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An Inception Report on the TOM Complex of the Amoeba *Acanthamoeba castellanii*, a Simple Model Protozoan in Mitochondria Studies

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It is suggested that in the course of the TOM complex evolution at least two lineages have appeared: the animal–fungal and green plant ones. The latter involves also the TOM complexes of algae and protozoans. The amoeba *Acanthamoeba castellanii* is a free-living nonphotosynthetic soil protozoan, whose mitochondria share many bioenergetic properties with mitochondria of plants, animals and fungi. Here, we report that a protein complex, identified electrophysiologically as the *A. castellanii* TOM complex, contains a homologue of yeast/animal Tom70. Further, molecular weight of the complex (about 500 kDa) also points to *A. castellanii* evolutionary relation with fungi and animal. Thus, the data indicates that the TOM complex of *A. castellanii* is not a typical example of the protozoan TOM complex.

KEY WORDS: *Acanthamoeba castellanii*; TOM complex; Tom70.

INTRODUCTION

Almost all mitochondrial proteins are encoded by nuclear genes, translated in the cytosol and imported into mitochondria. The only known entry point into mitochondria for these proteins is the TOM complex (the translocase of the outer membrane) located in the mitochondrial outer membrane (Paschen and Neupert, 2001; Pfanner and Chacińska, 2002; Rapaport, 2002; Rehling *et al*., 2003). The TOM complex decodes targeting signals within imported preproteins and mediates their translocation into or across the mitochondrial outer membrane. It also plays an active role in preprotein sorting for all sub-mitochondrial locations (Gabriel *et al*., 2003). Thus, the TOM complex is essential for protein import into mitochondria. Most of the knowledge about the TOM complex was obtained by studying *Saccharomyces cerevisiae* (Lill and Neupert, 1996; Dekker *et al*., 1998; Hill *et al*., 1998; Meisinger *et al*., 2001; Model *et al*., 2002) and *Neurospora crassa* mitochondria (Künkele *et al*., 1998a, 1998b; Ahting *et al*., 1999, 2001; Taylor *et al*., 2003) but there are also some data concerning the TOM complexes from mammalian and plant mitochondria (Braun and Schmitz, 1998; Jänsch *et al*., 1998; Mori and Terada, 1998; Macasev *et al*., 2000; Saeki *et al*., 2000; Suzuki *et al*., 2000; Werhahn *et al*., 2001; Werhahn and Braun, 2002; Hoogenraad *et al*., 2002) pointing at their similarities in overall structure to *S. cerevisiae* and *N. crassa* complexes. The TOM complex consists of surface receptors that recognize and bind imported preproteins and probably influence the structural organization of a second part of the complex, namely a general import/insertion pore (GIP), termed also the TOM

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Abbreviations used: DDM, *n*-dodecyl β-D-maltoside; DHFR, dihydrofolate reductase; ES-MS/MS, electrospray ionization tandem mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MOPS, 4-morpholinopropanesulfonic acid; TOM, translocase of the outer membrane; VDAC, voltage-dependent anion-selective channel.

complex channel, which mediates the protein translocation. In mitochondria of *S. cerevisiae*, which constitute a useful simple model system, two surface receptors have been identified, namely Tom70 and Tom20, whereas the TOM complex channel consists of five proteins: Tom40, Tom22, Tom7, Tom6 and Tom5. The major component of the TOM complex channel is Tom40 which forms two or even three oligomeric preprotein-conducting channels within the complex (Hill *et al*., 1998; Künkele *et al*., 1998a, 1998b; Ahting *et al*., 1999, 2001; Meisinger *et al*., 1999; Model *et al*., 2002; Taylor *et al*., 2003) while the central receptor Tom22 together with Tom7, Tom6 and Tom5 are associated tightly with the channels and modulates the TOM complex channel activity (Van Wilpe *et al*., 1999; Meisinger *et al*., 2001; Rapaport, 2002; Rehling *et al*., 2003).

Homologues of *S. cerevisiae* Tom proteins are present in *N. crassa* and animal mitochondria. However, in the case of plant mitochondria, only Tom40 displays an essential similarity to the yeast Tom40 while only limited and no sequence similarity is reported for the yeast Tom22, Tom6, Tom7 and Tom70, Tom20, Tom5, respectively (Braun and Schmitz, 1998; Jänsch *et al*., 1998; Mori and Terada, 1998; Saeki *et al*., 2000; Suzuki *et al*., 2000; Macasev *et al*., 2000, 2004; Werhahn *et al*., 2001; Hoogenraad *et al*., 2002). Thus, it is suggested that in the course of the TOM complex evolution at least two lineages have appeared: the animal–fungal and green plant ones. The latter lineage seems to involve also the TOM complexes of other eukaryotic groups such as algae and protozoans (Macasev *et al*., 2004). The amoeba *Acanthamoeba castellanii* is a free-living nonphotosynthetic soil protozoan, whose mitochondria share many bioenergetic properties with mitochondria of plants, animals and fungi (Jarmuszkiewicz *et al*., 2001; Sluse and Jarmuszkiewicz, 2001). Moreover, on the basis of ribosomal RNA analysis, *A. castellanii* is located in the molecular phylogenesis tree on a branch basal to the divergence points of the above kingdoms (Wainright *et al*., 1993; Gray *et al*., 1999). Therefore, it is interesting to study the TOM complex of *A. castellanii* mitochondria and to compare its properties with those described for other representatives of the eukaryotic kingdoms.

Here we report that *A. castellanii* mitochondria import the fusion protein pSu9-DHFR imported by *S. cerevisiae* mitochondria with comparable efficiency, which suggests some similarities of the targeting signal decoding machinery. Furthermore, the protein complex isolated from *A. castellanii* mitochondria and identified electrophysiologically as the TOM complex has a molecular weight comparable with that of *S. cerevisiae* (i.e. about 500 kDa) and distinctly higher than that reported for the TOM complex of plants (i.e. about 230 kDa). Finally, the TOM complex of *A. castellanii* contains ~45 kDa protein being probably a counterpart of Tom70 characteristic for the animal–fungal lineage of the TOM complex. Therefore it can be concluded that the TOM complex of *A. castellanii* is not a typical example of the protozoan TOM complex, which, in turn, is important conclusion for evolutionary analysis of the complexes.

MATERIALS AND METHODS

Amoeba and Yeast Strains

The soil amoeba *A. castellanii* strain (Neff and Neff, 1964), was cultured according to (Jarmuszkiewicz *et al*., 1997). Trophozoites of the amoeba were collected at the middle exponential phase. The yeast *S. cerevisiae* strain M3 (*MAT*a, *lys2 his4 trp1 ade2 leu2 ura3*) (Blachly-Dyson *et al*., 1997; Lee *et al*., 1998) was grown at 28°C in YPG medium (1% yeast extract, 2% peptone and 3% glycerol) at pH 5.5.

Isolation of Mitochondria

A. castellanii and *S. cerevisiae* mitochondria were isolated according to published procedures (Jarmuszkiewicz *et al*., 1997 and Daum *et al*., 1982, respectively). The estimation of the integrity of the outer membrane of mitochondria was based on the permeability of the membrane to exogenous cytochrome *c* (Douce *et al*., 1984). The calculated mean value of the degree of outer membrane intactness was 95 and 96% for *A. castellanii* and *S. cerevisiae* mitochondria, respectively

Synthesis of pSu9-DHFR

The fusion protein called pSu9-DHFR consists of the first 69 amino acid residues of *N. crassa* ATP synthase subunit 9 precursor comprising the mitochondrial targeting sequence (pSu9) and the entire mouse dihydrofolate reductase (DHFR). Radiolabelled form of pSu9-DHFR ([^{35}S] pSu9-DHFR) was synthesized in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S] methionine. The high level expression of pSu9-DHFR with C-terminal His_6-tag (pSu9-DHFRHis_6-tag) was performed according to QIAexpressionlist protocol (March 2001) and the protein was purified by nickel-nitrilotriacetic

acid-agarose (Ni-NTA) chromatography (Amersham Pharmacia Biotech).

Import of [^{35}S] pSu9-DHFR

Mitochondria (50 μg) were incubated in the import buffer (250 mM sucrose, 20 mM KCl, 10 mM 4-morpholinopropanesulfonic acid (MOPS)-KOH pH 7.2, 5 mM $MgCl_2$, 0,5% BSA, 2 mM ATP, 2 mM NADH) for 20 min at 25°C in the presence of [^{35}S]pSu9-DHFR according to Antos *et al*. (2001). After washing with SM buffer (250 mM sucrose, 10 mM MOPS-KOH pH 7.2), mitochondria were resuspended in the same buffer and treated with proteinase K (250 μg/ml, 10 min at 0°C) halted by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). After reisolation (10 min, 12000*g*), the organelles were subjected to SDS-PAGE. Import of [^{35}S] pSu9-DHFR was visualized by fluorography and quantified by ScanPack 3.0.

Isolation of the TOM Complex and VDAC

The TOM complex isolation was performed as in (Gabriel *et al*., 2003). The clarified extracts obtained after solubilisation of the mitochondrial outer membrane fraction in the presence of 0,01% *n*-dodecyl β-D-maltoside (DDM) was loaded onto a MonoQ (Amersham Pharmacia Biotech) anion-exchange column and the TOM complex was eluted by a linear 0–500 mM KCl gradient and then subjected to SDS-PAGE or Blue Native-PAGE followed by SDS-PAGE. The voltage dependent anion selective channel (VDAC) was isolated according to De Pinto *et al*. (1987). The outer membrane pellet was suspended in the solubilisation buffer containing 3% Triton X-100, 10 mM Tris–HCl (pH 7.0) and 1 mM EGTA, loaded onto a dry hydroxyapatite/celite column and eluted by the solubilisation buffer.

Conductance Measurements in Planar Phospholipid Membranes

The planar phospholipid membrane experiments were performed according to Benz *et al*. (1978). Membranes were formed from 2% (w/v) solution of soybean asolectin dissolved in *n*-decane, across a circular hole (surface area about 0.5 mm^2) in the thin wall of a Teflon chamber separating two compartments (*cis–trans*) filled with unbuffered 1 M KCl, pH 7.0. The chamber was connected with the recording equipment through calomel half-cells. All preparations were added in small aliquots (2–9 μl) to the *cis* compartment. *Cis* also refers to the compartment where the voltage was held. The amplified signal was monitored with an oscilloscope and recorded on a strip chart recorder.

Mass Spectrometry Analysis and Data Processing

The putative TOM complex of *A. castellanii* was separated by Blue Native-PAGE followed by SDS-PAGE and analysed by electrospray ionization tandem mass spectrometry (ES-MS/MS—Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS—Institut für Physiologische Chemie, Ludwig-Maximilians-Universität, München, Germany). The obtained data were used for searching the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/enterez). It should be mentioned that sequences of *A. castellanii* Tom proteins are not available.

Other Methods

Protein concentrations were measured by the method of Bradford. Respiration of mitochondria was monitored at 25°C with Rank oxygen electrode in the incubation volume of 0.5 ml. Changes of the inner membrane potential ($\Delta\psi$) were monitored with tetraphenylophosphonium (TPP^+)-specific electrode as described in Kamo *et al*. (1979).

RESULTS AND DISCUSSION

A. castellanii and *S. cerevisiae* Mitochondria Import [^{35}S] pSu9-DHFR with Comparable Efficiency but Differ in the Levels of the Protein Binding

The preliminary step in our study on the TOM complex of *A. castellanii* mitochondria consisted in functional analysis, i.e. we performed protein import to the mitochondria. Because, according to our knowledge, any sequence of *A. castellanii* mitochondrial protein encoded by a nuclear gene has not been published till now we used a well-known model protein, namely [^{35}S] pSu9-DHFR (see Materials and Methods). The import reaction was performed simultaneously for *A. castellanii* mitochondria and the yeast *S. cerevisiae* mitochondria, used as a control, in the

presence of the same amount of [^{35}S] pSu9-DHFR (Fig. 1A). The total amount of [^{35}S] pSu9-DHFR associated with both types of mitochondria (Fig. 1A and B) was determined by quantification and addition of bands representing the precursor (p), intermediate (i) and mature (m) forms of the protein obtained in the absence of proteinase K (−PK). The amounts of the imported protein (Fig. 1A and B) were determined by quantification of bands representing the mature form of the applied protein protected against externally added proteinase K (+PK). As shown in Fig. 1B, both the total amount of bound [^{35}S] pSu9-DHFR and imported [^{35}S] pSu9-DHFR were lower in the case of *A. castellanii* mitochondria. However, the efficiency of [^{35}S] pSu9-DHFR import, calculated as a ratio of the amount of the imported protein to the amount of the total protein bound, is comparable for *A. castellanii* and *S. cerevisiae* mitochondria (approximately 30%). Thus, it might be suggested that *A. castellanii* and *S. cerevisiae* mitochondria differ in capability to bind the imported protein while its translocation across the outer membrane proceeds with comparable efficiency. This, in turn, suggests the lack of distinct differences between the TOM complex channel of *A. castellanii* and *S. cerevisiae* mitochondria.

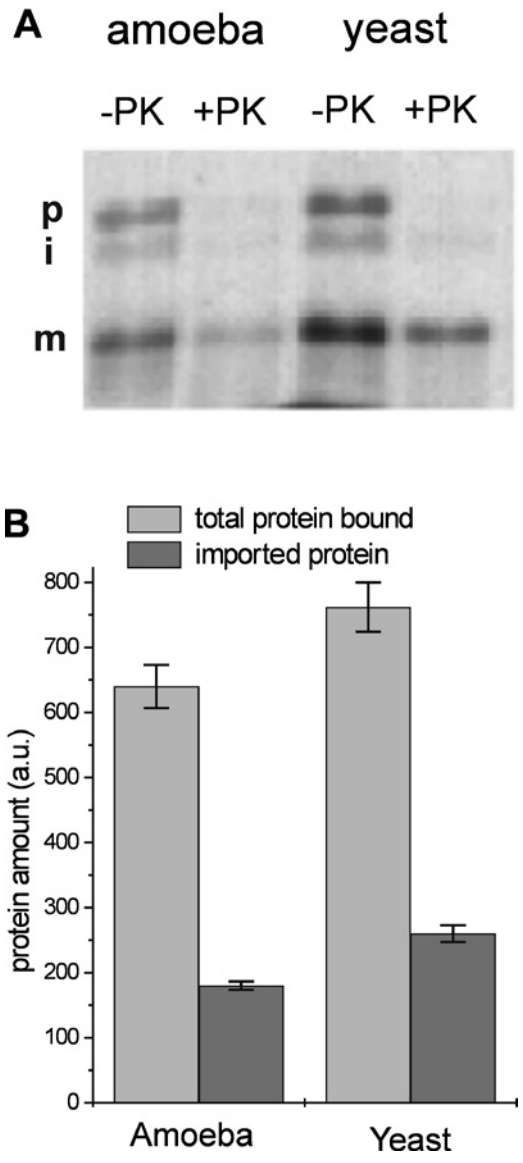

Fig. 1. Efficiency of pSu9-DHFR import into mitochondria of the amoeba *A. castellanii* and the yeast *S. cerevisiae*. (A) Typical results of [^{35}S] pSu9-DHFR import into *A. castellanii* (amoeba) and *S. cerevisiae* (yeast) mitochondria. (B) The levels of [^{35}S] pSu9-DHFR binding and its import into *A. castellanii* and *S. cerevisiae* mitochondria. Bands representing different forms of [^{35}S] pSu9-DHFR were visualised by fluorography and quantified by ScanPack 3.0. The terms p, i and m denote precursor, intermediate and mature forms of the imported protein [^{35}S] pSu9-DHFR, respectively.

Properties of the TOM Complex Channel of *A. castellanii* and *S. cerevisiae* are Essentially Similar

Fractionation of proteins of the outer membrane of *A. castellanii* mitochondria by ion-exchange chromatography (see Materials and Methods) led to isolation of a complex (Fig. 2A) which molecular weight of approximately 500 kDa resembles that of the TOM complex of *S. cerevisiae* (Meisinger *et al*., 2001; Model *et al*., 2002) and is distinctly higher than that reported for the TOM complex of plants (Jänsch *et al*., 1998; Werhahn *et al*., 2001). To test whether the obtained complex, termed here as the *A. castellanii* 500 kDa complex, displays channel activity corresponding to the TOM complex channel (Hill *et al*., 1998; Künkele *et al*., 1998a, b; Ahting *et al*., 1999, 2001; Stobienia *et al*., 2002), we analysed its channel forming activity after reconstitution into lipid membranes. As shown in Fig. 2B, a histogram of 106 insertion events, recorded in 1 M KCl at membrane potential of +20 mV, revealed an average conductance of 2.03 ± 0.02 nS. This is comparable to those calculated for the TOM complex isolated from *N. crassa* and *S. cerevisiae* mitochondria and corresponds to an open state of the channel (Künkele *et al*., 1998a, 1998b; Ahting *et al*., 1999; Stobienia *et al*., 2002). Thus, the *A. castellanii* 500 kDa complex might be the TOM complex. To check the hypothesis, we tested whether the observed channel activity can be affected by modulating factors described for the TOM complex channel. A well-known factor influencing permeability of the TOM complex channel is a mitochondrial-targeting signal termed a presequence. In reconstitution systems proteins containing presequences facilitate the transition of the TOM complex channel from open to closed states when

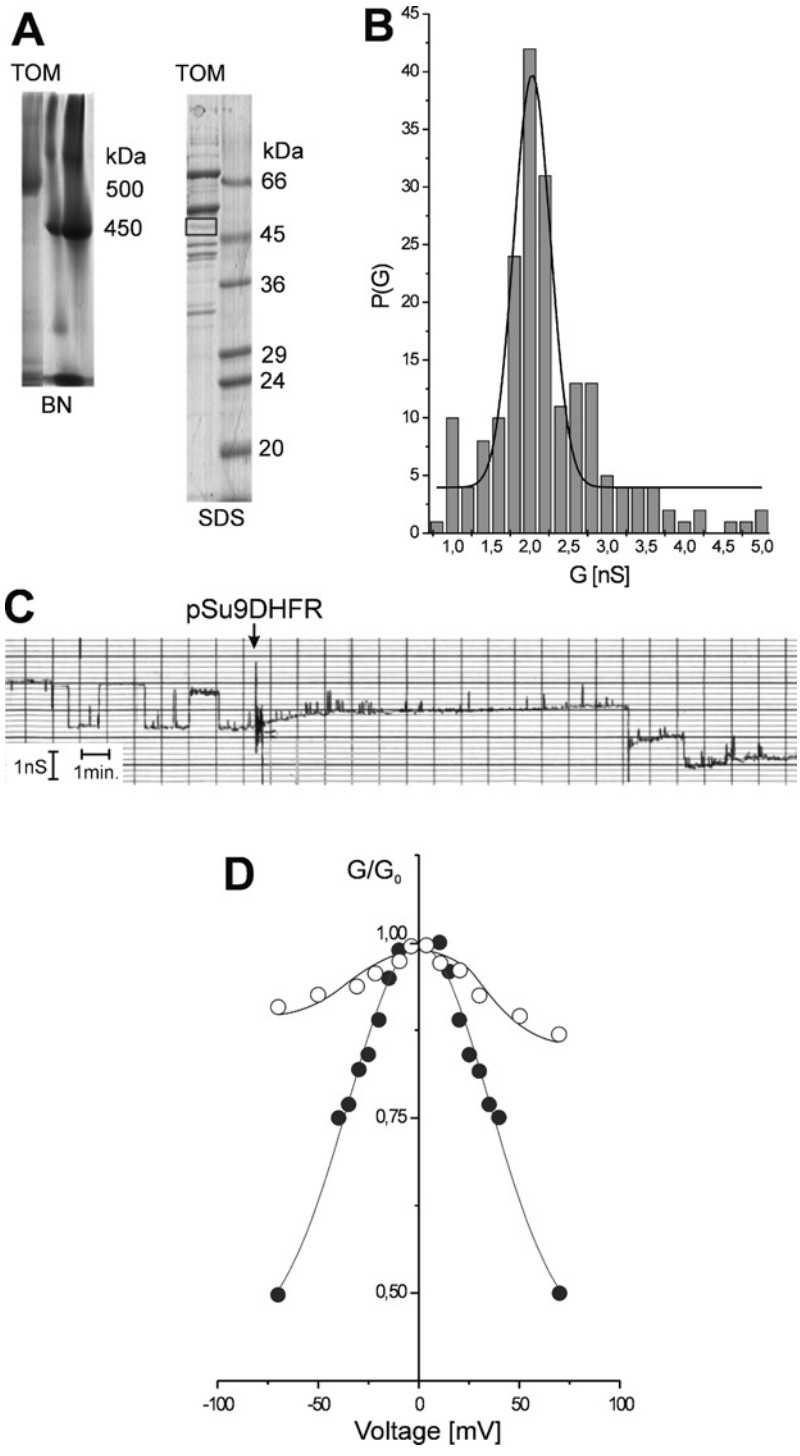

Fig. 2. Electrophysiological characteristic of the *A. castellanii* 500 kDa complex. (A) Two-dimensional Blue Native/SDS-PAGE of the *A. castellanii* 500 kDa complex. TOM, *A. castellanii* 500 kDa complex; BN, Blue Native—PAGE gel; SDS, SDS-PAGE gel. (B) Histogram of conductance fluctuations calculated for the *A. castellanii* 500 kDa complex. $P(G)$ is the probability that a given conductance increment G is observed. The data represent 106 insertion events. The applied potential was +20 mV. (C) Single-channel analysis of the *A. castellanii* 500 kDa complex in the presence of the fusion protein pSu9-DHFRHis$_6$-tag (see Materials and Methods). The protein (30 μM) was added to the site of +20 mV. (D) The dependence of the *A. castellanii* 500 kDa complex (*open circles*) and *A. castellanii* VDAC (*filled circles*) on the applied potential. G/G_0 is the ratio of the conductance at a given voltage (G) and the conductance at the lowest applied potential (5 mV) (G_0).

added from the site of positive potentials. The addition of the proteins from the site of negative potentials as well as the addition of control proteins devoid of a mitochondrial presequence from the site of both positive and negative potential do not impose any effect on the TOM complex channel conductance (Künkele *et al*., 1998b; Ahting *et al*., 2001; Stobienia *et al*., 2002). As shown in Fig. 2C, the addition of chemical amounts of the fusion protein pSu9-DHFRHis$_6$-tag (see Materials and Methods) to the site of +20 mV resulted in a strong reduction of the *A. castellanii* 500 kDa complex channel conductance, i.e. a distinct decrease in the channel open-state probability. When the same fusion protein was added at site of −20 mV or control proteins devoid of a mitochondrial presequence were added at site of ±20 mV, no effect on the studied channel open-state probability was observed (not shown). The next important feature of the TOM complex channel, observed in reconstitution systems, is voltage dependence, i.e. the probability of the channel occurrence in a given conductance state depends on the value of the applied potential (Hill *et al*., 1998; Künkele *et al*., 1998a, 1998b; Ahting *et al*., 1999, 2001). The dependence is described as a symmetrical one, which means that the sign of the applied potential is not important for the observed effect. As shown in Fig. 2D, an increase in the value of the applied potential facilitated closure of *A. castellanii* 500 kDa complex channel which resulted in a decrease of the value of an average conductance calculated for a given potential (G). This, in turn, caused a decrease in the value of the ratio G/G_o, where G_o denotes an average conductance at the lowest applied potential (5 mV). The sign of the applied potential did not influence these changes. Thus, the channel displays symmetrical voltage dependence, which is, however, much less pronounced than that of VDAC (the voltage dependent anion selective channel) (Mangan and Colombini, 1987). Taking into account the above data, it can be concluded that the *A. castellanii* 500 kDa complex channel shares important features with the TOM complex channel which allows the complex to be regarded as the *A. castellanii* TOM complex. To make the identification more convincing we decided to determine the channel selectivity and diameter.

To probe the selectivity of the *A. castellanii* 500 kDa complex channel, we calculated its average conductance in the presence of 1 M solutions of salts differing in electrochemical properties (Benz, 1994). The pair of K^+ and Cl^- was applied as a reference system displaying the highest mobility (a parameter which corresponds to the specific conductivity). In the presence of the transmembrane potential of 20 mV, these ions were replaced by ions of lower mobility (e.g. K^+ by TRIS while Cl^- by CH_3COO^- and MOPS), and the effects of these replacements on the channel average conductance were determined. For the combination of less mobile anions with K^+, only a slight decrease in the channel average conductance was observed (e.g. 1.71 ± 0.05 for CH_3COOK) which indicates that anions might move within the channel. However, the strongest decrease in the channel average conductance, to smaller than 0.5 nS, was observed when K^+ was replaced by TRIS, whose mobility is approximately two times lower that that of K^+. Thus, K^+ plays a crucial role in the channel conductance observed in the presence of KCl. This denotes that the *A. castellanii* 500 kDa complex channel displays a preference for cations, i.e. belongs to cation-selective channels like the TOM complex channel. Since an average conductance of 2.03 ± 0.02 nS, calculated for the *A. castellanii* 500 kDa complex channel (Fig. 2B and C) is comparable to those calculated for the TOM complex isolated from *N. crassa* (approximately 2.3 nS) and *S. cerevisiae* (approximately 2.2 nS) mitochondria and corresponds to an open state of the channel (Künkele *et al*., 1998a, 1998b; Ahting *et al*., 1999; Stobienia *et al*., 2002) we might assume that diameters of the channels should be almost the same. Thus, our electrophysiological data indicate that *A. castellanii* 500 kDa complex is indeed the TOM complex. To check the proposed identity of the complex we analyzed its components by mass spectrometry.

The TOM Complex of *A. castellanii* Contain a Homologue of Yeast/Animal Tom70

On preliminary analysis by mass spectrometry (see Materials and Methods) of a protein of ∼45 kDa involved in the putative *A. castellanii* TOM complex (Fig. 2A, marked with a box) a peptide containing 14 amino acids was detected. As shown in Fig. 3, sequence comparison revealed that this peptide perfectly matches a sequence in the C-terminal region of human (and other mammalian, e.g. rat, Suzuki *et al*., 2002) Tom70. The region contains the tetratricopeptide repeat (TPR), a motif comprising degenerate 34 amino acid sequence widely characterized as mediators of protein–protein interaction and found in different proteins, also in receptors of the TOM complex (Das *et al*., 1998; Terada *et al*., 2003). Because perfect matching of 12 amino acid peptide allows a protein to be identified (Burger *et al*., 2003; Sickmann *et al*., 2003) we can conclude that *A. castellanii* 45 kDa protein is probably a counterpart of Tom70. Although both proteins differ in their molecular weight this does not exclude the homology between them. In mammalian mitochondria, a protein termed Tom34 was identified which displays high level similarity to Tom70, also in the analysed region, and is consequently classified as a member of Tom70 family

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MAASKPVEAA VVAAAVPSSG SGVGGGGTAG PGTGGLPRWQ LALAVGAPLL  50
LGAGAIYLWS RQQRRREARG RGDASGLKRN SERKTPEGRA SPAPGSGHPE 100
GPGAHLDMNS LDRAQAAKNK GNKYFKAGKY EQAIQCYTEA ISLCPTEKNV 150
DLSTFYQNRA AAFEQLQKWK EVAQDCTKAV ELNPKYVKAL FRRAKAHEKL 200
DNKKECLEDV TAVCILEGFQ NQQSMLLADK VLKLLGKEKA KEKYKNREPL 250
MPSPQFIKSY FSSFTDDIIS QPMLKGEKSD EDKDKEGEAL EVKENSGYLK 300
AKQYMEEENY DKIISECSKE IDAEGKYMAE ALLLRATFYL LIGNANAAKP 350
DLDKVISLKE ANVKLRANAL IKRGSMYMQQ QQPLLSTQDF NMAADIDPQN 400
ADVYHHRGQL KILLDQVEEA VADFDECIRL RPESALAQAQ KCFALYRQAY 450
TGNNSSQIQA AMKGFEEVIK KFPRCAEGYA LYAQALTDQQ QFGKADEMYD 500
KCIDLEPDNA TTYVHKGLLQ LQWKQDLDRG LELISKAIEI DNKCDFAYET 550
MGTIEVQRGN MEKAIDMFNK AINLAKSEME MAHLYSLCDA AHAQTEVAKK 600
YGLKPPTL
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Fig. 3. Identification of a peptide sequence in ∼45 kDa protein of the *A. castellanii* 500 kDa complex regarded as the *A. castellanii* TOM complex. The peptide sequence perfectly matches amino acid sequence (*marked with a box*) in C-terminal region of human Tom70. Numbers refer to amino acid positions.

(Mori *et al*., 1998; Terada *et al*., 2003). Thus, it might be concluded that the detected peptide of *A. castellanii* is a part of a Tom protein of probably receptor function resembling that of Tom70. The conclusion indicates that the TOM complex of *A. castellanii* is not a typical example of the protozoan TOM complex. The latter, like other known TOM complexes of the green plant lineage, does not contain proteins with sequence similarity to Tom70 of the animal–fungal lineage (Macasev *et al*., 2004).

Summing up, the obtained data are important for studies of evolutionary history of the protozoan TOM complexes.

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