The death domain of Rab3 guanine nucleotide exchange protein in GDP/GTP exchange activity in living cells

Thierry COPPOLA*, Véronique PERRET-MENOUD*, Sonia GATTESCO*, Sarah MAGNIN*, Isabel POMBO†, Ulrich BLANK† and Romano REGAZZI*[1]

*Institut de Biologie Cellulaire et de Morphologie, University of Lausanne, Rue du Bugnon 9, 1005 Lausanne, Switzerland, and †Institut Pasteur, Unité d'Immuno-Allergie, Paris, France

Rab3 GTPases regulate exocytosis of neurons, endocrine and exocrine cells. In the present paper, we report a system to measure the guanine nucleotide status of Rab3 proteins in living cells. The assay is based on the ability of the Rab3 interacting molecule RIM to extract selectively the GTP-bound form of Rab3. Using this system, we found that approx. 20% of wild-type Rab3A, -B, -C or -D transfected in the insulin-secreting cell line HIT-T15 is in the GTP-bound conformation. The pool of activated Rab3 is decreased under conditions that stimulate exocytosis or by co-expression of the Rab3 GTPase-activating protein. In contrast, co-expression of Mss4 or Rab3-GEP (guanine nucleotide exchange protein) increases by approx. 3-fold the GTP-bound pool of Rab3 isoforms. Rab3-GEP is very similar to MADD, a death domain-containing protein that associates with the type 1 tumour necrosis factor receptor. We observed that the death domain of Rab3-GEP is involved in intramolecular interactions and that deletions or mutations that affect this domain of the protein impair the nucleotide exchange activity towards Rab3. We propose that the death domain of Rab3-GEP acts as a molecular switch and co-ordinates multiple functions of the protein by exchanging its binding partners.

Key words: cell death, exocytosis, insulin.

INTRODUCTION

Rab proteins constitute a large family of Ras-like GTPases that play an important role in the regulation of vesicle traffic in eukaryotic cells [1]. Each member of the Rab family is localized on specific cellular compartments and is in charge of a subset of transport steps in the secretory or in the endocytic pathways. The four isoforms of Rab3 (Rab3A, -B, -C and -D) are associated with the secretory vesicles of exocrine, endocrine and neuronal cells and are part of the machinery controlling exocytosis [1,2]. Like the other GTPases, Rab3 also switches between two functionally distinct conformational states. In the inactive GDP-bound conformation, Rab3 forms a cytosolic complex with RabGDI, a regulatory protein responsible for the delivery of Rab family members to their appropriate cellular location [3]. In the active GTP-bound conformation, Rab3 isoforms are associated with the secretory vesicle membrane and interact with several putative effectors [1,2]. The switch between the two activation states is controlled by a Rab3-specific GTPase-activating protein (Rab3-GAP) [4] and by guanine nucleotide exchange proteins (GEPs). Rab3-GAP stimulates the intrinsic GTPase activity of Rab3, promoting its inactivation and, indirectly, its dissociation from the membrane. GEPs catalyse the replacement of GDP by GTP and, consequently, favour the activation of Rab3. *In vitro*, two unrelated proteins have been shown to stimulate Rab3 GDP/GTP exchange: Mss4, a small protein (18 kDa) that catalyses guanine nucleotide exchange on several members of the Rab family [5,6], and Rab3-GEP, a large protein (200 kDa) whose exchange activity is restricted to Rab3 isoforms [6]. It is still unclear whether Mss4 regulates the activation of Rab3 proteins *in vivo*; in contrast, several observations indicate that Rab3-GEP controls Rab3 function in living cells. In fact, overexpression of Rab3-GEP in PC12 cells mimics the effect on exocytosis of the GTPase-deficient mutant of Rab3 [7]. Moreover, a mutation in *aex-3*, the gene encoding the *Caenorhabditis elegans* homologue of Rab3-GEP, results in an aberrant accumulation of Rab3 in neuronal cell bodies and prevents the normal localization on synaptic vesicles [8].

There is increasing evidence that the function of Rab3-GEP is not limited to the regulation of the Rab3 cycle. In fact, genetic studies in *C. elegans* revealed that *aex-3* mutants display defects in the defaecation motor programme, which are not observed in *rab-3* mutants [8–10]. Moreover, a protein very similar to Rab3-GEP, MADD [a death-domain containing protein that associates with the tumour necrosis factor receptor type 1 (TNFR1)] gets involved in mitogen-activated protein (MAP) kinase signalling [11]. The interaction with TNFR1 occurs through a death domain that is located at the C-terminus of MADD and is conserved in Rab3-GEP. Moreover, a splicing variant of MADD was identified as a substrate for c-Jun N-terminal kinase 3 and was shown to translocate to the nucleolus of hypoxia-sensitive neurons in patients undergoing acute hypoxia [12]. This led to the hypothesis that, under normal conditions, MADD and Rab3-GEP function as regulators of Rab3, but under stress conditions they become involved in the control of other signalling pathways [12].

In the present study, we have developed a new method to measure the fraction of Rab3 in the GTP-bound conformation. This assay was used to follow the activation state of Rab3 in living cells and to investigate the properties of human Rab3-GEP (human brain clone KIAA0358) which is very similar to rat

Abbreviations used: ΔC1, Rab3-GEP mutant lacking 127 amino acids at the C-terminus; ΔC2, Rab3-GEP mutant lacking 229 amino acids at the C-terminus; ΔDD, Rab3-GEP mutant lacking the death domain; GAP, GTPase-activating protein; GEP, guanine nucleotide exchange protein; GST, glutathione-S-transferase; GTPγS, guanosine 5′-[γ-thio]triphosphate; MADD, death domain-containing protein that associates with TNFR1; MAP, mitogen-activated protein; RIM, Rab3-interacting molecule; TNFR1, tumour necrosis factor receptor type 1.

[1] To whom correspondence should be addressed (e-mail Romano.Regazzi@ibcm.unil.ch).

Rab3-GEP and MADD. Our data demonstrate that an intact death domain is required not only for the interaction of the proteins of the Rab3-GEP/MADD family with TNFR1 but also for nucleotide exchange activity towards Rab3.

EXPERIMENTAL

Materials

The human brain cDNA clones KIAA0358 and KIAA0066 encoding human Rab3-GEP and Rab3-GAP, respectively were obtained from the Kazusa DNA Research Institute (Kizarazu, Chiba, Japan). The Mss4 cDNA was received from Dr P. De Camilli (Yale University). The plasmid encoding the glutathione-S-transferase (GST) fusion protein with the Rab3-binding domain of RIM (Rab3-interacting molecule) (amino acids 11–398) was provided by Dr T. C. Südhof (University of Texas). GDP and GTPγS (guanosine 5′-[γ-thio]triphosphate) were purchased from Roche. Glutathione-agarose beads were purchased from Sigma.

Mutagenesis and plasmid construction

The pcDNA3 constructs encoding myc-tagged Rab3A, -B, -C and -D have been described previously [13,14]. The full-length Rab3-GEP construct was prepared by first subcloning the *Xho*I/*Apa*I fragment of the KIAA0358 clone in pcDNA3 (Invitrogen). A DNA fragment encoding a myc epitope tag and the first 214 amino acids of Rab3-GEP were then amplified by PCR and inserted at the N-terminus of the protein. Site-directed mutagenesis was performed using the Quickchange kit (Stratagene) and was verified by sequencing the insert of the plasmids. The Rab3-GEP mutant lacking 127 amino acids at the C-terminus (ΔC1) was produced by excising the fragment between the unique *Eco*RV site (position 4546 of KIAA0358) and the *Apa*I site of the pcDNA3 polylinker. The sticky ends produced by *Apa*I were refilled and the plasmid ligated. The Rab3-GEP mutant lacking 229 amino acids at the C-terminus (ΔC2) was generated using an analogous procedure by deleting the fragment between the *Kpn*I site (position 4238 of KIAA0358) and *Apa*I. To generate the Rab3-GEP mutant lacking the death domain (ΔDD) we created a second *Eco*RV restriction site corresponding to amino acid 1307. We then excised the sequence between the two *Eco*RV sites (amino acids 1307–1454) and re-circularized the plasmid. The Rab3-GAP construct was obtained by subcloning a *Xho*I/*Hpa*I fragment of the KIAA0066 Kazusa clone containing the coding region of human Rab3-GAP in the pNeoSRα mammalian expression vector [15]. The bacterial expression vector encoding the GST-fusion protein with the death domain of Rab3-GEP was prepared by inserting the DNA fragment spanning between the *Bam*HI site at position 4067 and the *Eco*RV site at position 4546 of KIAA0358 in the corresponding sites of pGEX-KG [16]. The construct containing amino acids 1–1134 of Rab3-GEP was produced by cutting the DNA fragment spanning from a *Hin*dIII restriction site in the polylinker upstream to the myc epitope to the *Hin*dIII site at position 3587 of KIAA0358. The isolated fragment was then subcloned in pcDNA3. The Rab3-GEP construct including the amino acids 1031–1306 was amplified by PCR and was subcloned directly in pcDNA3.

Cell culture

HIT-T15 cells were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM glutamine, 32.5 μM glutathione and 0.1 μM selenium.

Assay for the measurement of the GTP-bound pool of Rab3

HIT-T15 cells (3×10^6) were transiently transfected by electroporation with 30 μg of plasmid encoding wild-type or mutated Rab3 isoforms and with 30 μg of plasmid encoding the proteins that regulate the Rab3 cycle. The cells were then seeded in a six-well plate and cultured for 2–3 days. On the day of the experiment, the cells were washed once in PBS and scraped in 200 μl of lysis buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 % (w/v) glycerol, 1 % (w/v) Triton X-100, 2 μg/ml leupeptin and 2 μg/ml aprotinin]. In the experiment presented in Figure 2, before lysis the cells were preincubated for 30 min at 37 °C in KB buffer (20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2.7 mM $CaCl_2$, 128 mM NaCl, 5 mM KCl). The medium was then removed and replaced for another 10 min period at 37 °C with KB or KS buffer (20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2.7 mM $CaCl_2$, 53 mM NaCl, 80 mM KCl and 10 mM glucose). The cell lysate was centrifuged at 4 °C for 5 min at 12000 ***g***. An aliquot of the supernatant (30 μl) was saved and used to verify the total amount of Rab3 expressed by the cells. The rest of the supernatant was mixed with 2–3 μg of GST-RIM previously attached to glutathione-agarose beads and incubated for 1 h at 4 °C. At the end of the incubation period the beads were washed four times in 400 μl lysis buffer and resuspended in SDS buffer [60 mM Tris/HCl, pH 6.8, 11 % (w/v) glycerol and 2 % SDS]. The totality of the proteins remaining associated with the beads was loaded on SDS/PAGE. The fraction of Rab3 bound to the affinity column was determined by Western blotting using a monoclonal antibody against the myc epitope tag (9E10). Quantitative analysis of the films was performed with the Scion Image software.

Interaction of Rab3-GEP with its own death domain

GST, GST-DD and GST-DD I/A produced in bacteria were immobilized on glutathione-agarose beads. The beads were resuspended in 20 mM Hepes, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 5 % glycerol, 0.05 % Tween-20 and 1 mg/ml BSA. They were then incubated with the indicated ^{35}S-labelled Rab3-GEP constructs generated by *in vitro* translation (Promega). The proteins that remained with the affinity columns after extensive washing were analysed by SDS/PAGE and identified by autoradiography.

RESULTS

We developed a new assay that permits assessment of the fraction of GTP-bound Rab3 in living cells. The assay is based on the property of RIM to associate selectively with the GTP-bound form of Rab3 [17]. The insulin-secreting cell line HIT-T15 was transiently transfected with myc-tagged wild-type Rab3A. Two days later, the cells were lysed and the homogenate incubated either with GDP or GTPγS. Rab3A was then isolated using a GST-RIM affinity column. As shown in Figure 1(A), GST-RIM was unable to bind to Rab3A loaded with GDP and associated exclusively with the GTP-bound form of the protein. Under our experimental conditions (1 h incubation at 4 °C), after loading with GTPγS, 10–30 % of the Rab3A present in the lysate was captured on the RIM affinity column. The percentage of GTP-bound Rab3A recovered on the column was not significantly influenced by the expression level of the GTPase achieved in each experiment. The cells were then transiently transfected with $Rab3A^{T36N}$ and $Rab3A^{Q81L}$, two mutants known to be locked in the GDP-bound and GTP-bound conformations, respectively [18]. After 2 days in culture the cells expressed the same amount

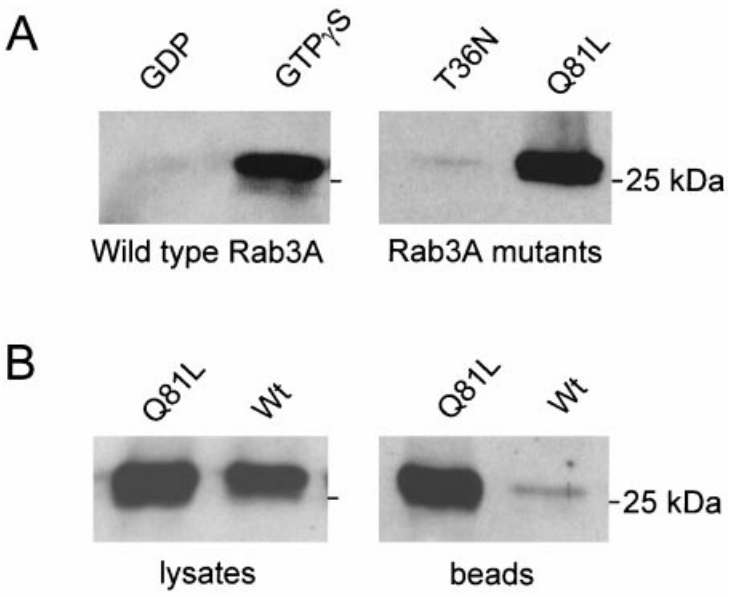

Figure 1 Isolation of the GTP-bound form of Rab3 from cell lysates

(**A**) Left panel: the insulin-secreting cell line HIT-T15 was transiently transfected with wild-type Rab3A. Two days later the cells were disrupted and the lysate incubated for 15 min at 30 °C in the presence of 1 mM GDP or GTPγS. Rab3A was then purified on a GST-RIM affinity column and visualized by Western blotting using an anti-myc antibody. Right panel: HIT-T15 cells were transfected with the T36N or the Q81L mutant of Rab3A. Two days later the mutants were isolated using a GST-RIM affinity column and visualized by Western blotting. (**B**) HIT-T15 cells were transiently transfected with wild-type (Wt) Rab3A or with the Q81L mutant. Two days later GTP-bound Rab3A was isolated using a GST-RIM affinity column and visualized by Western blotting. Right panel: gel loaded with an aliquot (1/10) of the cell lysate. Left panel: total amount of Rab3A remaining associated with the affinity column.

of Rab3A^{T36N} and Rab3A^{Q81L} (data not shown). As expected from the results of the previous experiment, only the mutant in the GTP-liganded state (Rab3A^{Q81L}) was isolated from the cell lysate using recombinant GST-RIM (Figure 1A). The percentage of Rab3A^{Q81L} remaining associated with the affinity column was very similar to that of Rab3A loaded with GTPγS, indicating that in HIT-T15 cells nearly 100 % of this mutant is in the GTP-bound conformation. When the wild-type protein was transfected in HIT-T15 cells, 5–10-fold less Rab3A was recovered on the GST-RIM column. This indicates that under these conditions approx. 10–20 % of wild-type Rab3A is in the active conformation (Figure 1B). Taken together, these results demonstrate that our assay allows the measurement of the pool of active Rab3 present in living cells.

The pool of active Rab3A was not significantly modified by short-term exposure of the cells to cAMP-raising agents. The fraction of GTP-bound Rab3A measured in lysates of cells treated for 30 min with a mixture of forskolin (10 μM) and 3-isobutyl-1-methylxanthine (1 mM) corresponded to 98 ± 30 % ($n = 4$) of the value obtained from untreated cells. In contrast, when the cells were exposed to glucose and depolarizing K^+ concentrations, a condition that causes a 4–6-fold stimulation of insulin exocytosis, the pool of activated Rab3A was decreased by about 70 % (Figure 2). The fraction of Rab3A in the GTP-bound conformation was also dramatically modified by co-expression of the protein with regulators of the Rab3 cycle. Thus, in cells transfected with Rab3-GAP the pool of active Rab3A was reduced by about 70 % (Figure 2). Overexpression of human Rab3-GEP (Kazusa clone KIAA0358) in HIT-T15 cells caused a 3-fold increase in the amount of GTP-bound Rab3A (Figure 2). The effect of Rab3-GEP was partially counteracted by exposing the cells to stimulatory conditions (Figure 2). Co-transfection of Mss4, a protein that displays nucleotide exchange activity on several members of the Rab family [5,6], stimulated a similar enhancement in the GTP-bound pool of Rab3A (Figure 3).

Taking advantage of our assay we then investigated whether Rab3-GEP displays differential GDP/GTP exchange activity towards the four Rab3 isoforms. Considering the respective Q81L mutants exclusively in the GTP-bound form, the fraction of wild-type Rab3B, -C and -D displaying an active conformation in transfected HIT-T15 cells was also close to 20 % (Figure 4). Co-expression of Rab3-GEP stimulated a 3-fold increase in the GTP-bound pool of each isoform indicating that there is no preferential activation of any of the Rab3 isoforms.

We then attempted to identify the domains of Rab3-GEP that are essential for the nucleotide exchange activity in living cells by generating different deletion mutants (Figure 5). Rab3-GEP (Kazusa clone KIAA0358) is a 1581 amino acid protein. Deletion of 127 amino acids at the C-terminus of the protein (ΔC1) did not impair the function of Rab3-GEP (Figure 6). In contrast, a C-terminal deletion of 229 amino acids (ΔC2) abolished the ability to catalyse GDP/GTP exchange (Figure 6). Rab3-GEP contains a death domain located close to the C-terminus of the protein (amino acids 1339–1415) (Figure 5). Most of this domain is lost in the ΔC2 mutant, but not in the ΔC1 deletion. To examine whether the death domain is required for Rab3-GEP activity we created a third mutant lacking the death domain (ΔDD). As shown in Figure 6 this mutant was unable to increase the pool of GTP-bound Rab3.

The importance of a functional death domain for the activity of Rab3-GEP was further investigated by replacing Ile^{1413} with Ala (Figure 5). We selected Ile^{1413} because this amino acid is conserved in AEX-3 and the corresponding residue in the death domain of TNFR1 plays a critical role for cell signalling [19]. As shown in Figure 7, the function of Rab3-GEP was partially impaired by the replacement of Ile^{1413} with Ala. In fact, in seven

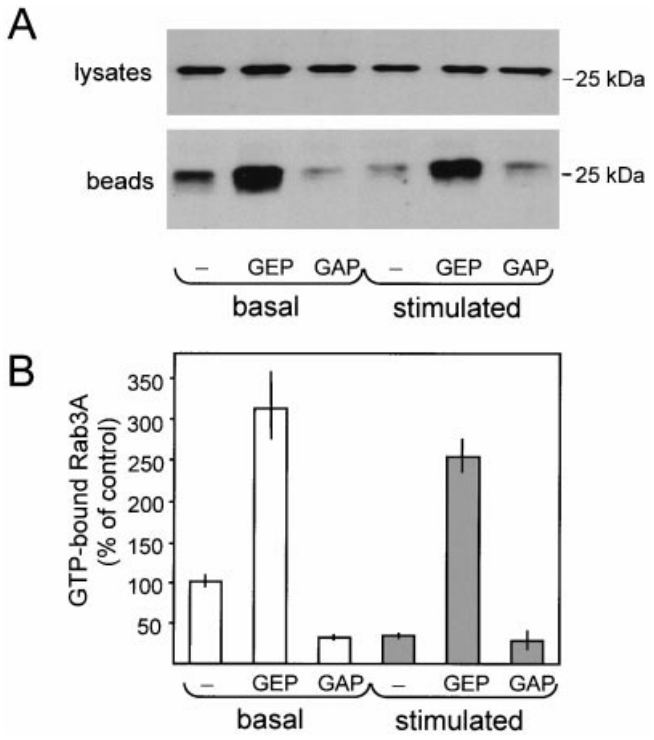

Figure 2 The pool of GTP-bound Rab3A modified by insulin secretagogues and regulators of the Rab3 cycle

HIT-T15 cells were transiently co-transfected with wild-type Rab3A and with an empty vector (−), GEP or GAP. Two days later the cells were washed and incubated for 30 min at 37 °C in KB buffer. The medium was then removed and the cells incubated in KB (basal) or in KS (stimulated) buffer for 10 min at 37 °C. At the end of the incubation period the GTP-bound form of Rab3A was isolated from the cell lysate using the GST-RIM affinity column. (**A**) Upper panel: Western blot analysis of an aliquot (1/20) of the cell lysates before purification. Lower panel: proteins associated with the agarose beads. (**B**) Data obtained after densitometric scanning of the films of three independent experiments. The results are expressed in terms of the means ± S.E.M. The pool of Rab3A associated with the GST-RIM column under basal conditions in cells co-transfected with Rab3A and an empty vector has been set to 100%.

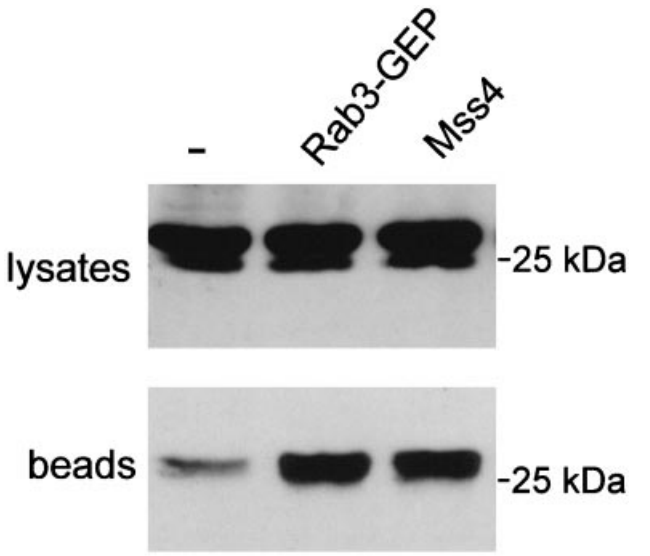

Figure 3 Increase in GTP-bound pool of Rab3A due to overexpression of Mss4

HIT-T15 cells were transiently co-transfected with wild-type Rab3A and with an empty vector (−), with Rab3-GEP or with Mss4. Two days later the cells were lysed and the GTP-bound form of Rab3A isolated with GST-RIM. Upper panel: aliquots (1/10) of the cell lysates before purification. Lower panel: totality of the proteins specifically associated with the beads analysed by Western blotting using an anti-myc antibody.

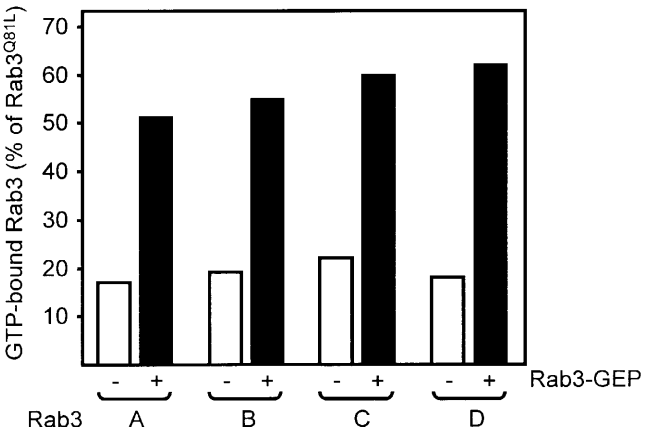

Figure 4 Human Rab3-GEP active on the four isoforms of Rab3

HIT-T15 cells were co-transfected with wild Rab3A, -B, -C or -D and with an empty vector (−) or with a vector encoding Rab3-GEP (+). In parallel, the cells were also transfected with the GTPase-deficient mutant (Q81L) of each Rab3 isoform. Two days later the cells were disrupted and the GTP-bound forms of the different Rab3 isoforms affinity-purified using GST-RIM. In each experiment, the amount of Rab3A-D^{Q81L} that remained associated with the affinity column was set to 100% and was used to normalize the results obtained with the corresponding wild-type Rab3 isoform.

independent experiments the increase in the GTP-bound pool of Rab3A elicited by this mutant was only about one-half (55 ± 9%) of that obtained in cells transfected with wild-type Rab3-GEP.

Death domains are well-known protein–protein interaction modules [20]. For this reason, we tested whether the death domain of Rab3-GEP is involved in inter- or intramolecular interactions. In yeast two-hybrid screening, a small protein fragment containing the death domain of Rab3-GEP/MADD was reported to bind to the death domain of the TNFR1 and to self-associate [11]. Surprisingly, we found that a fusion protein between GST and the death domain of Rab3-GEP (GST-DD) cannot interact with full-length Rab3-GEP (Figure 8A) or with the ΔC1 mutant (amino acids 1–1454) (not shown). This indicates that in full-length Rab3-GEP the death domain is not readily available for intermolecular interactions. In contrast, the deletion mutants lacking the death domain, ΔC2 (amino acids 1–1352) and ΔDD, were selectively retained on the GST-DD affinity column (Figure 8A). This suggests that the deletion of the C-terminus of Rab3-GEP unmasks a binding site for GST-DD that is normally occupied by the endogenous death domain. The interaction between ΔDD and GST-DD is likely to involve a complex three-dimensional folding of the first 1306 amino acids of Rab3-GEP because, independently, protein fragments ranging from amino acids 1–1134 or 1031–1306 were unable to bind efficiently to the affinity column (Figure 8B). The association of the ΔDD mutant with GST-DD was strongly impaired by the replacement of Ile^{1413} with Ala (Figure 8C), indicating that the interaction requires a functional death domain.

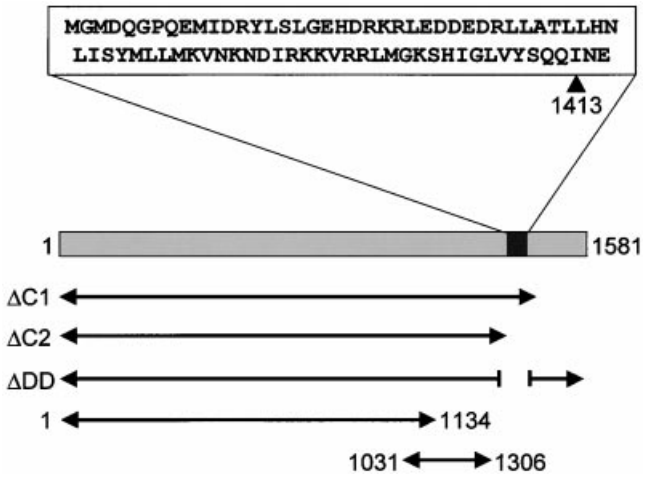

Figure 5 Schematic representation of the different Rab3-GEP constructs

Rab3-GEP is constituted of 1581 amino acids and contains a death domain (in black) spanning from amino acid 1339 to 1415. The amino-acid sequence of the death domain is shown in the box. The ΔC1 and ΔC2 mutants lack 127 and 229 amino acids, respectively at the C-terminus. In the ΔDD mutant the death domain has been deleted. The portion of Rab3-GEP included in the 1–1134 and 1031–1306 fragments is indicated by the lines. The position of Ile^{1413} is shown by the arrowhead.

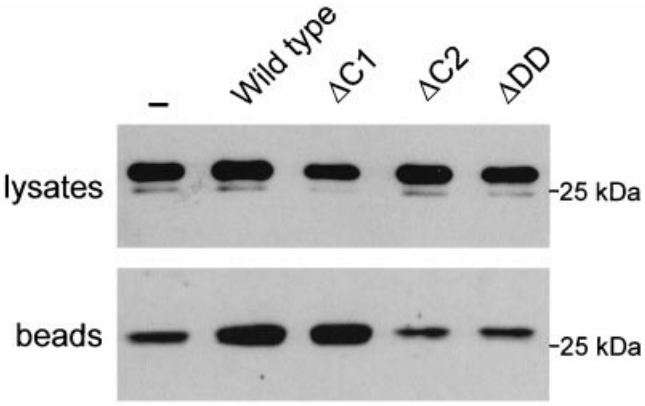

Figure 6 The death domain of Rab3-GEP is essential for its guanine nucleotide exchange activity

Wild-type Rab3A was co-transfected with an empty vector (—) or with vectors encoding wild-type Rab3-GEP, Rab3-GEPΔC1, Rab3-GEPΔC2 or Rab3-GEPΔDD. The pool of GTP-bound Rab3A present in the cells under each condition was determined using the GST-RIM pull-down assay. The figure shows Western blots of aliquots (1/10) of the cell lysates before purification and of the totality of the proteins remaining associated with the GST affinity column in one representative experiment out of three.

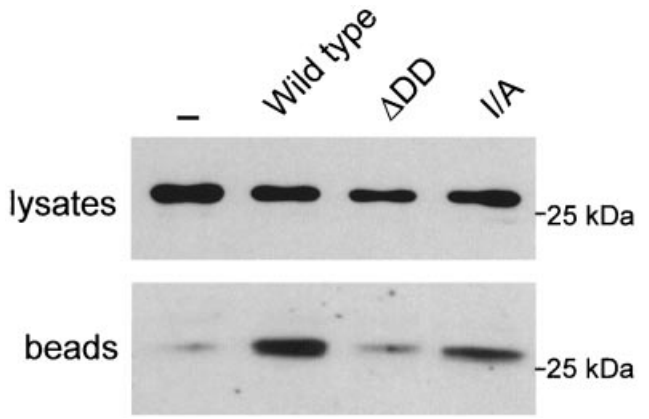

Figure 7 A point mutation within the death domain decreases the guanine nucleotide exchange activity

Wild-type Rab3A was co-transfected with an empty vector (—) or with vectors encoding wild-type Rab3-GEP, Rab3-GEPΔDD or a Rab3-GEP mutant in which Ile^{1413} was replaced by Ala (I/A). The pool of GTP-bound Rab3A present in the cells under each condition was determined using the GST-RIM pull-down assay. The figure exhibits one representative experiment out of seven. Upper panel: Western blot of an aliquot (1/10) of the cell lysates before purification. Lower panel: total amount of Rab3A retained on the affinity columns. The level of expression of the three Rab3-GEP constructs was the same (not shown).

DISCUSSION

Despite the recognized involvement of Rab3 in the regulation of exocytosis, few studies have attempted to follow the guanine nucleotide status of these GTPases in living cells [18,21,22]. This situation is mainly due to technical difficulties inherent in the conventional assays for measuring the nucleotide associated with Rab3. In fact, the standard protocols require metabolic labelling of the cells, immunoprecipitation of Rab3 and analysis of the guanine nucleotide bound to the protein by TLC [18,21,22]. The methodology developed in this study is based on a different approach and avoids most of the difficulties encountered with the traditional assays. In particular, our assay does not require preincubation of the cells with radioactive precursors and can be applied to both intact and permeabilized cells. For this reason, the method presented in this paper will be a valuable tool for a broad range of studies involving detailed analyses of Rab3 function. In the present study we have measured the activation state of Rab3 in transiently transfected cells. Assessment of the activation state of endogenous Rab3 proteins in HIT-T15 cells is difficult because these cells contain only very small amounts of Rab3A [13] and Rab3B, -C and -D give rather poor signals with commercially available antibodies. However, in other cell systems expressing higher levels of Rab3, such as neurons, our method can be easily adapted to the investigation of the nucleotide status of endogenous proteins.

Using our assay we found that the pool of GTP-bound Rab3 is diminished when HIT-T15 cells are exposed to compounds that elicit a strong secretory response. This is in agreement with the observations of Stahl et al. [21], who reported an increase in the GDP/GTP ratio of Rab3A in synaptosomes incubated with α-latrotoxin, a potent stimulator of exocytosis. We also observed that the activation state of Rab3 is modified by alterations in the balance between GAP and GEPs. Overexpression of Rab3-GAP mimicked the action of insulin secretagogues and strongly reduced the amount of GTP-bound Rab3. The effects of Rab3-GAP overexpression and of insulin secretagogues were not additive, suggesting that the decrease in the GTP-bound pool of Rab3A associated with insulin exocytosis is due to an increase in Rab3-GAP activity. The active fraction of Rab3 was augmented in cells overexpressing Rab3-GEP or Mss4. This indicates that both proteins can contribute to regulating the Rab3 cycle in living cells. Rab3-GEP and Mss4 are proteins of very different sizes (200 and 18 kDa, respectively) and share no significant sequence similarity. Mss4 displays *in vitro* nucleotide exchange activity towards a subset of Rab proteins [5,6]. However, the precise role of Mss4 and its yeast homologue Dss4 *in vivo* remain to be established [23,24]. In fact, Mss4 and Dss4 have been suggested to be chaperones of the guanine nucleotide-free form

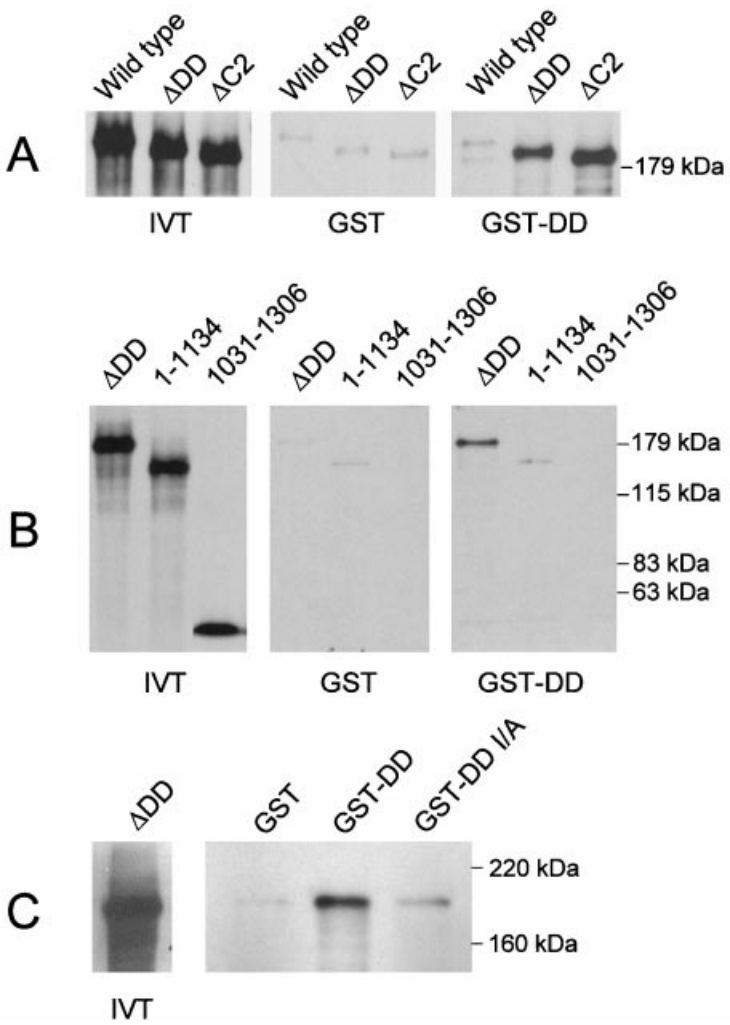

Figure 8 The death domain of Rab3-GEP is involved in protein–protein interactions

(**A**) Radioactively labelled wild-type Rab3-GEP, Rab3-GEPΔDD and Rab3-GEPΔC2 produced by *in vitro* translation (IVT) (left panel) were incubated with glutathione-agarose beads coated with GST alone (middle panel) or with GST-DD, a fusion protein between GST and the death domain of Rab3-GEP (right panel). The proteins attached to the GST affinity columns after extensive washing were resolved by SDS/PAGE and detected by autoradiography. The figure shows one representative experiment out of three. (**B**) Radioactively labelled Rab3-GEPΔDD and the Rab3-GEP fragments including amino acids 1–1134 and 1031–1306 were produced by *in vitro* translation. They were then incubated with glutathione-agarose beads coated with GST alone or with GST-DD. After extensive washing, the totality of the proteins remaining associated with the GST affinity columns were resolved by SDS/PAGE and detected by autoradiography. An aliquot of the *in vitro* translation (IVT) products (1/10) is shown in the left panel. Radioactively labelled Rab3-GEPΔDD produced by IVT was incubated with glutathione-agarose beads coated with GST alone, GST-DD or with GST-DD I/A a fusion protein in which Ile^{1413} of the death domain of Rab3-GEP was replaced by Ala. The totality of the proteins attached to the GST affinity columns after extensive washing and an aliquot (1/10) of the IVT product were resolved by SDS/PAGE and detected by autoradiography. The figure shows one representative experiment out of three.

of Rabs rather than GEPs [24]. Future studies will have to elucidate whether Mss4 can directly catalyse the increase in the GTP-bound form of Rab3 that we observe in transfected HIT-T15 cells or whether it favours the nucleotide exchange reaction of endogenous GEPs by stabilizing the nucleotide-free form of the GTPase.

Human Rab3-GEP and MADD are very similar proteins that differ only in small distinct segments corresponding to exon–intron boundaries, suggesting that they correspond to splicing variants of a single gene [25]. However, the proteins have been reported to control different cellular processes. Rab3-GEP and its *C. elegans* homologue AEX-3 have been shown to regulate the activation state of Rab3 and to play a role in exocytosis, as shown in [7,8,10] and confirmed by the present study. On the contrary, it was proposed in [11] that MADD was involved in TNFR1 signalling and was translocated into the nucleolus under stress conditions [12]. Despite these divergent observations the alignment of the sequences of Rab3-GEP and MADD indicates that they most probably perform similar, if not identical, functions. In fact, the fragment of MADD which causes activation of MAP kinases and is involved in TNFR1 signalling [11] is also included in the sequence of Rab3-GEP. If the members of the Rab3-GEP/MADD family participate in multiple cellular processes how are their different functions co-ordinated? The proteins of the Rab3-GEP/MADD family possess a death domain that mediates the binding to the TNFR1 and is responsible for the activation of MAP kinases [11]. The presence of a death domain suggests the possibility of a protein–protein interaction but does not always implicate a protein in the apoptotic process [20]. In this paper we demonstrate that the death domain of Rab3-GEP is involved in interactions with the N-terminal region of Rab3-GEP and that mutations or deletions in the death domain have

adverse effects on guanine nucleotide exchange activity. Our findings are consistent with the results obtained *in vitro* by Oishi et al. [7] with rat Rab3-GEP. In fact, Oishi et al. found that a Rab3-GEP mutant lacking 242 amino acids at the C-terminus of the protein displays no nucleotide exchange activity towards Rab3. Interestingly, this deletion involves part of the death domain and includes the Ile residue corresponding to Ile^{1413} of human Rab3-GEP. Based on these findings, we propose that the different activities of the members of the Rab3-GEP/MADD family are determined by the proteins that associate with their death domains. Under normal conditions, Rab3-GEP/MADD proteins are likely to control the function of Rab3 GTPases and to participate in the regulation of the exocytotic process. The guanine nucleotide exchange activity probably necessitates a three-dimensional conformation involving an interaction between the death domain and another region of the protein. Under stress conditions, this interaction may be perturbed by post-translational modifications (i.e. phosphorylations), by caspase cleavage, or by the availability of new potential binding partners. This would permit the association of the death domain with other proteins (such as the TNFR1) and would initiate signalling cascades that could eventually lead to cell death.

In conclusion, the development of a new assay for measuring the guanine nucleotide status of Rab3 enabled us to follow the activation state of the GTPase in living cells and to demonstrate that the nucleotide exchange activity of Rab3-GEP necessitates the presence of an intact death domain. Elaborate studies are needed to elucidate the mechanisms that operate under normal and pathophysiological conditions and regulate the availability of the death domain for intra- or intermolecular interactions.

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REFERENCES

1 Martinez, O. and Goud, B. (1998) Rab proteins. Biochim. Biophys. Acta **1404**, 101–112
2 Geppert, M. and Südhof, T. C. (1998) RAB3 and synaptotagmin: the yin and yang of synaptic membrane fusion. Annu. Rev. Neurosci. **21**, 75–95
3 Pfeffer, S. R., Dirac-Svejstrup, B. A. and Soldati, T. (1995) Rab GDP dissociation inhibitor: putting rab GTPases in the right place. J. Biol. Chem. **270**, 17057–17059
4 Fukui, K., Sasaki, T., Imazumi, K., Matsuura, Y., Nakanishi, H. and Takai, Y. (1997) Isolation and characterization of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. J. Biol. Chem. **272**, 4655–4658
5 Burton, J. L., Burns, M. E., Gatti, E., Augustine, G. J. and De Camilli, P. (1994) Specific interactions of Mss4 with members of the Rab GTPase family. EMBO J. **13**, 5547–5558
6 Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y. and Takai, Y. (1997) Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. J. Biol. Chem. **272**, 3875–3878
7 Oishi, H., Sasaki, T., Nagano, F., Ikeda, W., Ohya, T., Wada, M., Ide, N., Nakanishi, H. and Takai, Y. (1998) Localization of the Rab3 small G protein regulators in nerve terminals and their involvement in Ca^{2+}-dependent exocytosis. J. Biol. Chem. **273**, 34580–34585
8 Iwasaki, K., Staunton, J., Saifee, O., Nonet, M. and Thomas, J. H. (1997) *aex-3* encodes a novel regulator of presynaptic activity in *C. elegans*. Neuron **18**, 613–622
9 Nonet, M. L., Staunton, J. E., Kilgard, M. P., Fergestad, T., Hartwieg, E., Horvitz, H. R., Jorgensen, E. M. and Meyer, B. J. (1997) *Caenorhabditis elegans rab3* mutant synapses exhibit impaired function and are partially depleted of vesicles. J. Neurosci. **17**, 8061–8073
10 Iwasaki, K. and Toyonaga, R. (2000) The Rab3 GDP/GTP exchange factor homolog AEX-3 has a dual function in synaptic transmission. EMBO J. **19**, 4806–4816
11 Schievella, A. R., Chen, J. H., Graham, J. R. and Lin, L.-L. (1997) MADD, a novel death domain protein that interacts with the type I tumor necrosis factor receptor and activates mitogen-activated protein kinase. J. Biol. Chem. **272**, 12069–12075
12 Zhang, Y., Zhou, L. and Miller, C. A. (1998) A splicing variant of a death domain protein that is regulated by a mitogen-activated kinase is a substrate for c-Jun N-terminal kinase in the human central nervous system. Proc. Natl. Acad. Sci. U.S.A. **95**, 2586–2591
13 Regazzi, R., Ravazzola, M., Iezzi, M., Lang, J., Zahraoui, A., Andereggen, E., Morel, P., Takai, Y. and Wollheim, C. B. (1996) Expression, localization and functional role of small GTPases of the Rab3 family in insulin-secreting cells. J. Cell Sci. **109**, 2265–2273
14 Iezzi, M., Escher, G., Meda, M., Charollais, A., Baldini, G., Darchen, F., Wollheim, C. B. and Regazzi, R. (1999) Subcellular distribution and function of RabA, B, C, and D isoforms in insulin-secreting cells. Mol. Endocrinol. **13**, 202–212
15 Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. (1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. **8**, 466–472
16 Guan, K. L. and Dixon, J. E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192**, 262–267
17 Wang, Y., Okamoto, M., Schmitz, F., Hofmann, K. and Südhof, T. C. (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature (London) **388**, 593–598
18 Brondyk, W. H., McKiernan, C. J., Burstein, E. S. and Macara, I. G. (1993) Mutants of Rab3A analogous to oncogenic Ras mutants. J. Biol. Chem. **268**, 9410–9415
19 Tartaglia, L. A., Ayres, T. M., Wong, G. H. W. and Goeddel, D. V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. Cell **74**, 845–853
20 Feinstein, E., Kimchi, A., Wallach, D., Boldin, M. and Varfolomeev, E. (1995) The death domain: a module shared by proteins with diverse cellular functions. Trends Biochem. Sci. **20**, 342–344
21 Stahl, B., Fischer von Mollard, G., Walch-Solimena, C. and Jahn, R. (1994) GTP cleavage by the small GTP-binding protein Rab3A is associated with exocytosis of synaptic vesicles induced by α-Latrotoxin. J. Biol. Chem. **269**, 24770–24776
22 Ohnishi, H., Samuelson, L. C., Yule, D. I., Ernst, S. A. and Williams, J. A. (1997) Overexpression of Rab3D enhances regulated amylase secretion from pancreatic acini of transgenic mice. J. Clin. Invest. **100**, 3044–3052
23 Collins, R. N., Brennwald, P., Garrett, M., Lauring, A. and Novick, P. (1997) Interactions of nucleotide release factor Dss4p with Sec4p in the post-Golgi secretory pathway of yeast. J. Biol. Chem. **272**, 18281–18289
24 Nuoffer, C., Wu, S.-K., Dascher, C. and Balch, W. E. (1997) MSS4 does not function as an exchange factor for Rab in endoplasmic reticulum to Golgi transport. Mol. Biol. Cell **8**, 1305–1316
25 Brown, T. L. and Howe, P. H. (1998) MADD is highly homologous to a Rab3 guanine-nucleotide exchange protein (Rab3-GEP). Curr. Biol. **8**, R191

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