Rho family GTPase Rnd2 interacts and co-localizes with *MgcRacGAP* in male germ cells

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The male-germ-cell Rac GTPase-activating protein gene (*MgcRacGAP*) was initially described as a human RhoGAP gene highly expressed in male germ cells at spermatocyte stage, but exhibits significant levels of expression in most cell types. In somatic cells, MgcRacGAP protein was found to both concentrate in the midzone/midbody and be required for cytokinesis. As a RhoGAP, MgcRacGAP has been proposed to down-regulate RhoA, which is localized to the cleavage furrow and midbody during cytokinesis. Due to embryonic lethality in *MgcRacGAP*-null mutant mice and to the lack of an *in vitro* model of spermatogenesis, nothing is known regarding the role and mode of action of MgcRacGAP in male germ cells. We have analysed the expression, subcellular localization and molecular interactions of MgcRacGAP in male germ cells. Whereas MgcRacGAP was found only in spermatocytes and early spermatids, the widespread RhoGTPases RhoA, Rac1 and Cdc42 (which are, to various extents, *in vitro* substrates for MgcRacGAP activity) were, surprisingly, not detected at these stages. In contrast, Rnd2, a Rho family GTPase-deficient G-protein was found to be co-expressed with MgcRacGAP in spermatocytes and spermatids. MgcRacGAP was detected in the midzone of meiotic cells, but also, unexpectedly, in the Golgi-derived pro-acrosomal vesicle, co-localizing with Rnd2. In addition, a stable Rnd2–MgcRacGAP molecular complex could be evidenced by glutathione S-transferase pull-down and co-immunoprecipitation experiments. We conclude that Rnd2 is a probable physiological partner of MgcRacGAP in male germ cells and we propose that MgcRacGAP, and, quite possibly, other RhoGAPs, may participate in signalling pathways involving Rnd family proteins.

Key words: cytokinesis, RhoGAP, testis.

INTRODUCTION

RhoGTPases (Rho, Rac and Cdc42) are evolutionarily conserved signalling molecules belonging to the Ras superfamily of small G-proteins. In diverse organisms from yeast to mammals, they have been implicated in the regulation of multiple cellular processes that are essential for development and differentiation; these include cell-shape change, cell adhesion and migration, cell-cycle progression and cytokinesis, among others [1–3]. Involvement of RhoGTPases in so many diverse functions and in various cell types implies that they are regulated by multiple and cell-specific mechanisms. Indeed, a multiplicity of RhoGEFs (guanine-nucleotide-exchange factor for RhoGTPases) [4] and RhoGAPs (GTPase-activating protein for RhoGTPases) [5] have been described that contribute to activation and deactivation of RhoGTPases respectively. In addition, the recent finding that GTPase-deficient Rho proteins, such as RhoH and Rnd family proteins, antagonize RhoGTPase effects through specific signalling pathways suggests an additional level of regulation of RhoGTPases [6,7].

In recent years, we have described a RhoGAP gene that we have called male-germ-cell RacGAP (*MgcRacGAP*), as it is primarily expressed in male germ cells [8]. MgcRacGAP mRNA was also detected in lower amounts in many human tissues and was found to be highly expressed in the proliferative areas of mouse embryonic cerebral cortex [8,9]. Additionally, *MgcRacGAP* expression was found to be growth-regulated in terminally differentiating 3T3-L1 adipocytes [10] and was shown to control growth and differentiation in HL-60 leukaemic cells [11].

A critical role of MgcRacGAP in cell division was first demonstrated by genetic studies in *Caenorhabditis elegans*, as a missense mutation in CYK-4, the product of the orthologous gene of *MgcRacGAP* in *C. elegans*, was shown to preclude cytokinesis in embryonic cells [12]. Immunohistochemical studies revealed that CYK-4/MgcRacGAP, which is mainly localized in the nucleus of interphasic cells, spreads throughout the cytoplasm in prometaphase, accumulates in the mitotic spindle and concentrates in the midbody during cytokinesis. CYK-4/MgcRacGAP has recently been shown to associate with the mitotic kinesin-like protein ZEN-4/CeMKLP1 (MKLP1 orthologue in *C. elegans*) in the midzone/midbody of dividing somatic cells; this complex is required for microtubule bundling and for the completion of cytokinesis [12–14]. As a RhoGAP, MgcRacGAP has been proposed to inactivate RhoA, the only RhoGTPase which has been localized to the midbody and shown to have a key role in cytokinesis [15,16].

Recently, mice with a homozygous gene-trap vector insertion in the *MgcRacGAP* gene were reported to die during

Abbreviations used: COG, conserved oligomeric Golgi; ECL®, enhanced chemiluminescence; GST, glutathione S-transferase; GAP, GTPase-activating protein; MgcRacGAP, male-germ-cell RacGAP; MKLP1, mitotic kinesin-like protein 1.

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pre-implantation development; pre-implantation embryos were found to display both a dramatic decrease in cell numbers and frequent multinucleated blastomeres, indicating that MgcRacGAP cannot be replaced by other RhoGAPs during early cleavage of mammalian embryos [17].

Taken together, these results demonstrate that MgcRacGAP has an essential role *in vivo* in somatic cell division and indicate that it operates in evolutionarily conserved mechanisms controlling cytokinesis [15,18].

In adult human and mouse, *MgcRacGAP* genes have been found mostly expressed in male germ cells at spermatocyte stage; however, owing to embryonic lethality in *MgcRacGAP*-null mutant mice ([17], and N. Naud, M. A. Ripoche and L. Dandolo, unpublished work), the potential functions of MgcRacGAP in meiosis or post-meiotic maturation of germ cells could not be investigated in this model and have remained so far undetermined. Indeed, spermatogenesis provides an interesting model of 'variant' cytokinesis, since both mitotic and meiotic cell divisions are characterized by an incomplete cytokinesis with persistent intercellular cytoplasmic bridges [19,20].

As an approach to these issues, we have studied the stage-dependent expression, subcellular localization and molecular interactions of MgcRacGAP in male germ cells; in the present paper, we report that, surprisingly, the widespread RhoGTPases RhoA, RhoB, Rac1 and Cdc42 are not detected in spermatocytes and spermatids, whereas MgcRacGAP is specifically expressed in these cells, and Rnd2, an atypical Rho family GTPase, primarily expressed in testis, is a probable partner of MgcRacGAP in male germ cells.

EXPERIMENTAL

In situ hybridization experiments

A 684 bp fragment of *Rnd2* cDNA was subcloned in a Bluescript KS plasmid (Stratagene), allowing both T3 and T7 transcription; [^{35}S]UTP-labelled antisense or sense cRNA probes were obtained by linearizing the plasmid with *Pst*I or *Xho*I respectively and transcribing with T3 or T7 RNA polymerase. Paraffin sections (4 μm) of human adult testis were hybridized overnight at 55 °C, washed as described previously in [21] and exposed to Ilford K5 emulsion (Knutsford, Cheshire, U.K.) for 3 or 4 days. After development of the emulsion, sections were counterstained with Toluidine Blue and analysed.

Antibodies

The primary antibodies used for immunolocalization experiments or for immunohistology were chicken polyclonal antibodies against the GAP domain of human MgcRacGAP [8], rabbit polyclonal anti-Rnd2 antibodies, mouse monoclonal anti-α-tubulin antibodies (Sigma) and rabbit polyclonal anti-RhoA antibodies (Santa Cruz Biotechnology). Golgi apparatus was labelled with rabbit polyclonal anti-Rab6 (Santa Cruz Biotechnology) or mouse monoclonal anti-γ-adaptin antibodies (Transduction Laboratories, Lexington, KY, U.S.A.). For immunoprecipitation, we used mouse monoclonal antibodies against c-myc tag (9E10; Boehringer Mannheim) and FLAG tag (M2; Sigma). The secondary antibodies used in immunofluorescence studies were FITC-conjugated rabbit anti-chicken IgY (Jackson Immunoresearch Laboratories, Westgrove, PA, U.S.A.), Cy3-conjugated goat anti-rabbit IgG (Molecular Probes) and Texas Red-conjugated horse anti-mouse IgG (Vector Laboratories) antibodies.

Cell isolation and Western blot analysis

Purification of rat testis cell populations was performed according to described procedures [21]; degrees of purity of cell populations were as follows: greater than 99 % for Sertoli cells, greater than 98 % for peritubular cells and greater than 90 % for spermatogonia, pachytene spermatocytes and early spermatids. For Western blotting experiments, detergent extracts from these cells were resolved by SDS/PAGE (12 % gel) and electroblotted on to nitrocellulose. Filters were incubated sequentially with primary antibodies against MgcRacGAP or RhoGTPases and peroxidase-labelled secondary antibodies (Dako Ltd, Glostrup, Denmark). Proteins were detected by enhanced chemiluminescence (ECL®, Amersham Biosciences).

Immunohistochemistry

Testes of adult rats were carefully dissected out and immersed in Bouin's fixative solution for 24 h. Samples were then dehydrated through an increasing gradient of ethanol and acetone, before being embedded in paraffin wax. Testis sections (4 μm thick) were microwaved (2 × 3 min in PBS) after mounting. Sections were then incubated with anti-Rnd2 or anti-RhoA antibodies. Complexes were revealed using a goat anti-rabbit biotinylated antibody (Dako Ltd), coupled with a streptavidin–peroxidase amplification combination. Sections were counterstained with haematoxylin. After colour development, the samples were mounted with Glycergel (Dako Ltd) for microscopic observation.

Spermatogenic cell preparation by squash procedure and immunofluorescence microscopy

Freshly collected mouse testes were dissected and fixed in 2 % (w/v) formaldehyde, 0.02 % (w/v) SDS and 0.05 % (v/v) Triton X-100 for 20 min. The seminiferous tubules were gently squashed between slide (Superfrost Plus, Menzel-Glaser, Freiburg, Germany) and cover glass (Erie Scientific, Portsmouth, NH, U.S.A.) and rapidly frozen in liquid nitrogen. Spermatogenic cells were then washed briefly and blocked with PBS, 0.15 % (v/v) BSA and 0.1 % (v/v) Tween 20 for 1 h. Cells were then incubated with primary antibodies overnight at 4 °C, washed in PBS once for 10 min and twice for 5 min and incubated with secondary antibodies for 1 h at room temperature (20 °C). After washing the secondary antibody, slides were mounted with a drop of 0.2 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride/Vectashield (Vector Laboratories).

For cytokinesis observations, testes were dissected and fixed with 2 % (w/v) formaldehyde in microtubule stabilizing buffer (50 mM Pipes, pH 7.5, 5 mM EGTA and 5 mM $MgSO_4$) for 30 min, squashed and rapidly frozen in liquid nitrogen; cells were then extracted with cold methanol (20 min, − 20 °C) [22] and processed as above. Testes were taken from 7-day-old mice to analyse spermatogonia, which are the only spermatogenic cells present at this stage, and 21-day-old mice to analyse meiotic divisions. In adult testes, pachytene cells are easily distinguished from early spermatids based on cell size and chromatin configuration; hence pachytene spermatocytes are characterized by their large cell size and large chromatin cords or blotches occupying much of the nuclear interior, whereas early spermatids are smaller sized cells with nuclei showing a finely granular texture and a large dense-staining nucleolus [23].

Cell preparations were analysed under a Zeiss Axiophot epifluorescence microscope; images were digitally acquired with

a cooled charge-coupled-device ('CCD') camera (Hamamatsu) and processed using Adobe Photoshop 4.

Cell culture and transient transfections

HeLa cells or COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (w/v) foetal calf serum at 37 °C with an atmosphere containing 5 % CO_2. For *in vitro* binding assays, HeLa cells were transiently transfected (24 h) with a pRK5-myc-Rnd2 expression vector using the FuGENE 6 reagent (Roche Molecular Biochemicals). For co-immunoprecipitation experiments, COS-7 cells were co-transfected with pRK5-FLAG-MgcRacGAP and pRK5-myc-Rac L61 or Rnd2 expression vectors.

Immunoprecipitation

Transfected cells were washed in PBS and extracted in a lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 % (v/v) Triton X-100, 0.5 % (v/v) Nonidet P40 and protease inhibitors. The lysate was then centrifuged at 15 000 ***g*** for 10 min at 4 °C. Equal aliquots of the supernatant fractions (400 μg of protein) were incubated overnight with appropriate antibodies and Protein A–Sepharose CL-4B beads (Amersham Biosciences) at 4 °C. Beads were washed three times with lysis buffer, and bound proteins were resolved by SDS/PAGE (12 % gel) and electroblotted on to nitrocellulose. Filters were incubated with primary antibodies against c-myc tag or FLAG tag, followed by peroxidase-labelled secondary antibodies and developed using the ECL® detection system.

In vitro binding assay

Equimolar quantities of glutathione S-transferase (GST), the GAP domain of Bcr fused to GST [GST–Bcr(GAP)] or the GAP domain of MgcRacGAP fused to GST [GST–Mgc(GAP)] fusion proteins were coupled to 40 μl of glutathione–Sepharose beads and incubated with a HeLa cell lysate expressing myc-Rnd2 for 1.5 h at 4 °C, in a binding buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1 % (v/v) Triton X-100, 2 mg/ml BSA and protease inhibitors. Beads were washed twice with the binding buffer without BSA. Bound proteins were resolved by SDS/PAGE (12 % gel) and electroblotted on to nitrocellulose. Filters were incubated sequentially with anti-Rnd2 rabbit antibodies and peroxidase-labelled swine anti-rabbit immunoglobulins (Dako Ltd), and developed as above.

RESULTS

Expression of MgcRacGAP and Rnd2 in male germ cells

Previous results of Northern blotting and *in situ* hybridization experiments have demonstrated that MgcRacGAP mRNA is highly expressed in testis and accumulates in the male germline at spermatocyte stage [8]. Despite multiple attempts, immunodetection of MgcRacGAP on fixed testis sections remained unsuccessful; however, Western blot analysis of elutriated rat germ cells clearly demonstrated that MgcRacGAP is expressed in spermatocytes and spermatids, but not in spermatogonia nor in mature spermatozoa (Figure 1).

In a search for RhoGTPases that might be physiologically regulated by MgcRacGAP in male germ cells, we first analysed the expression of Rac, Cdc42, RhoA and RhoB in elutriated rat germ cells; surprisingly, although these widespread RhoGTPases were all detected in testis extracts, none of them were found expressed significantly in spermatocytes or spermatids (Table 1), thus suggesting that MgcRacGAP must have different partner(s) in these cells. Since Rnd2 mRNA has been found by Northern blot to accumulate primarily in testis in human ([24], and N. Naud and G. Gacon, unpublished work), we looked for a cell-specific expression of Rnd2 in this organ. *In situ* hybridization of an Rnd2 cRNA probe to sections of adult human testis showed that Rnd2 mRNA is restricted to germ cells at spermatocyte and spermatid stages (Figure 2A). In good agreement with this pattern of gene expression, immunodetection assays on testis sections revealed that Rnd2 protein accumulates in a sickle-shaped acrosome-like structure in early spermatids and in elongated spermatids (Figure 2B). As expected from the results of Western blot experiments, RhoA could not be detected in male germ cells at any stage of spermatogenesis on testis sections; in contrast, we observed a strong expression of RhoA in Leydig cells (results not shown).

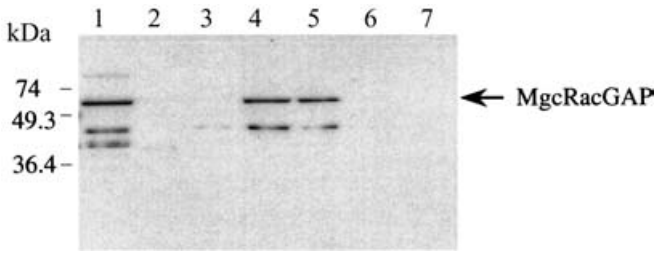

Figure 1 Western blotting analysis of MgcRacGAP in rat testis cell populations

Enriched cell populations of rat testis were obtained as described in the Experimental section; the immunoblot was probed for MgcRacGAP with an affinity-purified chicken polyclonal antibody against the Mgc-GAP domain. Lane 1, testis extract; lane 2, peritubular cells; lane 3, spermatogonia; lane 4, spermatocytes; lane 5, spermatids; lane 6, Sertoli cells; lane 7, residual bodies. (Low-molecular-mass bands correspond to MgcRacGAP-degradative products generated during testis dissection and cell purification.)

Table 1 Cell-specific expression of MgcRacGAP and RhoGTPases in testis

Each protein was detected by Western blotting in extracts from murine brain, rat testis and elutriated rat testis cells. Spg, spermatogonia; Spc, spermatocytes; Spt, spermatids; Spz, spermatozoa; –, no signal.

Protein	Brain	Testis	Peritubular cells	Sertoli cells	Spg	Spc	Spt	Spz
MgcRacGAP	++	++	–	–	–	++	++	–
Rac1	++	++	++	+	+	–	–	–
Cdc42	++	+	++	+	+	–	–	–
RhoA	++	+	+	+	–	–	–	–
RhoB	++	++	–	–	–	–	–	++

Subcellular localization of MgcRacGAP and Rnd2

Since, despite multiple trials, MgcRacGAP could not be immunodetected on testis sections, we attempted to detect the protein in male germ cells prepared by a squash procedure. Consistent with the results of expression studies ([8] and Figure 1), MgcRacGAP was not found in spermatogonia; in particular, no trace of the protein could be detected in the

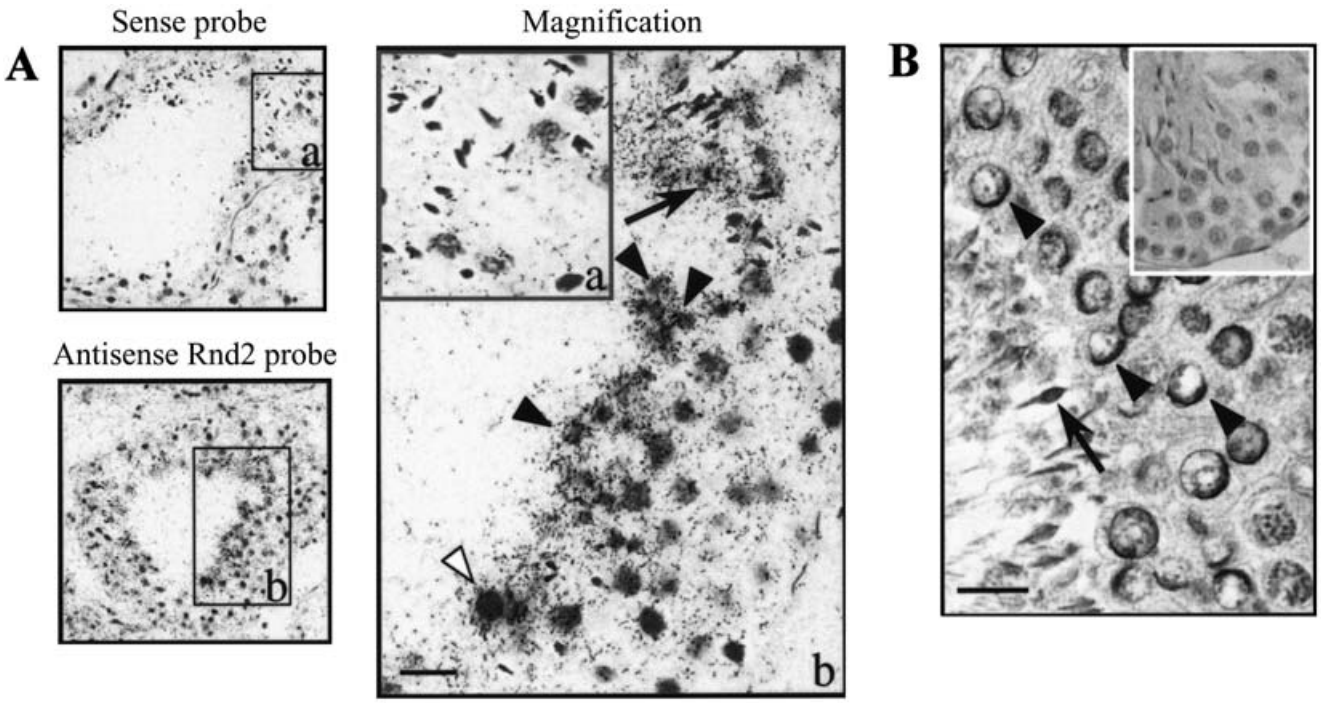

Figure 2 Expression of *Rnd2* in male germ cells

(**A**) *In situ* hybridization analysis of *Rnd2* in human adult testis. Paraffin sections of adult testis were hybridized with ^{35}S-labelled sense or antisense cRNA probe for *Rnd2*. Counterstaining of cell nuclei with Toluidine Blue and spatial localization of the cells in seminiferous tubules permit identification of spermatogonia (localized in the periphery of seminiferous tubules), spermatocytes (with large strongly stained nuclei, close to the lumen) and early or elongated spermatids (with small, lightly stained or oblong nuclei respectively). Under magnification (a and b), strong signals were evident over spermatocytes (white arrowhead), early spermatids (black arrowhead) and elongated spermatids (arrow). Scale bar, 30 μm. (**B**) Immunodetection of Rnd2 in adult rat testis paraffin sections. Rnd2 immunoreactivity is detected in early spermatids (black arrowheads) in a sickle-shaped acrosome-like structure and in elongated spermatids (arrow). Inset, pre-immune serum control. Scale bar, 10 μm.

midzone region of mitotic spermatogonia (Figure 3A). Also in agreement with Western blot data, MgcRacGAP was detected in spermatocytes and spermatids. Similar to the pattern previously observed in dividing somatic cells, MgcRacGAP was found associated to the midzone during cytokinesis of meiotic division as well (Figure 3B). Surprisingly, however, MgcRacGAP was not found to be concentrated in the nucleus of male germ cells, as observed in interphasic somatic cells ([13], and L. Morin, N. Naud, A. Touré and G. Gacon, unpublished work); instead, a staining for MgcRacGAP was visible in the whole cytoplasm and in a juxtanuclear structure appearing as a rounded shape in spermatocytes and flattened over the nucleus in early spermatids (Figure 4A). No significant amount of MgcRacGAP could be detected in elongated spermatids (results not shown).

In the same cell preparations, Rnd2 immunolabelling also showed a cytoplasmic pattern and juxtanuclear spots superimposable with MgcRacGAP staining, indicating similar localization of the two proteins in spermatocytes and early spermatids (Figure 4A). Unlike MgcRacGAP, Rnd2 could not be detected in the midzone of meiotic cells, but was found expressed in elongated spermatids, showing a perinuclear immunostaining suggestive of the acrosomal cap (results not shown).

Acrosome is known to derive from the *trans*-Golgi network as a spherical juxtanuclear vesicle in early spermatids [25,26]; then, the vesicle enlarges and flattens over the surface of the nucleus, leading to the acrosomal cap in elongated spermatids and spermatozoa. Indeed, two markers of the *trans*-Golgi network, i.e. Rab6 GTPase [27] and γ-adaptin (results not shown) revealed a clear staining of the juxtanuclear organelles containing MgcRacGAP and Rnd2 (Figure 4B).

Taken together, these results demonstrate that Rnd2 is co-expressed with MgcRacGAP in spermatocytes and early spermatids and that the two proteins co-localize in Golgi-derived pro-acrosomal vesicles and the acrosome.

Rnd2–MgcRacGAP interaction

As male germ cells are not easily amenable to cell culture, we performed interaction studies in Rnd2-transfected cells. Rnd2 was expressed in HeLa or COS-7 cells and the interaction with MgcRacGAP was analysed by GST pull-down and co-immunoprecipitation assays.

Although GST alone showed no significant association with Rnd2, GST–Mgc(GAP), previously described in [21], was capable of binding Rnd2 in a lysate from Rnd2-transfected HeLa cells (Figure 5A), indicating that Rnd2 stably interacts *in vitro* with the GAP domain of MgcRacGAP. Control experiments using a GST–Bcr(GAP) fusion protein, as described in [21], showed that the GAP domain of Bcr (amino acids 1050–1271) also binds Rnd2, suggesting that Rnd2 may associate with other RhoGAPs.

Association between MgcRacGAP and Rnd2 was also assessed by co-immunoprecipitation. Lysates from COS-7 cells expressing c-myc–Rnd2 and FLAG–MgcRacGAP were immunoprecipitated with anti-c-myc antibodies, followed by Western blotting with anti-FLAG antibodies. As shown in Figure 5(B), MgcRacGAP was co-immunoprecipitated with Rnd2, indicating that the two

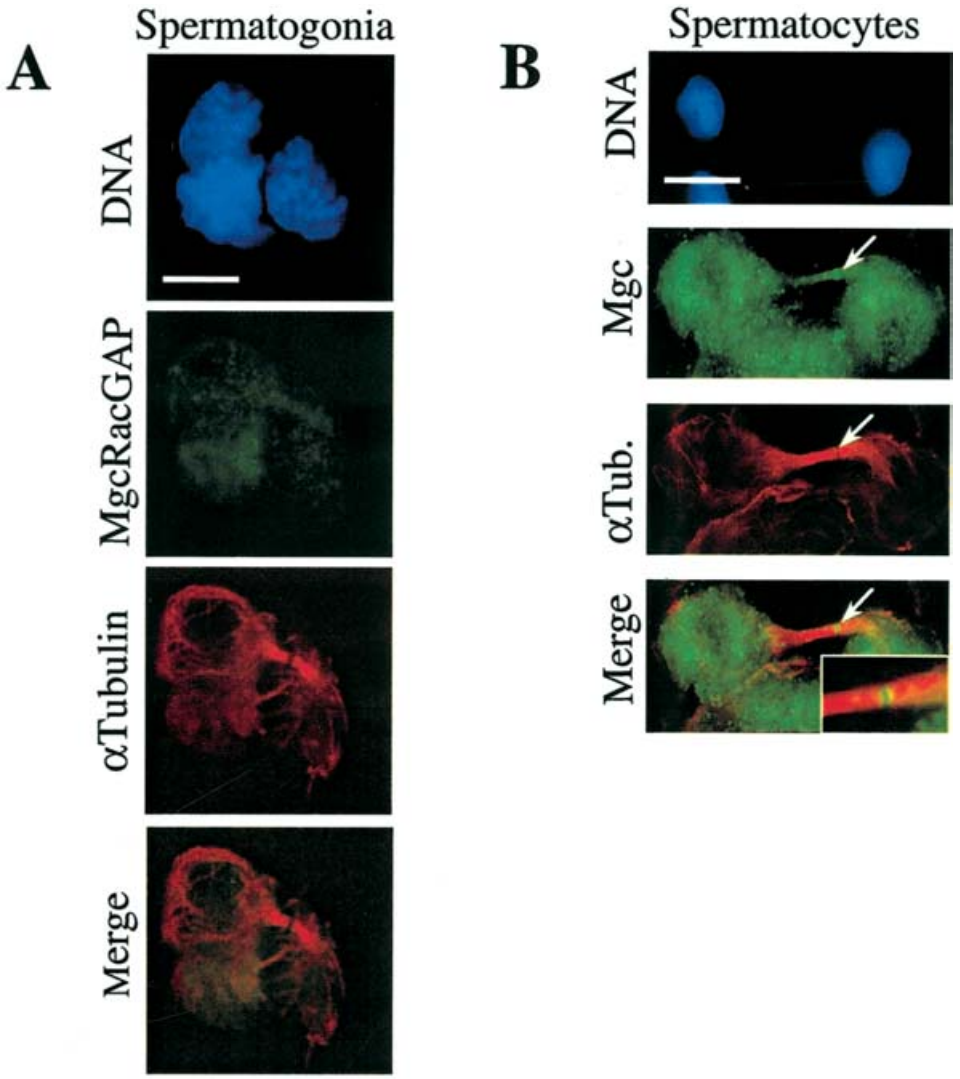

Figure 3 Detection of MgcRacGAP in dividing male germ cells

Late-stage telophase spermatogonia (**A**) or spermatocytes (**B**) were analysed following the squash procedure of 7-day-old and 21-day-old mouse testis, respectively. MgcRacGAP was detected with an affinity-purified antibody against the GAP domain, microtubules were detected with a monoclonal antibody against α-tubulin, and DNA was labelled with 4′,6-diamidino-2-phenylindole dihydrochloride. Late-stage cytokinetic cells are shown displaying an hourglass-shaped midzone spindle (red). As expected from Western blotting, MgcRacGAP is absent in spermatogonia (**A**; scale bar, 10 μm) and is detected in spermatocytes (**B**; scale bar, 15 μm) in cytosol and is also, as shown in the inset, concentrated in the midbody (arrow).

proteins associate within the cells. This result was confirmed by reciprocal immunoprecipitation: Rnd2 was co-immunoprecipitated with MgcRacGAP by anti-FLAG antibodies (Figure 5B). As judged from parallel experiments using RacL61 (a constitutively activated Rac mutant) as a partner of MgcRacGAP, Rnd2–MgcRacGAP and RacL61–MgcRacGAP molecular complexes exhibited similar stability (Figure 5B).

Taken together, the results of binding studies demonstrate that Rnd2 stably interacts with the GAP domain of MgcRacGAP and suggest that the two proteins associate within the cell. *In vitro* binding assays also indicate that other RhoGAP domains (Bcr) can associate with Rnd2 and also that Mgc(GAP) can bind Rnd3, another member of the Rnd family (results not shown); therefore, although additional studies are required to confirm these results, interaction between Rnd family proteins and RhoGAPs may prove to be a widespread mechanism.

DISCUSSION

We have shown here that the widespread or quasi-ubiquitous Rho family GTPases RhoA, Rac1 and Cdc42 are absent or very poorly expressed in the male germline at meiotic and post-meiotic stages. This result appears somewhat puzzling, since RhoGTPases are strongly implicated in many basic cellular functions (including regulation of cell shape and differentiation, cell migration, cell-cycle progression and cytokinesis), which must be tightly regulated through meiosis and spermiogenesis. Regulatory proteins of the RhoGAP family have already been found expressed in male germ cells [28–30]; in addition, in the present paper, we show that MgcRacGAP, initially found to be abundant in testis, is specifically expressed in spermatocytes and spermatids. MgcRacGAP is a bona fide RhoGAP for Rac and Cdc42, but is much less active *in vitro* on RhoA [8]; in dividing

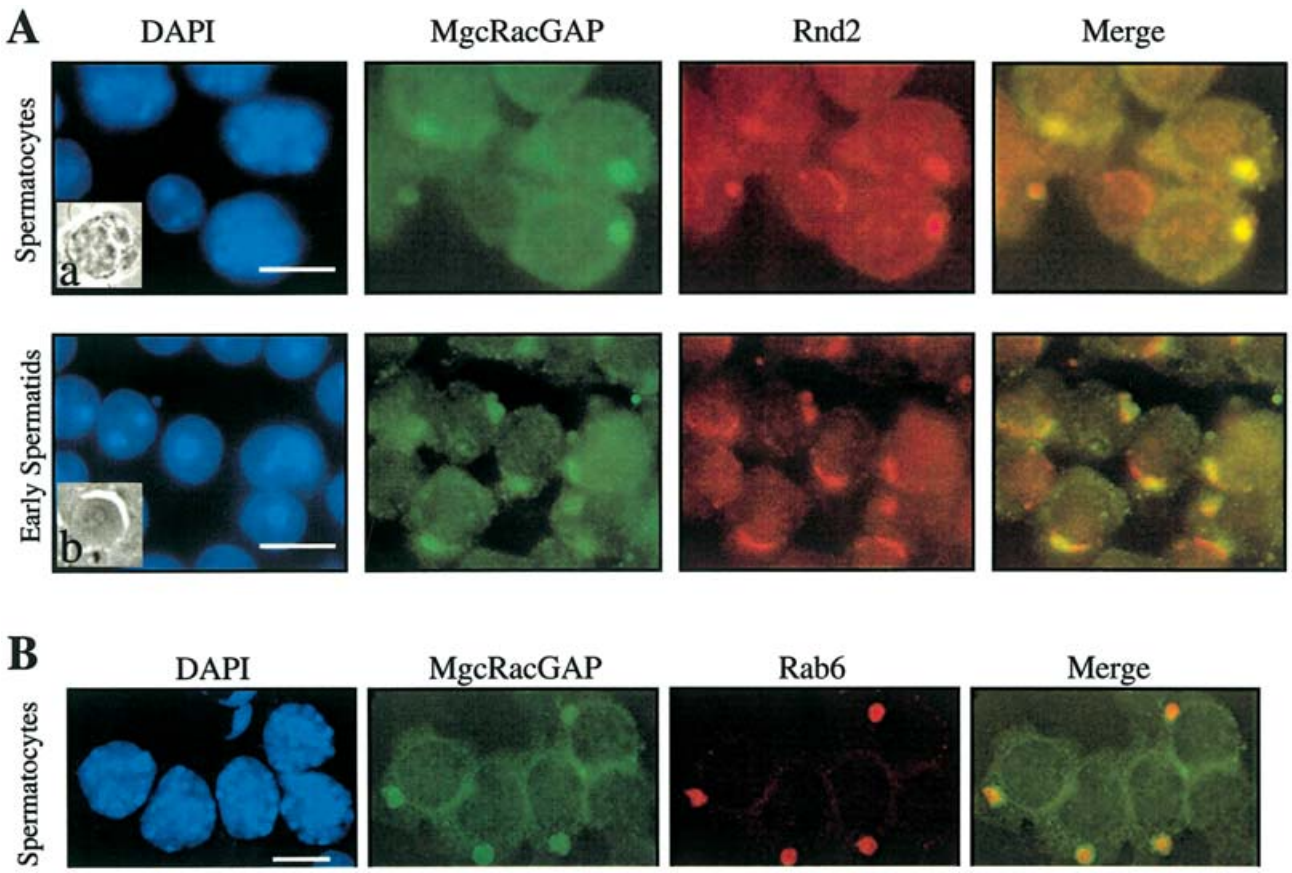

Figure 4 Detection of MgcRacGAP and Rnd2 in spermatogenic cells

(**A**) Spermatogenic cells were prepared from adult mouse testis by the squash procedure and stained for MgcRacGAP (chicken primary antibodies revealed by green fluorescence), Rnd2 (rabbit primary antibodies revealed by red fluorescence) and DNA (labelled with 4′,6-diamidino-2-phenylindole dihydrochloride, blue fluorescence). A strong label for MgcRacGAP and Rnd2 is detected in spermatocytes in Golgi apparatus and in early spermatids as a sickle-shaped acrosome-like structure. Control experiments performed with rabbit pre-immune serum for Rnd2 and chicken IgY to an irrelevant protein for MgcRacGAP showed no significant staining. Scale bar, 10 μm. Phase-contrast images of the cells are shown in insets a and b. (**B**) Using the same procedure as in (**A**), MgcRacGAP (green) appears to co-localize with the *trans*-Golgi network marker Rab6 (red). Scale bar, 10 μm.

somatic cells, MgcRacGAP has been shown to concentrate in the midbody and to be required for the completion of cytokinesis. Among the RhoGTPases tested, RhoA is the only one that is localized to the midbody and is clearly essential for cytokinesis: activation of RhoA is required for actomyosin-ring contraction and furrow ingression, and its down-regulation is essential for cytokinesis completion [31]. Thus it appears probable that, within the cell, RhoA is the key substrate of MgcRacGAP activity during cytokinesis [15,18].

Therefore the absence of RhoGTPases and particularly RhoA in spermatocytes and spermatids prompted us to analyse the expression, subcellular localization and molecular interactions of MgcRacGAP in these cells more accurately.

In the present paper, we show that the Rho family protein Rnd2 is also expressed in spermatocytes and spermatids and that a stable Rnd2–MgcRacGAP molecular complex forms both *in vitro* and in cells co-expressing the two proteins. This suggests that a physiological interaction between the two proteins could take place in male germ cells.

Rnd proteins, Rnd1, Rnd2 and Rnd3 (also known as RhoE) constitute a branch of RhoGTPases sharing around 60 % sequence identity and showing distinct structural, biochemical and functional properties [32–34]. Due to key amino-acid substitutions corresponding to Ras activating mutations, Rnd proteins lack intrinsic and GAP-stimulated GTPase activity and are supposed to be constitutively bound to GTP. Rnd1 and Rnd3 have been shown to inhibit the RhoA-dependent formation of actin stress fibres and focal adhesions in fibroblasts and to antagonize RhoA-mediated events in various other cell types. The molecular mechanism underlying these effects is not understood; however, a protein binding specifically to Rnd subfamily members, named Socius, has been recently found to be involved in the Rnd-induced disassembly of actin stress fibres [7]. Interestingly, the inhibitory effect of Socius requires its Rnd-dependent translocation to cell periphery [7]; this suggests that Socius is a downstream target of Rnd proteins and participates in a Rnd-induced signal transduction pathway leading to reorganization of the actin cytoskeleton.

The function of Rnd2 is still more elusive as, unlike Rnd1 and Rnd3, Rnd2 does not antagonize RhoA-dependent actin remodelling when expressed in fibroblasts, nor does it induce any obvious morphological effect. Moreover, recombinant Rnd2 produced in bacterial cells is highly unstable, thus precluding detailed analysis of its biochemical properties.

The finding that Rnd2 binds to MgcRacGAP in both GST pull-down and co-immunoprecipitation experiments is the first evidence that Rnd proteins can associate stably with RhoGAPs. Owing to the fact that purified recombinant Rnd2 is highly unstable ([32], and N. Naud, O. Dorseuil and G. Gacon,

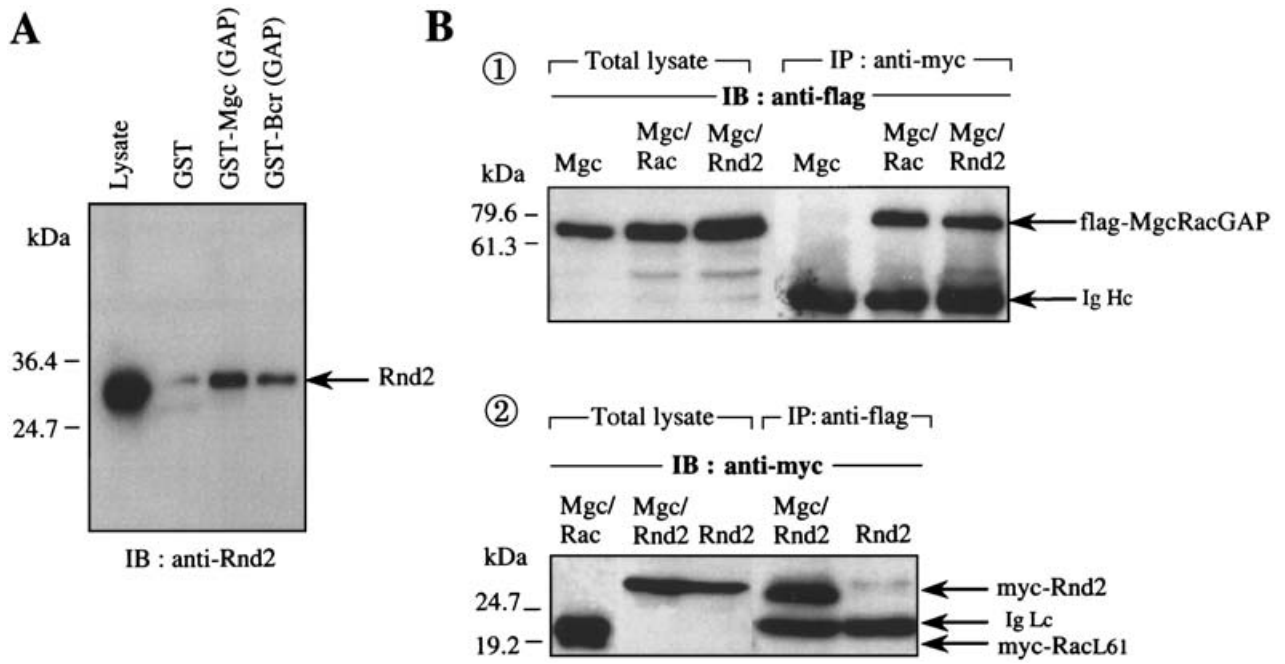

Figure 5 Interaction of MgcRacGAP with Rnd2

(**A**) GST pull-down assays were performed with lysates of HeLa cells transfected with the expression vector pRK5-myc-Rnd2. Lysates were incubated with GST, GST–Mgc(GAP) or GST–Bcr(GAP) bound to glutathione–Sepharose beads. Bound proteins were analysed by Western blotting using an anti-Rnd2 antibody. Results are representative of three independent experiments. (**B**) Panel 1, FLAG–MgcRacGAP was detected by immunoblotting with anti-FLAG antibodies in lysates from COS-7 cells transfected with MgcRacGAP alone (Mgc) or co-transfected with MgcRacGAP and either myc–RacL61(Mgc/Rac) or myc–Rnd2 (Mgc/Rnd2). Lysates were immunoprecipitated with anti-myc antibodies, followed by anti-FLAG immunoblotting. Panel 2, myc–Rnd2 and myc–RacL61 were detected by immunoblotting with anti-myc antibodies in lysates of COS-7 cells transfected with myc-Rnd2 alone or co-transfected with MgcRacGAP and either myc-Rnd2 (Mgc/Rnd) or myc-RacL61 (Mgc/Rac). Lysates were immunoprecipitated with anti-FLAG antibodies followed by anti-myc immunoblotting. Results are representative of three independent experiments. IP, immunoprecipitation; IB, immunoblotting. IgLc, immunoglobulin light chain; IgHc, immunoglobulin heavy chain.

unpublished work), we could not formally demonstrate a direct interaction between Rnd2 and MgcRacGAP; however, as indicated by GST pull-down experiments, Rnd2 binds to MgcRacGAP and Bcr through their isolated GAP domains, suggesting the possibility of a direct interaction of Rnd2 with the GTPase-binding site of RhoGAPs. The biological significance of such an interaction is not clear since Rnd proteins are devoid of GTPase activity and are insensitive to RhoGAPs. However, Rnd–RhoGAP interactions could be physiologically meaningful, if we assume that interacting RhoGAPs function as downstream targets of Rnd proteins.

The presence of Rnd2 and MgcRacGAP in the *trans*-Golgi network compartment and in Golgi-derived structures involved in acrosome biogenesis would support the hypothesis for a specific role of Rnd2–MgcRacGAP complex in this process. Previous studies [12–14] have demonstrated that MgcRacGAP is required for central spindle formation in cytokinetic somatic cells and that microtubules constitute a probable target for MgcRacGAP action. Thus MgcRacGAP has been shown to both directly bind to tubulin [13] and associate with the mitotic kinesin-like protein MKLP1 to form a complex with microtubule-bundling activity [14]. Since microtubules are involved in both the traffic of Golgi vesicles [35] and the early steps of acrosome differentiation [36,37], MgcRacGAP may operate in either or both of these functions by interfering with microtubules and vesicle trafficking.

Interestingly, genetic studies in *Drosophila melanogaster* have recently demonstrated that the *cog5* gene product, a subunit of the conserved oligomeric Golgi (COG) complex, involved in Golgi architecture and vesicle trafficking, is required for three functions during spermatogenesis: cytokinesis, acroblast formation and cell elongation [38]. This suggests a possible model for unifying MgcRacGAP functions in male germ cells.

Recently, Rnd2 has been shown to bind vacuolar protein sorting 4-A ('Vps-4A'), a protein involved in endosomal maturation; these results point to a role for Rnd2 in endosomal trafficking [39]. Since close contacts exist between the *trans*-Golgi network and endosomes [40], it will be interesting to determine whether Rnd2 and its partners may interfere with trafficking of vesicles in these two compartments.

MgcRacGAP, but not Rnd2, could also be detected in low amounts in the midzone of meiotic cells, consistent with its reported localization and proposed function in cytokinetic somatic cells. However, cytokinesis of male germ cells differs from that described in dividing somatic cells: throughout spermatogenesis, both mitotic and meiotic cell divisions are characterized by incomplete cytokinesis, which results in the persistence, between daughter cells, of cytoplasmic bridges called ring canals [19,20]. The mechanisms underlying this variation are not understood. Whether or not these particular aspects of male-germ-cell cytokinesis may be related to the absence (or low expression) of RhoA in these cells is a novel question raised by our observations and should be addressed in future studies.

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