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Distinct and specific GAP activities in rat pancreas act on the yeast GTP-binding proteins Ypt1 and Sec4

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Previous studies have demonstrated that Sec4, a 23.5 kDa guanine nucleotide-binding protein of the ras superfamily is required for exocytosis in the budding yeast *Saccharomyces cerevisiae*. Ypt1, another ras-like 23 kDa guanine nucleotide-binding protein in yeast has been found to be involved in ER-Golgi transport. A mammalian homologue of Ypt1 called rab1 has also been identified. Recent studies using purified Sec4 protein have identified a component of yeast lysate that specifically stimulates the hydrolysis of GTP bound to Sec4. In the present study, purified recombinant Sec4 and Ypt1 proteins expressed in *E. coli* have been used as substrates to determine if GTPase activating proteins (GAPs) directed toward these proteins are present in rat pancreas. Our studies showed that 65% of Sec4-GAP activity was associated with the 150,000 × *g* pancreatic particulate fraction with approximately 35% being found in the cytosol. On the other hand, more than 95% of Ypt1-GAP activity was found to associate with the particulate fraction. Sec4 and Ypt1 competition assays further demonstrated the specificity of the Sec4 and Ypt1 GAPs. The results from the present study suggest the presence of a distinct GAP in the pancreas that interacts with Sec4, and another that interacts with Ypt1.

Yeast GTP-binding protein: Ypt1 and Sec4; Pancreatic Sec4-GAP and Ypt1-GAP

1. INTRODUCTION

In recent years, a number of *ras*-like low-molecular-weight GTP-binding proteins have been identified and some have been implicated in distinct vesicular transport processes. In the budding yeast *Saccharomyces cerevisiae*, the association of a 23.5 kDa GTP-binding protein (Sec4) with post-Golgi secretory vesicles is required for exocytosis. Mutations in the *SEC4* gene have been shown to cause defects in fusion of vesicles with the plasma membrane, leading to their accumulation in the cytosol [1–3]. Similarly, Ypt1 of molecular weight 23 kDa has been implicated in protein transport from the endoplasmic reticulum (ER) to the Golgi complex in yeast [4,5]. Ypt1 is associated with the Golgi [4] as well as ER–Golgi carrier vesicles [6]. Extensive similarity is seen between the Sec4 and Ypt1 proteins [2,4–9].

Unlike the heterotrimeric GTP-binding G proteins that possess relatively high intrinsic GTPase activity, the small *ras*-like GTP-binding proteins have very low intrinsic GTPase activity. Small GTP-binding proteins associate with specific GTPase activating proteins (GAPs), resulting in the potentiation of hydrolysis of bound GTP. Site directed mutagenesis of the putative effector domain of Ypt1 (as defined from studies on *ras* as residues 32 through 40) suggests the interaction of GAP with this region is necessary for function of Ypt1 in regulating ER to Golgi transport. In these mutants small vesicles accumulate between the ER and Golgi [10]. Recently, a similar mutation in Sec4 [11], that results in the reduction of both intrinsic and stimulatable rates of GTP hydrolysis by Sec4, was shown to cause decrease in secretion, and resulted in an accumulation of post Golgi secretory vesicles. In addition, reports from studies with H-*ras* and *ras*-GAP [12–17] demonstrate the importance of hydrolysis and turnover of GTP bound to these small GTP-binding proteins.

Recent studies on mammalian *ras*-GAP demonstrate that the putative effector-binding site, defined as residues 32–40, is identical in all *ras* proteins from yeast to mammals, explaining why mammalian *ras*-GAP can functionally substitute for the yeast Ira1 and Ira2 proteins [16,17,19–21]. In addition, several studies [22–24] demonstrate that small GTP-binding proteins with identical effector domains such as yeast Ypt1 and the mammalian rab1, are able to functionally substitute for each other. Similarly, mammalian rab6 and *S. pombe* ryh1 are able to functionally substitute for each other. These studies suggest that the specific interaction of a GAP with its small GTP-binding protein is in part determined by the effector binding site on GAP as well as the effector domain on the GTP-binding protein. Functionally homologous GTP-binding proteins therefore

Abbreviations: SBTI, soybean trypsin inhibitor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol; PEI-TLC, polyethyleneimine cellulose thin layer chromatography; PMSF, phenylmethylsulfonyl fluoride.

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should substitute for each other, even between cells as divergent as yeast and mammals.

Although a GAP activity from porcine liver that accelerates GTPase activity of Ypt1 and rab1 proteins has been reported recently [10], no mammalian Sec4 homologue or a mammalian GAP activity directed toward Sec4 has been identified to date. Our study demonstrates, for the first time, the presence of a specific GAP activity directed toward Sec4 in the rat pancreas and suggests the presence of a Sec4-like substrate in mammals. The results presented in this study demonstrate the presence of specific Sec4- and Ypt1-GAP proteins. In addition, these studies describe the particulate and cytosolic distribution of rat pancreatic GAP directed toward purified recombinant Sec4 and Ypt1 proteins.

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]GTP and [γ-^{32}P]GTP were obtained from Amersham Corporation, Arlington Hts., IL. Male Sprague-Dawley rats were obtained from Camm Research Laboratory Animals, Wayne, NJ. *N*-Dodecyl octaethyleneglycol monoether ($C_{28}H_{58}O_9$), a non-ionic surfactant and SBTI were purchased from Calbiochem Co., La Jolla, CA. PEI-TLC plates, BSA, DTT, benzamidine and PMSF, were purchased from Sigma Chemical Co, St. Louis, MO. EDTA and sucrose were from J.T. Baker Chemical Co, Phillipsburg, NJ. HEPES, aprotinin, pepstatin, GTP, GDP, GMP, GMP-PNP and GTPγS were from Boehringer Mannheim, Indianapolis, IN. Type HA, 0.45 μm nitrocellulose filters were purchased from Millipore Corporation, Bedford, MA. G-50 Sephadex was obtained from Pharmacia, Piscataway, NJ.

Sec4 and *Ypt1*: native Sec4 and Ypt1 genes inserted into pET11d vector, were expressed in *E. coli* BL21(DE3), using a T7 expression system [25]. Details of expression, characterization and purification of the Sec4 and Ypt1 proteins have been published [11,26].

2.2. *Preparation of rat pancreatic fractions*

150,000 × *g* rat pancreatic particulate and cytosol fractions as well as a 1 M salt extract of the particulate fractions were prepared by the following method. Six male Sprague-Dawley rats (200–250 g each) were starved overnight and euthanasia was performed by cervical dislocation. Subsequent processing of the tissue was performed at 4°C. The pancreata were dissected out and diced into ~0.5 mm^3 pieces. The diced tissue was homogenized in 5 vols. of buffer A (25 mM HEPES, pH 7.5, 0.3 M sucrose, 0.5 mM $MgCl_2$, 1 mM benzamidine, 0.1% wt/vol Trasylol and 0.01% SBTI) using 10 strokes of a Teflon-glass homogenizer. The homogenate was then passed twice through an 18-gauge needle. A third of the homogenate was flash frozen in liquid nitrogen and saved at −70°C. The rest of the homogenate was centrifuged at 150,000 × *g* for 60 min to generate cytosol and particulate fractions that were similarly flash frozen and stored at −70°C.

Salt stripping of the 150,000 × *g* pancreatic particulate fraction was performed by incubating the particulate fraction in buffer B (Buffer A containing 1 M NaCl) for 60 min with mixing every 15 min. A fifth of the particulate fraction in buffer B was saved and the rest centrifuged at 150,000 × *g* for 60 min to obtain a particulate and soluble fraction. All three fractions were dialyzed for 12 h in 4 changes of 1000 vols. of buffer C (50 mM HEPES, pH 7.4, 0.5 mM PMSF and 1 mM benzamidine), prior to flash freezing and storage.

2.3. *GTPγS binding to Ypt1 and Sec4p*

The time course of binding GTPγS to Sec4 was performed by incubating ~300 nM Sec4p with 2.4 μM [^{35}S]GTPγS at 37°C in buffer D (40 mM HEPES, pH 8.5, mM $MgCl_2$ 1 mM DTT and 0.1% $C_{28}H_{58}O_9$). At each time point, two 5-μl aliquots were diluted into 2 ml of ice-cold TNMg buffer (20 mM Tris-HCl, pH 8, 100 mM NaCl and 25 mM $MgCl_2$) and rapidly filtered through a 0.45-μg type HA millipore nitrocellulose filters. The filters were washed with TNMG buffer and counted as described earlier [27]. The time course of GTPγS binding to Ypt1 was performed as for Sec4 binding except that the binding reaction was carried out in buffer E (40 mM HEPES, pH 8, 2 mM EDTA, 1 mM DTT and 0.1% $C_{28}H_{58}O_9$).

2.4. *Filter binding assays*

Sec4 and Ypt1 proteins were preincubated in [γ-^{32}P]GTP at 37°C for 30 min in their respective binding buffers D and E as described for GTPγS binding. All experiments were performed with the preincubation mixture containing 300 nM GTP and 300 nM Sec4 or Ypt1 (determined by their GTPγS binding). The Sec4-/Ypt1-[γ-^{32}P]GTP complexes were diluted 10-fold into 50 μl assay mixes containing either 1 mg/ml BSA or different pancreatic fractions. In addition, the assay mixes contained 0.5 mM GMP-PNP or 34 mM GTP in the Sec4-GAP, and 0.5 mM GMP-PNP or 34 mM GTP and 0.7 mM $MgCl_2$ in the Ypt1-GAP assays. Incubations were carried out for 30 or 60 min with at least 3 time points taken in duplicate. Five μl aliquots of the incubation mixes were diluted into 2 ml of ice-cold TNMg buffer and filtered as described earlier. Non-specific binding of radiolabel to the filter was accounted for by assaying a mix containing [γ-^{32}P]GTP in 1 mg/ml BSA in the absence of Sec4 or Ypt1. The rate of hydrolysis of [γ-^{32}P]GTP bound to Sec4 or Ypt1 in the presence of different pancreatic fractions was determined by plotting the percent total [γ-^{32}P]GTP bound to Ypt1 or Sec4 with time. The intrinsic rate of GTP hydrolysis by Sec4 or Ypt1 was similarly measured, but in the presence of 1 mg/ml BSA. First-order rate constants were determined from experimental curve fits of Sec4- or Ypt1-bound GTP hydrolysed with time, using the Cricket Graph software.

2.5. *Sec4-/Ypt1-bound GTP product analysis*

In order to determine the fate of GTP-bound to Sec4 or Ypt1 in the GTPase assays, with different pancreatic fractions, the following experiments were carried out. Hundred μl of mixture containing 1.6 μM of Ypt1 or Sec4 protein were incubated with 2.4 μM of [α-^{32}P]GTP for 30 min at 37°C in their respective binding buffers as described earlier. The Ypt1-[α-^{32}P]GTP and Sec4-[α-^{32}P]GTP complexes were separated from unbound nucleotide by gel filtration on a 2 ml Sephadex G-50 column equilibrated in buffer D containing 0.5 mg/ml BSA. The total incubation mix was loaded onto the column, which was gravity-run, and 100 μl fractions collected. The first peak of radioactivity, corresponding to protein-bound nucleotide, was pooled and later used as substrate in the GTPase assay. One mg/ml BSA in buffer D was prepared containing the same amount of radioactivity found in Sec4- or Ypt1-[α-^{32}P]GTP pools. Assays contained 30 nM Sec4-[α-^{32}P]GTP, 30 nM Ypt1-[α-^{32}P]GTP and or the same amount of radioactivity as [α-^{32}P]GTP in BSA and were carried out at 37°C both in the presence or absence of different pancreatic fractions. For each time point, 10 μl aliquots from assay mixes were added to 10 μl of prewarmed (65°C) stop solution (2% SDS in 40 mM EDTA). Nucleotides were resolved by performing TLC using PEI plates, developed in 1 M LiCl, followed by autoradiography. GTP, GDP and GMP were run simultaneously as standards.

2.6. *Sec4 and Ypt1 competition assays*

Competition assays were conducted to determine the specificity of the pancreatic GAP's toward Sec4-GTP and Ypt-GTP. Pancreatic homogenates as the GAP source were used in Sec4- and Ypt1-GAP assays both in the presence and absence of various concentrations of Sec4-GTPγS and Ypt1-GTPγS as competitors. The rapid filtration GTPase assay were performed as described previously except 0.01% $C_{28}H_{58}O_9$ was used. Sec4-GTPγS and Ypt1-GTPγS complexes were prepared as described earlier except that different concentrations of Sec4 and Ypt1 proteins (see Fig. 4 legend) were incubated with equimolar GTPγS, in their respective incubation buffers containing 0.01% $C_{28}H_{58}O_9$. Inhibition of Sec4- or Ypt1-GAP activity was deter-

mined following the addition of various concentrations of Sec4- or Ypt1-GTPγS (competitors) in the GAP assay.

3. RESULTS

GTPγS binding assays were performed to determine the time course of guanine nucleotide binding to Sec4 and Ypt1 before the complex was used in the GTPase assays. GTPγS, a non-hydrolyzable GTP analogue bound to Sec4 and Ypt1 and not to BSA (control), in a time-dependent manner. After 20 min the binding neared completion (data not shown).

The intrinsic hydrolysis rate of GTP bound to Sec4 or Ypt1 was determined in the presence of 1 mg/ml BSA. The intrinsic GTPase activity of these preparations of Sec4 and Ypt1 proteins was found to be 0.002 mol of P_i released/mol of protein/min, which is in good agreement with published rates for these proteins [26–28].

To determine the optimal concentrations of pancreatic homogenate protein in Sec4 and Ypt1 GTPase assays, different concentrations of pancreatic homogenates were assayed for their Sec4- and Ypt1-GAP activities. Results from these experiments demonstrate that the optimal pancreatic homogenate concentration for assaying Sec4- and Ypt1-GAP activity is 75 μg/assay (Fig. 1).

Analysis of the GAP activity associated with the 150,000 × *g* pancreatic particulate and cytosolic fractions, using Sec4-GTP and Ypt1-GTP (Fig. 2), demonstrated that while approximately 65% and 35% of the Sec4-GAP activity is associated with the particulate and cytosol fractions respectively, more than 95% of the Ypt1-GAP activity was associated with the particulate fraction. When the particulate fraction was stripped with 1 M salt, more than 96% of the Sec4 and Ypt1-GAP activities were found still associated with the salt-stripped particulate fractions (Fig. 2). These results suggest that Sec4- and Ypt1-GAP activities in the pancreas are different, and that the GAP's are tightly associated with structures of the particulate fraction rather than being adsorbed during homogenization and fractionation of the pancreas. NaF, a classic G-protein agonist, did not stimulate GTPase activity in our experiments (data not shown).

To determine if the observed loss of filter bound counts was due to GTP hydrolysis rather than to release of the bound nucleotide from Sec4 or Ypt1, the products of Sec4- and Ypt1-bound [α-^{32}P]GTP were analysed (Fig. 3). The results from these experiments correlate very well with the previous GAP assay data. Both Sec4 and Ypt1 intrinsic GTPase activities were low as indicated by negligible GTP hydrolysis in the absence of pancreatic fractions (Fig. 3). In addition, negligible GTP hydrolysis was observed when free [α-^{32}P]GTP was incubated with pancreatic homogenate alone. The negligible hydrolysis of [α-^{32}P]GTP by pancreatic homogenate seen in the assays was due to the presence of 34 mM unlabeled GTP in the assays as indicated in

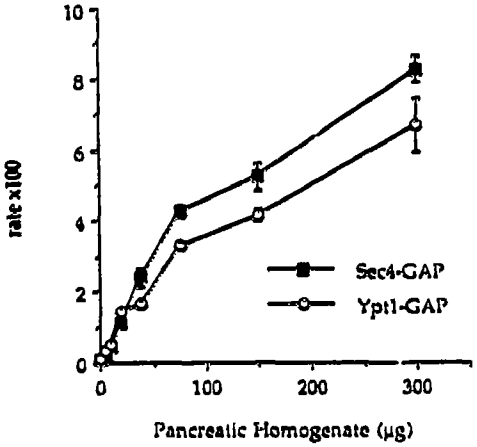

Fig. 1. Concentration dependence of GAP activity in pancreatic homogenates. Total pancreatic homogenate was used as a source of GAP activity. Sec4- and Ypt1-GAP activity was measured as described in section 2. The exponential rate of the loss of radioactivity bound to filters, as a function of time, was a measure of GAP activity. Sec4- and Ypt1-GAP activities are linear over a range of homogenate concentrations, up to 75 μg/assay. In the presence of 75 μg of pancreatic homogenate protein, the rate of loss of radioactivity bound to filters was found to be 36- and 50-fold higher for Ypt1 and Sec4, respectively, over their intrinsic rates. Values represent means ± S.E. from 3 separate experiments assayed in duplicate.

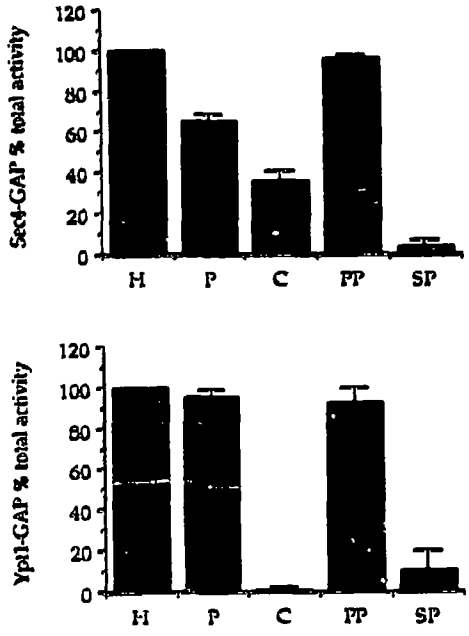

Fig. 2. GTPase activity for Ypt1 and Sec4 in pancreatic fractions. Pancreatic homogenate [H] (75 μg), 150,000 × *g* particulate [P] (50 μg), cytosol [C] (25 μg) as well as the 150,000 × *g* pellet [PP] (24 μg) and supernatant [SP] (22 μg) of the salt-stripped particulate fractions were incubated with 30 nM Ypt1- or Sec4-[γ-^{32}P]GTP, and the percent total activity measured as described in section 2. Protein was estimated by the method of Bradford [31]. Values represent means ± S.E. from 3 separate experiments, each assayed in duplicate.

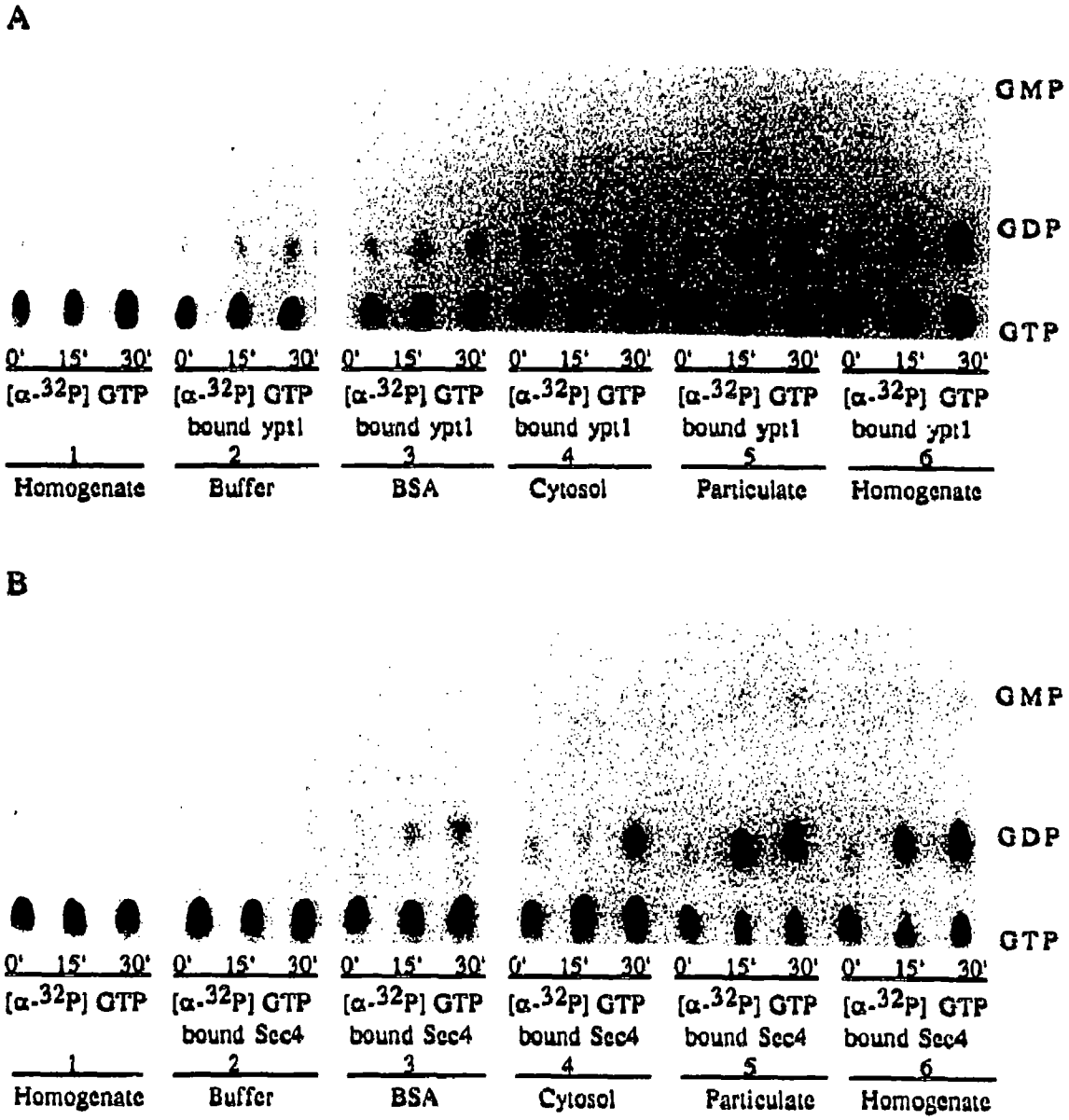

Fig. 3. Sec4 and Ypt1 bound [α-^{32}P]GTP hydrolysis. Autoradiogram of a thin-layer chromatogram from a time course of Sec4 and Ypt1 bound [α-^{32}P]GTP hydrolysis. Assays were carried out in the presence of BSA (40 μg), homogenate [H] (75 μg), particulate [P] (50 μg) and cytosol [C] (25 μg), as described in section 2. The figure represents one of three separate experiments.

section 2. This high concentration of unlabeled GTP was necessary to block non-specific endogenous nucleotidases present in the tissue. However, in the presence of pancreatic fractions, Sec4 hydrolysed the bound GTP to GDP in a time-dependent manner. In contrast, the majority of the Ypt1 GTPase activity was found to be present in the particulate fraction (Fig. 3). Very little Ypt1 GTPase activity was seen on addition of the 150,000 × *g* cytosol. These results demonstrate that the Sec4 and Ypt1 GTPase activities determined in the filter-binding assays were indeed due to hydrolysis of GTP bound to Sec4 or Ypt1 and not due to nucleotide dissociation from these proteins. These experiments also support the Ypt1-GAP data from filter-binding assays where low levels of Ypt1-GAP activity were seen.

To further establish that the Sec4 and Ypt1-GAP's activities are different, Sec4 and Ypt1 competition assays were performed as described in section 2. These studies demonstrated that both Ypt1-GTPγS and Sec4-GTPγS could compete out their respective GAP but not that of the other (Fig. 4). The differential centrifugation and competition studies therefore strongly suggest that the Sec4-GAP and Ypt1-GAP are distinct and specific activities in the rat pancreas.

4. DISCUSSION

Although very little is known about the effectors for small GTP-binding proteins, a number of GAP's each specific to a small GTP-binding protein have been re-

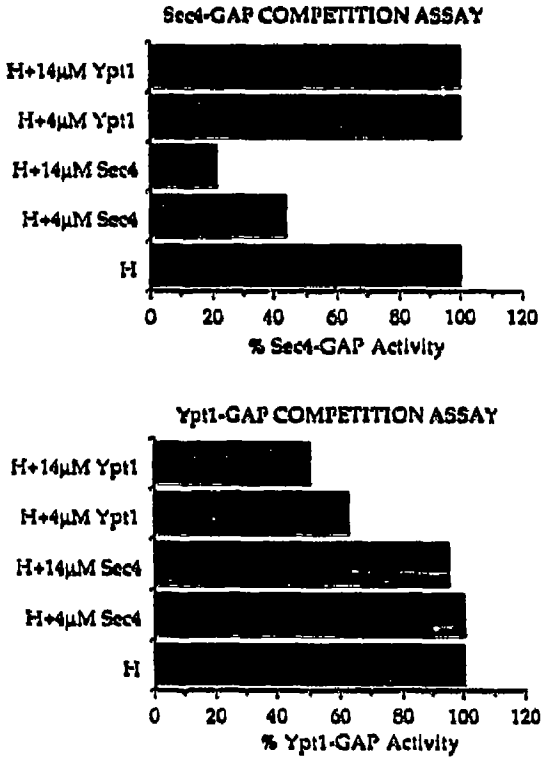

Fig. 4. Inhibition of Sec4- and Ypt1-GAP activity by Sec4-GTPγS and Ypt1-GTPγS. Competition experiments were performed as described in section 2, using pancreatic homogenate (37.5 μg) as the GAP source. GAP activities are expressed in percent of total. Sec4- and Ypt1-GTPγS were able to compete out Sec4- and Ypt1-GAP's respectively, in a concentration-dependent manner, but were unable to inhibit each other. Values represent one of two similar experiments, with similar values in each experiment.

ported [12,19,20]. Recently, various yeast homologues of small GTP-binding proteins along with proteins which regulate their activities have been identified in mammalian cells [10,16,17,19–24,29,30]. The mammalian rab1 protein is known to be closely related to Ypt1 and this is presumably the natural substrate for the Ypt1-specific GAP activity seen here. However, no homologue of Sec4 has been identified to date in mammalian cells. In this study, we report for the first time the presence of specific pancreatic GAPs that interact with the yeast Sec4 and Ypt1 proteins. Our results suggest that mammalian cells may possess a Sec4 homologue. Two of the rab proteins already identified, i.e. rab8 and rab10 show high conservation with Sec4 in their putative effector domains and therefore could be candidate substrates for the Sec4-GAP activity we are observing. The purification and subcellular distribution of these GAPs in the pancreas will help further determine the characteristics and function of their activities in this tissue.

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