0014-5793(00)01572-6
The Rab3-interacting molecule RIM is expressed in pancreatic β-cells and is implicated in insulin exocytosis

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Received 17 March 2000; received in revised form 25 April 2000  

Edited by Guido Tettamanti

Abstract The putative Rab3 effector RIM (Rab3-interacting molecule) was detected by Northern blotting, RT-PCR and Western blotting in native pancreatic β-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 β-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 exocytosis 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.  

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Key words: Exocytosis; Rab3A; Ca 2+-induced secretion; Confocal microscopy; INS-1E cell; Human; Rat islet

1. Introduction

In the pancreatic β-cell, insulin is stored in large dense core vesicles (secretory granules) and is released by exocytosis in a Ca 2+-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 zinc-finger motif, followed by an alanine/proline-rich sequence. The central portion of the protein contains a PDZ domain mediating the attachment to plasma membrane proteins [12]. 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 Ca 2+-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. Sudhof (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.
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), denatured 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 transcrip-
tase (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'-GTCGATTGTCTTTCTT-
CCG-3' (sense strand, positions 2031–2050 in reference [16]) and 5'-TACTATAGCCGGATGCAGGG-
3' (antisense strand, positions 5059–5079). The following primers were used for amplification of HIT-T15 cDNA: 5'-ACGTAGATGTCGATGACA-3' (sense strand, positions 2031–2050 in reference [16]) and 5'-TACTATAGCCGGATGCAGGG-3' (antisense strand, posi-
tions 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 Tfl DNA polymerase (Promega, Catalys AG, Wallisellen, Switzerland) with 1 μM of each primer. Control experiments demonstrated that amplification products were absent after reactions omitting reverse transcribe.

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, immunofluores-
cence using pre-immune serum showed no specific plasma membrane labelling. Affinity purification of the RIM antibody was performed by incubating 1 μl of serum sample with 250 μg of protein A Sepharose 4B (Sigma), followed by washing with PBS containing 0.1 M glycine pH buffer 2.5 and desalted using centrifugal filter Centricon (Millipore, Geneva, Switzerland). Alternatively, the serum was affinity purified by binding GST and GST-RIM fusion proteins to nitrocellulose filters by preparative Western transfer; these immob-

obilized filters were used to select antibodies specific to RIM epitopes as described [17]. Before performing Western blotting, the specificity of the antibody was tested by a competition assay between a denatured GST-RIM protein and the protein A-pu-
rified affinity antibody. A similar approach was also used before analy-
ysis of immunofluorescence by incubating a native GST-RIM fusion protein with the nitrocellulose affinity-purified antibody.

2.6. Preparation of homogenates, GST-Rab3A Q81L binding assay and protein with the nitrocellulose affinity-purified antibody.

The specificity of the antibody was tested by a competition assay between a denatured GST-RIM protein and the protein A-purified antibody. The coverslips were analyzed by confocal mi-

croscopy (Leica Lasertechnik, Heidelberg, Germany, model TCS NT). Images were taken with a 40× objective.

The cytosolic localization of the RIM fragment in transfected INS-
1E cells was analyzed as described [9].

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 mi-

croscopy (Leica Lasertechnik, 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 corre-

sponding to approximately 8 kb and 12 kb in all three prep-

arations. 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 condi-

tions 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 vari-

ants 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

~ 1 mg of rat brain or kidney homogenates (prepared in HB), in

the presence of 100 μM GTP-γS 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
attached to the beads were analyzed by Western blotting as previously described [18].

3.2. Cell culture, transient transfection and hGH release

The INS-1E cell clone [19] and the insulin-secreting cell line HIT-
T15 (kindly provided by R.F. Santerre and E. Lilly, INC, Indianap-
olis, USA) were cultured in RPMI 1640 medium supplemented with

30% 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 × 105 cells per well) and transfected 3 days later. To this end, cells were washed with Mg++ and Ca2+ 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 expres-
sion plasmids (residues 1–399 of RIM and Rab3a Q81L mutant cDNAs, inserted in the N-terminal myc-tagged pcDNA3) or the con-

trol vector myc-tagged pcDNA3 in 400 μl RPMI 1640 medium con-
taining 10 mM HEPES. The mixture was added to the cells after aspiration of PBS and exchanged for complete medium after 6 h.

Three days after transfection the cells were incubated at 37°C for 2 h in complete medium without glucose and washed three times at

37°C with modified Krebs–Ringer bicarbonate buffer (KRb) [6].

Thereafter cells were preincubated for 30 min in KRb containing

2.8 mM glucose and then incubated for 30 min in the same buffer or KRb supplemented with 12.8 mM glucose. After incubation, se-

cretion from transfected cells was assessed by enzyme linked immu-

nunorobant assay (ELISA) (Boehringer, Mannheim, Germany). Al-

ternatively, cells were permeabilized with α-toxin from Staphylococcus aureus using intracellular buffers as described [9].

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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 (Databank 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 $R_f$ of ~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.

Fig. 1. Expression of RIM in insulin-secreting cells. A: Poly A⁺ RNA from rat brain (RB), HIT-T15 (HIT) and INS-1E (INS) cells were analyzed by Northern blotting. Based on the ethidium-bromide staining and the spectrophotometric quantitation, similar amounts (~2–3 μg) of HIT-T15 and INS-1E mRNA were loaded on the agarose gel. In order to obtain a similar intensity of the signal, ~0.5 μg of brain mRNA was loaded. The molecular weight markers are indicated. B: PCR amplification of RIM cDNA from rat kidney (RK), rat brain (RB), rat islet β-cells (RI), INS-1E cells (INS) and HIT-T15 cells (HIT). PCR reactions in which reverse transcriptase was omitted are also shown (--). The sizes (bp) of the amplification products are indicated by arrows.

Fig. 2. Western blotting of the homogenates. A: GTP-dependent binding of RIM to GST-Rab3A Q81L. 1/4 of the volume of rat kidney (RK) and rat brain (RB) binding assay was loaded on each lane and immunoblotted with an antibody against RIM. B, C: Expression of RIM in insulin-secreting cells. Homogenates (100 μg) of rat kidney, rat brain, rat islet β-cells (RI), INS-1E (INS), HIT-T15 (HIT), BHK cells, human brain (HB), and human islets (HI) were separated by SDS-PAGE, electroblotted and incubated with the protein A-purified anti-RIM antibody (1:2000). The estimated molecular weight (kDa) of RIM is indicated by the arrow.
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](image)

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](image)

3.4. 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.

![Fig. 5. Effect of Rab3A Q81L and the N-terminal domain of RIM on exocytosis in intact and α-toxin permeabilized INS-1E cells](image)
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 Ca2+ signalling pathway or by directly enhancing the exocytotic process, we performed experiments in α-toxin permeabilized cells, to bypass the function of ion channels and other plasma membrane events. Ca2+ (10 μM) 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 β-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 Ca2+ channels [24,25]. Recently, functional interaction between the exocytotic machinery and this type of voltage-sensitive Ca2+ channel was reported [25]. Following membrane depolarization by glucose and other secretagogues, the Ca2+ concentration increases. The subsequent binding of Ca2+ 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 α-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 (Strasbourg, France) RGO197/1998-M.

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