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RT-PCR cloning of Rab3 isoforms expressed in peritoneal mast cells

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Abstract

Rab proteins are ras-like low molecular mass GTP-binding proteins, which are postulated to act as specific regulators of membrane trafficking in exocytosis and endocytosis. We have previously shown that synthetic peptides, corresponding to the effector domain of Rab3 proteins, stimulate a complete exocytotic response in mast cells. We have used a PCR-cloning strategy to investigate the presence of mRNA encoding Rab3 in mast cells. RNA based PCR was then performed on mast cell RNA using degenerate oligonucleotide primers based on two conserved sequences among Rab3 proteins. However, no PCR products were obtained, even for proteins known to be expressed in high copy numbers in mast cells (β-actin and Fc receptor). We have found that the problem resides in the presence of mast cell secretory granule derived heparin, that copurifies with the RNA; heparin has been shown to inhibit the activity of reverse transcriptase and Taq polymerase in PCR. After treating the RNA (obtained from about 500 mast cells) with heparinase, several PCR products of varying size were obtained using primers specific for Rab3 proteins. These products were cloned and sequenced. We have found clones containing sequences that had a 100% homology at the deduced amino acid level to a portion of Rab3B and Rab3D (amino acids 16 to 83).

Key words: Rab3 protein; RT-PCR; PCR cloning; Heparin inhibition; Peritoneal mast cell

1. Introduction

Recently, the diverse superfamily of small GTP-binding proteins have been implicated in the regulation of many aspects of cellular dynamics. Members of the Rab family have been implicated as important regulators of vesicle trafficking and membrane fusion [1–3]. More than 30 members of the Rab family have been identified. It is thought that each Rab protein controls a specific vesicular transport event [3]. For instance, Rab3A, one isoform of Rab3 has been implicated in neurotransmitter secretion because it has been found only in secretory cells [4,5], is localized to synaptic vesicles [6,7] and undergoes cellular redistribution during exocytosis [7]. It seems that there are different Rab3 isotypes controlling exocytosis in different cell types. Rab3D has been proposed to regulate the exocytotic fusion of vesicles carrying the glucose transporter GLUT-4 in adipocytes [8]. Rab3B has been shown to have an essential role in exocytosis in pituitary cells, because inhibition of expression of Rab3B with antisense oligonucleotides markedly reduced Ca^{2+}-dependent exocytotic fusion [9].

We have recently shown that synthetic oligopeptides, corresponding to the proposed effector domain of Rab3 proteins [10], stimulate a complete exocytotic response in mast cells [11]. These peptides have since been shown to produce a similar effect in several other cell types. For example in pancreatic acinar cells [12,13], adrenal chromaffin cells [14], insulin secreting cells [15], Helisoma cells [16] and in an exocytotis reconstitution system [17]. The mechanism of action of Rab3 peptides is not known; a plausible hypothesis is that the peptides directly activate the Rab3 effector proteins. We proposed that a sustained activation of a Rab3 protein was a sufficient stimulus for exocytosis in mast cells, and that this protein must be an integral part of the scaffold of proteins that causes exocytotic fusion [11,18]. An essential component of this hypothesis is the presence of Rab3 proteins in peritoneal mast cells. Their identification will permit the design of electrophysiological experiments to test their role in exocytosis. Peritoneal mast cells have been notoriously difficult to use in molecular biological experiments, requiring large numbers of animals and with modest results [19,20]. In this paper we report a simple methodology that allows RNA based PCR (RT-PCR) cloning from a small number of peritoneal mast cells (100 to 500). Using these methodologies we have detected the expression, in mast cells, of two members of the Rab3 family, Rab3B and Rab3D.

2. Materials and methods

2.1. Cell preparation

Total peritoneal cells (about 50% macrophages and 2% mast cells) were isolated from mouse or rat by peritoneal lavage [21]. Mast cells were purified using a metrizamide gradient (about 95% pure) [22]. To obtain a small number of mast cells, the purified cells were plated on glass bottom chambers, and individual mast cells were aspirated into a glass micropipette under Normaski optics. Mast cells were readily

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distinguished from other cell types by their characteristic morphology. The rat basophilic leukemia cell line RBL-1 was obtained from ATCC.

2.2 RNA preparation

Total RNA was extracted using an RNA STAT-60 kit (Tel-Test 'B' Inc. TEXAS) which is based on the method developed by Chomczynski and Sacchi [23]. To obtain RNA from a small number of mast cells, a glass micropipette containing mast cells was immersed in lysis buffer and sonicated (Branson model 450 sonifier, Branson Ultrasonics corporation, Danbury CT. Settings: Duty cycle 22%, Output control 25% for 6–7 pulses until the glass micropipette was broken to small pieces). Total RNA was then isolated according to kit instructions, using 10–20 μg of *E. coli* transfer RNA (Boehringer Mannheim) as a carrier.

2.3. Heparinase treatment of RNA

Total RNA was incubated with heparinase I (Sigma, 5 units/μg RNA) in 5 mM Tris pH 7.5, 1 mM $CaCl_2$, 50 units of RNasin (Promega) for 2 h at 25°C to remove native mast cell heparin.

2.4 Reverse transcription and PCR

Approximately 1 μg of RNA was reverse transcribed at 37°C for 60 min with a mixture of 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 5 μM random primers and 1 mM of each dNTP. The cDNA product was subjected to PCR using primers for Rab3, β-actin, and FcεRI. The Rab3 primers target a region that is specific for Rab3 proteins (DQNFDYM) and a region that is highly conserved between Rab proteins (WDTAGQE) [2,8,24]. The Rab3 primers are degenerate to accommodate for the codon uncertainty and flank a region of 201 bp. The primers for Rab3: 5′-GAY-CAR-AAY-TTY-GAY-TAY-ATG-3′ and 5′-YTC-YTG-NCC-NGC-NGT-RTC-CCA-3′. R is A or G; Y is C or T and N is any. For the PCR the cDNA and 100 μM of each primer in 100 μl of reaction mix (Gene Amp PCR core reagents, Perkin-Elmer) containing 1.5 mM $MgCl_2$ were used. The temperature cycles were 15 s at 94°C, 30 s at 45°C, and 20 s at 72°C. β-Actin and Fc receptors were used as positive controls. Primers for β-actin: 5′-ATG-CCT-CTG-GTC-GTA-CCA-CGG-GCA-TTG-3′ and 5′-CTT-GCT-GAT-CCA-CAT-CTG-CTG-GAA-GGT-3′ (flanking a region of 650 bp) [25]. Primers for FcεRI: 5′-TKG-GTC-ATT-GTG-AGT-GCC-ACC-3′ and 5′-GTG-TCC-ACA-GCA-AAC-AGA-ATC-3′ (flanking a region of 430 bp) [26]. We routinely included negative controls in the RT-PCR protocol ('no template' (no cDNA template) and 'no RT' (no reverse transcriptase) to rule out amplification of DNA from contaminating sources. In addition, in some experiments the purified RNA was pretreated with DNase I (5 U/μg RNA for 15 min) to eliminate any trace contaminant genomic DNA. However, this treatment did not change the results. PCR products were detected by electrophoresis in ethidium bromide stained agarose gels.

2.5 Subcloning and sequencing of PCR products

The PCR products were spliced into the PCR ready pCR II plasmid (TA cloning kit, Invitrogen, San Diego, CA), cloned and sequenced using a dideoxy termination sequencing kit (United States Biochemical Corp. Cleveland, OH).

2.6. Denaturing gel electrophoresis for RNA

The quality of the isolated RNA was checked using formaldehyde, denaturing agarose gel electrophoresis (1.5 h, 70 V) [27]. 1–3 μg samples of total RNA were applied to the gels.

3. Results

Total peritoneal cells (~ 50% macrophages, ~ 2% mast cells) were isolated from a mouse or a rat by peritoneal lavage. RT-PCR was performed as previously described [25]. 1–3 μg of RNA were reverse transcribed with a mixture Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and random hexamer primers. However, when the cDNA product was subjected to PCR using primers for β-actin, no bands were detected after

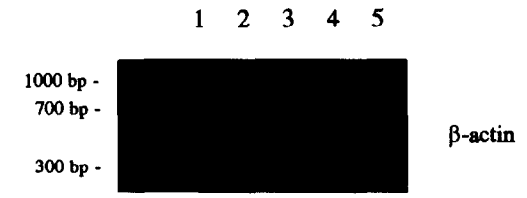


Fig. 1. RT-PCR in peritoneal cells. Agarose gel electrophoresis of RT-PCR products for β-actin mRNA from total peritoneal cells and rat basophilic leukemia (RBL) cells. First lane, DNA size standards; lane 1, PCR products from peritoneal cells RNA (3 μg) obtained after 60 cycles at 95°C 15 s, 50°C 25 s, 72°C 20 s; lane 2, PCR products from RBL RNA (0.5 μg) obtained after 30 PCR cycles; lane 3, PCR products from a 1.1 mixture of RBL and peritoneal cells RNA (1 μg of each); lane 4, PCR products from peritoneal cell RNA (1 μg) treated with heparinase; lane 5, PCR products from RBL cell RNA (0.5 μg) treated with heparinase. The PCR products in lanes 3 to 6 were obtained after 30 cycles at 95°C 15 s, 54°C 25 s, 72°C 20 s.

electrophoresis in ethidium bromide stained agarose gels, even after 60 PCR cycles (Fig. 1, lane 1). This was unexpected, since β-actin is an ubiquitous gene product that has been shown to be expressed in high copy numbers in mouse peritoneal macrophages [25]. Amplification of β-actin fragments was however possible from RNA isolated from a 'mast cell like' cell line, rat basophilic leukemia cells (RBL) (Fig. 1, lane 2). Surprisingly, we were unable to amplify β-actin fragments upon mixing RNA from freshly isolated peritoneal cells and RBL cells in equal amounts (Fig. 1, lane 3). One possible explanation for these results would be that the RNA is degraded due to contamination of the mast cell RNA with RNases. We therefore analyzed the RNA integrity by denaturing agarose electrophoresis. This analysis showed that the 28 S and 18 S ribosomal bands of peritoneal cells RNA were intact, indicating very little or no RNA degradation during the isolation (not shown). So, it is unlikely that the lack of amplification shown in lanes 1 and 3 of Fig. 1 was due to RNase contamination. These results suggest that the peritoneal mast cell RNA contain inhibitors of PCR.

Several inhibitors of PCR have been described [28]. Heparin has been shown to inhibit the activity of MMLV reverse transcriptase and Taq polymerase [29]. As peritoneal mast cell have large quantities of the heparin proteoglycan in their secretory granules, we therefore investigated if treating peritoneal mast cell RNA with heparinase would allow RT-PCR. We have found that after incubation of the RNA with heparinase I (5 units per μg RNA) for 2 h at 25°C, amplification of cDNA fragments by PCR was easily accomplished (Fig. 1, lane 4).

Using this method we were able to obtain PCR products with degenerate primers for Rab3 proteins, using the RNA isolated from a large number of peritoneal mast cells (about 10^6 cells) (Fig. 2A). However, although the purity of the mast cell samples was very high (about 95%) it was difficult to be certain about the origin of the PCR products. To demonstrate the presence of Rab3 mRNA

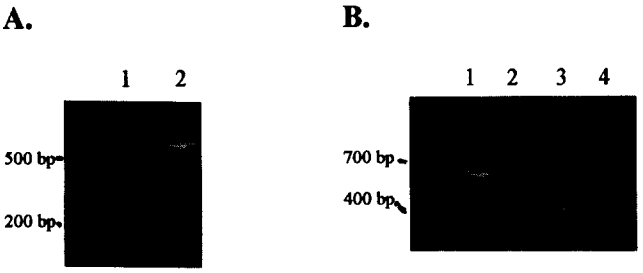
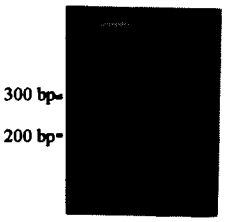


Fig. 2. (A) Detection of Rab3 (lane 1) and β-actin (lane 2) PCR products from RNA isolated from 10^6 purified rat peritoneal mast cells. The PCR consisted of 30 cycles of 94°C 15s, 45°C 30 s, 72°C 20 s, with 1 μM of degenerate primers for Rab3 proteins. (B) Detection of β-actin (lanes 1,2) and FcεRI (lanes 3,4) cDNA from about 500 individually picked peritoneal mast cells from a rat (lanes 1,3) and a mouse (lanes 2,4), after 45 cycles of PCR at 95°C 15 s, 54°C 25 s, 72°C 20 s.

in peritoneal mast cells, we developed a procedure to isolate RNA from a small number of mast cells. Mast cells were purified using a metrizamide gradient, plated in glass bottomed chambers and individual mast cells aspirated into a glass micropipette under Normarski optics. Mast cells were readily distinguished from other cell types by their characteristic morphology. After collecting about 500 cells, the pipette tip was immersed into the lysis buffer and was broken by sonication. Total RNA was isolated in the presence of 10–20 μg of *E. coli* transfer RNA as a carrier [25], and then treated with heparinase I. Fig. 2B shows that this method resulted in successful PCR amplification of several gene products from both rat and mouse peritoneal mast cells, including β-actin and the high affinity IgE receptor (FcεRI), a protein that is uniquely expressed in mast cells and basophils [26,30]. As Fig. 3 shows, we were also able to obtain Rab3 PCR products from a few peritoneal mast cells. As expected, the products of the PCR reaction obtained with the Rab3 degenerate primers migrated in a 3% agarose gel as a band of approximately 200 bases. These PCR products were ligated into a TA cloning pCRII vector (Invitrogen). About 100 positive clones were obtained and 5 were sequenced. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3B. Three of these clones were 100% identical to Rab3B and the other two were identical to Rab3D (amino acids 16 to 83). To demonstrate the specificity of this method, we subcloned and sequenced Rab3 PCR products obtained from total mouse brain RNA. Two out of a total of 25 positive clones were sequenced and found that they were 100% identical to the mouse Rab3a nucleotide sequence [5].

4. Discussion

We have presented evidence showing that peritoneal mast cells express two isotypes of Rab3 proteins, Rab3B and Rab3D. These results strengthen the hypothesis that Rab3 proteins play a role in exocytosis in mast cells. We have previously shown that synthetic peptides corresponding to the Rab3A effector domain caused complete exocytotic degranulation in patch-clamped mast cells [11]. However, since the putative effector domains for the different Rab3 isotypes are very similar we could not implicate a specific member of the Rab3 subfamily. We can now postulate that the Rab3 peptides are probably mimicking the action of Rab3B or Rab3D. It is not known which of the two isotypes regulates oxocytosis in mast cells; Rab3B and Rab3D have been shown to play a role in exocytosis in pituitary cells [9] and in adipocytes



B.

Rab3B:

GACCAGAATTTTGATTATATGTTCAAACTGCTCATCATTGGCAACAGCAGCGTCGGGAAG
ACCTCCTTCCTTTCCGCTATGCTGATGACACCTTCACCCCTGCCTTTGTCAGCACTGTGG
GTATCGACTTCAAAGTGAAGACAGTTTACCGTCATGAGAAGCGTGTGAAGCTGCAGATAT
GGGACACGGCGGGTCAGGAA

DQNFDYMFKLLIIGNSSVGKTSFLFRYADDTFTPAFVSTVGIDFKVKTVYRHEKRVK
LQIWDTAGQE

Rab3D:

GACCAGAATTTTGATTATATGTTCAAACTGCTTTTGATCGGGAACAGCAGCGTAGGCAAG
ACCTCCTTCCTGTTCCGCTATGCCGATGACTCCTTCACCCCCGCCTTCGTGAGCACAGTGG
GCATTGACTTCAAGGTCAAGACAGTCTACAGACATGACAAGAGGATCAAGCTGCAGATCT
GGGACACAGCAGGACAAGAA

DQNFDYMFKLLLIGNSSVGKTSFLFRYADDSFTPAFVSTVGIDFKVKTVYRHDKRIK
LQIWDTAGQE

Fig. 3. PCR cloning and sequencing of Rab3 RNA transcripts in peritoneal mast cells. (A) PCR products obtained with specific primers for Rab3 proteins. The cDNA isolated from about 500 rat peritoneal mast cells was subjected to 45 cycles of PCR at 94°C 15 s, 45°C 30 s, 72°C 20 s. The PCR products obtained with the Rab3 degenerate primers were subcloned into a pCRII vector. As shown, the subcloned PCR products migrated as a single band of 230 bp in a 3% agarose gel (where the additional bases correspond to the two pCRII segments between the mast cell inserts and the *Eco*RI sites in the cloning vector). (B) Nucleotide and predicted amino acid sequence of the Rab3 PCR products obtained from rat peritoneal mast cells were 100% identical to Rab 3B and Rab 3D. The areas where Rab 3B and Rab 3D are distinct from each other are underlined. Differences both at the nucleotide and predicted amino acid level are shown.

[8], respectively. Using specific antibodies, it has been shown that human platelets [31] and rat mast cells [32] express Rab3B but not Rab3A. Interestingly, it was shown that secretion of histamine was associated with the phosphorylation of Rab3B [32]. This raises the possibility that Rab3B may be the isotype that participates in exocytosis in mast cells. However, it is also possible that both proteins, Rab3B and Rab3D, participate in different branches of regulated exocytosis in the mast cell. Further experimentation is required to resolve this question.

We found that the heparin contained in the mast cell secretory granules inhibits RT-PCR. This result may explain why it has been so frustrating to study mast cells using molecular biological techniques. It is difficult to prepare acceptable yields of high quality RNA from mast cells using conventional RNA isolation techniques such as guanidinium isothiocyanate and centrifugation through cesium chloride [19,20]. The isolated RNA has been used for Northern blot analysis [33], in vitro translation of proteins [20] and amplification of cDNA fragments using PCR [26]. However, in order to prepare acceptable quantities of good quality RNA a surprisingly large number of mast cells were needed in all these studies (more than 2×10^7 cells or 20–50 rats per experiment!). It was therefore suggested that mast cells have a limited protein synthetic capacity and contain extremely low amounts of mRNA [19]. Because of these problems, almost all the molecular biology of mast cell gene products has been performed using transformed (e.g. rat basophilic leukemia) and non-transformed (e.g. bone marrow derived mast cells) 'mast cell like' cell lines. However, these cells are only distant models for the highly differentiated mature peritoneal mast cells. We now have shown that the difficulties in using PCR in the peritoneal mast cells are created by heparin that copurifies with the RNA. Heparin has been shown to bind nonspecifically to oligonucleotides [27], several oligonucleotide binding proteins (like RNA polymerase [34]), inhibit reverse transcription and PCR [29], and inhibit gene expression [35]. Analysis of RNA from tissues rich in proteoglycans such as skeletal tissues has also been shown to be difficult since proteoglycans copurify with the RNA and decrease the sensivitity of Northern blots [36]. The methods described here should simplify studies of the function of gene products in native mast cells.

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