Rab37 is a novel mast cell specific GTPase localized to secretory granules

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Abstract GTPases regulate a myriad of cellular functions including signal transduction, cytoskeletal organization and membrane trafficking. Rab GTPases act to coordinate the membrane dynamics of cells by organizing and regulating the activity of effector proteins important in vesicle trafficking. Rab37 is a novel Rab GTPase specifically expressed in the MC-9 mast cell line and bone marrow mast cells. Rab37 is 74% identical to Rab26 and 47% identical to Rab8, a GTPase important in Golgi to plasma membrane vesicle trafficking in mammalian cells. When green fluorescent protein tagged Rab37 is expressed in bone marrow mast cells, the secretory granules are labeled. These data suggest that Rab37 may play an important role in mast cell degranulation making this protein a potentially important target for therapeutic intervention in the treatment of allergy.

Key words: GTPase; Exocytosis; Mast cell; Allergy

1. Introduction

Eukaryotic cells rely on a series of membranous organelles to compartmentalize biochemical reactions and to communicate with the extracellular environment. Transport between organelles occurs via the formation of a vesicle laden with cargo, transport of the vesicle to a target membrane and fusion of the vesicle and target membranes. In the final step of the secretory pathway, exocytosis, secretory vesicles often derived from the *trans*-Golgi network fuse with the plasma membrane. Molecules such as receptors or extracellular matrix components are deposited on the cell surface by constitutive exocytosis. In contrast, release of neurotransmitters, secretion of hormones, or secretion of inflammatory mediators from mast cells, occurs in a highly regulated fashion. The action potential, releasing factors or hormones, or crosslinking of the IgE receptor triggers these secretory events respectively. The close coupling of secretion to a signaling event requires regulatory proteins in addition to the basic components that mediate vesicle trafficking.

Many of the proteins important for vesicle trafficking have been characterized and their functions are becoming well defined [1]. The Rab proteins comprise an important family of low molecular weight GTPases critical for regulating vesicle trafficking [2]. Over 35 Rabs have been identified from mammalian species and each localizes to a particular membrane compartment. Rab proteins act upstream of the membrane fusion machinery by regulating the localization and activity of effector proteins. Rab effector proteins have been proposed to serve many functions. For example, Rab6 interacts with a protein that has a molecular motor-like ATPase domain and may function to translocate vesicles to target sites [3] while Rab5 interacts with the early endosomal antigen 1 which may be important in tethering organelles prior to the membrane fusion event [4]. Defining the spectrum and function of Rab proteins and their effectors is an important area of investigation in membrane trafficking.

Mast cells and basophils are hematopoietic lineage derived cells that mediate allergic inflammation [5]. The inflammatory response is due, in part, to the exocytosis of a variety of molecules contained within dense core granules including histamine, serotonin, and various proteases. While the widely conserved mechanisms of exocytosis are likely to be utilized for mast cell secretion, few of the specific molecules involved have been clearly identified and their functions assigned. In an attempt to better understand secretory processes in mast cells, we identified a novel Rab protein that is specifically expressed in mast cells and is selectively localized to the secretory granules. This molecule may be a target, or may be useful in defining targets, for molecules of therapeutic value in modulating mast cell secretion.

2. Materials and methods

2.1. Cloning of Rab37

Using yeast two-hybrid screening, a novel cDNA clone interacting with human Rab5 was identified out of eight million independent yeast transformants derived from a murine mast cell line (MC9) library. Nucleotide sequencing revealed that the clone encoded the 101 carboxy-terminal amino acids of a novel member of the Rab family of proteins. Clones containing the 5′ sequence of the cDNA were isolated by PCR from the MC-9 cDNA library using nested primers. The nucleotide sequence was verified by checking it against clones from over four independent PCR reactions. Northern blotting was performed on a blot using 2 μg poly(A)$^+$ RNA per lane. A PCR product amplified from nucleotides 385 to 749 of the Rab37 coding region was used as a probe. As a control probe, a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA fragment (Clontech) was used.

2.2. Antibody production

Custom rabbit antisera and the corresponding affinity purified antibodies were generated against the following peptides (Zymed Laboratories): TAGDGEAPERSPPFSPNYD(C) (amino-terminal peptide) and (C)KYRAGRQPDEPSFQIRDY (carboxy-terminal peptide), where the cysteines in parentheses were added for single site conjugations to keyhole limpet hemocyanin. Antibodies were used to immu-

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noprecipitate detergent extracts from cells. The resulting precipitates were then analyzed by Western blotting. The bands were visualized by enhanced chemiluminescence (Amersham).

2.3. Green fluorescent protein (GFP)–Rab37 fusion protein expression

All GFP fusion constructs were based on the pRGL103 retroviral expression vector, which contains *Bst*XI cloning sites at the 3′-end of the GFP encoding sequence. The coding region for Rab37 was amplified by PCR and directionally cloned in frame at the 3′-end of the GFP sequence into pRGL103 using flanking *Bst*XI sites within the following primers: 5′-CTGCAGAACCACCACCATGGCTGGCACACCAGGAGCTGCTACC-3′ and 5′-CAATGCATCCAATTTAATGGCAGCTTCTCCTAGTTGGTGTGGC-3′. Rab37(T43N) and Rab37(Q89L) mutants were generated by standard PCR techniques using the above primers and in combination with the following primers: for T43N, sense 5′-CGTCGGCAAGAACTGTTTCCTGATCC-3′ and antisense 5′-GGATCAGGAAACAGTTCTTGCCGACG-3′; for Q89L, sense 5′-TGCAGGACTCGAGCGCTTCCGCAGTGTGA-3′ and antisense 5′-AAGCGCTCGAGTCCTGCAGTGTCCCAG-3′

Viral constructs, infection protocols and lysotracker granule labeling have been described previously [6].

3. Results

Rab5 was used as a yeast two-hybrid bait to screen 8×10^6 independent clones from an MC9 mast cell line. Nucleotide sequencing of the interacting clones revealed that two of the clones encode Rab5 and Rab2 while the third appeared to encode a novel Rab protein. A full-length clone for the novel protein was isolated and characterized (see Section 2). The clone encodes a predicted protein of 224 amino acids with all of the characteristics of a Rab GTPase (Fig. 1). The sequence motifs predicted to be critical for guanine nucleotide binding are highly conserved when Rab37 is compared to other low molecular weight GTPases. In addition, a characteristic pair of cysteine residues is found at the carboxy-terminus of the protein. These residues likely serve as sites for isoprenylation, a modification important in membrane association. Deletion of either five or 10 residues from the carboxy-terminal end of the protein results in a Rab37–GFP fusion protein that is cytosolic not vesicle associated (data not shown). The predicted sequence is most similar to Rab26, a GTPase of unknown function that is broadly expressed in somatic tissues [7]. Rab37 has a 32 amino acid amino-terminal extension compared to Rab26. One of our peptide antibodies was raised to a region corresponding to the amino-terminal extension. This antibody recognizes a band of the expected molecular weight confirming that this predicted sequence is indeed translated. Rab37 may actually be a larger protein than Rab26, or alternatively the predicted reading frame of Rab26 may be incomplete at the amino-terminus. As is characteristic of Rab proteins, the carboxy-terminal 30 residues comprise the most divergent domains of the molecule when compared to related sequences.

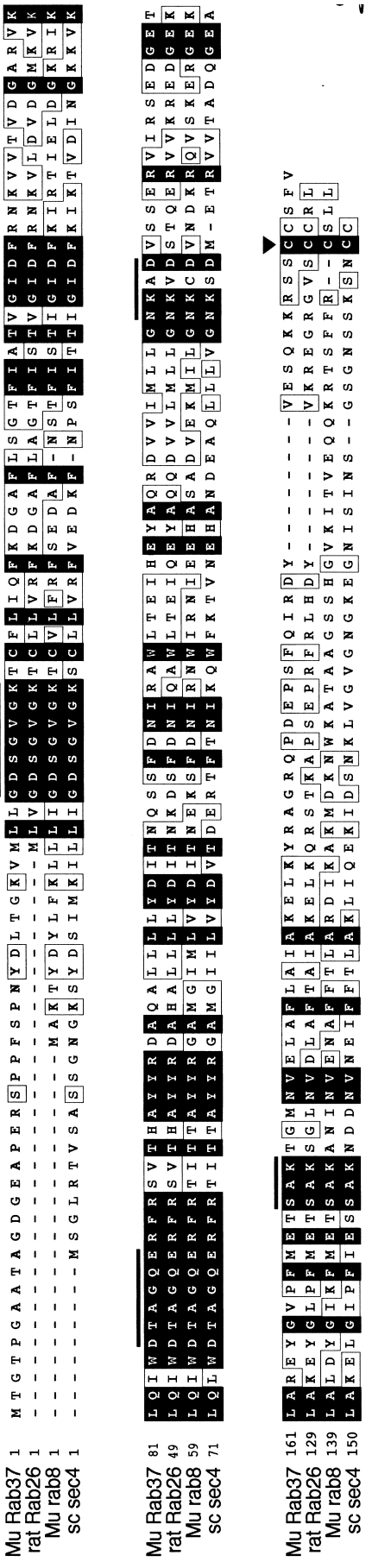

Fig. 1. Rab37 is a novel GTPase. The predicted amino acid sequence of murine Rab37 (GenBank AF233582 accession number) aligned to rat Rab26 (74% identical), murine Rab8 (47% identical) and yeast sec4 (36% identical). Residues that are conserved in all four sequences are indicated in black and boxed residues are conserved between two or more sequences. Bars over the sequences indicate GTP binding domains and the arrow at the carboxy-terminus is a conserved cysteine residue likely important for isoprenylation. The two gray shaded sequences in Rab37 correspond to the peptides used to raise antibodies.

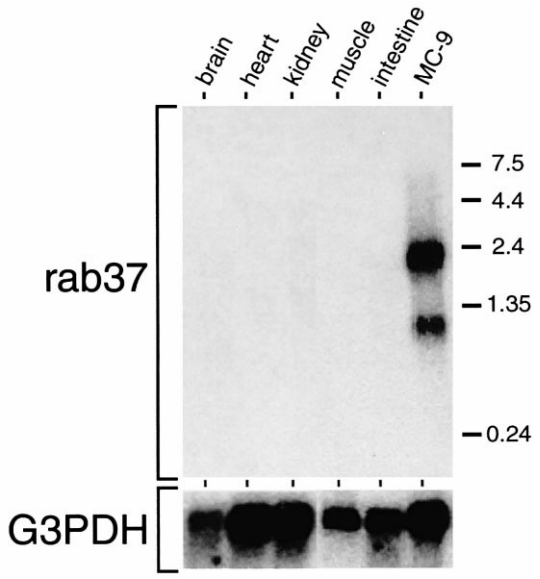

Fig. 2. Rab37 mRNA is specifically expressed in the MC-9 mast cell line. Six RNA samples were fractionated by gel electrophoresis, transferred to nitrocellulose and probed for either Rab37 or G3PDH transcripts. All lanes contain intact mRNA as indicated by the G3PDH hybridization, however transcripts encoding Rab37 are found specifically in mast cells. Sizes in kilobases are indicated to the right.

To better understand the potential function of Rab37, we investigated the tissue distribution of expression and subcellular localization of the protein. RNA was prepared from five tissues and the MC-9 mast cell line. These RNAs were probed for the presence of Rab37 or G3PDH transcripts (Fig. 2). While all tissues and cells examined contain transcripts encoding G3PDH, transcripts for Rab37 were only detected in the MC-9 cell line. Two Rab37 transcripts of 2.3 and 1.2 kb are observed. Since we only observe one protein, the difference in mRNA size may be due to alternative RNA splicing in the untranslated region or alternative poly(A) addition sites.

To further investigate the distribution of Rab37, we generated two peptide antibodies. One antibody was raised against a 19 amino acid peptide from the amino-terminal region of the protein and the second was raised against an 18 amino acid peptide from the carboxy-terminal region of Rab37. To confirm the specificity of the antibodies we performed Western blots on 293T cells transfected with vector or with a Rab37 expressing construct. The appropriate size band was only detected in transfected cells (Fig. 3A). Immunoprecipitation with the amino-terminal antibody followed by Western blots with the carboxy-terminal antibody revealed a band of 34 kDa in bone marrow mast cells but not in any of the other 16 tissues tested (Fig. 3B). If we first immunoprecipitate with the carboxy-terminal antibody then probe with the amino-terminal antibody similar results are obtained confirming the specificity of the antibodies and the identity of the band.

The most dramatic feature of the mast cell cytoplasm is the presence of a number of large secretory granules. Furthermore, the sequence homology of Rab37 to Rabs 26 [7] and 8 [8], as well as the yeast sec4 GTPase [9], suggests a function late in the secretory pathway, perhaps at the step of secretory granule exocytosis. To begin to test this prediction, we generated a retroviral expression construct containing Rab37 fused to GFP. Expression of this construct in bone marrow mast cells revealed intense labeling of the secretory granules (Fig. 4). Expression of GFP alone results in a diffuse cytoplasmic labeling as expected for a soluble protein. Furthermore, expression of a GDP stabilized mutant (T43N) of Rab37–GFP also results in diffuse cytoplasmic labeling, while expression of a GTP stabilized mutant (Q89L) localizes to granules (Fig. 4A). The identity of the GFP–Rab37 labeled organelles was confirmed by co-localization with lysotracker, a marker of acidic secretory granules [10] (Fig. 4B). In addition, the mast cells contain a very high density of secretory granules that are labeled by the SNARE protein GFP–VAMP2. These data suggest that Rab37 is localized to the

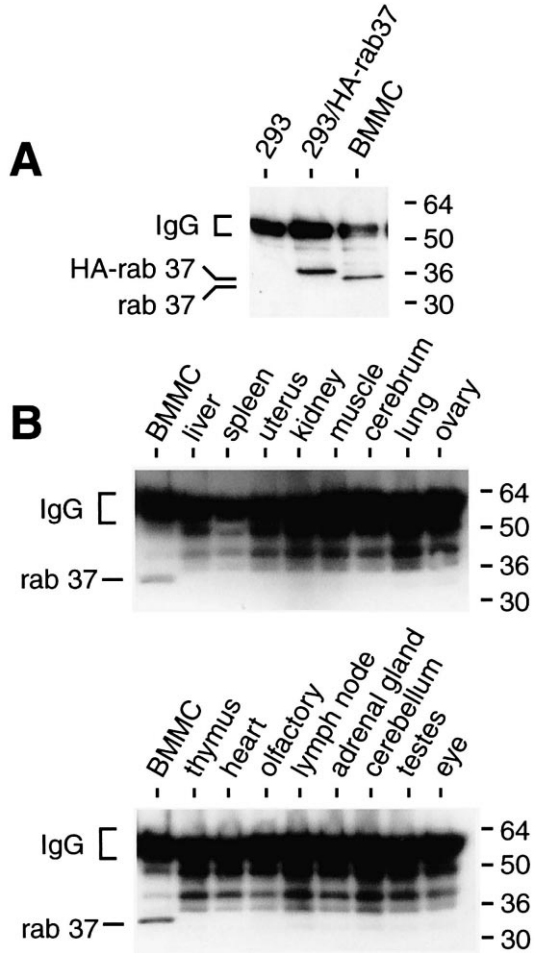

Fig. 3. Rab37 is present in bone marrow mast cells but not detected in 16 other tissues. A: HA–Rab37 is detected in transfected but not untransfected 293T cells. Extracts were immunoprecipitated with the amino-terminal peptide antibody and probed with the carboxy-terminal antibody. Untransfected 293T cells (293) only show reactivity with IgG while 293 cells expressing HA–Rab37 (293/HA–rab37) have an additional band at 36 kDa. The HA–Rab37 protein is slightly larger than endogenous Rab37 from bone marrow mast cells (BMMC). B: The antibody raised against the amino-terminal Rab37 peptide was used to immunoprecipitate 17 different protein samples. The immunoprecipitates were then probed for the presence of Rab37 using the antibody raised against the carboxy-terminal peptide. Only bone marrow mast cells (BMMC) express the protein at a molecular weight of 34 kDa. The bands above 36 kDa, present in all samples, arise from reactivity with the antibodies used for the immunoprecipitations. If the order of the antibodies is reversed a similar result is obtained. Molecular weights in kilodaltons are indicated to the right.

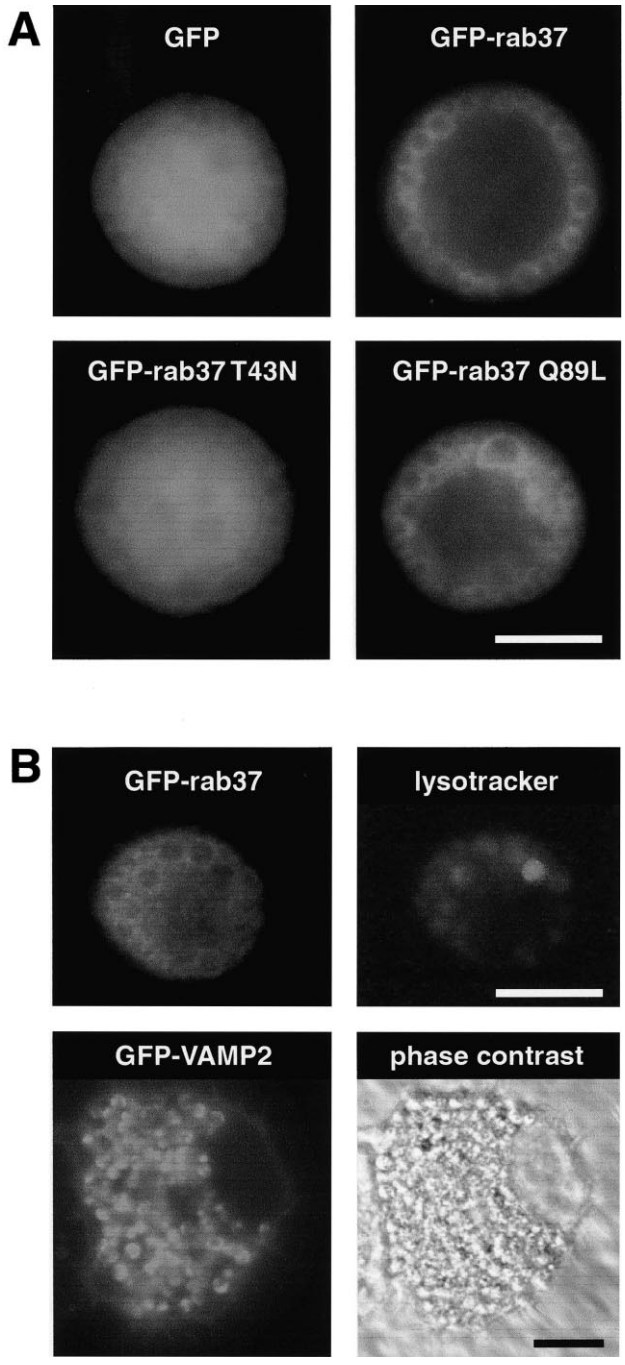

Fig. 4. Rab37 is localized to bone marrow mast cell granules. A: GFP expression in bone marrow mast cells results in cytoplasmic labeling. GFP–Rab37 expression in bone marrow mast cells results in secretory granule labeling. The GFP–Rab37 T43N (GDP stabilized) mutant expressed in bone marrow mast cells results in cytoplasmic labeling. The GFP–Rab37 Q89L (GTP stabilized) mutant expressed in bone marrow mast cells results in secretory granule labeling. B: GFP–Rab37 and lysotracker label the same organelles confirming the identity of secretory granules. GFP–VAMP2 also labels granules that are packed at high density in the bone marrow mast cells. Size bar = 3 μm.

secretory granules of mast cells, however conformation of this hypothesis awaits direct detection of the endogenous protein. The antibodies we have produced to date have proven inadequate for immunohistochemical analysis.

4. Discussion

Rab37 is a low molecular weight GTPase specifically expressed in bone marrow mast cells and mast cell lines. When expressed in mast cells, GFP tagged Rab37 is localized to secretory granules. These observations are most consistent with the hypothesis that Rab37 plays a role in exocytosis of mast cell dense core granules. Perhaps Rab37 is important in recruiting effector proteins to the membrane of the mast cell granule. Rab37 and its effector proteins are likely to function upstream of the actual membrane fusion event that releases the granule contents. Rab37 may function in regulating vesicle attachment to the cytoskeleton, granule translocation to the plasma membrane, or organizing the vesicle and target membrane associated fusion machinery.

The specificity of Rab37 expression suggests this GTPase may interact with mast cell specific effector proteins. The specificity of expression further suggests that Rab37, or associated proteins, may be useful target proteins for the development of therapeutic agents that will block mast cell degranulation. These agents could be useful in the treatment of allergy.

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