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Recombinant SEC14-like proteins (TAP) possess GTPase activity

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Abstract

The three human SEC14-like proteins TAP1, TAP2, and TAP3 were expressed in *Escherichia coli* and purified by means of an amino-terminal His-tag. The recombinant TAP proteins bound α-, β-, γ-, and δ-tocopherol, certain phospholipids, and squalene. Intriguingly, the TAP proteins showed considerable GTPase activity that was comparable to that of small GTP-binding proteins of the Rab family. Although the TAP proteins contain important motifs to provide GTPase activity, the surrounding secondary structure markedly differed from common G-protein domains. However, these motifs are located in close proximity in the TAP structure and may therefore form an active site for GTP-binding and hydrolysis.

Keywords: SEC14p; CRAL-TRIO; GTPase; Vitamin E; Tocopherol; Phospholipids

In recent years, a number of SEC14-like proteins have been identified and investigated. Members of this family share a CRAL-TRIO domain, which refers to similar sequences in the cellular retinaldehyde binding protein (CRALBP) and the triple function domain of the Trio protein. Apart from CRALBP, further members of this family are the yeast phosphatidylinositol transfer protein SEC14p, the α-tocopherol transfer protein (α-TTP), the MEG2 phosphatase, neurofibromin, and the tocopherol associated protein (TAP/SPF). Crystal structures show that the CRAL-TRIO domain contains a large hydrophobic pocket [1,2] and mutations in this structure were implicated in the pathogenesis of certain degenerative diseases.

CRALBP is found in the retina and the pineal gland where it binds and transports 11-*cis*-retinaldehyde and 11-*cis*-retinol. Thereby it modulates the interactions of these retinoids with enzymes of the visual cycle [3], and mutations in its gene lead to autosomal recessive retinitis pigmentosa [4]. α-TTP has been shown to selectively incorporate α-tocopherol during the assembly of VLDL in the liver [5]. Consequently, mutations in this gene result in highly reduced α-tocopherol levels in plasma and tissues [6], and cause a neuromuscular dysfunction termed ataxia with vitamin E deficiency (AVED) [7]. In yeast, SEC14p serves as a transfer protein for phosphatidylinositol and phosphatidylcholine, and is required for the secretory pathway via the Golgi complex [8]. Genomic mapping identified further genes that encode SEC14-like proteins associated with hereditary diseases. For instance, mutations in the gene encoding a SEC14-like protein called caytaxin were proposed to be responsible for the recessive congenital Cayman ataxia [9].

TAP1 was isolated from bovine liver as a protein that bound radioactive tocopherol [10]. Further investigations led to the discovery of two highly similar genes in close proximity of TAP1, all three genes being located on chromosome 22 in region 22q12.1-ter [11]. While the TAP proteins were shown to bind a number of lipid molecules in vitro (tocopherols, squalene, phosphatidylinositol, phosphatidylcholine, and phosphatidylglycerol), a predominant in vivo ligand could not yet be identified.

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Intriguingly, TAP1 was found to be identical with a previously described supernatant protein factor (SPF), first investigated over 30 years ago by Bloch et al. They found this protein due to its ability to stimulate in vitro microsomal squalene epoxidase, which catalyzes a downstream reaction of the cholesterol biosynthesis [12]. Although SPF was shown to facilitate the exchange of squalene between membranous compartments [13], direct binding to squalene or another involved reaction product could not be demonstrated in early studies [14]. More recently, SPF/TAP was shown to have a weak affinity towards squalene, though it is, as suggested by the authors, probably insufficient for a physiological function as a transfer protein [15,16]. Thus, the mechanism by which TAP/SPF promotes cholesterol synthesis is still unknown.

Studies about SPF/TAP as well as rSEC, a rat homologue of the human TAP2, suggested that nucleotide binding might be involved in the function of TAP [17–19]; thus we investigated a possible GTPase activity of the three human TAP proteins.

Materials and methods

Expression and purification of recombinant TAP. The amino-terminal hexa-histidine tag was attached by cloning cDNAs of human TAP1, TAP2, and TAP3 in-frame into the plasmid pET100/D-TOPO (Invitrogen), respectively. The precise protocol of expression, harvesting, cell lysis, and FPLC purification has been described elsewhere [11,20].

To achieve additionally purified TAP1, a second purification step was performed. TAP1 as obtained by the procedure described above was separated with a Sephacryl-column (HiPrep 16/90, Sephacryl S100, high resolution) using 20 mM ammonium acetate, 2 mM Tris–HCl, pH 7.0, as a running buffer.

Isoelectric point mobility shift assay (IPMS). Stock solutions were 5 mM in chloroform for the phospholipids (Sigma) and 50 mM in ethanol for the tocopherols and their derivatives (Cognis). To prepare liposomes, 50 μl of the desired phospholipid stock and/or 5 μl of the tocopherol stock were combined in a glass tube. The solvent was removed under nitrogen and the remaining lipids were taken up in 200 μl liposome buffer (50 mM Tris–HCl, 50 mM KCl, and 10 mM dithiothreitol (DTT), pH 7.5). The tube was filled with nitrogen and bath sonicated 10 times for 5–10 s with intermittent cooling on ice. To achieve mixtures with distinct ratios of different compounds, the amounts of added stocks were adjusted appropriately, keeping the phospholipid with the highest concentration at the original 1.25 mM in the liposome preparation (final concentration 125 μM).

Fifty micrograms of protein (~1 nmol, final concentration 10 μM) was incubated in a final volume of 100 μl with 10 μl of the liposome preparation or 10 μl buffer for the control at 37 °C for 30 min with vigorous shaking. The samples were separated on Precast IEF Ready Gels, pH 3–10 (Bio-Rad), as previously described [11].

GTPase activity. GTPase activity was measured essentially according to the method of Tavitian and Zahraoui [21]. Purified protein at a concentration of 10 μM was preincubated for 3 min at 37 °C in a total volume of 100 μl incubation buffer (50 mM Tris–HCl, 2 mM EDTA, 1 mM DTT, and 100 nM [α-^{32}P]GTP (3000 Ci/mmol) (Perkin–Elmer), pH 7.8). The reaction was started by addition of $MgCl_2$ to a final concentration of 10 mM. At various time points, 5 μl samples were removed and transferred to the same volume of a stop solution containing 0.2% (v/v) SDS, 5 mM EDTA, 50 mM GDP, and 50 mM GTP (nucleotides from Sigma). The mixture was immediately vortexed, spun down, and frozen on dry ice.

The frozen samples were heated to 70 °C for 2 min, retransferred to ice, and 1 μl aliquots were spotted on polyethyleneimine–cellulose thin layer chromatography plates (Merck, PEI Cellulose F). Plates were developed in 0.6 M sodium phosphate buffer, pH 3.4, dried, and subjected to autoradiography with Kodak X-OMAT UV films. Quantitative analysis was performed using the dot-blot analysis of the Lumi-Imager (Roche).

Results and discussion

Protein expression and purification

Previous studies in our laboratory investigated recombinant TAP proteins furnished with a carboxy-terminal His-tag. To exclude a possible influence of the carboxy-terminal His-tag on the described properties [11], new constructs were prepared carrying the His-tag at the amino-terminus.

After extraction from *E. coli*, all three TAP proteins confirmed their expected molecular weight of ~50 kDa including the His-tag (data not shown). Furthermore, TAP1 and TAP3 migrated to their calculated p*I*s of 6.95 and 6.14, respectively, when loaded on an IEF-gel. Unlike the former construct with the carboxy-terminal His-tag the new recombinant TAP2 could be shown to enter the IEF-gel and showed a p*I* of 6.0 to 6.5, the originally calculated p*I* being 5.9 (data not shown).

Ligand binding

To examine the binding of possible ligands, an isoelectric point mobility shift (IPMS) assay was used [16]. Amino-terminal tagged TAP exhibited binding affinities that were similar to those observed with the former protein preparation [11]. In general, TAP (10 μM) bound all four tocopherol analogues (α-, β-, γ-, and δ-tocopherol), the phospholipids phosphatidylinositol (PI), and phosphatidylcholine (PC) but also squalene and certain derivatives of tocopherol such as tocopherylsuccinate at concentrations of 125 μM. Fig. 1 shows the IPMS analysis of TAP1 after incubation with liposome preparations containing PI and PC in the indicated ratios. Incubation with the charged ligand PI resulted in a clear shift to a p*I* of approximately 5.1 which could be reversed by competition with the uncharged PC. These results confirm previous observations indicating that the binding properties were not decisively altered by the amino-terminal His-tag.

GTPase activity

The closest relative of human TAP within the family of the SEC14-like proteins is a 45 kDa protein from the

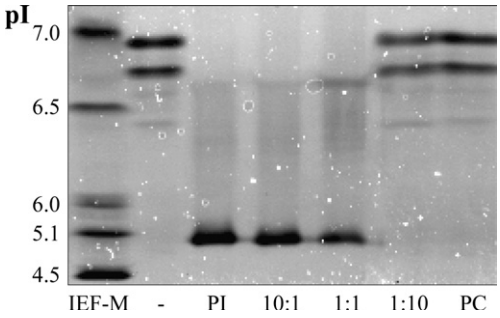

Fig. 1. Isoelectric point mobility shift (IPMS)-assay with TAP1. The first lane shows the IEF marker. In the following lanes, 50 μg of protein was incubated with liposome buffer only as a control (lane 2), phosphatidylinositol (PI), phosphatidylcholine (PC), and mixtures of these in the indicated ratios, respectively. The final concentrations of the ligands were 125 μM.

rat olfactory epithelium (rSEC) [18]. In addition to the CRAL-TRIO domain these proteins exclusively share also a carboxy-terminal GOLD domain, a structure that has been suggested to mediate protein–protein interactions in other proteins [22]. Moreover, both proteins contain a proposed GTP-binding site which as well has not been described for other members of the SEC14-like family. In fact, rSEC was first detected by antibodies, raised against a peptide fragment common to the G-protein α-subunit [23]. While its physiological function is still unknown, further studies of the same group confirmed a high affinity towards GTP in contrast to ATP, along with a low GTPase activity [17].

Interestingly, early studies by Senjo et al. [24] showed already that the ability of SPF/TAP to stimulate microsomal squalene epoxidase was abolished in the presence of nucleotides. More recently, Mokashi et al. [19] investigated rSEC and found a similar albeit weaker potential to stimulate this step of the cholesterol biosynthesis compared to human SPF/TAP. Nevertheless, they confirmed for rSEC, that the addition of GTP or GDP inhibited the activating effect of rSEC while the competition with α-tocopherol in turn prevented the inhibiting effect of the guanine nucleotides. To further elucidate the potential influence of guanine nucleotides on TAP functions the present study tried to investigate whether the three human TAP proteins might also exert GTPase activity.

The ability of the TAP proteins to hydrolyze GTP was assessed as described in Materials and methods. Fig. 2A depicts a typical result, comparing the GTPase activity of TAP1-3 with that of a negative control done without addition of protein. While an increase in the GDP band was evident, the appearance of two additional bands could be observed. It can be assumed that these show GMP and phosphate, resulting from further hydrolysis and/or insufficient restriction of the ^{32}P to the α-phosphate of GTP.

For statistical analysis, all visible bands were quantified with the Lumi-Imager and each was determined as percentage of total radioactivity per sample. For the plots depicted here, GTPase activity was assessed as the ability to raise the percentage of GDP with respect to the total radioactivity. All three TAP proteins were able to exert a GTPase activity that increased the amount of GDP to about 190% of that present at preincubation (see Fig. 2B).

Based on these results a molar GTPase activity was calculated. The values refer to the decrease in the amount of GTP and express the molar amount of GTP hydrolyzed in one minute by 1 mol of protein. Hence, the GTPase activity was for TAP1 0.0038, for TAP2 0.0035, and for TAP3 0.0027 mol/min/mol. These values can be compared to those of small GTP-binding proteins such as members of the Rab family, displaying un-stimulated GTPase activities between 0.0005 and 0.05 mol/min/mol protein [25,26]. The results are also in line with the observations of Kempna et al. [11] who found a GTPase activity of 0.0033 mol/min/mol for the carboxy-terminal tagged TAP1.

The obtained plots indicate that the GTPase activity was highest in the initial phase and rather slowed down afterwards. In general, GTP-binding proteins (GNBP) serve as molecular switches that control a variety of physiologic processes and require the presence of GEFs and GAPs (guanine nucleotide exchange factor and GTPase activating protein, respectively) to achieve their full GTPase activity. Therefore, the limiting factor at the basis of those observations might be the absence of appropriate co-effectors, especially a GEF that may promote the release of hydrolyzed GDP.

To exclude that the observed GTPase activity was caused by accidentally co-purified bacterial proteins, we investigated a TAP1 that had been subjected to additional purification by a Sephacryl column. The GTPase activity of the extensively purified TAP1 could not only be confirmed but was found to be higher than before. This preparation displayed a 1.5-fold higher GTPase activity of 0.0056 mol/min/mol indicating that the observed effect is due to intrinsic properties of TAP (Fig. 3).

We speculated that the association with potential ligands such as phospholipids or tocopherols could have an influence on the observed GTPase activity. Panagabko et al. [15] reported that the binding affinities of TAP1 towards several ligands were not altered in the presence of guanine nucleotides. Therefore, prior to the GTPase assay the proteins were incubated in a procedure similar to that of the binding studies and the GTPase activity was subsequently determined as previously described. PI, PC, α-tocopherol, δ-tocopherol, α-tocopherylquinone, and combinations of these were used to prepare the liposomes.

In general, under the applied experimental conditions none of the ligands tested so far caused a significant change in the GTPase activity of TAP. However, minor increases or decreases could be noticed upon incubation

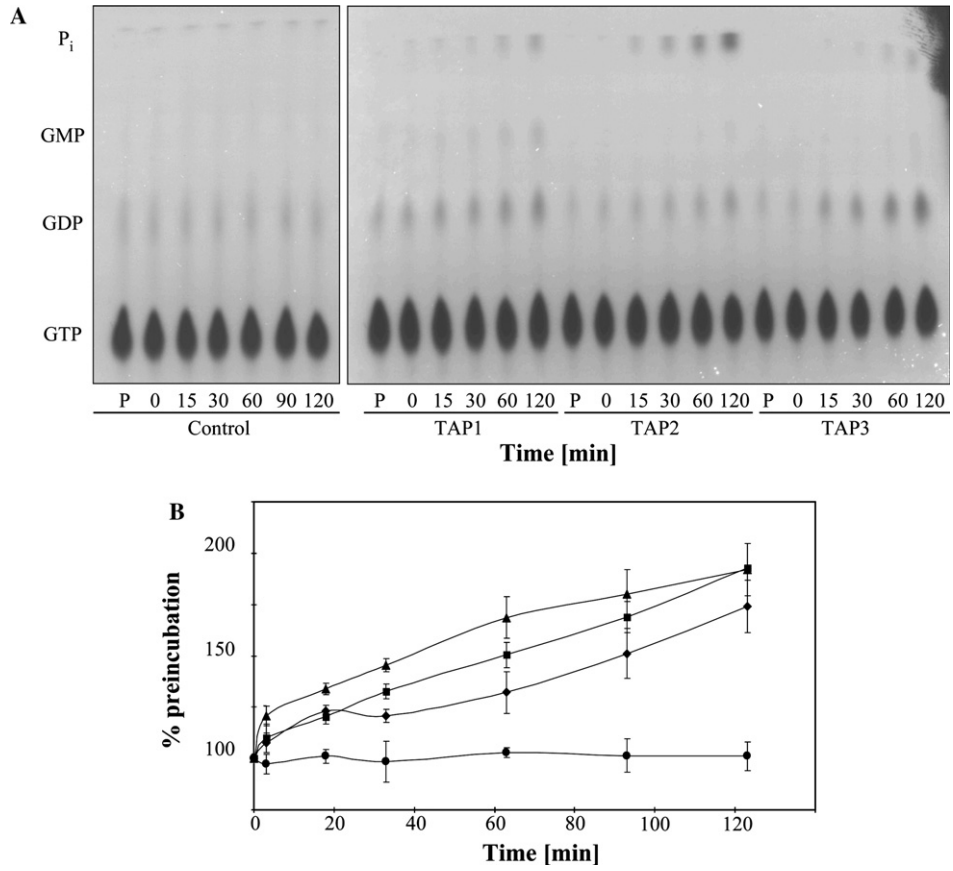

Fig. 2. GTPase activity of the recombinant human TAP proteins. Protein (10 μM) was incubated with [α-^{32}]GTP. Samples were taken after preincubation (P) and at the indicated time points, followed by addition of a stop solution. Control was done without protein. (A) One microliter aliquots of the samples were spotted on TLC-plates, developed, and analyzed by autoradiography. (B) The bands were quantified with the Lumi-Imager (Roche) and the GTPase activity was displayed as the increase in the amount of GDP over time. Values are means ± SD of three independent experiments. ●, control; ▲, TAP1; ♦, TAP2; and ■, TAP3.

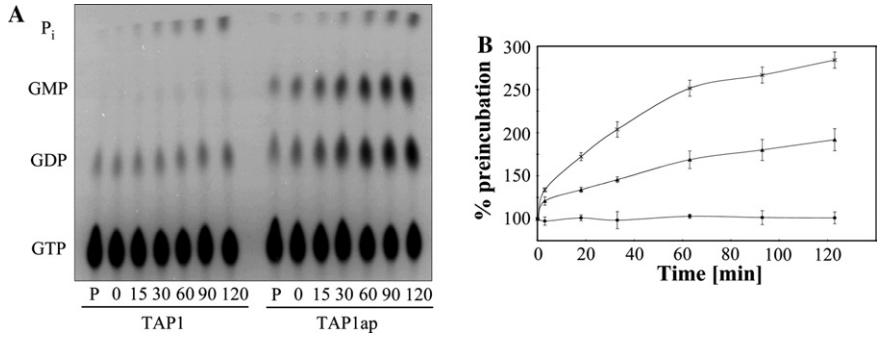

Fig. 3. Comparison of GTPase activities of TAP1 and additionally purified TAP1 (TAP1ap). The experiment was done essentially as described in Fig. 2. ●, control; ▲, TAP1; and ×, TAP1ap.

with phospholipids. While it had no influence per se, α-tocopherol was able to abolish these effects when incubated in a mixture together with the respective phospholipid. These observations indicate that the binding and especially the competition of ligands may modulate the GTPase activity of TAP.

Model of the proposed GTP-binding site

In TAP, the presence of a structure that is very similar to that of the G_α-common peptide in rSEC has been confirmed previously [11]. This so-called P-loop (phosphate-binding loop) or Walker A motif is characteristic

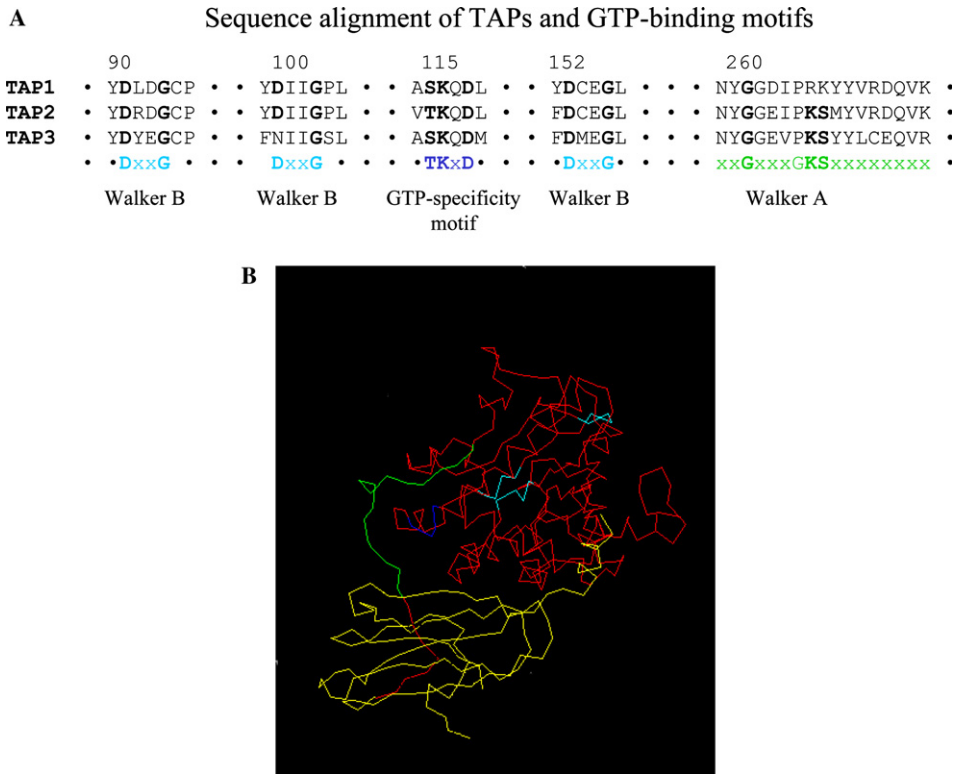

Fig. 4. Hypothetical GTP-binding domain of TAP. (A) Sequence alignment of TAP proteins and GTP-binding motifs (for a recent review see [27]): GxxxGK(S/T)—Walker A (phosphate-binding loop) within the G_{α}-common peptide (green); DxxG—Walker B (light blue); and (N/T)KxD—GTP-specificity motif (blue). (B) Conserved motifs present in the three dimensional structure of TAP1. The lipid-binding SEC14-like core domain is shown in red, the GOLD-domain in yellow. The conserved motifs, colored as indicated in (A), are all located within a hypothetical GTP-binding pocket. The three-dimensional structure of human TAP (PDB code 1O6U [2]) was generated using the quick PDB viewer of the RCSB PDB Protein Databank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

for a number of NTPases serving as a binding site for the nucleotide β- and γ-phosphates [27]. Further sequence analysis revealed now that the TAP proteins contain additional structures that were reported to be involved in specific binding and hydrolysis of GTP. The two motifs [N/T]KxD and DxxG (where x is any amino acid) are required for specificity for guanine as well as binding of Mg^{2+} [28] and could both be found in the TAP proteins (Fig. 4A). In variance to the composition of described consensus G-domains, the P-loop of TAP is found carboxy-terminal, the DxxG motif is detected in triplicate, and the entire encompassing secondary structure does not reflect the described pattern for G-domains [29]. However, all motifs can eventually be found in close proximity in the known crystal structure of TAP, thus suggesting that this may allow the formation of a functional active site for GTP hydrolysis (Fig. 4B). The difference of domain order suggests an evolutionary origin different from the described GTPase domains.

This proposed GTP-binding site can be located to the area that connects the two main motifs, the CRAL-TRIO and the GOLD domain, and the relative positions of these two domains may be flexible. Due to this position, it can be hypothesized that the structure of the GTP-binding pocket and the observed GTPase activity might be modulated by the binding of a ligand, the localization within the cell or the interaction with further proteins. Alternatively, GTP- and GDP-binding may lead to structural changes in TAP; the GTP-bound form may represent the active, membrane-bound TAP with the ability to exchange lipids, whereas the GDP-bound form may be the cytosolic, transport form of TAP.

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