Characterization of Rab3A, Rab3B and Rab3C: different biochemical properties and intracellular localization in bovine chromaffin cells

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In this study we examined the biochemical properties and subcellular localization of Rab3A, Rab3B and Rab3C in bovine adrenal chromaffin cells. The K_d for guanosine 5'-[γ -thio]triphosphate (GTP[S]) of the three Rab3 proteins was 15, 2700 and 204 nM for Rab3A, Rab3B and Rab3C respectively. The intrinsic GTPase activity of the three Rab3 proteins seemed similar and was increased approx. 3-fold by bovine chromaffin cell lysate. Truncation of the C-terminal 31 amino acid residues decreased the binding affinity for GTP[S] of the three Rab3 proteins. When the C-terminus of Rab3C was replaced with that

of Rab3A, the binding affinity of Rab3C for GTP[S] was decreased, but the replacement did not affect the affinity of Rab3B for GTP[S]. Immunostaining experiments showed that Rab3A, Rab3B and Rab3C are localized separately within chromaffin cells. Anti-Rab3A and anti-Rab3C antibodies stained vesicle-like structures, whereas anti-Rab3B antibody distinctly stained the plasma membrane. In summary, bovine chromaffin cells express the three Rab3 proteins but the subcellular localization and biochemical properties of the three Rab3 proteins are distinct.

INTRODUCTION

Rab family proteins have been shown to be associated with specific membranes of the endocytotic and exocytotic pathways. Evidence suggests that virtually each step in the intracellular membrane trafficking requires unique Rab protein [1]. So far more than 30 members of the Rab family have been identified. Among these, only proteins of the Rab3 family have been implicated in regulated exocytotic pathways. The Rab3 family consists of at least four members, namely Rab3A, Rab3B, Rab3C and Rab3D. The different Rab3 proteins are very similar and contain 77–85% identical amino acid residues (Figure 1). The most divergent regions are in the C-terminal region [2].

In contrast with other members of Rab family, which are found in most tissues examined, Rab3 proteins are expressed exclusively in cells that have a high activity of regulated exocytosis [3–6]. Rab3A is expressed in neuronal and neuroendocrine cells, where it is associated with synaptic vesicles and secretory granules [3–5, 7–8]. Several lines of evidence suggest that Rab3A is involved in synaptic vesicle recruitment and acts as a barrier to prevent exocytosis in bovine chromaffin cells until exocytosis is triggered [9–12]. Suppression of Rab3B by using an anti-sense oligonucleotide technique results in an inhibition of secretion from pituitary cells [13]. Rab3D is expressed predominantly in adipocytes and is induced during adipogenesis [6]. Rab3D has also been suggested to regulate the exocytosis of glucose transporter GLUT 4-containing granules [14]. Furthermore although Rab3D and Rab3A are co-localized in adipocytes, they are associated with different membranes [6, 14].

The physiological function of low-molecular-mass G-proteins is thought to depend on the interconversion between GDP and GTP that is controlled by several regulatory proteins [15]. Several of these proteins for Rab3A have been identified and characterized: GTPase-activating protein (GAP), guanine nucleotide-releasing factor (GRF) and GDP dissociation inhibitor [15].

However, the physiological significance of these proteins remains to be characterized.

In this study we show that Rab3A, Rab3B and Rab3C are co-localized in bovine chromaffin cells and we have further characterized the biochemical properties and subcellular localization of the three Rab3 proteins and their GAPs.

MATERIALS AND METHODS

Expression and purification of recombinant Rab3 proteins

The gene for Rab3A was isolated from an NG108-15 cell cDNA library as previously described [12]. Rab3B and Rab3C genes were from bovine adrenal chromaffin cell cDNA library (Clontech, Palo Alto, CA, U.S.A.). It should be noted that there are 8 extra amino acid residues in the N-terminus of the deduced Rab3C sequence compared with those of Rab3A and Rab3B [2]. In this study these extra amino acids were not included when Rab3C was expressed because our goal was to examine the influence of the C-terminal variable region on the biochemical properties of the three Rab3 proteins (see below). Adding the extra amino acids could have complicated the results.

For expression in pET3a system, new *NdeI* restriction sites were added by PCR to the open reading frames encoding Rab3 proteins by using the following primers: Rab3A, 5'-GCCATATGGCCTCAGCCACAGACTCTCGATATGGGACAG-3' and 5'-GCCATATGGTCAGCAGGCGCAATCCTGATGAGGT-3'; Rab3B, 5'-GCCATATGGCTAGCAGCAGCAGTCTGCTG-3' and 5'-GCCATATGGCTTACGACCGATGGTAAGC-3'; Rab3C, 5'-GCCATATGGCTAGCCACAGTTGGGGTGC-3' and 5'-GCCATATGGCCTCTGCCAAGATGCAGGTATG-3'.

The PCR products were subcloned into pGEM-T (Promega) for sequencing and then subcloned into the *NdeI* site of the expression vector pET3a, and expressed in *Escherichia coli* (BL21/DE3) as described previously [16]. All sequences of the

Abbreviations used: D β H, dopamine- β -hydroxylase; GAP, GTPase-activating protein; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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	I									
Rab3A	MASATDARYG	QKESSDQNF	YMFKILLIIGN	SSVGKTSFLF	RYADDSFTPA					
Rab3B	MASVTDGKAG	VKDASDQNF	YMFKLLIIGN	SSVGKTSFLF	RYADDTFTPA					
Rab3C	MASAQDARYG	QKDSDDQNF	YMFKLLIIGN	SSVGKTSFLF	RYADDSFTSA					
	1				50					
	II									
Rab3A	FVSTVGIDFK	VKTIYRNDKR	IKLQIWDTAG	QERYRTITTA	YYRGAMGFIL					
Rab3B	FVSTVGIDFK	VKTIVYRHEKR	VKLQIWDTAG	QERYRTITTA	YYRGAMGFIL					
Rab3C	FVSTVGIDFK	VKTIVYRHEKR	IKLQIWDTAG	QERYRTITTA	YYRGAMGFIL					
	51				100					
	III									
Rab3A	MYDITNEESF	NAVQDWSQI	KTYSWDNAQV	LLVGNKCDME	DERVVSSESG					
Rab3B	MYDITNEESF	NAVQDWSQI	KTYSWDNAQV	LLVGNKCDME	DERVVSSESG					
Rab3C	MYDITNEESF	NAVQDWSQI	KTYSWDNAQV	LLVGNKCDME	DERVVSSESG					
	101				150					
	IV									
Rab3A	RQLADHLGFE	FFEASAKDNI	NVKQTFERLV	DVICEKMSSE	LDTADPAVTG					
Rab3B	RLLAEQLGFD	FFEASAKDNI	SVRQAFERLV	DAICDKMSDT	LDT.DPSSLG					
Rab3C	GHLEHLGFE	FFETSADKNI	NVKQTFERLV	DIICDKMSSE	LET.DPPITA					
	151				200					
	V									
Rab3A	AKQGPQLTDQ	QAPPHQDCAC								
Rab3B	TSKNTRLSDT	PPLLQONCSC								
Rab3C	AKQNTRLKET	PPPPHNCGC								
	201	220								

Figure 1 Comparison of deduced amino acid sequences and functional domains of the bovine Rab3 proteins

Sequences were obtained from the GenEMBL database. The regions corresponding to the GTP/GDP binding and GTPase catalytic domains (I–IV) and those that have been implicated in the interaction with effectors (effector region, α -helix and loop 7) are indicated by solid bars above the sequences [30–33]. Bold type indicates the residues that are different from Rab3A.

PCR products have been verified as being the sequences of *rab3* genes. Recombinant Rab3 proteins were purified as described by Matteoli et al. [17] and Tucker et al. [18], with slight modifications. The purification was based on the binding activity of Rab3 for guanosine 5'-[γ -thio]triphosphate (GTP[S]). Recombinant Rab3 proteins were purified by this procedure to near homogeneity (see Figure 2).

Expression and purification of His-tagged Rab3 proteins

To express His-tagged proteins, new *NdeI* restriction sites were added to Rab3 open reading frames by PCR by using the following primers. Primers for Rab3A, Rab3B and Rab3C were the same as those used for the wild-type proteins. For truncated Rab3 the C-terminal 31 amino acids were truncated: truncated Rab3A_(1–189), 5'-GGGGGGATCCCTACGACATCTTCTCAC-AGA-3' and 5'-GCCATATGGCCTCAGCCACAGACTCTC-GATATGGGCAG-3'; truncated Rab3B_(1–189), 5'-GGGGGGA-TCCCTAAGACATCTTGTGCGCAA-3' and 5'-GCCATAT-GGCTTCAGTGACCGATGGTAAAGC-3'; truncated Rab3C_(1–189), 5'-GGGGGGATCCCTAGGACATCTTGTAC-AGA-3' and 5'-GCCATATGGCCTCTGCCCAAGATGCCA-GGTATG-3'.

For chimaeric Rab3B/A (B_{1–189}/A_{190–220}) and chimaeric Rab3C/A (C_{1–189}/A_{190–220}), the 190–220 regions of Rab3B and Rab3C respectively were replaced with that of Rab3A and constructed by methods previously described [19]. These chimaeric Rab3s were generated by two-step PCR by using the following primers: Rab3B/A, (1) 5'-TGCCATTTGCGACAA-GATGTCGGAGTCCCT-3' and 5'-CTAGGGACTCCGACA-TCTTGTGCGCAAATGG-3', and (2) 5'-GCCATATGGTCAG-CAGGCGCAATCCTGATGAGGT-3' and 5'-GCCATATGG-CTTCAGTGACCGATGGTAAAGC-3'; Rab3C/A, (1) 5'-GC-CATATGGTCAGCAGGCGCAATCCTGATGAGGT-3' and

5'-GCCATATGGCCTCTGCCCAAGATGCCAGGTATG-3', and (2) 5'-CATCTGTGACAAGATGTCGGAGTCCCTAGA-3' and 5'-TAGGGACTCCGACATCTTGTACAGATGATA-TCCACGA-3'.

The PCR products were subcloned into pGEM-T (Promega) for sequencing and then subcloned into the *NdeI* sites of the expression vector His-pET11d. The sequences of the PCR products were verified as being the mutants, which were constructed as expected. Expression and lysate preparation were the same as those described for full-length Rab3 proteins. His-tagged proteins were purified by a Ni²⁺ column as indicated in the manufacturer's protocol (Novagen, Madison, WI, U.S.A.).

[³⁵S]GTP[S] binding assay

[³⁵S]GTP[S] binding activity was assayed as described previously [20] with slight modifications. Rab3 proteins (Rab3A, 1 pmol; Rab3B, 100 pmol; Rab3C, 10 pmol) in 5 μ l aliquots were added to 45 μ l of GTP[S] binding buffer containing 100 μ g/ml BSA, 0.6% CHAPS, 20 mM Hepes, 1 mM EDTA, 1 mM D,L-dithiothreitol, 0.8 M NaCl, 6 mM MgCl₂, pH 8.0, and various concentrations of [³⁵S]GTP[S] (250 Ci/mol; Du Pont–New England Nuclear). After incubation for 2 h at room temperature, 2 ml of ice-cold stop solution [100 mM NaCl/25 mM MgCl₂/20 mM Tris/HCl (pH 8.0)] was added to the reaction mixture and the reaction mixture filtered through 0.45 μ m nitrocellulose filters (BA-85, 25 mm; Schleicher and Schuell, Dassel, Germany). Each filter was washed five times with 2 ml of ice-cold stop solution, after which 5 ml of scintillation fluid was added for radioactivity determination, which was taken to be a measure of bound nucleotides.

GTPase assay

Assays for GTPase activity were performed as described previously [21], with modifications. For formation of [α -³²P]GTP-bound Rab3 complex, 10–30 pmol of recombinant Rab3 protein was incubated in 10 μ l of complex buffer {0.6% CHAPS/20 mM Tris/HCl (pH 7.5)/30 mM MgCl₂/20 μ Ci of [α -³²P]GTP/0.05 μ M GTP} for 5 min at room temperature. The mixture was added to 190 μ l of ice-cold high-Mg²⁺ buffer [25 mM Tris/HCl (pH 7.5)/0.5 mM GTP/0.6% CHAPS/5 mM MgCl₂] and incubated for 5 min on ice. Excess (unbound) [α -³²P]GTP was removed by passage through a G-50 Sephadex column.

For the measurement of GTPase activity, the preformed GTP–Rab3 complex (5 μ l) was incubated with 30 μ l of high-Mg²⁺ buffer. For the GAP assay, the cell lysate was included in the reaction mixture. The cell lysate was prepared by resuspending the freshly isolated chromaffin cells in high-Mg²⁺ buffer and then centrifuging at 600 g for 5 min. The supernatant was adjusted to a protein concentration of 35 mg/ml. Most of the guanine nucleotide-releasing factor activity was inhibited by including 0.6% CHAPS in the reaction mixture [22]. Indeed, more than 80% of Rab3 protein was present in the GTP-bound form during the assay. Thus a change in the rate of GTP hydrolysis by adding cell lysate was taken as a measure of the GAP activity in the lysate. Aliquots (5 μ l) of reaction mixture were removed at timed intervals and 25 μ l of extraction buffer [50 mM EDTA/2% (w/v) SDS/0.5 mM GTP/0.5 mM GDP] was added to terminate the reaction. An aliquot (1–5 μ l) was removed and subjected to polyethyleneimine-cellulose (PEI-cellulose; Merck, Germany) thin-layer chromatography (developing time 1 h; the mobile phase was 0.25 M PBS, pH 6.6) to separate GTP and GDP. The ratio of GTP to GDP was then determined by a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Preparation of antibody

For anti-Rab3A antibody preparation, full-length recombinant Rab3A purified by SDS/PAGE was used as an antigen for immunization. Purified Rab3A in polyacrylamide gel was cut into pieces and mixed with equal volumes of adjuvant and $5 \times$ PBS immediately before injection. For preparation of anti-Rab3B and anti-Rab3C antibodies, oligopeptides corresponding to residues 199–218 of Rab3B and 207–220 of Rab3C were synthesized by using a peptide synthesizer (430A Applied Biosystems, Forster, CA, U.S.A.) and conjugated with BSA by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by Harlow and Lane [23]. To generate the antibodies, male New Zealand white rabbits (approx. 2.5 kg) were immunized by intramuscular injections of the antigen by using standard procedures [23]. Anti-dopamine- β -hydroxylase (anti-D β H) antibody was obtained commercially (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

SDS/PAGE, transblotting, immunoblotting and [α - 32 P]GTP binding

SDS/PAGE was performed by the method of Laemmli [24] with a 14% (w/v) polyacrylamide gel. The proteins were subsequently transblotted (200 mA for 1 h) to a PVDF membrane (Immobilon-P; Millipore Corporation, Bedford, MA, U.S.A.). Binding of [α - 32 P]GTP to Rab3 protein blotted on PVDF membrane was performed as described previously [25]. For Western blotting, the antibodies were used at a dilution of 1:1000 for Rab3A, 1:100 for Rab3B and 1:500 for Rab3C. The proteins were revealed by alkaline phosphatase-linked reaction.

Isolation and culture of bovine chromaffin cells

Bovine adrenal glands were supplied by a local slaughterhouse. The adrenal glands were removed immediately after the animals had been killed, and they were kept on ice. Bovine chromaffin cells were isolated and cultured as previously described [26]. Cells were used immediately for preparation of cell lysate or plated at a density of 2×10^5 cells/ml on poly(L-lysine)-coated coverslips for immunostaining experiments. Media of the cultured cells were changed every other day and experiments were performed on cells that had been cultured for between 3 and 7 days.

Immunostaining

Immunostaining was performed as previously described [27]. Briefly, cultured bovine chromaffin cells on a coverslip were fixed by a mixture of methanol/acetone (1:1, v/v). Fixed cells were washed with PBS containing 50 mM NH_4Cl for 15 min at room temperature and then permeabilized by 0.2% Triton X-100 in PBS for 5 min. The permeabilized cells were then stained with anti-Rab3 antibodies (anti-Rab3A antibody, 1:1000; anti-Rab3B antibody, 1:100; anti-Rab3C antibody, 1:500) in the presence of 5% (w/v) BSA for 1 h. Cells were washed twice, for 5 min each time, with PBS and then incubated with fluorescein-conjugated goat anti-rabbit IgG for 30 min at 37°C. The stained cells were mounted in 50% (v/v) glycerol in PBS for observation by fluorescence microscope.

RESULTS

Characterization of recombinant Rab3 proteins and antibodies

To characterize the three Rab3 proteins, the molecular tools were first established. Wild-type, C-terminally truncated (Rab3₁₋₁₈₉) and chimaeric Rab3 proteins (Rab3B₁₋₁₈₉/A₁₉₀₋₂₂₀)

Figure 2 Characterization of recombinant Rab3 proteins

(A) SDS/PAGE analysis, with 2 μ g of protein in each lane; (B) immunoblot analysis, with different antibodies as indicated; (C) [α - 32 P]GTP binding to Rab3 protein blot. Lanes 1, Rab3A; lanes 2, Rab3B; lanes 3, Rab3C. Experiments were performed twice with similar results.

Figure 3 Characterization of chimaeric and truncated Rab3 proteins

(A) SDS/PAGE analysis, with 2 μ g of protein in each lane; (B) immunoblot analysis, with different antibodies as indicated; (C) [α - 32 P]GTP binding to Rab3 protein blot. Lanes 1, chimaeric Rab3B/A; lanes 2, chimaeric Rab3C/A; lanes 3, truncated Rab3A; lanes 4, truncated Rab3B; lanes 5, truncated Rab3C. Experiments were performed three times with similar results.

and Rab3C₁₋₁₈₉/A₁₉₀₋₂₂₀) were expressed in *E. coli* and then purified to near homogeneity. Specific antibodies for the three Rab3 proteins were also generated. Figures 2 and 3 show the protein pattern and [α - 32 P]GTP binding of the recombinant proteins. The mobilities of Rab3A and Rab3C on an SDS/polyacrylamide gel are similar, whereas that of Rab3B was slightly faster than Rab3A or Rab3C. The activity of [α - 32 P]GTP binding on a PVDF blot was higher for Rab3A than for Rab3B and Rab3C. The [α - 32 P]GTP binding of Rab3 proteins after blotting shows that the Rab3B/A chimaera failed to bind any [α - 32 P]GTP, and the Rab3C/A chimaera bound more [α - 32 P]GTP than the wild-type Rab3C. Interestingly, the three C-terminally truncated proteins bound [α - 32 P]GTP equally well (Figure 3C). Minor bands of lower molecular masses are shown in the PVDF blots (Figures 2C and 3C). These minor bands are possibly the hydrolysed product of Rab3 proteins because they were largely eliminated when protease inhibitors were included during purification (results not shown).

Specific antibodies against the recombinant protein (Rab3A) or C-terminal variable regions (Rab3B and Rab3C) of the three proteins were generated. Western blot analysis showed that each antibody specifically recognized its own Rab3 protein (Figure 2B). The truncated proteins of Rab3A, Rab3B and Rab3C are expected to have molecular masses of approx. 22 kDa, which correlate with their positions on the SDS/polyacrylamide gel. Because antibodies were specific for the C-terminal region of Rab3 proteins, C-terminally truncated Rab3 proteins were not detected by these antibodies. The chimaeric proteins have similar

Figure 4 GTP[S]-binding affinity of Rab3 proteins

Rab3 proteins (Rab3A, 1 pmol; Rab3B, 100 pmol; Rab3C, 10 pmol) were incubated with various concentrations of [35 S]GTP[S] (GTP γ S) and the amount of [35 S]GTP[S] bound was determined as described in the Materials and methods section. Results are expressed as the percentage of maximal binding. Experiments were carried out six times with similar results; one representative result is shown.

Table 1 Binding affinity for GTP[S], and k_{cat} for intrinsic and GAP-induced GTPase activities of Rab3 mutant proteins

Results are means $\pm$ S.E.M. for three experiments. Numbers in parentheses are the fold increase in k_{cat} . * $P < 0.01$; ** $P < 0.001$, significantly different from the values of the full-length proteins.

Mutant	K_d (GTP[S]) (nM)	k_{cat} (min^{-1}) (fold increase)	
		— Lysate	+ Lysate
Rab3A	15 $\pm$ 3	0.022 $\pm$ 0.006	0.062 $\pm$ 0.012 (2.82)
His-Rab3A	16 $\pm$ 4	0.024 $\pm$ 0.004	0.059 $\pm$ 0.009 (2.46)
His-C-truncated Rab3A†	25 $\pm$ 3*	0.022 $\pm$ 0.006	0.065 $\pm$ 0.014 (2.95)
Rab3B	2700 $\pm$ 263	0.029 $\pm$ 0.007	0.090 $\pm$ 0.018 (3.10)
His-Rab3B	2680 $\pm$ 327	0.012 $\pm$ 0.006	0.042 $\pm$ 0.008 (3.50)
His-C-truncated Rab3B†	11950 $\pm$ 72**	0.026 $\pm$ 0.006	0.065 $\pm$ 0.012 (2.50)
His-Rab3B/A‡	2437 $\pm$ 295	0.006 $\pm$ 0.003	0.012 $\pm$ 0.004 (2.00)
Rab3C	204 $\pm$ 45	0.021 $\pm$ 0.003	0.060 $\pm$ 0.003 (2.86)
His-Rab3C	225 $\pm$ 38	0.023 $\pm$ 0.001	0.063 $\pm$ 0.012 (2.74)
His-C-truncated Rab3C†	667 $\pm$ 42	0.024 $\pm$ 0.006	0.057 $\pm$ 0.01 (2.38)
His-Rab3C/A‡	628 $\pm$ 36	0.027 $\pm$ 0.004	0.061 $\pm$ 0.015 (2.26)

† C-terminal amino acids 190–220 were truncated.

‡ C-terminal amino acids 190–220 were replaced by the corresponding region in Rab3A.

molecular masses to the wild-type proteins and are recognized exclusively by anti-Rab3A antibody (Figure 3B). On the basis of the above results it seems that the anti-Rab3A antibody specifically recognized the C-terminus of Rab3A although full-length Rab3A was used as the antigen to generate antibody.

Biochemical characterization of recombinant Rab3 proteins

The biochemical properties of the wild-type, truncated and chimaeric Rab3 proteins were subsequently characterized. The concentration dependence of GTP[S] binding to Rab3 proteins is shown in Figure 4. The K_d values for GTP[S], estimated by Scatchard plot analysis, are 15, 2700 and 204 nM for Rab3A, Rab3B and Rab3C respectively (Table 1). The K_d of Rab3A is in a similar range to that of the Rab3A purified from bovine brain [28]. It is surprising that the K_d values of Rab3B and Rab3C were

Figure 5 GTPase activity of Rab3

GTPase activity of the three Rab3 proteins was measured in the absence (○) or presence (●) of cell lysate. The radioactivity associated with GDP and GTP spots was quantified and used to calculate the k_{cat} values shown in Table 1. Experiments were performed three times with similar results; one representative result is shown.

Figure 6 GTP[S]-bound Rab3A as competitor for Rab3-GAP activity

Various concentrations of GTP[S] (GTP γ S)-bound Rab3A were included in the reaction mixture for Rab3-GAP assay as described in the Materials and methods section. Results are shown as the percentage of the GTPase activity in the absence of GTP[S]-bound Rab3A. Similar results were obtained from two experiments in triplicate with different batches of recombinant Rab3 proteins.

200-fold and 15-fold that of Rab3A because the domains that are thought to be responsible for guanine nucleotide binding are almost identical in the three proteins (Figure 1).

The intrinsic GTPase activity of Rab3 proteins was determined by the rate of GTP hydrolysis (Figure 5) [21]. The three Rab3 proteins had similar intrinsic GTPase activities, with a k_{cat} (Table 1) similar to that of the Rab3A purified from bovine brain [29]. When bovine chromaffin cells were solubilized and used as a source for GAP, the intrinsic GTPase activities of the three Rab3 proteins were enhanced by the crude GAP by approximately 3-fold (Figure 5 and Table 1).

To examine whether there are different GAPs for different Rab3 proteins, GTP[S]-bound Rab3A was used as a competitor in the GAP assay. The GTP[S]-bound Rab3A inhibited the GAP-induced GTPase activity of the three Rab3 proteins with IC_{50} of 40, 12.5 and 25 μ M (averaged from two experiments) for Rab3A-GAP, Rab3B-GAP and Rab3C-GAP respectively (Figure 6). The IC_{50} of GTP[S]-Rab3A for Rab3A-GAP activity is

Figure 7 Localization of Rab3 proteins and D/βH by immunofluorescence in chromaffin cells

Chromaffin cells were fixed and stained with various antibodies (A, anti-Rab3A; B, anti-Rab3B; C, anti-Rab3C; D, anti-D/βH; E, normal serum) as described in the Materials and methods section. Experiments were performed three times with similar results. Scale bar, 10 μ m.

similar to that reported previously with GMPPNP-bound Rab3A as a competitor [30].

C-terminal region modulates the biochemical properties of Rab3 proteins

The above results show that the three Rab3 proteins have different affinities for GTP[S] and GAP. However, the three Rab3 proteins have almost identical putative functional domains for GTP/GDP binding and GTPase catalytic activity, and share more than 85 % similarity in their deduced amino acid sequences except in the C-terminal variable region (Figure 1). To determine whether the C-terminus has any contribution in the different biochemical characteristics of Rab3 proteins, the C-terminus of the three proteins was truncated, and the C-termini of Rab3B and Rab3C were replaced with that of Rab3A. For easy purification, a tag of eight histidine residues was added to the N-terminus. Our results show that the K_d values for GTP[S] and the intrinsic GTPase activities of Rab3A and Rab3C are not significantly changed by the His-tag (Table 1). However, for His-tagged Rab3B, the k_{cat} of its intrinsic GTPase activity was decreased by 60 %, whereas its K_d for GTP[S] and the enhancement of its GTPase activity by crude GAP are similar to those of the wild-type Rab3B protein.

Deletion of C-terminal 31 amino acid residues decreased the affinity for GTP[S] of all three Rab3 proteins; in particular the K_d for truncated Rab3B increased almost 5-fold compared with the wild type (Table 1). When the C-terminal 31 residues of Rab3B and Rab3C were replaced with the corresponding regions of Rab3A, the K_d of the Rab3B/A chimaera for GTP[S] was similar to that of the wild type, whereas that for the Rab3C/A

chimaera remained unchanged. Neither truncation of the C-terminus nor swapping the C-terminus for the Rab3A C-terminus altered the intrinsic GTPase activity and the GAP activity of Rab3C or Rab3A. In contrast, the intrinsic GTPase activity and activation of GTPase by crude GAP were decreased in His-tagged Rab3B. The results suggest that C-terminal variable regions affect the biochemical properties of the three Rab3 proteins in different ways.

Subcellular distribution of Rab3 proteins

The subcellular localization of the three Rab3 proteins was studied by immunostaining cultured bovine adrenal chromaffin cells with specific polyclonal antibodies (Figure 7). All three Rab3 protein antibodies stained the bovine chromaffin cells but in different regions of the cells. Anti-Rab3A and anti-Rab3C antibodies stained vesicle-like structures, similar to those reported previously [7]. Anti-Rab3B antibody distinctly stained the plasma membrane with patches near the membrane. In the cytoplasm the staining by anti-Rab3B antibody did not exhibit the spotty pattern produced by anti-Rab3A or anti-Rab3C antibodies. The immunofluorescence staining of chromaffin cell by anti-D/βH, a marker of chromaffin granules, is also shown for comparison (Figure 7). The pattern of anti-D/βH antibody showed a similar pattern to that of Rab3A and Rab3C.

DISCUSSION

The functional domains of small G-proteins that are responsible for guanine nucleotide binding, GTPase activity and interactions with regulatory proteins and effector proteins have been identified (Figure 1) [28–34]. On the basis of the predicted amino acid sequences of the previously cloned small G-proteins, these functional domains are very similar. It is therefore expected that the three Rab3 proteins should have similar biochemical properties and physiological functions. Indeed, the Rab3 proteins have been shown to be involved in exocytosis in different cell systems, although it is not clear at which step of exocytosis they might act [9–14].

In this study we show that bovine chromaffin cells express the three Rab3 proteins. The three Rab3 proteins are different in their affinities for guanine nucleotides and in their subcellular distributions. These differences reside at least in part in the C-terminal variable region of the proteins. The localization and biochemical characteristics are the main determinants for specific functions of small G-proteins. It is generally thought that the C-terminal region is responsible for the subcellular localization of the small G-proteins after proper post-translational modification [1]. However, post-translational modification is not the only factor to determine the location of the proteins because the three Rab3 proteins are thought to have the same modification at the C-terminal Cys-Xaa-Cys residues, but their intracellular distributions were different (Figure 7). Evidence from a study by exchanging different regions between Rab5 and Rab6 suggests that geranylgeranylation of the C-terminal cysteine residues is required only for the association of the Rab proteins with membrane; the correct localization depends on another part in the hypervariable region [34].

In addition to the different localizations of Rab3 proteins, the biochemical properties of these proteins were also different. His-tagged Rab3 proteins were used to study the biochemical properties of the Rab3 proteins. For Rab3A and Rab3C, adding histidine residues had no effect on their biochemical properties. However, the intrinsic GTPase activity of Rab3B was sig-

nificantly decreased by histidine tagging. The cause of this phenomenon is unclear.

According to the results of GTP[S] binding, the binding affinity of Rab3A for GTP[S] was 200-fold that of Rab3B and 150-fold that of Rab3C. Truncation of the C-terminal region in general decreased the affinity for GTP[S] of Rab3 proteins. Exchanging the C-terminus for that of Rab3A decreased the binding affinity of Rab3C for GTP[S] but had no effect on that of Rab3B. In both truncated and chimaeric Rab3 proteins the order of affinity for GTP[S] of Rab3 proteins did not change: Rab3A > Rab3B > Rab3C. It seems that the amino acid residues outside the putative functional domains (Figure 1) might also be important in determining the affinity for guanine nucleotide and that the C-terminal variable region is not necessary for the binding of guanine nucleotide but modulates the affinity for GTP[S]. From the three-dimensional structure of Ras, the C-terminal region might be close to the N-terminal region, which is linked with the G-I region (I in Figure 1) via a β -sheet structure [32]. The G-I region is involved in the interaction between protein and nucleotide. It is possible that interaction between the C-terminal and N-terminal regions somehow affects the conformation of the guanine nucleotide-binding site [30].

Our results show that changes in the C-terminal region affect [α - 32 P]GTP binding to Rab3 proteins on PVDF blots. The binding for GTP of Rab3 proteins after blotting depends on the affinity for the nucleotide and the extent of renaturation of the blotted proteins. Because our results show that C-terminal truncation in general decreased the affinity for GTP[S] (Table 1), truncation should increase the renaturation of the Rab3 and thus cause a net increase in [α - 32 P]GTP binding to truncated Rab3 proteins. In contrast with our results when the C-terminal 23 residues of c-Ha-Ras were truncated it failed to bind [α - 32 P]GTP after blotting [25]. Nevertheless both results suggest that the C-terminal variable region of small G-proteins is important in determining the renaturation of the proteins after blotting.

The functions of small G-proteins are regulated by the interconversion between GTP and GDP. In bovine chromaffin cells there is a GAP for Rab3 that in general increases approximately 3-fold the intrinsic Rab3 GTPase activity. With GTP[S]-bound Rab3A as a competitor in Rab3-GAP assay, the IC_{50} is different for each of the three Rab3 proteins (Figure 5). It is interesting that the IC_{50} for Rab3A-GAP was higher than that of Rab3B-GAP and Rab3C-GAP. Rap1a shows 1000-fold higher affinity for Ras-GAP than Ras itself [35]. On the basis of our results, it is possible that there are different GAPs for different Rab3 proteins. The effector region (Figure 1), which is thought to interact with GAP [32], is identical in the three Rab3 proteins. If there is only one GAP for the three Rab3 proteins, there should be other parts of the Rab3 that are involved in the interaction with GAP.

We conclude that the three Rab3 proteins have different biochemical properties and subcellular distribution. These findings suggest that Rab3 proteins are functionally distinct. Our results provide a basis for the study of the physiological role of Rab3 proteins.

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