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IDENTIFICATION OF RAB 5 BUT NOT RAB 3A IN RAT PANCREATIC ZYMOGEN GRANULE MEMBRANES

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SUMMARY: Low molecular weight GTP-binding (LMWG) proteins are known to be present on secretory organelles and a role for Rab 3A in exocytosis of synaptic vesicles has been postulated. Pancreatic zymogen granules possess multiple LMWG proteins which were shown to be on the outside of the granules by pronase susceptability. Rab 3A, however, could not be identified in rat pancreatic acinar cells or on zymogen granules by immunoblots or by northern blots or PCR amplification although all three techniques demonstrated its presence in rat brain, RINm5F and AR42J cells. Immunoblots revealed expression of Rab 5, Rab 7, Rab 11 and ARF in acini. Of these, Rab 5 was identified in purified zymogen granule membranes where it may be involved in the endocytotic retrieval of granule membrane following exocytosis.

Pancreatic acinar cells synthesize and secrete digestive enzymes or proenzymes which are stored in zymogen granules located in the apical pole of the cell. Stimulation with secretagogues triggers a series of events resulting in fusion of the zymogen granule membrane with the apical plasma membrane and subsequent release of the zymogen granule content into the pancreatic duct. Following exocytosis, the granule membrane is retrieved by endocytosis and recycled (1). Although early events in stimulus-secretion coupling are well understood (2), little is known about the processes involved in fusion of zymogen granule membranes with the apical plasma membrane and exocytosis. The characterization of zymogen granule membrane components, particularly its proteins, can therefore be expected to yield insights into these processes.

In recent years, low molecular weight GTP-binding proteins (LMWGP) have emerged as a large superfamily of proteins regulating diverse cellular functions including secretion and vesicular trafficking (3, 4). In yeast, the LMWG protein SEC4 regulates a post-Golgi event in constitutive secretion (5), while in mammalian cells members of the Rab subfamily regulate vesicular trafficking (6). One of the first Rab's for which a possible function was shown is the Rab 3A protein. It is a component of synaptic vesicle membranes (7) and has been implicated in the

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regulation of neurotransmitter release (8). LMWG proteins have also been found on secretory organelles in other tissues (9, 10).

Evidence indicates that pancreatic enzyme secretion may also be regulated by LMWGP's. Studies on streptolysin-O permeabilized pancreatic acini have shown that GTPγS increases Ca^{2+}-dependent amylase secretion, supporting the concept that a G-protein may regulate a terminal event in stimulus-secretion coupling (11, 12). In addition, [α-P^{32}]GTP or [α^{35}S]GTP labeling of nitrocellulose membranes after western transfer has shown multiple LMWG proteins in pancreas, some of which have also been found in purified zymogen granule membranes (13, 14). However, none of the LMWGP's found in zymogen granule membranes have yet been identified. In this study we show that LMWGP's present in pancreatic zymogen granule membranes are exposed on the outer surface of the granules. We also show that while Rab 3A is present in RINm5F and AR42J cells, its expression in rat pancreatic acini could not be established at either the protein or nucleic acid level. We found that ARF, Rab 5, Rab 7 and Rab 11 are expressed in rat pancreas, and have identified the presence of Rab 5 in zymogen granule membranes.

MATERIALS AND METHODS

Chemicals: Detection reagents for enhanced chemiluminescence (ECL) were from Amersham (Arlington Heights, IL); Pronase was from Boehringer Mannheim (Indianapolis, IN). One rat pancreatic cDNA and one AR42J cell library were from Clontech (Palo Alto, CA). The other rat pancreatic and AR42J cell cDNA libraries were gifts from C. Logsdon; the RINm5F cell library was a gift from J. Dixon. Antibodies against Rab 5, 7 and 11 were generous gifts from M. Zerial, EMBL, Heidelberg, Germany, antibodies against ARF were a generous gift from R. Kahn, NIH, Bethesda, MD, antibodies against Rab 3A were a generous gift from I. Macara, University of Vermont.

Purification of zymogen granules and zymogen granule membranes: Purified zymogen granules and zymogen granule membranes were prepared by Percoll gradient separation following previously published procedures with slight modifications (15). Protein content was determined with the BioRad protein assay kit using bovine serum albumin as standard. SDS-PAGE, Western analysis and pronase digestion (15) as well as [α^{35}S]GTP labeling of low molecular weight proteins (14) were carried out as described.

Polymerase chain reaction: Primers were designed according to the published rat brain Rab 3A sequence (16) with the following sequences: Primer I: 5'-AAGGATCCATGGCCTCAG-CACAGACTCTCG-3' and Primer II: 5'- AAGAATTCTCAGCAGGCGCAATCCTGAT-3'. For efficient subcloning a BamHI site was added to the 5' end of primer I and an EcoRI site to the 5' end of primer II. Seperate PCR reactions were carried out using 50 pmol of each of the primers together with $3x10^5$ clones from the different cDNA libraries. Reactions were run on a thermal cycler for 40 cycles of 94°C denaturing, 62°C annealing and 72°C extending temperature using Taq DNA polymerase as described by the manufacturer (Perkin-Elmer Cetus Instruments). PCR products were then subcloned into pUC18 plasmids and sequenced with the dideoxynucleotide chain termination method using a commercially available kit (Amersham).

Preparation of RNA: RNA from whole pancreas and isolated acini was prepared as previously described (17) with two additional ethanol-precipitation steps added to improve RNA quality. For isolation of rat brain RNA, freshly removed brain without cerebellum was minced on ice and RNA prepared as described (18). Two additional phenol-chloroform extractions and isopropanol precipitations were performed in order to obtain high quality RNA. The final RNA was dissolved in TE-buffer pH 7.6 containing 0.5% (w/vol) SDS, and stored at -70°C, after 2.5 vol ethanol were added.

Northern transfer: For Northern analysis, 50 µg of RNA were electrophoresed on 1% agarose gels containing 1.8% formaldehyde at 40 mA for 6 hours and blotted onto nitrocellulose-membranes overnight. Membranes were then dried at room temperature, baked in a vacuum oven for 2 hours at 80°C and prehybridized for 10 hours at 42°C in 50% (vol/vol) formamide, 5x Denhardt's reagent, 100 µg/ml denatured salmon sperm DNA, 5% (w/vol) dextran sulphate, 0.1% (w/vol) SDS and 10 mM vanadyl ribonucleotide complex. 50 ng α-^{32}P-labelled cDNA probe (spec. activity 10^9 cpm/µg DNA) was then added and hybridization continued overnight. Probes were labelled using random hexamer primers. Membranes were then washed twice for 15 min. in 1xSSC and 0.1% SDS, 2x15 min. in 0.25xSSC and 0.1% (vol./vol.) SDS at room temp. and 1-2 times in 0.25xSSC and 0.1% SDS at 42°C and autoradiographed with intensifying screens at -70°C.

RESULTS AND DISCUSSION

Low molecular weight GTP-binding proteins (LMWGP's) have previously been found on pancreatic zymogen granule membranes (13, 14). In order for the LMWGP's to function as molecular switches regulating vesicular trafficking and possibly exocytosis in acinar cells, these proteins should be exposed on the cytoplasmic surface of the zymogen granules. We used the nonspecific protease pronase as a tool to determine the sidedness of zymogen granule membrane LMWG proteins. When intact granules are treated with pronase only proteins exposed on the outer surface of the granules will be digested, while the proteins anchored in the inner leaflet of the zymogen granule membrane will be protected. Since labeling of LMWGP's with [α^{35}S]GTP is lost when intact granules are treated with pronase (Fig.1), it is evident that all LMWGP's detected by [α^{35}S]GTP-labeling reside on the cytoplasmic surface of the granules. Pronase treatment does not destroy the granules themselves since we have previously shown that two zymogen granule membrane glycoproteins, GP-2 and GP-3, are protected from the action of pronase as long as the granules remain intact (15).

To understand the function of these LMWGP's and their possible involvement in regulating exocytosis, identification of these proteins is required. The LMWGP Rab 3A has been implicated in the regulation of neurotransmitter release from synaptic vesicles (8). Using a monoclonal antibody for immunocytochemistry, Rab 3A has further been reported to be present in exocrine and endocrine rat pancreatic tissue (19). Moreover, peptides similar to the putative effector domain of Rab 3A (Rab 3AL peptide) were shown to stimulate both amylase release in SLO-permeabilized rat pancreatic acini (20) as well as fusion of purified zymogen granules with purified plasma membranes (21). Since Rab 3A thus appeared as a plausible candidate for a Rab protein regulating acinar exocytosis, we have investigated its expression in pancreatic acini. However, using a polyclonal antibody raised against a unique peptide sequence near the C-terminus of rat Rab 3A for western analysis (22) we failed to identify Rab 3A in whole acinar cell lysates and in purified zymogen granule membranes (Fig. 2). As a positive control the antibody detected Rab 3A as a 27 kDa protein in rat brain. We also detected Rab 3A in RINm5F and AR42J cells. Expression of Rab 3A in RINm5F cells has also been found by northern analysis (23). We also investigated the expression of Rab 3A at the nucleic acid level. Oligonucleotides designed according to the rat brain Rab 3A sequence (16) were used for PCR amplification using a RINm5F as well as two different pancreatic and two different AR42J cell libraries as templates.

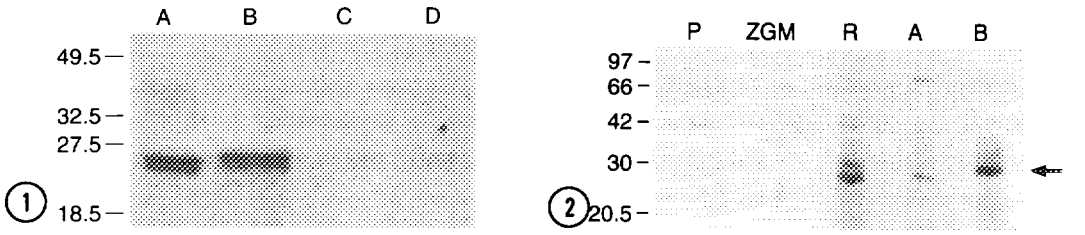

Figure 1. LMWG proteins in zymogen granule membranes are exposed on the cytoplasmic surface of the granules. Intact granules were incubated alone (lane A), with the nonionic detergent NP-40 (B), with pronase (C) and with pronase and NP-40 (D). Five μg protein were then resolved on 8 % minigels, transferred to nitrocellulose membranes and LMWGP's labeled by incubation with [α^{35}S]GTP. Molecular mass markers in kDa are indicated on the left.

Figure 2. Rab 3A is expressed in RINm5F and AR42J cells but not in pancreatic acini. Five μg protein of pancreatic acinar lysate (P), zymogen granule membranes (ZGM), RINm5F cells (R), AR42J cells (A) and rat brain homogenate (B) were separated and transferred as in Fig 1. Membranes were then probed with affinity-purified anti-Rab 3A antibody followed by detection with peroxidase coupled anti-rabbit IgG visualized by ECL.

PCR products of the expected size were obtained from RINm5F and AR42J cells but not from pancreas (Fig. 3, left panel). We subcloned the PCR products and determined that their sequence was identical to the published rat brain Rab 3A sequence (not shown). Using the cloned Rab 3A as probe for northern analysis we obtained a strong positive signal from rat brain but not from whole pancreas or pancreatic acinar total RNA (Fig. 3, middle panel). To ensure that our pancreatic and acinar RNA was of good quality, we also used a cDNA coding for GP-3, a recently cloned rat pancreatic zymogen granule membrane glycoprotein (24), as a probe for northern analysis and obtained a strong signal with RNA from whole pancreas and isolated acini but not from brain (Fig. 3, right panel).

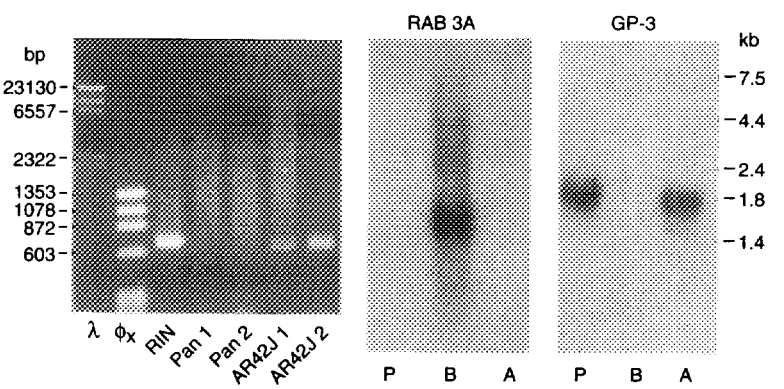

Figure 3. Polymerase chain reaction and northern analysis confirm that Rab 3A is not expressed in exocrine pancreas. Left panel: PCR was performed using a RINm5F (RIN), two different AR42J (AR42J 1 and 2) and two different rat pancreatic (Pan 1 and 2) cDNA-libraries as template for amplification of Rab 3A. Molecular size standards (λ/Hind III (λ) and φX174RF/Hae III (φ) fragments) are indicated on the left. Middle panel: Rab 3A cloned from RINm5F cells was used for northern analysis of total pancreatic (P) total rat brain (B) and total pancreatic acinar (A) RNA. Right panel: The pancreas-specific GP-3 cDNA was used for northern analysis of total RNA from rat pancreas, brain and acini.

Taken together our data clearly indicates that Rab 3A is not expressed in rat pancreas. This is in accordance with earlier studies based on northern (25) as well as western (22) analysis of multiple organs which reported that Rab 3A is expressed exclusively in brain, although pancreas was not investigated in these studies. Interestingly, studies using different monoclonal antibodies against Rab 3A and Rab 3B have identified a Rab 3-like protein in rat pancreatic acini which is neither Rab 3A nor Rab 3B (27,28). The presence of such a Rab 3-like protein might explain the positive reaction of some anti-Rab 3A antibodies with extraneuronal tissue (19). Recent evidence also suggests that results obtained with the Rab 3AL peptide have to be interpreted with caution since this peptide has been shown to stimulate secretion in mast cells via a pertussis toxin sensitive pathway by activating a heterotrimeric GTP-binding protein in a fashion similar to mastoparan (26). Thus, data obtained with this peptide cannot be taken as evidence for Rab 3-mediated mechanisms. It seems surprising that although Rab 3A is expressed in RINm5F cells we were unable to amplify Rab 3A from pancreatic cDNA libraries. This can be explained by findings that Rab 3A is expressed in human insulinomas but not in normal human pancreatic islets (29), indicating that expression of Rab 3A in endocrine cells occurs only after transformation.

In an attempt to further characterize pancreatic LMWG proteins we have also used antibodies against other LMWG proteins for western analysis of whole acinar cell lysate, purified zymogen granules and zymogen granule membranes (Fig 4, lanes P, ZG and ZGM, respectively). Using a previously characterized polyclonal antibody directed against residues of 23-36 of bovine ADP-ribosylation factor (ARF) (29) we could identify two species of ARF with molecular weights of 20 and 22 kDa in acini (Fig. 4, left upper panel). Recognition of two species of ARF is in accordance with earlier data using this antibody (29). We have similarly used specific polyclonal antipeptide antibodies (30) against Rab 7, Rab 11 and Rab 5 proteins, showing that all of these LMWGP's are expressed in pancreatic acini with molecular weights of 27, 26 and 25 kDa

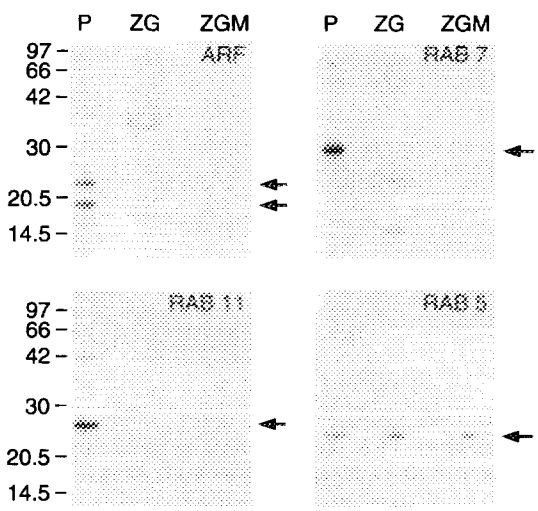

Figure 4. Identification of several LMWG proteins in pancreatic acini. Five μg protein of acinar lysate (P), purified zymogen granules (ZG) and purified zymogen granule membranes (ZGM) were separated and transferred as in Fig 1. Membranes were then probed with antibodies against ADP-ribosylation factor (ARF), Rab 7, Rab 11 and Rab 5.

respectively (Fig. 4). The only LMWG protein we also identified in our zymogen granule and zymogen granule membrane preparations was Rab 5 (Fig. 4). Thus, Rab 5 is the first LMWG protein which has been identified in zymogen granule membranes. Rab 5 has been previously shown to be localized to early endosomes in MDCK cells (30) and to regulate early endosome fusion in vitro (31). One could therefore hypothesize that Rab 5 might be involved in the endocytotic retrieval of zymogen granule membranes after exocytosis has occurred rather than in the regulation of acinar exocytosis.

In conclusion, we have shown that LMWG proteins, identified by [α^{35}S]GTP-labeling in rat pancreatic zymogen granule membranes are exposed on the cytoplasmic surface of the granules. We have also shown that Rab 3A is expressed in RINm5F and AR42J cells but not in pancreatic acinar cells. We have finally shown that several other LMWG proteins are expressed in rat pancreatic acinar cells and have identified one of the LMWG proteins present in zymogen granule membranes as Rab 5.

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