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Unique Composition of the Preprotein Translocase of the Outer Mitochondrial Membrane from Plants*

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Transport of most nuclear encoded mitochondrial proteins into mitochondria is mediated by heteropolymeric translocases in the membranes of the organelles. The translocase of the outer mitochondrial membrane (TOM) was characterized in fungi, and it was shown that TOM from yeast comprises nine different subunits. This publication is the first report on the preparation of the TOM complex from plant mitochondria. The protein complex from potato was purified by (*a*) blue native polyacrylamide gel electrophoresis and (*b*) by immunoaffinity chromatography. On blue native gels, the potato TOM complex runs close to cytochrome *c* oxidase at 230 kDa and hence only comprises about half of the size of fungal TOM complexes. Analysis of the TOM complex from potato by SDS-polyacrylamide gel electrophoresis allows separation of seven different subunits of 70, 36, 23, 9, 8, 7, and 6 kDa. The 23-kDa protein is identical to the previously characterized potato TOM20 receptor, as shown by *in vitro* assembly of this protein into the 230-kDa complex, by immunoblotting and by direct protein sequencing. Partial amino acid sequence data of the other subunits allowed us to identify sequence similarity between the 36-kDa protein and fungal TOM40. Sequence analysis of cDNAs encoding the 7-kDa protein revealed significant sequence homology of this protein to TOM7 from yeast. However, potato TOM7 has a N-terminal extension, which is very rich in basic amino acids. Counterparts to the TOM22 and TOM37 proteins from yeast seem to be absent in the potato TOM complex, whereas an additional low molecular mass subunit occurs. Functional implications of these findings are discussed.

Most mitochondrial proteins are nuclear encoded, synthesized on cytoplasmic ribosomes, and posttranslationally transported into the organelles (1, 2). The transport of proteins into mitochondria relies on two prerequisites: (i) targeting information of the proteins destined for mitochondria, which in most cases is encoded by N-terminal extensions called presequences, and (ii) a protein import apparatus within the mitochondrial membranes that recognizes the targeting information and translocates proteins from the cytoplasm to their subcellular destination. Central components of the protein import apparatus are two polymeric protein complexes called the "translocase of the outer mitochondrial membrane" (TOM)[1] and the "translocase of the inner mitochondrial membrane" (TIM) (3). During protein transport, the translocases are believed to interact dynamically as most nuclear encoded mitochondrial proteins must cross both mitochondrial membranes.

The TOM complex was isolated from outer mitochondrial membranes from *Neurospora crassa* and yeast (4–6). The yeast translocase consists of nine subunits designated TOM72, TOM70, TOM40, TOM37, TOM22, TOM20, TOM7, TOM6, and TOM5 according to their calculated molecular masses (for nomenclature, see Ref. 7). Five of these proteins (TOM72, TOM70, TOM37, TOM22, and TOM20) are involved in the recognition of nuclear encoded mitochondrial proteins on the surface of the organelles forming two heterodimeric receptors (TOM20-TOM22 and TOM37-TOM70/TOM72) (8–10). TOM40 is the main component of the pore for the translocation of proteins across the outer mitochondrial membrane (4, 11). It is associated with the three low molecular weight subunits: TOM7, TOM6, and TOM5 (12–14). The TOM complex from *N. crassa* has a similar subunit composition; TOM complexes from other organisms have not been characterized so far.

In a structural sense, the TOM complex is a rather dynamic association of polypeptide components. The receptors are partially present within TOM complexes but also occur independently within the outer mitochondrial membrane. Consequently, it is a difficult task to purify this protein complex. The TOM complexes from yeast and *Neurospora* were prepared by immunoprecipitation using antibodies directed against individual subunits of the translocase (4–6). The overall molecular mass of the TOM complex from *Neurospora* was estimated by gel filtration and lies in the range of 500 kDa (4).

Little is known about the transport of proteins into plant mitochondria. Some components of the protein import apparatus could be defined, namely the mitochondrial processing peptidase, which excises the presequences of nuclear encoded mitochondrial proteins upon their import into the organelles. In plants only is this enzyme membrane-bound and an integral part of the cytochrome *c* reductase complex of the respiratory chain (15–17). Recently, an additional processing activity in the soluble fraction of plant mitochondria was described, which might be distinct from the membrane-bound mitochondrial processing peptidase (18). Furthermore, the mitochondrial heat stress proteins HSP70, HSP60, and HSP10 were characterized, which are involved in the terminal steps of protein

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[1] The abbreviations used are: TOM, translocase of the outer mitochondrial membrane; TIM translocase of the inner mitochondrial membrane; PMSF, phenylmethylsulfonyl fluoride; OM, outer mitochondrial membrane; MOPS, 4-morpholinepropanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BN, blue native; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine.

transport into mitochondria (19–21). Finally, one component of the TOM complex, TOM20 from potato, has been characterized at the molecular level (22). TOM20 from potato is a 23-kDa protein of the outer mitochondrial membrane that exhibits significant sequence identity to the TOM20 proteins from fungi and mammals. Antibodies directed against potato TOM20 specifically inhibit protein transport into mitochondria, as demonstrated by *in vitro* import experiments (22).

Here, we describe the purification of the TOM complex from potato by native gel electrophoresis and by immunoaffinity chromatography. The protein complex has a molecular mass of 230 kDa and comprises seven different subunits that are characterized by direct protein sequencing and by sequencing corresponding clones encoding a 7-kDa polypeptide. TOM20 forms part of this complex, as shown by *in vitro* assembly of this protein into the 230-kDa complex, by direct sequence determination and by immunoblotting. The TOM complex from potato has a unique subunit composition.

EXPERIMENTAL PROCEDURES

Purification of the Outer Mitochondrial Membrane from Potato—Mitochondria from potato tuber were prepared on Percoll step gradients as described by Braun *et al.* (23) with the following modifications: (i) 0.1 mM PMSF was added to all buffers, and (ii) after the gradient centrifugation the organelles were washed and suspended in a buffer containing 0.4 M mannitol, 1 mM EGTA, 0.1% bovine serum albumin, 0.1 mM PMSF, and 10 mM KH_2PO_4, pH 7.2. The isolated mitochondria were resuspended in 7.5 ml of swelling buffer (5 mM K_iPO_4, 1 mM PMSF, pH 7.2)/g of organelles for 8 min at 4 °C. Subsequently, the same volume of swelling buffer was added and mitochondria were ruptured in a Potter homogenizer. Separation of outer mitochondrial membranes (OM) and mitoplasts was achieved by sucrose step gradient centrifugation, as reported by Heins *et al.* (24). One gram of potato mitochondria (100 mg of mitochondrial protein) yielded about 1 mg of OM protein. The OMs were stored in a buffer containing 10 mM EDTA, 0.4 mM PMSF, 10% glycerol, 100 mM MOPS-KOH, pH 7.2, at a protein concentration of 5 mg/ml at −80 °C.

Purification of the Potato TOM Complex by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) and Electroelution—Starting material for the purification of the potato TOM complex by blue native polyacrylamide gel electrophoresis were 40-μl OM aliquots (200 μg of OM protein) per lane of the gels. The aliquots were diluted by 100 μl of double-distilled H_2O; the OMs were sedimented by ultracentrifugation at 100,000 × *g* for 45 min and resuspended in 75 μl of ACA buffer (750 mM aminocaproic acid, 0.5 mM EDTA, 50 mM Bis-Tris, pH 7.0). Membrane proteins were solubilized by addition of 75 μl of ice-cold digitonin solution (10% digitonin, 750 mM aminocaproic acid, 0.5 mM EDTA, 50 mM Bis-Tris, pH 7.0), and insoluble material was removed by ultracentrifugation at 100,000 × *g* for 20 min. Supernatants were supplemented with 15 μl of Coomassie solution (750 mM aminocaproic acid, 5% Coomassie Blue) and directly loaded onto the gel. BN-PAGE was carried out as described previously (25, 26). The potato TOM complex forms a band at about 230 kDa, which is visible without staining. The band was cut out, and the protein complex was electroeluted in a buffer containing 25 mM Tricine, 7.5 mM Bis-Tris, pH 7.0, 0.1 mM PMSF using the electroeluter from CBS Scientific Co. (Del Mar, CA).

Determination of the Molecular Weight of the Potato TOM Complex—For molecular weight determination of the potato TOM complex, 7 mg of mitochondria (0.7 mg of mitochondrial protein) from potato were resuspended in 75 μl of ACA buffer (see above) and supplemented with either 25 μl of a 10% digitonin solution or 15 μl of a 10% laurylmaltoside solution. The solubilized protein complexes in both fractions were separated by BN-PAGE as described above and subsequently blotted onto nitrocellulose membranes as outlined by Jänsch *et al.* (26). The respiratory protein complexes were directly visible due to bound Coomassie Blue, whereas the potato TOM complex was identified by immunostaining. The size of the potato TOM complex was determined by comparison to the sizes of the well characterized respiratory protein complexes from potato.

Immunoaffinity Purification of the Potato TOM Complex—Antibodies directed against TOM20 from potato (22) were covalently bound to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Sweden) according to the supplier's instructions. Potato outer mitochondrial membranes prepared from 0.8 g of isolated organelles were sedimented by ultracentrifugation at 70,000 × *g* for 90 min and resuspended in 375 μl of coupling buffer (20 mM Tris acetate, 10 mM NaCl, pH 7.0). Membrane proteins were solubilized by addition of 375 μl of ice-cold digitonin solution (10% digitonin, 20 mM Tris acetate, 10 mM NaCl, pH 7.0), and insoluble material was removed by ultracentrifugation at 100,000 × *g* for 20 min. About 2 ml of the anti-TOM20-Sepharose were equilibrated in a column with coupling buffer. After applying the solubilized membrane proteins to the column, it was washed with coupling buffer and bound protein was eluted with 5 ml of elution buffer (100 mM glycine, pH 2.5). Eluted proteins were neutralized by addition of 400 μl of 1 M Tris-HCl, pH 8.0. The proteins were precipitated by the Roti-Collect system (Roth GmbH, Karlsruhe, Germany), supplemented with loading buffer and directly analyzed by Tricine-SDS-PAGE (27).

In Organello Assembly Assay—The TOM20 protein from potato was synthesized *in vitro* by the coupled transcription/translation system from Stratagene (Heidelberg, Germany) in the presence of [^{35}S]methionine. Mitochondria were prepared on Percoll step gradients as described above. For an assembly assay, 600 μl of mitochondria (10 mg of protein/ml) were combined with 2.4 ml of assembly buffer (0.25 M mannitol, 80 mM KCl, 1 mM K_2HPO_4, 1 mM ATP, 1 mM malate, 2 mM NADH, 1 mM dithiothreitol, and 20 mM HEPES, pH 7.5) and 37.5 μl of radiolabeled TOM20 protein in transcription/translation lysate. The assembly mixture was incubated for 20 min at 25 °C. Mitochondria were reisolated by centrifugation for 10 min at 13,000 × *g*, resuspended in 600 μl of resuspension buffer (0.4 M mannitol, 1 mM EGTA, 0.1% bovine serum albumin, 0.1 mM PMSF, and 10 mM KH_2PO_4, pH 7.2) and supplemented with 3.4 ml of untreated potato mitochondria (10 mg of protein/ml) in the same buffer. Subsequently, outer mitochondrial membranes were prepared as described above and proteins were separated by two-dimensional BN/SDS-polyacrylamide gel electrophoresis. Gels were treated with Amplify (Amersham Pharmacia Biotech, Braunschweig, Germany) and exposed on x-ray films.

Identification of Subunits of the Potato TOM Complex by Direct Sequence Determination and Immunoblotting—The subunits of the potato TOM complex were separated either by SDS-PAGE of the electroeluted protein complex or directly by electrophoresis of a BN gel stripe in a second gel dimension in the presence of SDS. Tricine-SDS-PAGE according to Schägger and von Jagow (27) gives best resolution because the TOM complex contains several very small subunits with similar molecular masses. The separated TOM subunits were blotted onto polyvinylidene difluoride membranes, and TOM20 was identified by immunostaining using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Bands corresponding to other TOM subunits were cut out from the blots and analyzed directly on a Procise-HT protein sequencer (model ABI 494A, Applied Biosystems, Foster City, CA). N-terminally blocked proteins were digested with endoprotease LysC, and the generated peptides were separated by high performance liquid chromatography and analyzed by direct protein sequencing.

Isolation of Clones Encoding the Potato TOM7 Subunit—Clones encoding TOM7 from potato were isolated by probing a λ ZAP-II cDNA library with a mixture of 23-nucleotide oligomers that were made degeneratively to an amino acid sequence close to the N terminus of the protein (Lys-Gly-Lys-Asn-Thr-Lys-Phe).

RESULTS

Purification of the Potato TOM Complex—Compared with the protein complexes of the respiratory chain, the TOM complex must be considered to be a dynamic supramolecular structure. Consequently, classical biochemical approaches to isolate the TOM complex has only had limited success. Recently, BN-PAGE was shown to be an alternative approach to characterize the translocase of the outer mitochondrial membrane from yeast (28). BN-PAGE was first used by Schägger and von Jagow (25) to isolate the respiratory protein complexes from beef and yeast and was later adapted to characterize the respiratory and photosynthetic protein complexes from plant organelles (26, 29).

Prerequisite for the isolation of the potato TOM complex was a fraction containing highly pure outer mitochondrial membranes, which was generated as described in the experimental procedures. Tricine-SDS-PAGE of potato outer mitochondrial membranes allows to separate about 30 different proteins (Fig. 1*C*). The two most dominant bands represent two isoforms of the pore-forming protein "porin," which run at 30 kDa on Tricine SDS gels (34–36 kDa on glycine SDS gels according to

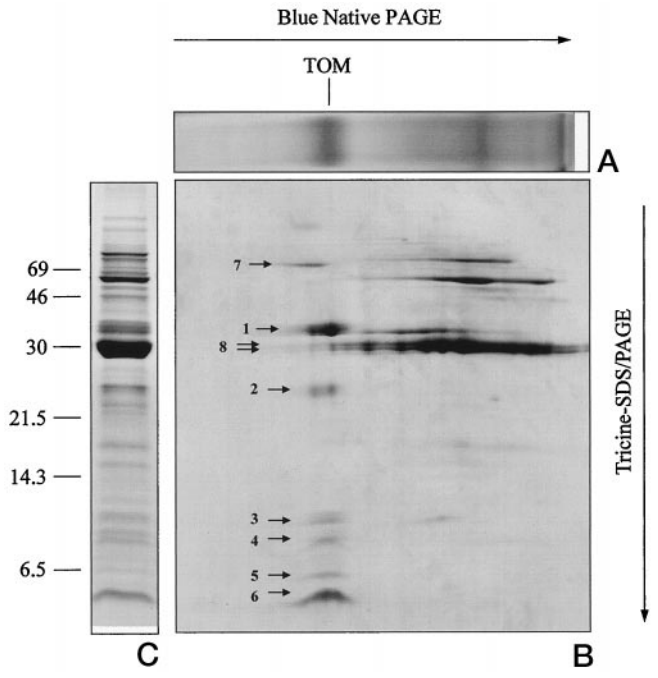

FIG. 1. **Purification of the potato TOM complex by BN-PAGE.** Potato OM proteins were solubilized by digitonin and subsequently analyzed by BN-PAGE as described under "Experimental Procedures." *A*, Coomassie-stained gel of potato OM proteins after BN-PAGE. *B*, Coomassie-stained gel of potato OM proteins after two-dimensional resolution by BN-PAGE (first dimension) and Tricine-SDS-PAGE (second dimension). *C*, Coomassie-stained gel of potato OM proteins after solubilization with SDS and one-dimensional Tricine-SDS-PAGE. The *arrows* indicate proteins that were subjected to direct protein sequencing (see Table I). The molecular masses of standard proteins are given on the *left* in kDa. *TOM*, translocase of the outer mitochondrial membrane from potato.

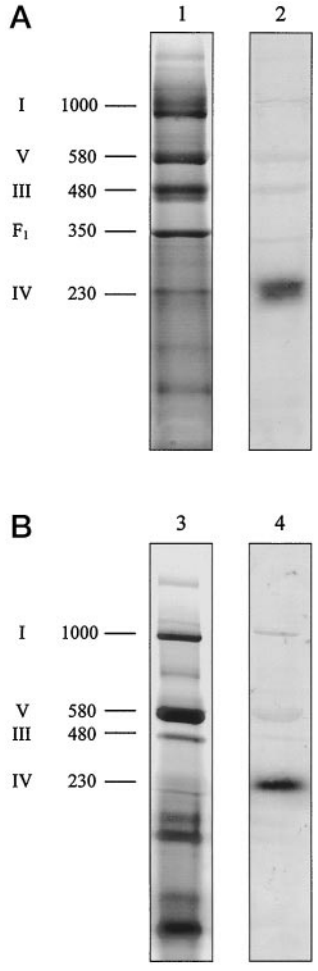

FIG. 2. **Determination of the molecular weight of the potato TOM complex.** Mitochondrial protein complexes were solubilized by laurylmaltoside (*A*) or digitonin (*B*) as described under "Experimental Procedures," separated by BN-PAGE, and blotted onto nitrocellulose membranes. *Lanes 1* and *3,* blot strips after staining with Coomassie Blue; *lanes 2* and *4*, blot strips after immunostaining using an antibody directed against potato TOM20. The designations on the *left* indicate the identity of some respiratory protein complexes and their molecular weights as determined previously (26).

Laemmli; Ref. 30) and which have been characterized previously (24). BN-PAGE of digitonin-lysed outer mitochondrial membranes from potato revealed the presence of one dominant protein complex, which, under the conditions applied, migrates in the central part of the gel (Fig. 1*A*). Furthermore some faint bands with higher electrophoretic mobility are visible on the BN gel. To characterize the subunit compositions of the separated protein complexes, a strip of the blue native gel was transferred horizontally onto a second gel dimension and electrophoresed in the presence of SDS. On Tricine SDS gels, the dominant protein complex could be resolved into seven subunits with apparent molecular masses of 70, 36, 23, 9, 8, 7, and 6 kDa (Fig. 1*B*). If analyzed by the glycine SDS-PAGE system, the apparent molecular mass of the 36-kDa protein lies at 39 kDa. The subunit composition resembles the composition of the TOM complex from fungi, which was characterized previously. The protein complexes with higher electrophoretic mobility on the BN gel turned out to be aggregates of varying numbers of porin proteins (Fig. 1*B*, proteins number 8).

Determination of the Molecular Weight of the Potato TOM Complex—Blue native polyacrylamide gel electrophoresis is a suitable method for the size determination of protein complexes solubilized by laurylmaltoside (31). To determine the apparent molecular weight of the putative potato TOM complex, isolated mitochondria from potato were treated with laurylmaltoside and directly analyzed by BN-PAGE (Fig. 2*A*). The separated protein complexes were transferred onto nitrocellulose membranes and stained by immunoblotting. The respiratory protein complexes of the inner mitochondrial membrane, which have well defined apparent molecular sizes, could be identified. An antibody directed against the potato TOM20 protein specifically reacted with a protein complex migrating close to the cytochrome *c* oxidase complex at about 230 kDa. Electrophoresis of the same sample on a second gel dimension in the presence of SDS revealed that the immunoreactive protein complex had an identical protein composition as described above for the putative TOM complex from potato after digitonin solubilization (data not shown). Determination of the molecular mass of the potato TOM complex after solubilization of mitochondria with digitonin gave similar results (Fig. 2*B*). The separation of the respiratory protein complexes is slightly different if compared with the separation of laurylmaltoside lysed mitochondria. However, the TOM complex again migrates close to the cytochrome *c* oxidase at 230 kDa.

The Identification of TOM20 as Part of the Potato TOM Complex—So far, the TOM20 protein from potato is the only component of the preprotein translocase from plants that has been characterized at the molecular and physiological level (22). To prove that the TOM20 protein forms part of the putative potato TOM complex, antibodies directed against pTOM20 were used to immunoaffinity-purify the proteins of a fraction containing outer mitochondrial membranes from potato. The antibody was covalently bound to CNBr-activated Sepharose and immunoaffinity chromatography was carried out using a

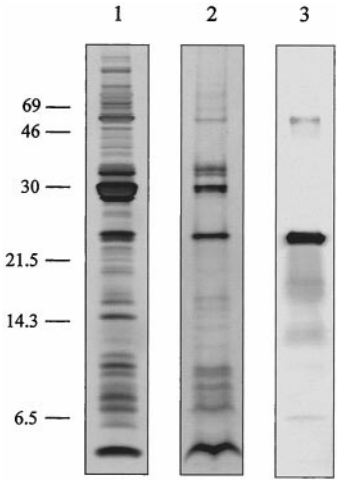

FIG. 3. **Purification of the potato TOM complex by immunoaffinity chromatography using antibodies directed against pTOM20.** Protein fractions were separated by Tricine-SDS-PAGE. *Lane 1*, silver-stained gel of separated OM proteins from potato. *Lane 2*, silver-stained gel of digitonin-lysed OM proteins after the immunoaffinity purification step. *Lane 3*, immunostained Western blot of the affinity-purified OM proteins using the pTOM20 antibody. The molecular masses of standard proteins are given on the *left* in kDa.

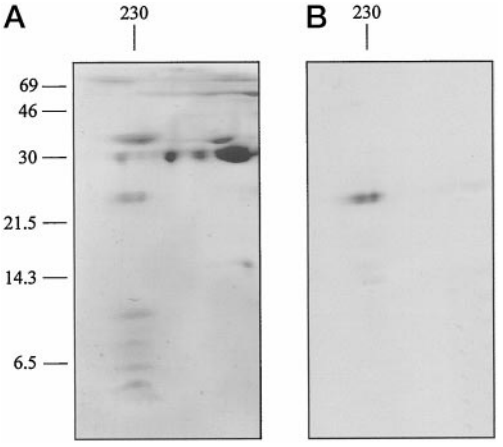

FIG. 4. ***In vitro* assembly of potato TOM20 into the potato TOM complex.** pTOM20 was synthesized in the presence of [^{35}S]methionine and incubated with freshly isolated potato mitochondria as described under "Experimental Procedures." Subsequently, the potato TOM complex was isolated by two-dimensional BN/SDS-PAGE. *A*, Coomassie-stained gel of the purified TOM complex; *B*, autoradiography of the same gel. The designations on the *top* and on the *left* indicate the sizes of standard proteins in kDa.

small column. Bound protein was eluted with 100 mM glycine at pH 2.5 as described under "Experimental Procedures" and analyzed on Tricine-SDS-PAGE (Fig. 3). The eluted fraction contains predominant proteins of 36, 30, 23, 9, 8, 7, and 6 kDa (Fig. 3, *lane 2*). The 23-kDa protein was immunologically identified to be TOM20 (Fig. 3, *lane 3*), and the 30-kDa protein turned out to be the most abundant protein of the mitochondrial outer membrane, porin (data not shown). The remaining proteins have identical molecular masses as the subunits of the potato TOM complex after purification by BN-PAGE. Hence, TOM20 seems to form part of the TOM complex from potato.

In a second approach to monitor the presence of TOM20 in the 230-kDa protein complex of the outer mitochondrial membrane from potato, *in vitro* assembly of the TOM20 protein into the 230-kDa complex was tested. Radiolabeled TOM20 from potato was incubated with isolated mitochondria as described under "Experimental Procedures." Subsequently, the outer mitochondrial membrane was prepared and the proteins of this fraction were analyzed by two-dimensional BN/SDS-PAGE and autoradiography. Indeed, the 23-kDa TOM20 protein from potato assembles into the 230-kDa protein complex as documented in Fig. 4. We conclude that the 230-kDa protein complex in the outer membrane from potato mitochondria corresponds to the preprotein translocase designated "TOM" in fungi.

Identification of Subunits of the Potato TOM Complex by Direct Sequence Determination—In an attempt to characterize the components of the potato TOM complex, the proteins of the outer mitochondrial membrane from potato were separated by two-dimensional BN/SDS-PAGE and blotted onto filter membranes. The subunits of the TOM complex were visualized by staining with Ponceau S, cut out, and subjected to direct protein sequencing. Subunits blocked for direct protein sequence determination were digested with endoprotease LysC to generate peptides, which were separated by high performance liquid chromatography and also analyzed by direct protein sequence determination. Table I summarizes the obtained amino acid data for the 36-, 23-, 9-, 8-, 7-, and 6-kDa subunits of the potato TOM complex. As expected, the peptide sequence for the 23-kDa subunit is identical to an internal amino acid stretch of potato TOM20. The peptide sequences for the 36-kDa protein exhibit some sequence similarity to the published TOM40 sequences from fungi. Sequence conservation was highest between peptide P1 and the fungal TOM40 proteins (Fig. 5). The partial sequence data generated for the 9-, 8-, 7-, and 6-kDa proteins of the potato TOM complex did not exhibit any significant sequence similarity to components of the TOM complex from other organisms.

Isolation of Clones Encoding the TOM7 Protein from Potato—In order to obtain complete sequences for the 9-, 8-, 7-, and 6-kDa proteins of the potato TOM complex, degenerate oligonucleotides designed from amino acid stretches of each protein were used to screen a cDNA library for potato tuber and to isolate corresponding clones. Screening with an oligonucleotide deduced from the N-terminal sequence of the 7-kDa protein of the potato TOM complex led to the isolation of two clones designated pTOM221 and pTOM222. Both clones contained inserts of 653 base pairs with identical sequences (Fig. 6). The inserts include an open reading frame of 216 base pairs encoding a protein of 72 amino acids with a calculated molecular mass of 7716 Da. The N-terminal amino acid sequence of the deduced protein is identical to the sequence directly determined by cyclic Edman degradation of the 7-kDa protein of the potato TOM complex, with the exception of the initiator methionine, which is absent in the mature protein. Comparison between the complete amino acid sequence of the potato 7-kDa protein and the sequence entries of protein data bases revealed striking similarities to the sequence of the TOM7 protein from yeast (Fig. 7). The overall sequence identity lies at 25% and sequence similarity at 50%. Interestingly, the sequence conservation is rather low in the N-terminal half of the protein but high in the C-terminal half. We conclude that the 7-kDa protein of the potato TOM complex is the plant counterpart to TOM7 from yeast. Further analyses with nucleotide sequence data bases led to the discovery of some unidentified plant sequences, which encode proteins with high homology to the sequence of potato TOM7, including the expressed sequence tag D22755 from rice and L35838 from cabbage (Fig. 7). On the basis of amino acid sequences for TOM7 proteins from five different organisms, a consensus sequence for TOM7 could be defined, which should be useful for analysis of the function of TOM7 by site-directed mutagenesis.

TABLE I
Partial amino acid sequences of the TOM subunits from potato

No.[a]	Mass[b]	Region[c]	Amino acid sequence[d]	Identity[e]
	kDa			
1	36	N	. . . . .	
		P1	SGIGYAARYNTDK	TOM40
		P2	ANGQLTGE	
		P3	FGFGLTVGE	
		P4	GLNQRFSLSHSVFMGPTE	
2	23	N	. . . . .	
		P1	QMISDATSK	TOM20
3	9	N	. . . . .	
		P1	GGVSLPDRPGAGDGIISRFSSSIXESPIVYQGK	
4	8	N	LSAVSLKNDGVLARIS	
		P1	NDGVLARISXSXIVTK	
5	7	N	AKGKNTKKFAAVVDEEGGAVTAXYXF	TOM7
6	6	N	ADSVISVDKVKAFYTLQ	

[a] The proteins are numbered according to Fig. 1. (No amino acid sequences could be determined for the protein number 7.)
[b] Apparent molecular weights as determined by Tricine-SDS-PAGE (Fig. 1).
[c] N, N termini of mature proteins; P1, P2,. . ., N termini of peptides.
[d] The amino acid data are given in one-letter code; . . . . . indicates blocked N termini.
[e] The identity was determined by sequence comparisons with the TOM proteins from yeast (see Figs. 5 and 7).

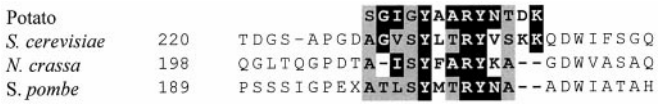

FIG. 5. **The 36-kDa subunit of the TOM complex from potato is homologous to TOM40 from fungi.** An internal sequence (P1 in Table I) of the 36-kDa subunit from potato was aligned with TOM40 sequences from *Saccharomyces cerevisiae* (P23644), *Neurospora crass*a (P24391), and *Schizosaccharomyces pombe* (AB004538). Amino acids conserved between the potato sequence and one or several fungal sequences are underlaid in *black*, amino acids that are similar in potato and one or several fungal sequences are underlaid in *gray*. The *numbers* to the *left* of the three fungal sequences indicate the position of the first amino acid within the protein sequences, as deduced from the open reading frames of the corresponding genes.

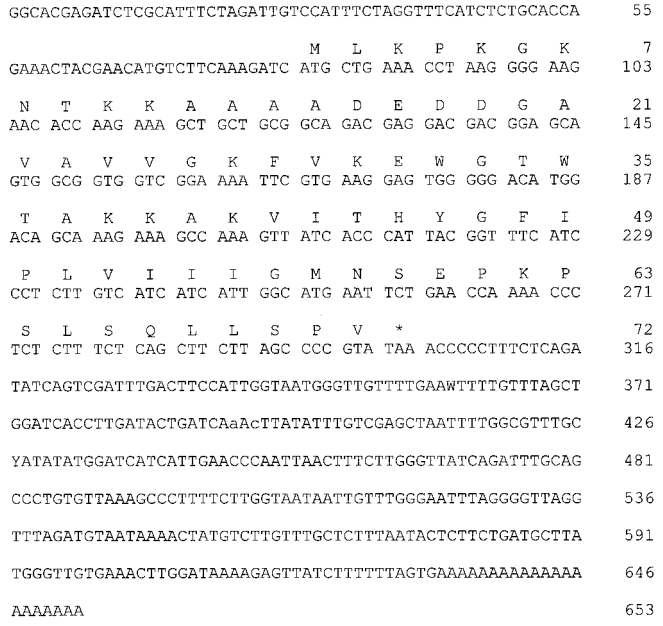

FIG. 6. **Nucleotide sequence and deduced amino acid sequence of the insert of clone pTOM221.** The sequence data have been submitted to the EMBL sequence data banks and are available under the accession number Y16228.

DISCUSSION

Solubilization of outer mitochondrial membranes from potato with digitonin and separation of the solubilized proteins by blue native polyacrylamide gel electrophoresis allows the purification of a complex of 230 kDa that consists of seven different subunits of 70, 36, 23, 9, 8, 7, and 6 kDa. The subunit composition resembles the one reported for fungal TOM complexes. TOM20 from potato, a well defined receptor for the transport of nuclear encoded mitochondrial proteins into mitochondria, forms part of the 230-kDa complex as shown by *in vitro* assembly of this protein into the 230-kDa complex, by direct protein sequencing and by immunoblotting. Hence, the 230-kDa complex represents the potato TOM complex. Blue native polyacrylamide gel electrophoresis was employed previously to analyze the TOM complex from yeast (14, 28). The procedure allows separation of two subcomplexes of the yeast TOM complex of 400 and 120 kDa. The larger subcomplex contains TOM40, TOM20, TOM5, and presumably TOM22, TOM7, and TOM6, whereas the smaller subcomplex contains TOM70 and possibly TOM37, as shown by immunoblotting. In contrast, the potato translocase seems to be stable during solubilization and native gel electrophoresis as no subcomplexes or singular subunits of the TOM complex are visible on the two-dimensional gels (Fig. 1).

The TOM complexes from yeast and *Neurospora* were isolated by immunoprecipitations using antibodies against individual subunits of the TOM complex (4, 6). However, immunoprecipitation often does not lead to biochemically pure proteins or protein complexes because the antibodies can be present in the final fractions and because cross-reactions can occur. In contrast, antibodies are not needed during blue native gel electrophoresis and the capacity of this procedure to resolve proteins is high. BN-PAGE seems to be a powerful tool for the isolation of the potato TOM complex. The 230-kDa protein complex can be electroeluted from BN gels and used for further investigations. In fact, it was shown that the protein complexes of the respiratory chain from beef and potato are physiologically active after electroelution from BN gels (25, 32). This also may be valid for the potato TOM complex after purification by BN-PAGE and electroelution.

Sequence analyses of the subunits of the potato TOM complex allowed us to relate its subunits to the components of the TOM complex from yeast. The 23-kDa protein is homologous to fungal TOM20 and the 7-kDa protein to TOM7 from yeast. The 36-kDa protein from potato exhibits sequence similarity to fungal TOM40. It was previously shown that antibodies directed against TOM40 from *Neurospora* cross-react with a 42-kDa protein of the outer membrane of *Vicia faba* mitochondria and that antibodies against this 42-kDa protein inhibit protein import into mitochondria from *V. faba* in *in vitro* import experiments (33). Most likely, the 36-kDa protein from potato and

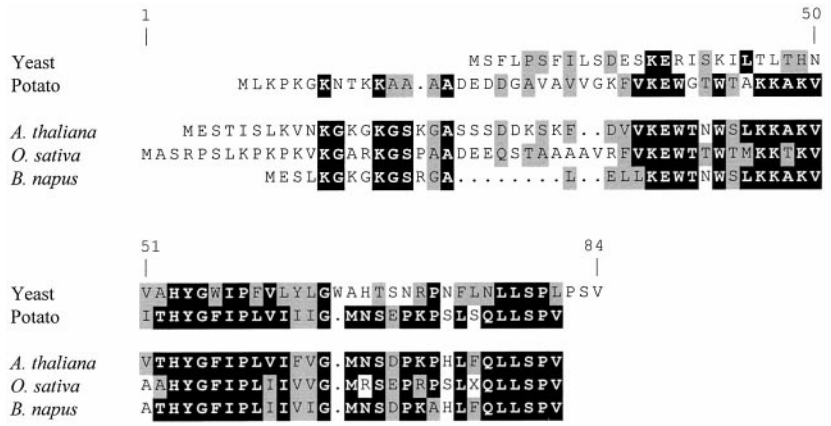

FIG. 7. **The potato 7-kDa protein is homologous to yeast TOM7.** Sequence comparison of the 7-kDa protein of the mitochondrial outer membrane from potato with TOM7 from yeast (S63002) and with amino acid sequences deduced from unidentified expressed sequence tags from the plants *Oryza sativa* (rice, D22755) and *Brassica napus* (cabbage, L35838) and with an unidentified open reading frame of a genomic region of *A. thaliana* (AB005233). Identical amino acids are underlaid in *black* if conserved in at least three organisms. Similar amino acids are underlaid in *gray*.

the 42-kDa protein from *V. faba* are homologues. Furthermore, we speculate that the 70-kDa protein of the TOM complex from potato is the counterpart of TOM70 from fungi and that two of the three unidentified small proteins represent the TOM5 and TOM6 proteins.

Based on these conclusions, the potato TOM complex differs in several respects from the translocase from fungi: (i) the TOM complex from potato is only about half the size of the fungal TOM complex, (ii) the TOM complex from potato comprises four instead of three low molecular mass subunits, and (iii) two of the four receptor proteins defined for yeast (TOM22 and TOM37) seem to be absent in potato. However, it cannot be excluded that the TOM complex from potato contains additional subunits *in vivo* that are lost during solubilization of the outer mitochondrial membrane with digitonin or during native gel electrophoresis. On the other hand, there are several arguments indicating that the TOM complex from potato was isolated in complete form; (*a*) different concentrations of digitonin during solubilization always led to the isolation of a protein complex with the same subunit composition (data not shown), (*b*) solubilization of outer mitochondrial membranes from potato with laurylmaltoside also gave identical results, and (*c*) two different purification procedures for the potato TOM complex (BN-PAGE and immunoaffinity chromatography) led to the isolation of a protein complex with identical subunit composition. Furthermore, the TOM37 protein has so far only been identified in yeast but not in *Neurospora*. Hence, the occurrence of two heterodimeric receptors (one of which is composed of the TOM20 and TOM22 proteins, the other of the TOM37 and TOM70/TOM72 proteins; see Refs. 8–10) might be an unique feature of yeast mitochondria. Possibly protein transport into plant mitochondria is based on the presence of two monomeric receptors of 23 kDa (TOM20) and 70 kDa (TOM70?). However, further experiments are needed to precisely define the structure and function of the plant TOM complex, including isolation of the translocase from other organisms. Preliminary characterization of the TOM complex from *Arabidopsis thaliana* also reveals the presence of four low molecular mass subunits.[2]

The small proteins of the TOM complex are best characterized in yeast (13, 14, 34). All of them are associated with TOM40 forming part of the "general insertion pore" for the transfer of nuclear encoded mitochondrial proteins into mitochondria. TOM5 functionally links the receptors and TOM40, whereas TOM6 and TOM7 have antagonistic functions in the modulation of the assembly and dissociation of the TOM machinery: TOM6 supports the binding of the receptors with TOM40, whereas TOM7 destabilizes the same interactions. (If TOM7 from potato has a similar function, its presence within the 230-kDa TOM complex together with TOM20 and TOM70 would add a further argument supporting the absence of heterodimeric receptors in plants *in vivo*.) The mature TOM7 proteins from potato and yeast both lack the N-terminal methionine and have slightly different calculated molecular masses of 7.6 kDa (potato) and 6.7 kDa (yeast). The size difference is due to an N-terminal extension of the potato protein, which is very rich in basic amino acids (Fig. 7). TOM7 from potato is more hydrophilic than the corresponding yeast protein. Most striking is the presence of 11 lysines in the potato protein (yeast: 2 lysines). Similarly to TOM7 from yeast, the potato protein lacks a putative hydrophobic membrane-spanning helix and most likely is bound to the outer mitochondrial membrane by protein-protein interactions. Identification of some additional TOM7 sequences from plants allowed to define a consensus sequence for TOM7 proteins. There are two highly conserved amino acid stretches within the C-terminal half of TOM7 proteins (-H-Y-G-(W/F)-I-P-(F/L)-V- and -(N/Q)-L-L-S-P-(L/V)-). Site-directed mutagenesis may allow elucidation of the function of these sequence motifs in protein translocation across the outer mitochondrial membrane.

Due to its dynamic structure, the translocase of the outer mitochondrial membrane is difficult to characterize. Immunopurifications and blue native gel electrophoresis were employed to characterize both the TOM complex from fungi and the TOM complex from potato. The purified TOM complexes have several characteristics in common, but also differ in some respects. So far, it cannot be excluded that the differences are due to isolation artifacts. On the other hand, the structure and function of the mitochondrial protein import apparatus from different organisms is known to vary to some extent. One example is the mitochondrial processing peptidase, which is a soluble enzyme in fungi and mammals but forms an integral part of the cytochrome *c* reductase complex in plants. It should be interesting to analyze the structure and function of the TOM complex from some further organisms.

[2] L. Jänsch, U. K. Schmitz, and H.-P. Braun, manuscript in preparation.

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