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Identification and partial purification of GTPase-activating proteins from yeast and mammalian cells that preferentially act on Ypt1/Rab1 proteins

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Two GTPase-activating proteins of apparent molecular mass of 100 kDa and 30 kDa have been partially purified from porcine liver cytosol using mammalian Ypt1/Rab1 protein as substrate Both proteins act most efficiently on Ypt1/Rab1p, but are inactive with H-Ras p21 From the budding yeast *Saccharomyces cerevisiae*, a cytosolic 40 kDa yptGAP was partially purified It accelerates the intrinsic GTPase activity of wild-type Ypt1p but not of H-Ras p21 or a mutant ypt1p with an amino acid substitution of the effector domain which renders the protein functionally inactive in yeast cells

GTPase-activating protein, Rab1 protein, Ypt1 protein, Porcine liver, *Saccharomyces cerevisiae*

1 INTRODUCTION

Ypt1p is a 23 kDa GTP-binding protein which belongs to the Ras superfamily of proteins It was first discovered in the budding yeast *Saccharomyces cerevisiae* [1] where it fulfills an essential function [2,3], most likely in vesicular protein transport between the endoplasmic reticulum and the Golgi complex [4–8] A structurally closely related protein, able to functionally replace Ypt1p in yeast [9], has been isolated from mammalian sources and is referred to as Ypt1p/Rab1p [10,11] By indirect immunofluorescence [4] and immunoelectron microscopy using different anti-Ypt1p/Rab1p antibodies (M Puzicha and D.G , unpublished), this protein has been shown to be predominantly associated with Golgi membranes, suggesting a similar regulatory function in the exocytic pathway of mammalian cells Several other of the many proteins belonging to the Ypt/Rab branch of the Ras superfamily also localize to different compartments of the exo- and endocytic pathways in yeast [12] and mammals [13–15], implying their involvement in vesicular trafficking between those compartments.

It is thought that in contrast to Ras proteins, Ypt1p and related proteins do not act by amplifying some kind of signal [16,17] However, like the well characterized Ras proteins, Ypt1p cycles between a GDP- and a GTP-bound state and it possesses a slow intrinsic GTPase activity [18] GDP/GTP exchange and GTPase activity of Ras proteins are regulated by different proteins [16,17,19] This is particularly well documented in the case of yeast, where a combined genetic and biochemical approach has led to the identification of *CDC25* and *SDC25* gene products promoting the exchange of guanine nucleotides [20,21] and of Ira1 en Ira2 proteins accelerating the intrinsic GTPase activity of Ras1,2p [22–24]

In the past few years, several GTPase-activating proteins (GAP), seemingly specific for different members of the Ras and Rho subfamilies of proteins, have been found [25–33] and some of them cloned The question arises whether GAPs with specificity for the multitude of Ypt/Rab proteins exist, and if so, how might they be integrated and regulated in interorganelle protein traffic An activity from rat brain able to accelerate the GTPase activity of the synaptic vesicle-associated Rab3Ap has been described recently [34] Furthermore, by studying yeast ypt1 mutant proteins with substitutions in the so-called effector region, which in Ras proteins is a site for the interaction with rasGAP, we obtained circumstantial evidence for the existence of a GAP activity acting on Ypt1p in *S. cerevisiae* [35].

We report here the partial purification of two distinct molecular weight GTPase-activating proteins from porcine liver and one from budding yeast which use Ypt1p/Rab1p as the preferred substrates and which are totally inactive with Ras proteins

Abbreviations GAP, GTPase-activating protein; PLI-cellulose, poly(ethyleneimine)-cellulose, PMSF, phenylmethylsulfonyl fluoride, Ypt1p, Ypt1 protein

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2. EXPERIMENTAL

2.1 *Materials*

[γ-^{32}P]GTP (5000 Ci/mmol) and [α-^{32}P]GTP (2500 Ci/mmol) were purchased from Dupont-New England Nuclear Molecular weight protein standards, protein assay reagents (Bradford) and Silver Stain Kit were obtained from Bio-Rad, PEI-cellulose F plastic foils from Merck, Darmstadt Nitrocellulose filters (0,45 μm pore size, 25 mm diameter) were from Schleicher & Schuell

2.2 *Methods*

2.2.1 *Bacterial production of GTP-binding proteins*

Yeast Ypt1p [1] and Ryh1p [36], mouse Ypt1/Rab1p [10] and Rab2p (W Laufer, unpublished) were produced in *E coli* using the pLN expression vector and purified as described previously [18] Bacterially produced H-Ras p21 [7] was kindly provided by A Wittinghofer, Heidelberg The GTP-binding proteins used were purified to a degree exceeding 90%

2.2.2 *GTP loading and GTPase assay*

The exchange of protein-bound GDP with ^{32}P-labelled GTP was performed in the absence of Mg^{2+} ions A solution of 50 mM Tris-HCl pH 8, 2 mM EDTA, 1 mM DTT, 0.5 μM of the respective GTPase, 25 nM of either [γ-^{32}P]GTP or [α-^{32}P]GTP, 0.5 μM unlabelled GTP and 500 μg/ml BSA was incubated at 30°C for 10 min and after adding $MgCl_2$ (5 mM final concentration) was stored on ice for use in the GAP assay To monitor GAP activity during purification, 15 μl of the column fractions were mixed with 22 μl of buffer A (50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 1 mM DTT), 2.5 μl BSA (10 mg/ml), 0.5 μl of 0.1 M ATP and 10 μl of [γ-^{32}P]GTP-loaded Ypt1p (0.1 μM final concentration) were mixed on ice and immediately transferred to 30°C and incubated for 30 min For kinetic experiments, the incubation mix was scaled up to 200 μl At each time point, two 10-μl aliquots were immediately passed through nitrocellulose filters and washed with ice-cold buffer B (20 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 10 mM NH_4Cl, 0.1 M KCl, 1 mM 2-mercaptoethanol) Filters were dried at 80°C for 10 min and the radioactivity of the remaining protein-bound GTP was measured by scintillation counting Thin-layer chromatography of the reaction products was performed as described previously [35]

2.2.3 *Preparation of soluble proteins from porcine liver and yeast*

One kg of porcine liver, obtained from a local slaughterhouse, was cut into small pieces and homogenized in 2.5 liters of buffer C (10 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) using a Waring blender The homogenate was filtered in turn through 2 and 4 layers of cheese cloth, and the supernatant obtained after a spin at 5000 × *g* (20 min) was centrifuged at 8000 × *g* (20 min) and finally at 100 000 × *g* for 1 h Commercially available, pressed bakers yeast (1 kg) was suspended in 4 liters of YEPD (1% yeast extract, 2% peptone, 2% glucose) and incubated at 30°C for 3 h under vigorous shaking Cells were pelleted, resuspended in 500 ml of buffer C containing 1 mM pepstatin A and broken in 70 ml aliquots using a French Press (Aminco) at 1100 bar About 50% of the cells were disrupted Soluble proteins were obtained after centrifugation as outlined above

2.2.4 *GAP purification*

About 140 g of soluble protein (90 mg/ml in buffer C) from 1 kg of porcine liver were slowly passed at 4°C through a 5 × 35 cm DEAE-Sephacel column precquilibrated with buffer C The column was washed successively with 0.5 liters of buffer C and 5 liters of 0.1 M NaCl in the same buffer Bound proteins were eluted with a 0.1–0.5 M NaCl gradient in buffer C at a flow rate of 60 ml/h and 20 ml fractions were collected yptGAP was assayed with mouse Ypt1/Rab1p Active fractions eluting as a broad peak with a maximum at 0.2 M NaCl were pooled (1.6 g protein in 220 ml) and the protein was concentrated by Amicon YM10 membrane filtration to about 15 ml After centrifugation at 10 000 × *g* for 10 min, the clear supernatant was chromatographed at 4°C on a 3.5 × 60 cm Superdex-200 column (Pharmacia) which had been precequilibrated with buffer C containing 0.6 M KCl. Two clearly separated activities, one eluting at about 100 kDa, the other at 30 kDa, were obtained The peak fractions of both activities were separately pooled (74 and 42 mg protein, respectively), dialyzed overnight against 10 mM Na-phosphate buffer, pH 8, and separately chromatographed at room temperature on a 7.8 × 100 mm hydroxylapatite HPLC column (Biogel-HPHT, Bio-Rad), applying a 10–250 mM Na- phosphate gradient in 60 min at a flow rate of 0.5 ml/h The 100 kDa GAP activity eluted at 60–105 mM, the 30 kDa species at 85–135 mM Na-phosphate Pooled peak fractions (14 mg of 100 kDa yptGAP- and 7.1 mg of 30 kDa yptGAP-containing protein) were dialyzed at 4°C against buffer D (buffer C containing 10%, v/v, of glycerol and lacking PMSF) and chromatographed separately at room temperature on an FPLC Mono-Q HR 10/10 column (Pharmacia) using a 0.15–0.45 M NaCl gradient in buffer D The column was developed at a flow rate of 1ml/min and 1 ml fractions were collected The 100 kDa and 30 kDa yptGAP activities eluted as single peaks at 0.33–0.36 M and 0.36–0.38 M NaCl, respectively, well behind the bulk of proteins

yptGAP from yeast was separated following the same protocol About 30 g of soluble protein from 1 kg of yeast (wet weight) served as starting material A significant portion of the GAP activity was eluted in the void volume of the Superdex-200 and was not further analyzed The remaining activity (roughly 60%) was eluted as a single peak with an apparent molecular mass of 40 kDa The GAP activity was eluted at about 0.12 M Na-phosphate from hydroxylapatite and at about 300 mM NaCl from a Mono-Q HR 10/10 An additional run was performed on an FPLC Mono-Q HR 5/5 column (Pharmacia) and the yptGAP was eluted at the trailing edge of the main protein peak

2.2.5 *Protein estimation and gel electrophoresis*

Protein concentrations were determined by the method of Bradford (Bio-Rad), polyacrylamide gel electrophoresis was performed according to Laemmli [38] Electrophoretically separated proteins were silver-stained (Bio-Rad)

3 RESULTS AND DISCUSSION

3.1 *Two distinct yptGAP activities from porcine liver*

The purification of cytosolic GAP activities was followed by using [γ-^{32}P]GTP-loaded mouse Ypt1/Rab1p as substrate Two activities could be clearly resolved by gel filtration (Fig 1) After the several purification steps outlined above, the 100 kDa species was purified about 750-fold, the 30 kDa species was enriched by a factor of about 2000 As revealed by SDS-polyacrylamide gel electrophoresis and silver staining, none of the GAPs was pure, but a predominantly stained protein of 32 kDa was observed with the 30 kDa GAP activity (data not shown) Because of their substrate specificity discussed below, we provisionally refer to the two proteins as yptGAP

The partially purified yptGAPs were tested with different Ras superfamily proteins having distinct effector domain sequences As shown in Fig 2, Ypt1/Rab1p was clearly the preferred substrate for both activities. The intrinsic GTPase activity of yeast Ypt1p, which shares an identical effector region sequence with mammalian Ypt1/Rab1p [1,9,10], was accelerated with similar kinetics by the two porcine liver yptGAPs (data not shown). Most importantly, H-Ras p21 did not serve as a substrate (Fig. 2). However, two other small GTPases of the Ypt/Rab subfamily tested, Ryh1p of the fission yeast *S pombe* which is the likely functional homologue of mammalian Rab6p [36], and mouse Rab2p reacted

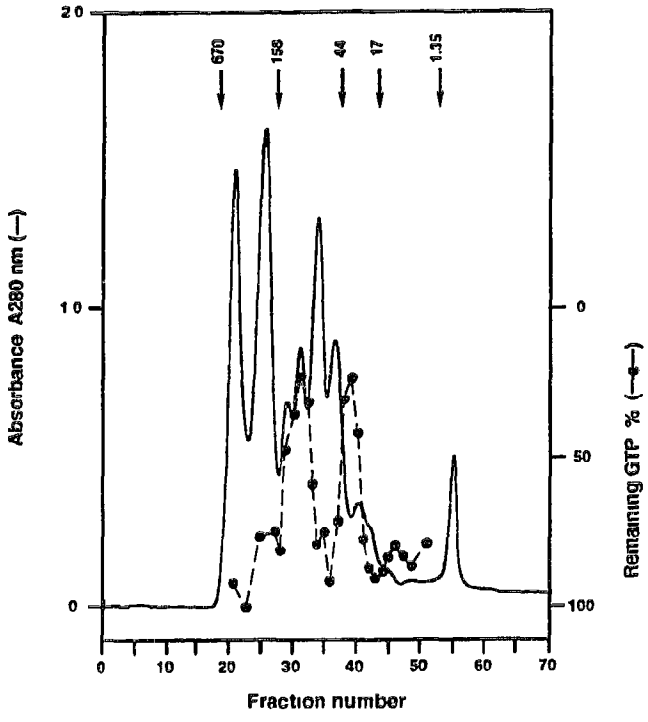

Fig 1 Separation of two yptGAP activities from porcine liver by gel filtration through Superdex-200 [γ-^{32}P]GTP-loaded mouse Ypt1/Rab1p was used as substrate The column was previously calibrated with molecular weight standards whose positions are indicated

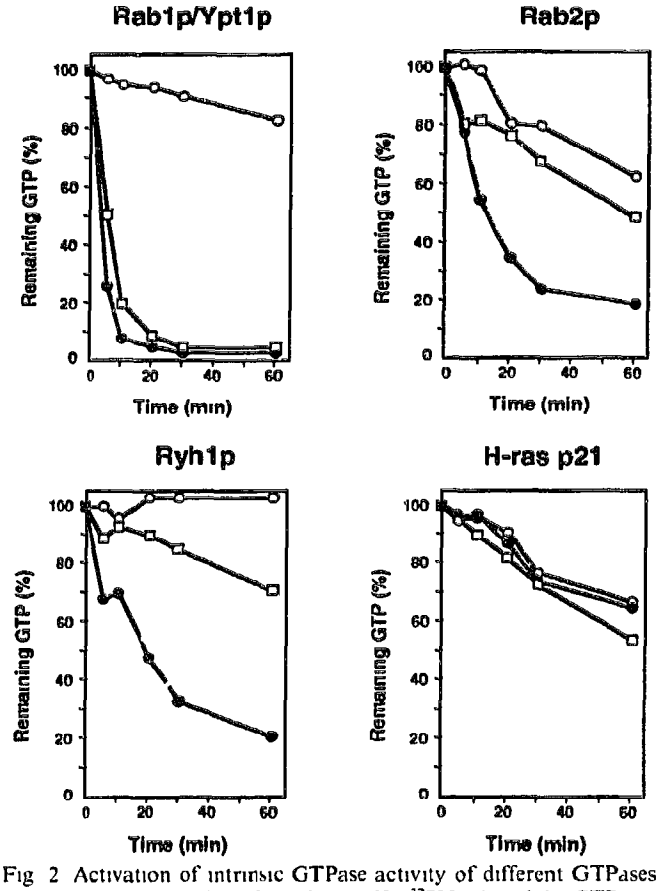

Fig 2 Activation of intrinsic GTPase activity of different GTPases by porcine liver yptGAPs Complexes of [γ-^{32}P]GTP and the GTPases indicated (final concentration 0 1 μM) were incubated in the absence (○) or in the presence of 100 kDa yptGAP (□) or 30 kDa yptGAP (●) GTPase activity is presented as % of labelled GTP remaining bound to the protein at the times indicated

with the 100 kDa and the 30 kDa yptGAP, but their intrinsic GTPase activities were activated to a lesser extent than that of Ypt1/Rab1p This seems to apply to the 100 kDa yptGAP in particular (Fig 2)

In this context, we would like to emphasize that the 30 kDa yptGAP could be a degradation product of the 100 kDa species since we repeatedly observed that in preparations with low 100 kDa activity, a significantly larger 30 kDa activity was present and *vice versa* Although this issue can be resolved only after sequence information on the two proteins becomes available, it might well be that the 30 kDa yptGAP has lost some of its substrate specificity. It is also worth mentioning that we obtained evidence for the existence of a GTPase-activating protein whose preferred substrate is Rab2p (W Laufer and D.G , unpublished).

That the product of the yptGAP-accelerated GTP hydrolysis was indeed GDP, was shown by analyzing the nucleotides bound to the different GTPases after incubation in the absence and presence of 100 kDa and 30 kDa yptGAP (Fig. 3). The kinetics of GDP formation perfectly reflected the time-dependent loss of [γ-^{32}P]GTP bound to the different GTPases (Figs. 2,3)

3 2 *A 40 kDa yptGAP from yeast*

Following the same purification scheme as that described for porcine liver yptGAPs, a cytosolic yptGAP with an apparent molecular mass of about 40 kDa was purified well above 100-fold from the budding yeast *S cerevisiae* At least 500 g of yeast cells had to be used as starting material to obtain an appreciable activity after the last purification step by FPLC on a Mono-Q column

yptGAP from yeast did not act on H-Ras p21 (Fig 4) and it exhibited only a marginal activity with Ryh1p (data not shown). The reaction product with Ypt1p from yeast and mammalian cells was GDP We had previously shown that the 30 kDa yptGAP from porcine liver did not increase the intrinsic GTPase activity of a mutant ypt1p unable to sustain viability of yeast cells [35]. This mutant protein, ypt1(I41M)p, and an other, ypt1(D44N)p, causing a ts phenotype, have a single amino acid substitution in the effector region but are not defective in nucleotide binding [35] The two yeast mutant proteins were used as substrates for the partially purified yeast yptGAP It was found that the intrinsic GTPase activity of ypt1(I41M)p was not increased by yptGAP whereas ypt1(D44N)p served as a less efficient substrate for yptGAP than wild-type Ypt1p (Fig 4) The impaired activity of yptGAP on the two mutant

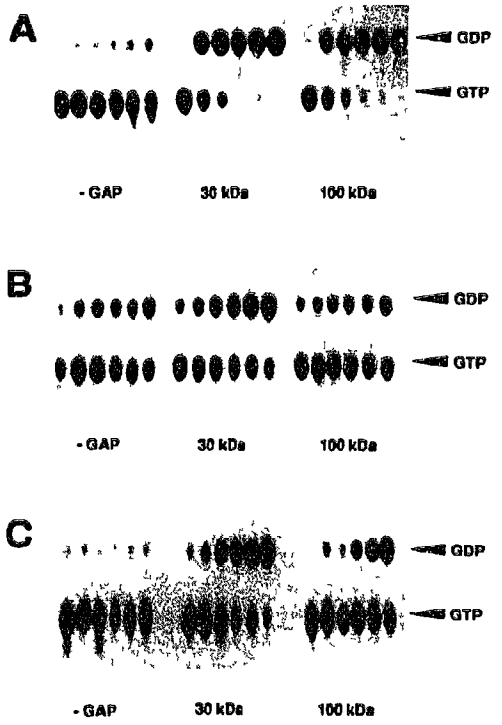

Fig 3 Thin-layer chromatography of reaction products of yptGAP-accelerated GTPase activity [γ-^{32}P]GTP-loaded mouse Ypt1/Rab1p (A), mouse Rab2p (B) and yeast Ryh1p (C) were incubated without yptGAP (−GAP) or in the presence of either 30 kDa or 100 kDa yptGAP from porcine liver Reaction products were analyzed on PEI-cellulose plates at zero, 5, 10, 20, 30 and 60 min (from left to right) of incubation at 30°C Note the different intrinsic GTPase activities of the three GTPases

proteins nicely mirrors their defective biological function, suggesting that yptGAP, like rasGAP, interacts with the effector domain of its substrate GTPases

In summary, yeast and mammalian cells have been shown to possess GTPase-activating proteins exhibiting some specificity for Ypt1/Rab1 proteins. This work, together with the finding that GAPs exist in mammalian cells which act preferentially on Rab3Ap [34] and Rab2p (W Laufer and D G , unpublished), lends credit to the notion that several GTPase-activating proteins specific for the different GTPases may in fact be involved in intracellular membrane traffic

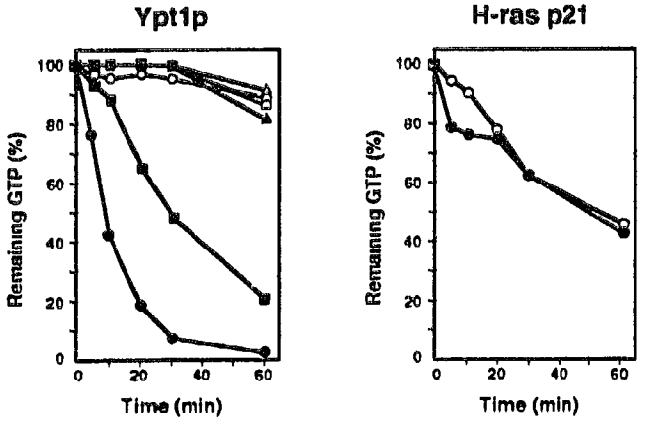

Fig 4 Intrinsic and yeast yptGAP-accelerated GTPase activity of yeast Ypt1 and mammalian H-Ras proteins [γ-^{32}P]GTP-loaded H-Ras p21 and Ypt1p (●, ○), ypt1(I41M)p (▲, △) and ypt1(D44N)p (■, □) at a final concentration of 0 1 μM were incubated without (open symbols) or with Mono-Q HR 5/5-purified 40 kDa yeast yptGAP (closed symbols) at 30°C At the times indicated, labelled GTP remaining bound to protein was measured by a nitrocellulose filter test

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