Identification of a novel member of the Rab8 family from the rat basophilic leukaemia cell line, RBL.2H3

John Armstrong, Nicola Thompson, Jane H. Squire, Janet Smith, Brian Hayes and Roberto Solari*

Cell Biology Unit, Glaxo Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK

*Author for correspondence (e-mail: rces4402@ggr.co.uk)

INTRODUCTION

The culmination of many biochemical and genetic studies have led to the development of the SNARE hypothesis which seeks to provide a model to explain protein transport between membrane compartments in the cell (Pryer et al., 1992; Bennett and Scheller, 1994; Rothman and Warren, 1994; Rothman, 1994). Many of the molecular components of the model have been identified in organisms as diverse as mammals and yeast, and the fundamental concept of protein transport mediated by vesicle budding and fusion has become a unifying theme in cell biology. One molecular component of the vesicle budding and fusion process is a large family of small GTP-binding proteins known as the Rab family (Pfeffer, 1992; Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993; Zerial and Stenmark, 1993). Members of the Rab family, of which there are over thirty, are thought to regulate docking and possibly fusion of transport vesicles with their specific target membrane. A number of different Rabs have been shown to have specificity both in terms of their tissue distributions and within the cell they are known to localise 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 (Nuoffer and Balch, 1994; Pfeffer, 1994).

Mast cells and basophils are specialised granulocytes which store an array of pre-formed inflammatory mediators in intracellular secretory granules. Upon activation through high affinity cell surface receptors for IgE, these cells undergo rapid degranulation releasing these mediators which include histamine, proteoglycans and mast cell neutral proteinases (Schwartz, 1994). We are investigating the intracellular molecular machinery of secretory granule fusion in mast cells and basophils, and as an initial study we have sought to identify novel members of the Rab family which may be specifically involved with this process. We have chosen to study the rat basophilic leukaemia cell line, RBL.2H3, as a model system since these cells perform comparably to mast cells and basophils in terms of IgE mediated degranulation. We have used a cloning strategy similar to one which has been used previously and which has proved successful for the identification of a number of novel Rabs (Chavrier et al., 1992). Here we report on the cloning and initial characterisation of a novel Rab from RBL.2H3 cells which is a member of the Rab8 family.

MATERIALS AND METHODS

Materials

All reagents unless stated otherwise were from the following suppliers: chemicals from Sigma; cell culture reagents were from HyClone; molecular biologicals from Promega.

Polymerase chain reaction (PCR)

The standard conditions for PCR were 50 mM KCl, 10 mM Tris-HCl, pH 9.0 (at 25°C), 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.125 units/µl Taq DNA polymerase (Promega) cycles 35 times using the following protocol: 95°C for 30 seconds; 42°C for 30 seconds and 72°C for 30 seconds). All PCR reactions were performed using an Hybaid OmniGene apparatus.
cDNA cloning

RBL.2H3 cells were grown to confluence (1×10⁶ cells) and total RNA was extracted using a RNaid kit according to the manufacturer’s instructions (BIO101 Inc.). Poly(A)+ mRNA was extracted by oligo(dT) affinity purification using Dynabeads Oligo(dT)25 (Dynal, Norway). This poly(A)+ mRNA was used to construct an EcoRI/XhoI directional cDNA library in the lambdaZAPII vector as described by the manufacturer (Stratagene). The library contained greater than 2×10⁶ primary recombinants with a background of less than 10%. The average insert size was approximately 1.5 kb and the largest inserts were estimated to be greater than 5 kb. Phage (2×10⁶) were plated in L broth containing 0.7% top agarose at 250,000 pfu in 23 cm × 23 cm dishes (NUNC bioassay dish) on a lawn of XL1 Blue MRF+ (Stratagene) plating cells and duplicate lifts were transferred onto Hybond N* membrane (Amersham Int.) according to the manufacturer’s instructions. The membranes were cross-linked using a Stratalinkr and then pre-hybridised in Hybrisol I (10% dextran sulphate, 1% SDS, 50% formamide, 6× SSC, sheared DNA and modified Denhardt’s solution) (Oncor Inc.) for at least 4 hours at 42°C using a Hybrid-Micro-4 oven. A degenerate cDNA probe common to all Rabs was generated by PCR on RBL.2H3 cDNA using modifications to the primers as previously described (Chavrier et al., 1992): (sense) 5¢-GGCGGCCGCTCCAGGGG(G50/A50)(G50/A50)IG(A33/G33/C33)(T33/G33/C33)(T50/A50)GGI-3¢; (antisense) 5¢-TTC(T75/C25)TGICC(T75/A25)GC(T75/C25)(T75/C25)G-3¢. This cDNA product was gel purified using a QIAquick kit (QIAGEN), and radio-labelled with [32P]dCTP using a Megaprime labelling kit (Amersham Int.). Denatured radiolabelled probe was added and the membranes incubated overnight at 37°C. The blots were washed twice for 30 minutes at room temperature in 6× SSC, then twice at 55°C for 20 minutes and finally twice at 55°C in 2× SSC for 20 minutes. The blots were exposed to Kodak XAR film at −70°C for up to 21 days.

A specific oligonucleotide probe to canine rab8 was synthesised, 5¢-GATGGACAAAAATTTGAAGGCAACGTCCAAAAGGAGCAACAGGGA-3¢ and end-labelled with [32P] ATP using a T4 polynucleotide kinase kit (Stratagene). The membrane was prehybridised overnight in 6× SSPE at 38°C using tRNA (50 µg/ml, Sigma) as a blocking agent and hybridised with kinase labelled oligonucleotide for 3 hours. The blots were washed in 6× SSPE from room temperature to 36°C and exposed to Kodak XAR5 film at −70°C overnight.

Epitope tagging

To set up a general system for epitope tagging the identified clones, the phagemid vector pBK-RSV (Stratagene) was modified by insertion of an oligonucleotide cassette containing a Kozak sequence followed by an initiating methionine and then sequence encoding the c-myc peptide EQKLISEEDL. This cassette was flanked by unique restriction sites, at the 5¢ end and (antisense) 5¢-ATAGAGAAGGAGCTTTTTTTAC-3¢ and (antisense) 5¢-CCCTCTAGATGTTCTGAG-3¢ using as template a cDNA clone of Rab3a from rat brain. The blots were washed three times at 50°C in 6× SSC, then twice at 55°C for 20 minutes and finally twice at 55°C in 2× SSC for 20 minutes. The blots were exposed to Kodak XAR5 film at −70°C for up to 21 days.

Sequence analysis

Analysis of amino-acid sequence homologies was performed using the FASTA (Genetics Computing Co., Madison, WI) and WDNASIS (Hitachi) programmes and the GenBank data base.

Northern blot analysis

Northern blot analysis was performed using a commercial rat multiple tissue northern blot (Clontech) containing approximately 2 µg of poly(A)+ RNA per lane from eight rat tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis). The blot was prehybridised overnight at 42°C in Hybrisol I and hybridised overnight at 38°C with fresh Hybrisol I containing radiolabelled probe. All hybridisations and washes were performed using a Hybaid Micro4 hybridisation oven. A cDNA probe for rat GAPDH was produced using PCR primers (sense) 5¢-CTTGGCCAAGTGCTCATTG-CAAC-3¢ and (antisense) 5¢-CATGAGGTCACACCTGT-GTGGTG-3¢ from RBL.2H3 cDNA. A rat β-actin cDNA probe was produced using primers (sense) 5¢-GAGAGGATCTTCAGGCTGTA-3¢ and (antisense) 5¢-ATAGGAAGATTGTTGGAC-3¢ with RBL.2H3 cDNA. A cDNA probe corresponding specifically to the unique 3¢ end of Rab8b was produced by PCR using the following primers: (sense) 5¢-TGTAGAAGAGGACATTTTTTAC-3¢ and (antisense) 5¢-CCCTCTAGATGTTCTGAG-3¢ using in the in vivo excised Rab8b plasmid as template. A cDNA probe corresponding to the unique 3¢ cDNA sequence from rat Rab3a was produced using the following primers; (sense) 5¢-AGACCCTTGAAGCTGGTGG-3¢ and (antisense) 5¢-TCAGCAGGGCATCCTGAT-3¢ using as template a cDNA clone of Rab3a from rat brain. The blots were washed twice for 30 minutes at room temperature in 6× SSC, then twice at 55°C for 20 minutes and finally twice at 55°C in 2× SSC for 20 minutes. The blots were exposed to Kodak XAR5 film at −70°C for up to 21 days.

Transient transfections

PC12 or RBL.2H3 cells were trypsinized, washed once in complete medium (RPMI 1640, 10% foetal calf serum, 2 mM glutamate), once in serum free complete medium and resuspended at 10⁵ cells in 0.4 ml RPMI 1640 and placed in an electro-cuvette with 20 µg of plasmid prepared using QIAGEN megaprep columns as described by the manufacturer’s protocol. After incubation at 4°C for 10 minutes the cells were electroporated at 250 mV, 960 µF (Bio-Rad Gene-Pulser) and replaced at 4°C for 10 minutes. The remaining cells were washed in 10 ml of warm complete medium and 250 µl of this suspension added to each well of a 4-well chamber slide (NUNC). The slides were incubated at 37°C and after 24 hours, the cells were washed with fresh medium and cultured for a further 24 hours at 37°C.

Confocal microscopy

The slides were washed with PBS and fixed (3.7% formaldehyde in 1x PBS) for 15 minutes at room temperature. After three washes in TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 25 mM Trizma base, pH 7.4), the slides were blocked with solution B (TD buffer containing 0.25% BSA, 0.2% Triton X-100) for 30 minutes at
Identification of a novel Rab8 isotype

RESULTS

Rab cloning strategy

In order to investigate the possibility that mast cells and basophils may express novel members of the Rab family, we constructed a cDNA library from the RBL.2H3 cell line. The library was mass excised from the lambdaZapII phagemid vector and the cDNA was amplified with a pair of degenerate PCR primers designed to correspond to the most highly conserved regions of the Ras and Rab families (Chavrier et al., 1992). Primer 1 (sense) corresponds to the GXXXGKS/T phosphate and magnesium binding region (PM1) and primer 2 (antisense) corresponds to the WDTAGQE (PM3) region. The PCR reaction generated a 180 bp DNA fragment which was purified and radiolabelled as described. The radiolabelled 180 bp fragment was used to probe the RBL.2H3 library by plaque hybridisation at low stringency, and 294 positives were recovered from the primary screening of 2x10^6 clones. Twenty of these primary positives were progressed through secondary and tertiary hybridisation to single positive plaques. The cDNA inserts were in vivo excised and sequenced. Of the first twenty clones sequenced, two previously identified members of the Ras family were found, namely Ran1/TC4 and Rho A. These cDNA clones were fully sequenced, and PCR primers were designed against unique regions of these clones. These primers were then used to screen the remaining 274 primary positives by PCR. Using this approach we identified 183 isolates of Ran1/TC4 and 51 isolates of Rho A and consequently we could eliminate these from our screen. From the remaining 60 primary positives, twenty more were progressed to single positive plaques by hybridisation, and sequenced. From this second round of cloning and sequencing additional members of the Ras superfamily were found, and PCR primers were designed to eliminate these clones from the remaining pool of primaries as described above. This iterative process of cloning and elimination by PCR screening was repeated until all 294 original positives had been accounted for. From this strategy we recovered five different members of the Ras superfamily, Ran1/TC4 (183 clones), Rho A (51 clones), Rab7 (7 clones), Arf1 (1 clone) and one clone corresponding to a novel isotype of the Rab8 family, which we have named Rab8b (b for basophil). The 51 remaining clones were eliminated since they could not be amplified with the Rab PCR primers and were consequently considered to be non-Rab clones. The nucleotide and predicted amino acid sequence for the novel Rab8b clone is shown in Fig. 1. Alignment of the predicted amino acid sequence of Rab8b revealed an overall identity of 82.6% with dog and human Rab8 and an 83.0% identity with another member of the Rab8 family called MEL (Nimmo et al., 1991). A similar degree of identity is seen with a partial sequence of rat Rab8 which is also in the database. As shown in Table 1, the highest degree of identity of Rat Rab8b with a known member of the Rab8 family is 86.7% with the Rab8 isotype cloned from the electric ray (Discopyge ommata) called Ora2 (Fig. 2; Ngsee et al., 1991). Analysis of the known Rab8 family members shows that based on their amino acid sequence, they can now be tentatively arranged into groups. Rab8 and MEL show 97.6% sequence identity to one another but only about 83% identity to Rab8b and about 80% identity to Ora2. The sequence identities are greatest over residues 1-153 which represent the highly conserved N-terminal domain of all Ras family members. However, following the third guanine base binding region (G3), the sequence identity decreases markedly and over the C-terminal a-helix 5 region from residues 154-207, the closest homology is with Ora2 (56% identity) and Rab8 (52% identity).

Analysis of Rab8b tissue distribution

To investigate the tissue distribution of Rab8b we hybridised northern blots at high stringency with specific cDNA or

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Human Rab8</th>
<th>Human MEL</th>
<th>Rat Rab8b</th>
<th>D. ommata Ora2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Rab8</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Human MEL</td>
<td>97.6</td>
<td>100</td>
<td>83.0</td>
<td>86.7</td>
</tr>
<tr>
<td>Rat Rab8b</td>
<td>82.6</td>
<td>83.0</td>
<td>80.1</td>
<td>86.7</td>
</tr>
<tr>
<td>D. ommata Ora2</td>
<td>81.2</td>
<td>80.1</td>
<td>86.7</td>
<td>100</td>
</tr>
</tbody>
</table>
Rab8b sequence alignments. The predicted amino acid sequences of human Rab8, rat Rab8b and D. ommata Ora2 were aligned using the PILEUP programme (Genetics Computing Co., Madison, WI). Residues in Rab8b and Ora2 which are different from Rab8 are shown in italics. Sequence motifs involved in binding Mg\(^{2+}\) are underlined (based on data from Valencia et al., 1991).

Intracellular distribution of Rab8b

In order to study the intracellular distribution of Rab8b, we sub-cloned the cDNA into a mammalian expression vector and introduced a nine amino acid c-myc epitope tag at the N terminus which could be recognised by the monoclonal antibody 9E10. This epitope tagging approach has been extensively used for subcellular localisation studies as it avoids the problems of antibody cross-reactivity with other closely related members of the Rab family. The myc-tagged Rab8b expression vector was transfected into both RBL.2H3 and PC12 cells, and the cells further cultured for 48 hours prior to fixation and processing for immunofluorescence microscopy. As negative controls, cells were either transfected with the empty expression vector, or were transfected with the myc-tagged Rab vector followed by immunostaining with an irrelevant control antibody (data not shown). As a positive control, cells were transfected with a myc-tagged Rab3a expression vector since Rab3a is known to localise to vesicles on the regulated secretory pathway (Fischer von Mollard et al., 1990). Fig. 4 shows a representative example of RBL.2H3 and PC12 cells transiently expressing myc-Rab3a. In PC12 cells, the myc-Rab3a is clearly localized to discrete punctate vesicles in addition to vesicular structures in the juxtanuclear region of the cell, whereas in RBL cells the vesicular staining was apparent but less well defined. There was also some cytosolic staining in both cases, although the relative levels of cytosolic compared to vesicular staining was variable from transfection to transfection. In PC12 cells transiently transfected with myc-Rab8b (Fig. 5), staining with the 9E10 antibody shows up most prominently at the plasma membrane and occasionally on cytoplasmic vesicles, but in all cases was quite distinct from the staining previously shown for myc-Rab3a (Fig. 4). A number
Identification of a novel Rab8 isotype

Examples of myc-Rab8b transfections in PC12 cells are shown to demonstrate that different levels of expression of the protein can give slightly different staining patterns. At low levels of expression the staining is almost exclusively at the plasma membrane (Fig. 5A,B,D) and with increasing expression levels staining is seen at both the plasma membrane and occasionally in ill-defined vesicular structures (Fig. 5C).

In the RBL.2H3 cells (Fig. 6), the staining was also prominent both in the cytosol and on the plasma membrane and some ill-defined staining could also be detected on vesicular structures, however overexpression of myc-Rab8b in these cells appeared to induce a profound morphological change to the plasma membrane. The cells flatten onto the surface of the slide and produce a number of branched plasma membrane extrusions and ruffles. In some cases the myc-Rab8b staining was highly concentrated into these membrane extensions (Fig. 6D). Dual staining of the cells with 9E10 and an antibody to serotonin, which is a marker for the secretory granule, demonstrates that Rab8b does not co-localize with the granules (Fig. 7). In addition we show by dual labelling with an antibody to TGN38 that Rab8b staining does not co-localize with the Golgi. Overexpression of Rab8b often resulted in the RBL cell producing prominent plasma membrane ruffles and as can be seen in Fig. 7 these ruffles show strong staining for Rab8b.

In our experience RBL.2H3 cells can show a high degree of morphological variability, and to be more certain that the

![Fig. 4. Immunofluorescence detection of Rab3a in PC12 and RBL.2H3 cells.](image)

![Fig. 5. Immunofluorescence detection of Rab8b in PC12 cells.](image)
observed changes were indeed due to overexpression of Rab8b we examined examples of transfected and non transfected cells in the same field by fluorescence and phase contrast microscopy (Fig. 8). The untransfected RBL.2H3 cells normally grow adherent and appear rounded with one or two plasma membrane extensions. Occasionally, when the cells are seeded at low densities one can see examples of cells with more than two plasma membrane extensions, although these are usually regular and fine. The Rab8b transfected cells, on the other hand, are consistently more flattened and with pro-

**Fig. 6.** Immunofluorescence detection of Rab8b in RBL.2H3 cells. RBL.2H3 cells were transfected with a c-myc epitope tagged version of rat Rab8b in a modified pBK-RSV vector. The cells were incubated for two days prior to fixation and immunostaining with the 9E10 monoclonal anti-myc antibody followed by FITC-conjugated sheep anti-mouse second antibody. Fluorescence staining was detected by confocal scanning microscopy. Controls were performed by omission of the 9E10 antibody or by staining cells transfected with empty vector (data not shown). Bars, 10 μm. (A to D) A number of examples of the staining pattern and morphological changes seen. The myc-Rab8b staining was found both at the plasma membrane (arrow) and on numerous intracellular vesicles. The plasma membrane of the cells was in all cases extended into a number of extensions, which could be finely branched. In some examples (D) the Rab8b immunoreactivity was localized to the extremities of these branches (arrowheads).

**Fig. 7.** Rab8b, secretory granule and Golgi dual labelling studies in transiently transfected RBL cells. RBL.2H3 cells were transiently transfected with the myc-Rab8b expression vector and immunostained with 9E10 followed by FITC-conjugated anti-mouse second antibody (a) and with a rabbit anti-serotonin antibody followed by a TRITC conjugated anti-rabbit second antibody (b). The cells were examined by confocal scanning microscopy and a single confocal section is shown (section depth = 0.4 μm). The Rab8b staining is localised to plasma membrane ruffles (arrows), cytosol and some ill defined intracellular vesicles, however there is no co-localisation with serotonin which is a secretory granule marker. Similarly, transiently transfected cells were double stained with 9E10 (c) and a rabbit antiserum to TGN38 (d) showing that there is no co-localisation of Rab8b with Golgi membranes.
nounced irregular plasma membrane extensions. In order to confirm that this morphological change was specific to the overexpression of Rab8b, we transiently transfected RBL.2H3 cells with cDNAs corresponding to myc-tagged versions of Rac-2, VAMP-2 and Rab3a (Fig. 9). The transiently transfected cells were then double-stained with 9E10 and with rhodamine phalloidin to reveal the characteristic cortical ring of actin seen in these cells. In almost all cases the untransfected cells appear either rounded or with one or two plasma membrane extensions. As shown previously, overexpression of Rab8b induced an abnormal plasma membrane morphology whereas overexpression of Rab3a, Rac-2 or VAMP-2 had no apparent effect. From these double labelling experiments it can clearly be seen that the Rab8b co-localises with the cortical
actin ring beneath the plasma membrane. As a final control to verify that the changes in the appearance of the plasma membrane were due to Rab8b overexpression we quantified cell morphology in untransfected cells and cells transiently transfected with Rab3a, Rab8b or VAMP-2. In each case between 500 and 1,000 cells were examined microscopically and the number of plasma membrane extensions per cell was scored as either zero (a round cell), one, two, three, four or greater than four. As shown in Fig. 10, in control cells or cells transfected with VAMP-2 or Rab3a greater than 85% of the cells are either round or have one or two extensions and less than 4% had greater than four extensions. In Rab8b transfected cells, on the other hand, 55% of the cells had multiple plasma membrane extensions and only 33% were round or had one or two extensions.

**DISCUSSION**

Regulated secretion or exocytosis is the final vesicular transport step whereby preformed mediators are released from a cell following an activation signal (Burgoyne and Morgan, 1993). In this respect, regulated secretion is distinguishable from constitutive secretion which is a continuous process of vesicular transport of proteins from their site of synthesis to their final destination. Although many of the molecular components involved in regulated and constitutive secretion are common, there must be additional or specific components which control the vesicular fusion in response to extracellular signals. The aim of our studies is to develop an understanding of regulated secretion in leukocytes, particularly mast cells, basophils and eosinophils. These cells have numerous granules which contain an array of pre-formed inflammatory mediators, and degranulation of these cells in response to activation stimuli contributes to the pathology of allergic inflammation.

The basic intracellular requirements for both constitutive secretion (Rothman, 1994) and regulated secretion have been characterised in a number of cell types (Tatham and Gomperts, 1991; Burgoyne and Morgan, 1993). Studies in permeabilized cells and reconstituted cell free systems have shown that there is an absolute requirement for a number of cytosolic proteins for secretion to occur, and that guanine nucleotides play an important role in controlling the processes of vesicle formation and fusion. In regulated secretion, Ca\(^{2+}\) and ATP may be required for optimal granule fusion with the plasma membrane, depending upon the cell type; however, it is clear that GTP-binding proteins are crucial for these late events to occur (Lillie and Gomperts, 1992). The best characterised of these GTP-binding proteins are members of the Rab family, and there is good evidence for the role of Rab3 in regulated secretion in neuroendocrine cells (Fischer von Mollard et al., 1994a,b). However, in mast cells both heterotrimeric G-proteins (Lillie and Gomperts, 1992; Aridor et al., 1993) and members of the Rab family have been implicated in the control of exocytosis (Oberhauser et al., 1992). It has been shown by specific antibody reactivity that rat mast cells express the Rab3b isoform but not Rab3a (Izushi et al., 1992), and PCR based analysis of these cells has detected Rab3b and 3d (Oberhauser et al., 1994). Rab3b has also been detected in human platelets, and in the same study it was shown that Rab8 and Rab6 were localized to the plasma membrane and \(\alpha\)-granules (Karniguian et al., 1993). However, there have been relatively few studies on the distribution of Rab proteins in haematopoietic cells. Consequently, the aim of this present study was to screen a basophil cell line, RBL.2H3, for members of the Rab family using a modification of a PCR based cloning approach that has been successful in identifying novel Rabs (Chavrier et al., 1992).

Amplification of an RBL.2H3 cDNA library with degenerate PCR primers designed against the conserved regions of the Rab and Rho families produced the expected 180 bp product, which was subsequently used to screen the library by low stringency plaque hybridisation. Unlike previous studies (Chavrier et al., 1992), our cloning strategy produced predominantly two cDNAs which accounted for 62% (Ran1/TC4) and 17% (RhoA) of the recovered clones. The frequency of Ran1/TC4 clones recovered may be due to two factors. Northern blot analysis revealed that the Ran1/TC4 mRNA is abundant in the RBL.2H3 cells (data not shown), and the sequences of the degenerate PCR primers used are highly favourable for the amplification of this transcript. Both factors would contribute to the fact that the 180 bp PCR product that was used to probe the library was enriched in Ran1/TC4 sequences. Consequently, such PCR based strategies for the discovery of novel Rabs can have a number of serious flaws. Nevertheless, there were clearly two alternative approaches we could take to PCR based screening for novel Rab family members. The first was to perform the PCR with degenerate primers, then clone and sequence the products. If novel products are identified, they can be used to obtain full length clones by library hybridisation (Chavrier et al., 1992). This approach was highly successful at identifying novel Rabs; however, since the PCR product generated is across the highly conserved domain of the Rabs, it is less likely that such an approach would identify...
isotypes of previously identified Rabs which only diverge from one another outside of this conserved domain. The second approach is to isolate full length clones directly by using the degenerate PCR product to screen a library at low stringency. We decided to use this second approach because we reasoned that a number of exhaustive searches for novel Rabs had already been performed, and consequently we were more likely to discover novel isotypes than completely novel Rabs. Our strategy produced 294 primary clones from which we only identified five small GTP-binding proteins, one of which is a novel isotype of Rab8 which we have named Rab8b. We developed a screening procedure to rapidly work through this large number of clones which involved an iterative elimination of known cDNAs from the pool by PCR with primers designed to unique regions of each sequence.

The Rab8b clone that we have identified represents an additional member of the mammalian Rab8 family which now includes Rab8, MEL, Rab8b and Or2 from the electric ray. The sequence identity of Rab8b to other members of the family is around 80%, and it also shows 70% identity to the closely related Rab10. Other members of the Rab family also exist as multiple variants, notably Rab3a, b, c and d and Rab5a, b and c, and the sequence identity of the different isotypes to one another is commonly about 80-90%. The differences between Rab8 and Rab8b are almost all clustered in the variable C-terminal domain, and between residues 154-207 the identity decreases to about 50%.

To date there is only limited information about specific tissue or organelle distribution for different Rab isotypes. The best examples are those of Rab3a,b and c which are predominantly expressed in neuroendocrine tissues (Zahraoui et al., 1989; Lledo et al., 1993; Matsu et al., 1988; Fischer von Mollard et al., 1994a,b) and Rab3d which is expressed in adipose tissue (Baldini et al., 1992). To investigate the tissue distribution of Rab8b we have performed northern blot analysis using probes against the unique C-terminal domains of Rab8 and 8b. The results revealed the highest levels of expression of 8b in brain, spleen and testis which was in marked contrast to the distribution of Rab8 which was most abundant in lung, kidney and skeletal muscle. The finding that 8b is the predominant isotype in brain may have significance for the interpretation of a number of studies that have addressed the function of Rab8 in neuronal tissues (Huber et al., 1993a,b, 1995; Chen et al., 1993). The function of mammalian Rab8 in TGN to plasma membrane vesicle traffic is strengthened by the finding that the ypt2 gene in Schizosaccharomyces pombe functions at the last stage of vesicle transport to the plasma membrane and that temperature sensitive mutants of ypt2 can be rescued by mammalian Rab8 (Craighead et al., 1993).

In our transient transfections we observe variable levels of protein expression both between experiments and between individual cells. At the lowest level of expression Rab8b staining in PC12 cells is almost exclusively plasma membrane, and at higher expression levels both cytosol and ill-defined vesicular structures begin to accumulate Rab8b staining. We interpret this observation as the protein being predominantly localized to the plasma membrane at low expression levels and that overexpression results in it accumulating along the secretory or endocytic pathway. Recent studies to address the function of Rab8 have revealed that treatment of hippocampal neurons with antisense to Rab8 inhibits neurite outgrowth (Huber et al., 1995). The depletion of Rab8 dramatically reduced the number of vesicles undergoing anterograde transport the consequence of which was reduced outgrowth of neuronal processes. In our studies overexpression of Rab8b in RBL.2H3 cells induced a striking alteration in plasma membrane morphology. The transfected cells appeared flattened compared to non-transfected cells, and the plasma membrane displayed spectacular branching outgrowths and ruffles. Furthermore, we demonstrated that this morphological change was specific for Rab8b, since expression of Rab3a, Rac-2 or VAMP-2 did not result in a similar change. We confirmed these qualitative studies with a quantitative analysis which clearly demonstrated that the morphological change was seen in greater than 55% of RBL cells transiently transfected with Rab8b but was not seen in cells overexpressing Rab3a or VAMP-2. These results appear to confirm the antisense experiments showing that Rab8 and Rab8b are involved in plasma membrane expansion. Our finding that the Rab8b isotype is more abundant in brain than Rab8 may be significant for future studies on the regulation of neurite outgrowth.

REFERENCES


(Received 1 June 1995 - Accepted, in revised form, 11 March 1996)