Rat basophilic leukaemia (RBL) cells overexpressing Rab3a have a reversible block in antigen-stimulated exocytosis

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The rat basophilic leukaemia (RBL) cell line has been widely used as a convenient model system to study regulated secretion in mast cells. Activation of these cells through the high-affinity receptor for IgE (Fcϵ-RI) results in degranulation and the extracellular release of mediators. There is good evidence of a role for GTPases in mast cell degranulation, and a number of studies with peptides derived from the Rab3a effector domain have suggested that Rab3a may function in this process. However, in neuroendocrine cells, overexpression of Rab3a can act as a negative regulator of stimulated exocytosis [Holz, Brondyk, Senter, Kuizon and Macara (1994) J. Biol. Chem. **269**, 10229–10234; Johanes, Lledo, Roa, Vincent, Henry and Darchen (1994) EMBO J. **13**, 2029–2037]. In order to study the function of Rab3a in RBL degranulation, we have generated clones of RBL cells stably expressing Rab3a, and show that in these haematopoietic cells Rab3a can also function as a negative regulator of exocytosis. Overexpression of a mutant form of Rab3a (Asn-135 to Ile), which is predicted to be predominantly GTP-bound, also inhibited degranulation. However, overexpression of a mutant form of Rab3a that was truncated at the C-terminus to remove the sites for geranylgeranylation failed to inhibit degranulation. The effect of Rab3a is specific to secretion, and we observe no effect of Rab3a on receptor-mediated endocytosis. The Rab3a-induced block in degranulation can be bypassed by stimulation of streptolysin-O-permeabilized cells with guanosine 5′-[γ-thio]triphosphate. We conclude from these studies that Rab3a is implicated in an early stage of granule targeting, whereas fusion of granules with the plasma membrane is regulated by a distinct downstream GTP-binding protein or proteins.

INTRODUCTION

Regulated secretion is an essential process in neuronal communication, the control of glucose uptake and the release of hormones and digestive enzymes. In addition to these processes, it is of fundamental importance to host defence mechanisms, which include the immune system, haemostasis and inflammation. One of the primary functions of many haematopoietic cell lineages is to release preformed mediators upon activation by specific stimuli. Despite the central importance of regulated secretion to the effective functioning of granulocytes, relatively little is known about the mechanism of exocytosis when compared with cells of the neuroendocrine system. A combination of yeast genetics and biochemical *in vitro* assays that reconstitute vesicle fusion have provided a powerful mechanistic working model for how the secretory pathway operates. This 'SNARE' hypothesis seeks to provide a model to explain protein transport between membrane compartments in the cell, and appears to relate to both the constitutive and regulated secretory pathways [1–4]. One molecular component of the vesicle budding and fusion process is a large family of small GTP-binding proteins known as the Rabs [5–8]. Members of the Rab family, of which there are approximately 30, are thought to regulate the docking and possibly fusion of transport vesicles with their specific target membrane. A number of different Rabs have been shown to have specificity in terms of both their tissue distribution and within the cell, where they are known to be localized to specific intracellular organelles. Based on the organelle-specific distribution of Rabs, it has been suggested that they may regulate in some way the fidelity of vesicular traffic within the cell. Functional studies in both yeast and mammalian cells have largely confirmed the role of Rabs as one of the key regulators of specific vesicle traffic [9,10].

In the regulated secretory pathway, there is substantial evidence that members of the Rab3 family play an important role in vesicle docking and/or fusion with the plasma membrane [11]. The Rab3 family has at present four members: Rab3a, b, c and d. These four proteins share between 77 and 85% amino acid identity and appear to be localized to secretory vesicles involved in regulated exocytosis. Rab3a is the most abundant member of the family and is predominantly expressed in neuronal and neuroendocrine tissues. The exact tissue distributions and functions of all four members are not known, and many cell types express more than one Rab3 isotype [11,12]. Despite considerable circumstantial evidence implicating the Rab3 family in regulated exocytosis, there is still little clear evidence for their mode of action. In a number of cells that perform regulated secretion, including mast cells, a peptide corresponding to the effector domain of Rab3a has been shown to stimulate exocytosis, suggesting that Rab3a functions to activate secretion [13–19]. Experiments in which an antisense RNA to Rab3b was microinjected into anterior pituitary cells have demonstrated that the

Abbreviations used: RBL cells, rat basophilic leukaemia cells; SL-O, streptolysin-O; wt-3a, wild-type Rab3a; N135I-3a, site-directed mutant of Rab3a with Asn-135 changed to Ile; ΔCAC-3a, truncated mutant of Rab3a which has the C-terminal sequence Cys-Ala-Cys removed; DNP, dinitrophenol; KLH–DNP, keyhole-limpet haemocyanin conjugated to DNP; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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decrease in Rab3b expression was correlated with an inhibition of exocytosis [20], and studies in which Rab3b was overexpressed in PC12 cells showed that noradrenaline exocytosis was greatly enhanced [21]. However, not all evidence is consistent with this active function for Rab3. Studies with transgenic mice in which the Rab3a gene was knocked out showed that expression of this protein was not essential for synaptic vesicle exocytosis [22], and overexpression of Rab3a in neuroendocrine cells appeared to have a suppressive effect on regulated secretion [21,23,24].

Our main interest is in the study of granulocyte exocytosis, and we use the rat basophilic leukaemia (RBL) cell line as a convenient model. This cell performs regulated exocytosis of 5-hydroxytryptamine (serotonin) and hexosaminidase in response to cross-linking of cell-surface IgE receptors. It is not known which Rab3 isotypes are expressed in RBL cells, although a PCR-based study suggested that the predominant isotypes expressed in rat peritoneal mast cells are Rab3b and Rab3d [25]. However, despite a concerted effort, we have been unable to obtain Rab3 clones by hybridization of an RBL cDNA library [26]. Nevertheless, we wished to determine the effects of Rab3a overexpression in RBL cells for two reasons. Firstly, a previous study has shown that Rab3a effector domain peptides were capable of inducing mast cell degranulation [13], suggesting a possible role for Rab3a in these cells. Secondly, and most significantly, the RBL cell provides a convenient and experimentally tractable system for the study of Rab3a function. The various stages of regulated exocytosis from receptor activation to granule–plasma-membrane fusion are amenable to biochemical analysis, and this has allowed us to define a step at which Rab3a is functioning prior to granule–plasma-membrane fusion. From our studies we conclude that there is a GTP-dependent step downstream of Rab3a that is required for granule–plasma-membrane fusion.

MATERIALS AND METHODS

Materials

All materials were obtained from the following suppliers unless specifically stated otherwise. Chemicals were obtained from Sigma (Poole, Dorset, U.K.), cell culture reagents from HyClone and molecular biology reagents from Promega. The 9E10 anti-Myc monoclonal antibody was purchased from Babco (Berkley Antibodies), the anti-Rab3 antibody (clone 42.1; anti-Rab3a/b/c) was purchased from Dr. R. Jahn (Yale University School of Medicine), and the anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Upstate Biotech Inc.

cDNA cloning

A cDNA clone for rat *rab3a* was generated by PCR as follows. Rat brain poly(A)$^+$ mRNA (Clontech) was reverse-transcribed into cDNA using a reverse transcriptase kit according to the manufacturer's instructions (Promega). The cDNA was amplified by PCR with primers designed from the published sequence for rat *rab3a*, and the PCR product was cloned into the pCRscript vector (Stratagene). The 5′ sequence of the *rab3a* insert was modified by single-stranded overlap extension PCR, which incorporated an *Nde*I site in-frame over the initiating methionine. The *rab3a* cDNA was excised from the pCRscript vector by digestion with *Nde*I and *Xho*I and cloned into a modified pBK-RSV vector (Stratagene). The pBK-RSV vector was modified by insertion of an oligonucleotide cassette containing a Kozak sequence followed by an initiating methionine and then a sequence encoding the c-Myc epitope recognized by the 9E10 antibody (-EQKLISEEDL-). This cassette was flanked by unique restriction sites, a *Not*I site at the 5′ end and an *Nde*I site at the 3′ end. This recombinant phagemid vector was termed pBKCmyc, and cloning inserts into the *Nde*I site added an in-frame Myc-tag to the extreme N-terminus of the encoded protein sequence. The Asn-135 to Ile mutation (N135I) was generated by sequence overlap extension PCR using an overlapping pair of primers that both encoded the base pair change: 5′ CTGGTGGGG*AA*CAAGTGCGAC 3′ (Asn-135); 5′ CTGGTGGGG***AT***CAAGTGCGAC 3′ (Ile-135 sense primer); 5′ GACCACCCC***TA***GTTCACGCAG 3′ (Ile-135 antisense primer).

A truncated form of Rab3a was produced by removal of the three C-terminal amino acids, Cys-Ala-Cys (ΔCAC). The ΔCAC mutant was made by amplification of a *rab3a* cDNA template using a 3′ antisense primer in which the codons for Cys-Ala-Cys were replaced by two stop codons and a *Sma*I site as follows: 5′ TCCCCCGGGTTATACATCCTGATGAGGTGGTGC 3′. The truncated Rab3a cDNA PCR product was cloned into the pCRscript vector then subcloned into the pBKCmyc vector using the *Nde*I and *Sma*I sites.

A cDNA clone for human Rac2 was obtained by screening a human eosinophil cDNA library and subcloned into the pBKCmyc vector as described above.

All cDNA clones were checked by double-stranded sequencing using standard protocols.

Transfection and generation of stable cell lines

RBL.2H3 cells (hereafter called RBL cells) were transfected by electroporation using a Bio-Rad GenePulser as follows. Cells were trypsinized, washed once in complete medium [RPMI 1640, 10% (v/v) foetal calf serum, 2 mM glutamate] and once in serum-free complete medium, and then resuspended at a density of 10^7 cells in 0.4 ml of RPMI 1640 and placed in an electrocuvette with 20 μg of plasmid prepared using QIAGEN Megaprep columns as described by the manufacturer's protocol. After incubation at 4 °C for 10 min, the cells were electroporated at 250 mV/960 μF and then incubated again at 4 °C for 10 min. The cells were washed in 10 ml of warm complete medium and then transferred to tissue culture flasks and incubated at 37 °C. After 24 h, the cells were washed and incubated with fresh medium containing 1 mg/ml G418 (Gibco-BRL). Control transfections were performed either with no plasmid (mock transfections) or with the pBKCmyc plasmid containing no cDNA insert (vector controls). Transfected cells were incubated for a period of approx. 3 weeks in G418-containing medium, by which time the mock-transfected cells had all died. Single-cell cloning of stable cell lines was performed in two rounds. The first round was by using cloning rings, and the second round was by limiting dilution in Terasaki plates (Nunc).

Analysis of secretion and endocytosis

Degranulation of RBL cells was quantified by release of the enzyme hexosaminidase. Release was always measured as a percentage of the total cellular content of the enzyme, which was determined by lysis of the cells in 0.1% Triton X-100. For antigen stimulation, the cells were seeded into 96-well plates at a density of 50000 cells per well in RPMI medium containing 10% foetal calf serum and 1 μg/ml anti-dinitrophenol (DNP) IgE (Sigma clone SPE-7), and incubated overnight. The adherent cells were washed in PBS prior to addition of 100 μl of KLH–DNP antigen (keyhole-limpet haemocyanin conjugated to DNP; usually at 1 μg/ml final concentration) in Phenol Red-free RPMI containing 1 mg/ml BSA and 2 mg/ml glucose. The cells were stimulated for various periods of time prior to the removal of 50 μl of cell supernatant for quantification of hexosaminidase

release. In order to determine the level of hexosaminidase release in unstimulated controls, all steps were identical to the above with the omission of the KLH–DNP antigen. To determine the total cellular content of hexosaminidase, 100 μl of Phenol Red-free RPMI containing 1 mg/ml BSA, 2 mg/ml glucose and 0.1 % Triton X-100 was added to the wells.

To determine the hexosaminidase enzyme activity, 50 μl of cell supernatant or 50 μl of cell lysate was added to 50 μl of substrate in a 96-well plate, and the reaction was allowed to proceed for 3 h at 37 °C. The substrate was 2 mM *p*-nitrophenyl *N*-acetyl-D-glucosaminide in 0.2 M citrate buffer, pH 4.5. The reaction was stopped by the addition of 150 μl of 1 M Tris buffer, pH 9.0, and the absorbance was read at 405 nm on a Molecular Devices ThermoMax plate reader against a substrate/buffer blank.

To measure receptor-mediated endocytosis, anti-DNP IgE (Sigma; clone SPE-7) was conjugated to the Cy5 fluorescent dye using a Fluorolink antibody labelling kit (Biological Detection Systems Inc., Pittsburg, PA, U.S.A.) according to the manufacturer's instructions. Conjugation of Cy5 to the IgE resulted in cross-linking of the IgE, as determined by liquid chromatography (results not shown). RBL cells were grown in chamber slides (Nunc) and incubated at 4 °C for 2 h in the dark with the Cy5-conjugated IgE at a concentration of 10 μg/ml. Unbound Cy5–IgE was washed with ice-cold medium prior to warming the cells to 37 °C for various periods of time. The time course was terminated by removal of the culture medium and the addition of fixative (3.7 % formaldehyde in phosphate buffer). The fixed cells were washed three times, mounted and analysed by scanning laser confocal microscopy using a Leica TCS4D microscope with the 63 × objective, which revealed about 20 cells per field. The *z* plane of the microscope was adjusted to take images through the centres of the cells, and the pinhole was adjusted to give confocal sections about 0.4 μm thick. For each time point the microscope captured images of the Cy5 fluorescence at 647 nm excitation and 680 nm emission. The images were transferred to a Leica Q500MC image analyser in order to automatically count the number of Cy5-fluorescent endosomes per cell. The fluorescence was detected from the background by grey thresholding in the image analyser, and the resulting binary image was edited where necessary so that only vesicles were present in the binary image. The number of vesicles per microscope field was then counted automatically by the image analyser, and the number of cells per microscope field was counted interactively.

Degranulation of streptolysin-O (SL-O)-permeabilized cells

RBL cells were plated into 96-well plates on the day preceding the degranulation experiment, at an appropriate density to give 50–70 % confluence by the following day. On the morning of the experiment, the cells were washed with Pipes/Cl buffer (50 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 20 mM Pipes, 1 mg/ml BSA, pH 6.8) and placed on ice. To each well was immediately added 50 μl of ice-cold Pipes/Cl buffer containing 0.5 unit/ml SL-O (Murex). After an 8 min preincubation on ice to allow the SL-O to bind, the cells were washed twice with ice-cold Pipes/Cl buffer to remove unbound SL-O and contaminants in the SL-O solution. Still on ice, stimulation buffers were added to the appropriate wells.

Different calcium concentrations were achieved by using calcium/EGTA buffers at a final concentration of 3 mM EGTA in Pipes/Cl buffer supplemented with 5 mM ATP. A range of guanosine 5′-[γ-thio]triphosphate (GTP[S]) concentrations was obtained by diluting a 100 mM stock of GTP[S] (Gibco-BRL) in Pipes/Cl buffer supplemented with 5 mM ATP. To obtain values for 100 % secretion, 1 % Triton X-100 was added to some wells. Cells were placed in a water bath at 37 °C to trigger the permeabilizing activity of SL-O and to allow entry of the secretogogues. Exocytosis was terminated after 20 min by addition of 100 μl of 95.6 mM EDTA. The 96-well plates were then centrifuged at 5000 ***g*** for 5 min to pellet any detached cells. Samples of 50 μl of supernatant from each well were transferred to a clean black 96-well microtitre plate, to which was added 50 μl of the fluorogenic hexosaminidase substrate 4-methylumbelliferyl *N*-acetyl-D-glucosaminide in citrate buffer, pH 4. The plate was incubated at 37 °C for 2 h before the reaction was terminated by the addition of 300 μl of 0.2 M tris(hydroxymethyl)methylamine. Fluorescence was measured by excitation at 355 nm and emission at 460 nm in a microtitre plate fluorimeter.

Analytical techniques

SDS/PAGE was performed using precast gels (Novex) and proteins were transferred to nitrocellulose filters electrophoretically by standard techniques. For Western blotting, nitrocellulose filters were first incubated in blocking buffer [PBS, 0.1 % Tween-20 and 5 % (w/v) freeze-dried milk powder] prior to incubation with specific primary antisera. Filters were washed four times in PBS containing 0.1 % Tween-20, followed by incubation with a goat anti-mouse second antibody conjugated to horseradish peroxidase. After washing, the blots were revealed using the ECL reagent as directed by the manufacturer (Amersham International).

RESULTS

Generation of RBL cell lines stably expressing Rab3a

A cDNA clone for rat Rab3a was generated by PCR and cloned into a modified pBK-RSV expression vector. The cloning strategy introduced a 10-amino-acid tag at the extreme N-terminus of the reading frame, corresponding to the c-Myc epitope that is recognized by the 9E10 monoclonal antibody. Two variants of the wild-type Rab3a clone (wt-3a) were produced by mutagenesis. The first mutant converted Asn-135 to Ile (N135I-3a), which is analogous to codon 116 in Ras. Based on biochemical studies, this mutant form of Rab3a was expected to act in a dominant fashion, i.e. to be predominantly GTP-bound *in vivo* [27]. The second mutant removed the C-terminal tripeptide -Cys-Ala-Cys (ΔCAC-3a), which is the site for post-translational modification by geranylgeranylation [28]. As an additional control, we also cloned human Rac2 into the Myc-tagged expression vector.

RBL cells were transfected by electroporation with vectors containing cDNAs for wt-3a, N135I-3a, ΔCAC-3a or Rac2 and, as controls, were also either transfected with an empty vector or mock-transfected with no vector at all. The transfected cells were selected in G418 for approx. 2 weeks, by which time the mock-transfected cells had all died, and were then analysed for their ability to degranulate following the cross-linking of high-affinity IgE receptors (Fcϵ-RI). Our preliminary findings were that RBL cells transfected with wt-3a and N135I-3a were impaired in their ability to degranulate following antigenic stimulation, whereas cells transfected with ΔCAC-3a, Rac2 or the empty vector all degranulated similarly to untransfected controls (Table 1). In all cases, protein expression was confirmed by Western blotting (results not shown).

Following these initial studies, we proceeded to generate single-cell clones from the mixed population of RBL cells transfected with wt-3a (clones called RBL.wt-3a). Six clones were isolated, and expression of the Myc-tagged wt-3a was confirmed by probing Western blots of cell extracts with the anti-Myc (9E10)

Table 1 Degranulation of RBL cells expressing Rab3a

RBL cells were transfected with expression vectors encoding wt-3a, ΔCAC-3a, N135I-3a and wild-type Rac2 (wt-rac2). Also included were untransfected RBL cells (Control) and cells transfected with an empty expression vector (Vector). The cells were preincubated with IgE anti-DNP and subsequently triggered to degranulate by the addition of the cross-linking reagent KLH–DNP. The results show degranulation, expressed as a percentage of the total cellular hexosaminidase released (means ± S.D. of quadruplicate determinations), with and without the addition of KLH–DNP antigen.

Cell type	KLH–DNP antigen	Degranulation (%)
Control	+	45.4 ± 1.9
	−	2.4 ± 0.2
Vector	+	35.4 ± 0.7
	−	1.7 ± 0.1
wt-3a	+	10.2 ± 0.4
	−	1.6 ± 0.1
N135I-3a	+	1.9 ± 0.2
	−	1.6 ± 0.1
ΔCAC-3a	+	35.7 ± 0.7
	−	1.9 ± 0.1
wt-rac2	+	30.5 ± 0.7
	−	1.6 ± 0.2

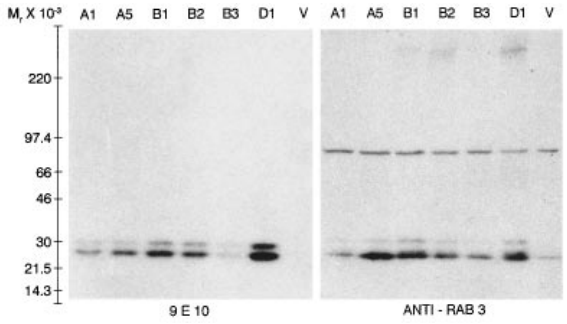


Figure 1 Western blot analysis of RBL clones stably expressing wt-3a

Extracts were prepared from the six clones indicated and from a control clone transfected with an empty expression vector (V). Duplicate extracts were run on SDS/polyacrylamide gels and transferred to nitrocellulose filters. The filters were probed either with the monoclonal anti-Myc antibody 9E10 or with a polyclonal antibody to Rab3.

and anti-Rab3 antibodies (Figure 1). The vector control cells (lane V) clearly expressed endogenous Rab3 but not any transfected Myc-tagged protein, whereas all six of the wt-3a-transfected clones expressed protein that was detected by both the 9E10 and anti-Rab3 antibodies. Although RBL cells clearly express Rab3, as shown by this Western blotting, the antibody used is not isotype-specific, and we have been unable to confirm the endogenous expression of Rab3a. To further characterize the Rab3a-transfected cells, we examined the intracellular distribution by immunofluorescence confocal microscopy [26]. Staining of transfected cells with the 9E10 monoclonal to detect the Myc-tagged protein showed that Rab3a was localized to the granules but, in addition, there was staining in the juxtanuclear region of the cell and the cytosol.

Analysis of degranulation in RBL clones stably expressing wild-type Rab3a

The six RBL clones overexpressing wt-3a were analysed for their ability to degranulate in response to antigen. Figures 2(a) and

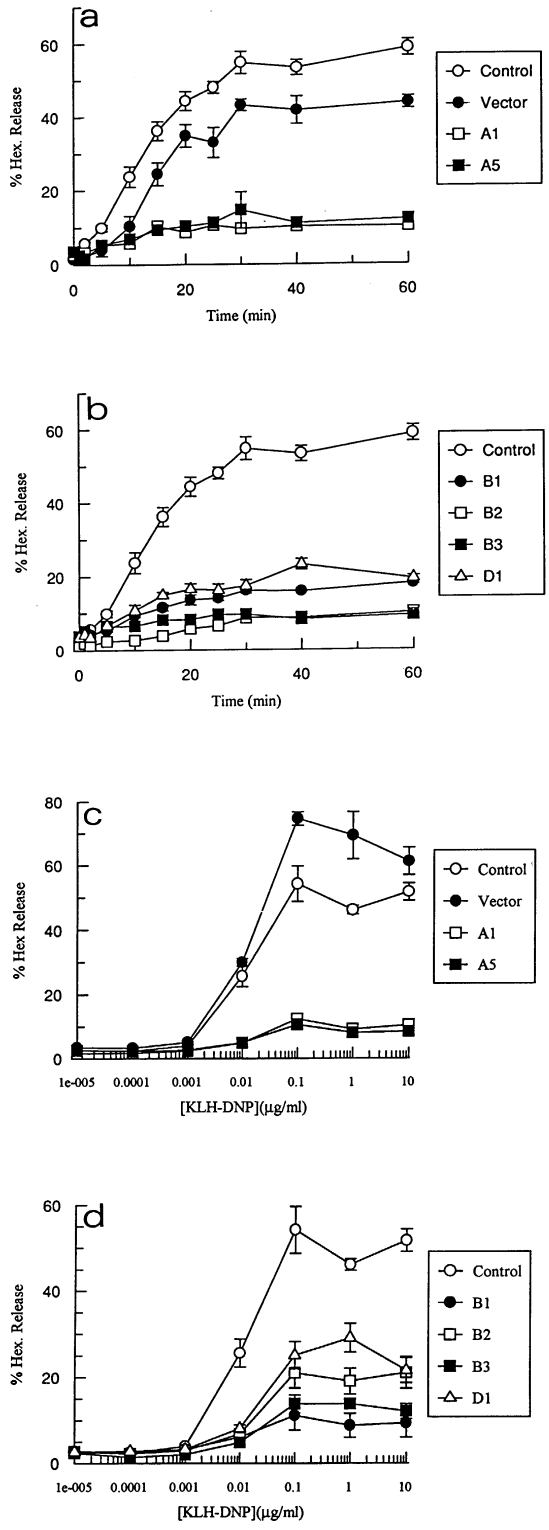


Figure 2 Degranulation time courses (a, b) and dose–response curves (c, d) for RBL cells stably expressing Rab3a

(**a**, **b**) The results shown are for six RBL.wt-3a clones (A1, A5, B1, B2, B3 and D1) which stably express Rab3a, plus a clone transfected with empty vector (vector) and untransfected RBL cells (control). Cells were preincubated with IgE anti-DNP and triggered at time zero with KLH–DNP. The results show degranulation expressed as a percentage of the total cellular hexosaminidase (Hex.) released (means ± S.E.M. of triplicate determinations). (**c**, **d**) The same clones were triggered with increasing doses of KLH–DNP and degranulation was quantified as for (**a**) and (**b**). 1e-005 = 1.0×10^{-5}.

Table 2 Degranulation by transfected RBL cells in response to various stimuli

Untransfected RBL cells (Control), RBL cells transfected with an empty vector (Vector) or cells stably expressing Rab3a (wt-3a) were triggered to degranulate by cross-linking of cell-surface IgE receptors (antigen stimulation) or by treatment with 10 μM ionomycin + 50 nM PMA. Unstimulated cells are shown as a control (None). The results show degranulation, expressed as a percentage of the total cellular hexosaminidase released (means ± S.D. of quadruplicate determinations), 30 min after administration of the stimulus.

Cell type	Stimulus	Degranulation (%)
Control	None	1.8 ± 0.5
	Antigen	82.1 ± 4.4
	Ionomycin + PMA	86.0 ± 7.0
Vector	None	2.5 ± 0.2
	Antigen	79.3 ± 3.4
	Ionomycin + PMA	85.0 ± 7.1
wt-3a	None	0.8 ± 0.2
	Antigen	14.5 ± 2.2
	Ionomycin + PMA	23.2 ± 1.3

2(b) show typical time courses of degranulation of untransfected RBL cells, in which exocytosis routinely peaks 30 min after stimulation and between 40–80 % of the total cell content of hexosaminidase is released. (There was some variability between experiments in the extent of RBL degranulation, and consequently each experiment was always internally controlled; in addition, where comparisons were to be made, all assays were performed with the same batch of cells at the same time.) In these studies, cells transfected with an empty vector showed a slight decrease in degranulation compared with untransfected cells; however, all of the wt-3a-expressing clones (RBL.wt-3a) showed a dramatic decrease to below 20 % release. We also analysed the response of these clones to increasing doses of antigen (Figures 2c and 2d). At very high concentrations of antigen, some of the clones, particularly RBL.wt-3a.D1, showed a partial degranulation response, whereas other clones such as RBL.wt-3a.A1 showed no response even at the highest doses.

From the above study it was evident that the RBL clones overexpressing wt-3a were impaired in their degranulation response following triggering of the IgE receptor. This may have been caused by some defect in the signal transduction cascade downstream from receptor activation. To test this hypothesis, we stimulated exocytosis by addition of PMA and ionomycin, which is known to mimic the signals generated by IgE receptor cross-linking (Table 2). These results clearly demonstrate that, whereas the control and vector-transfected RBL cells degranulated effectively in response to such stimulation, the cells transfected with wt-3a showed greatly reduced degranulation. The second characteristic signalling cascade that is triggered upon IgE receptor cross-linking is the stimulation of tyrosine kinase activity. To determine if this activity was altered following overexpression of Rab3a, cell extracts were prepared from cells 5 min after receptor stimulation and analysed by Western blotting with an anti-phosphotyrosine antibody (Figure 3). Comparison of RBL.wt-3a cells with control cells showed that there were no qualitative or quantitative differences detectable in the pattern of tyrosine phosphorylation.

Having established that the upstream signals were unlikely to be the cause of the blocked degranulation, we chose to examine the granule–plasma-membrane fusion event. It is possible to bypass the upstream signals that trigger degranulation by using cells that have been permeabilized with SL-O. In these semi-intact cells, degranulation can be induced by the addition of

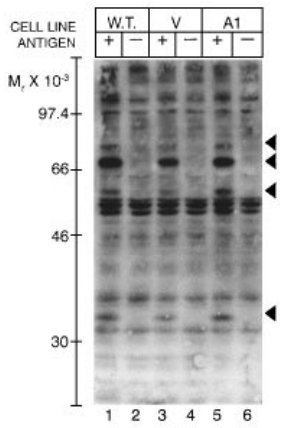


Figure 3 Effect of Rab3a overexpression on tyrosine kinase activity

Untransfected RBL cells (W.T.), RBL cells transfected with an empty vector (V) or RBL cells expressing Rab3a (A1) were preincubated with IgE anti-DNP and subsequently treated with (+; lanes 1, 3 and 5) or without (−; lanes 2, 4 and 6) antigen (KLH–DNP). Cell extracts were prepared 5 min after antigen stimulation and analysed by SDS/PAGE and Western blotting. The nitrocellulose filter was probed with an anti-phoshotyrosine antibody, and the major inducible tyrosine-phosphorylated substrates are indicated by arrowheads.

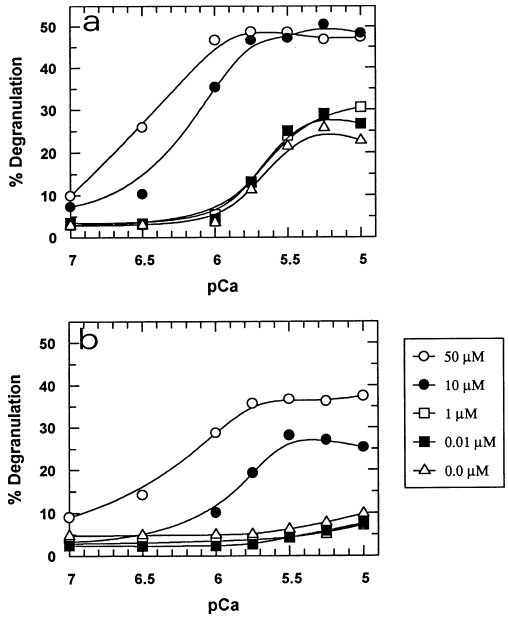
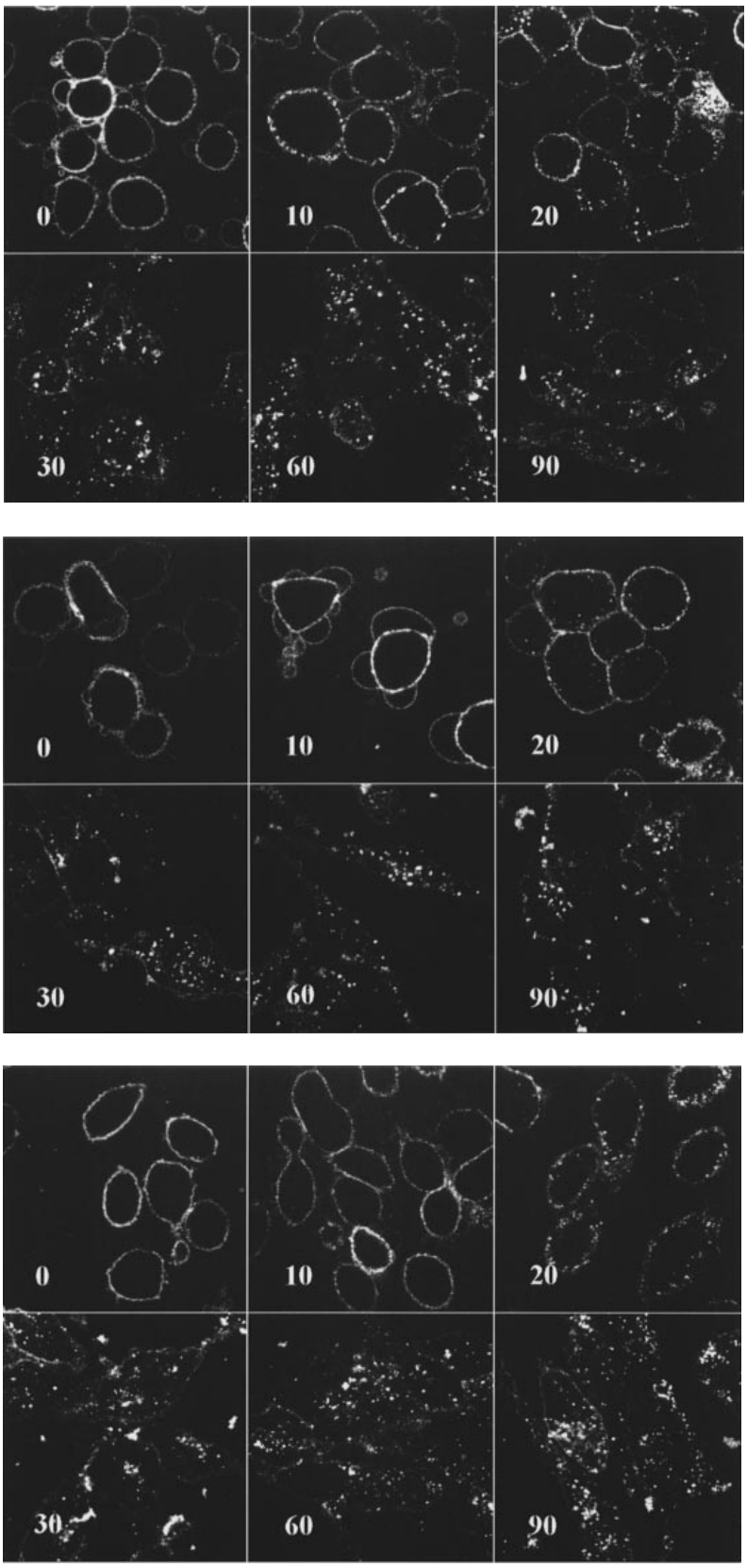


Figure 4 Degranulation of SLO-permeabilized cells triggered by GTP[S]

Untransfected RBL cells (**a**) or cells stably expressing Rab3a (**b**) were permeabilized with SL-O, as described in the Materials and methods section, and triggered to degranulate by the addition of GTP[S] in the presence of increasing concentrations of calcium in the buffer. A range of GTP[S] concentrations was used as indicated, from 50 μM (○) down to 0 μM (△). The results show degranulation, expressed as a percentage of the total cellular hexosaminidase released (means ± S.E.M. of triplicate determinations).

calcium and non-hydrolysable analogues of GTP, which provide a sufficient stimulus for granule–plasma-membrane fusion [29,30]. Figure 4 shows that SL-O-permeabilized untransfected cells, when triggered with 50 μM GTP[S] and at optimal pCa, can release approx. 50 % of their stored hexosaminidase. The SL-O-permeabilized RBL.wt-3a cells can also be induced to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degranulate by GTP[S], and at a concentration of 50 μM almost 40 % release can be achieved.

Analysis of receptor-mediated endocytosis in RBL.wt-3a.A1 cells

Having established that the RBL.wt-3a.A1 clone was blocked in antigen-triggered degranulation, we proceeded to investigate another membrane traffic event in these cells to determine if the defect was selective for the secretory pathway or whether endocytosis was also affected. To measure receptor-mediated endocytosis, we prepared a fluorescent conjugate of IgE coupled to the fluorophore Cy5. The conjugation protocol resulted in the formation of cross-linked IgE which was capable of inducing RBL cell degranulation without the addition of antigen. This is significant, because monomeric IgE is internalized very slowly by Fcϵ receptors. Consequently, the IgE–Cy5 conjugate was bound at 4 °C to the surface of untransfected cells, vector controls or RBL.wt-3a.A1 cells which were growing adherent to glass slides. Unbound IgE–Cy5 was washed off and receptor-bound ligand was allowed to internalize by warming the cells to 37 °C for up to 90 min. At timed intervals the cells were fixed and ligand internalization was monitored by scanning laser confocal microscopy (Figure 5). There were no detectable differences in the rate, quantity or pattern of receptor-mediated endocytosis. These data also confirm that the RBL.wt-3a.A1 cells express high-affinity IgE receptors on their surface, and consequently that the observed defect in antigen-triggered exocytosis cannot be due to a loss of surface receptors.

DISCUSSION

From early studies on regulated secretion in mast cells, it was observed that degranulation could be induced when a non-hydrolysable analogue of GTP (GTP[S]) was introduced into cells either via a patch pipette [29] or by permeabilization with SL-O [31,32]. These observations clearly placed a GTP-binding protein late in the granule–plasma-membrane fusion event, and the unidentified protein was termed G_E [30]. Circumstantial evidence predicted that G_E would be a heterotrimeric G-protein, and previous studies have shown that G_{i3} functions to stimulate the final steps in exocytosis in mast cells [33], whereas G_o may act to suppress exocytosis in adrenal chromaffin cells [34]. There is also a substantial body of evidence that implicates low-M_r GTP-binding proteins of the Rab family in the secretory pathway; in particular, members of the Rab3 family are thought to be involved in regulated secretion (for reviews, see [11,12]).

Exactly what role Rab3 plays in regulated secretion is still unclear. Peptides derived from the effector domain of Rab3a have given conflicting results, as they have been shown to stimulate [13–19,35] or inhibit [36] exocytosis. The stimulation of mast cell exocytosis by the Rab3a effector domain peptide clearly raised the possibility that Rab3a may play an important role in these cells [13]. However, the validity of all of these studies has been called into question [37,38]. Antisense experiments have been used to deplete cells of Rab3a, and it was concluded that there was a reduction in response habituation or desensitization in adrenal chromaffin cells [24], whereas similar experiments with Rab3b antisense oligonucleotides in anterior pituitary cells appeared to inhibit secretion [20]. A transgenic mouse in which the Rab3a gene was knocked out was still capable of neurotransmitter release, and the only clear defect that could be detected was in synaptic function following repetitive stimulation [22]. The conclusion drawn from these studies was that Rab3a was not essential for synaptic vesicle exocytosis, but probably played a role in the recruitment or recycling of synaptic vesicles. Perhaps the most convincing evidence to date that sheds light on the role of Rab3a in exocytosis has come from studies in which the protein was transiently transfected into adrenal chromaffin cells or PC12 cells, which resulted in a significant inhibition of exocytosis [21,23,24], whereas overexpression of Rab3b enhanced exocytosis [21]. Recently, a complementary study has demonstrated that overexpression of the Rab3a binding protein Rabphilin had the opposite effect and enhanced exocytosis [39]. However, these studies were performed on transiently transfected cells, and consequently there was only limited analysis of the phenotype resulting from Rab3a or Rabphilin overexpression.

The Rab3 family has four known members at present, Rab3a, b, c and d, and there is some limited evidence for tissue-specific distribution of these isotypes. Rab3a and Rab3c are shown to be predominantly expressed in neuroendocrine tissue [40,41], and Rab3d was originally found in adipocytes [42,43]. A PCR-based analysis of peritoneal mast cells revealed that these cells express Rab3b and Rab3d, but the authors were unable to detect Rab3a or Rab3c [25]. From our own studies, we can clearly detect Rab3 expression in RBL cells by Western blotting; however, the antibody used is not isotype-specific. This is unlike the situation in eosinophils, where it was impossible to detect Rab3 expression by Western blotting [44]. We have generated a cDNA library from RBL cells and undertaken an extensive Rab cloning exercise [26]. However, we failed to isolate any Rab3 clones from the library, and consequently we were unable to confirm the Rab3 isotype expression pattern suggested by previous studies on mast cells [25].

Although uncertain as to whether RBL cells express Rab3a, we proceeded to study the effects of Rab3a overexpression. Firstly, there was clear evidence that this protein may be a negative regulator of exocytosis, whereas the only available information on Rab3b suggested that it is a positive regulator [20,21]. Secondly, the evidence from Rab3a-derived peptides suggested a link with mast cell exocytosis. Finally, the RBL cell is an excellent experimental model to study the mechanism of action of Rab3a. From our studies and from published observations [23,24], there is now strong evidence to support the conclusion that overexpression of Rab3a can act as a negative regulator of exocytosis. However, in previous studies using transient transfection of Rab3a into chromaffin cells, the inhibitory effect was mainly seen on the early rate of secretion in the first 5 min following stimulation [23], whereas in our stable transfections in RBL cells the block of exocytosis persisted throughout the whole time course of degranulation. The production of stable cell lines was also important because it enabled us to validate biochemically that other membrane traffic events, such as receptor-mediated endocytosis, are not altered by overexpression of Rab3a. We were also able to confirm that the block in degranulation was not due to defective signal transduction or to a lack of cell-surface IgE receptors. However, most im-

Figure 5 Receptor-mediated endocytosis of Cy5-conjugated IgE by control RBL cells (top), RBL cells stably expressing Rab3a (middle) or RBL cells transfected with an empty vector (bottom)

Cy5–IgE was incubated with the cells at 4 °C for 1 h, followed by extensive washing to remove unbound ligand. The cells were subsequently warmed to 37 °C for timed periods of 10, 20, 30, 60 and 90 min, as indicated. Following the warm-up period, the cells were washed in ice-cold buffer and fixed prior to analysis by laser scanning confocal microscopy. Magnification $\times$ 400.

portantly, it allowed us to show that the block was reversible by the addition of GTP[S] and Ca^{2+} to SL-O-permeabilized cells. At the highest dose of GTP[S] (50 μM) there was slightly less exocytosis from Rab3a-overexpressing cells, although it was quite clear that the granules were capable of fusion. However, at lower doses of GTP[S] the difference between the cell lines became more marked. If our observations are valid, overexpression of the GTP-bound form of Rab3a effectively blocks exocytosis, so why does administration of GTP[S] to SL-O-permeabilized secretory cells induce degranulation? The simplest explanation is that there is another GTP-binding protein acting downstream of Rab3a which is responsible for regulating the final stage of granule fusion. Taken together, our data suggest that overexpression of Rab3a in RBL cells does not inhibit the final step of plasma-membrane–granule fusion and that this final fusion step may be regulated by another GTP-binding protein.

There are two other significant observations from the transfection studies that help us to understand the mechanism of the Rab3a block of exocytosis: firstly, the N135I mutant of Rab3a is profoundly inhibitory and, secondly, the mutant lacking the C-terminal -Cys-Ala-Cys motif (ΔCAC) required for geranylgeranylation is not inhibitory. The proposed cycle for Rab3 predicts that, when associated with the vesicle membrane, Rab3 is loaded with GTP; following hydrolysis of the nucleotide, the GDP-bound form is removed from the membrane by the action of GDP dissociation inhibitor [11,12]. Since the N135I mutant is predicted to be preferentially GTP-bound and the ΔCAC mutant is unable to insert into membranes, the data from transfection of these mutants suggest that it is the membrane-associated, GTP-bound form of the Rab3a that is exerting the inhibitory effect. This is further supported by the findings that the Thr-36 → Asn mutant of Rab3a, which is predicted to be preferentially GDP-bound, does not inhibit exocytosis in chromaffin or PC12 cells, whereas the GTP-loaded Gln-81 → Leu mutant is strongly inhibitory [24]. One potential effector for the GTP-bound form of Rab3a is Rabphilin3a [45,46], and increasing the concentration of GTP–Rab3a on the granule membrane would be expected to recruit Rabphilin3a to the membrane [47]. Recent studies showed that overexpression of full-length Rabphilin3a enhanced secretion, whereas expression of the Rab3a binding domain alone was inhibitory [39]. One working hypothesis that can account for these data is that exocytosis is a multistep process that requires the formation of the Rab3a–Rabphilin3a complex on the vesicle membrane, and that for exocytosis to occur this complex must subsequently disassociate. Since Rab3a exists in a dynamic equilibrium between a GDP-bound cytosolic form and a GTP-bound vesicular-associated form, one would expect that overexpression of Rab3a will increase the concentrations of both cytosolic Rab3a–GDP and Rab3a–GTP on the vesicle membrane. Increasing the concentration of GTP–Rab3a on the vesicle membrane may effectively sequester Rabphilin3a, so blocking exocytosis.

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