Isolation and Characterization of the Principal ATPase Associated with Transitional Endoplasmic Reticulum of Rat Liver

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Abstract. The transfer of membranes from the endoplasmic reticulum to the Golgi apparatus occurs via 50–70 nm transition vesicles which derive from part-rough, part-smooth transitional elements of the endoplasmic reticulum (TER). Vesicle budding from the TER is an ATP-dependent process both in vivo and in vitro. An ATPase with a monomer molecular weight of 100 kD by SDS-PAGE has been isolated from TER and designated as TER ATPase. The native TER ATPase has been characterized as a hexamer of six 100-kD subunits by gel filtration. The protein catalyzes the hydrolysis of \( \gamma^{32-P} \text{ATP} \) and is phosphorylated in the presence of Mg\(^{2+} \). It is distinct from the classical transport ATPases based on pH optima, ion effects, and inhibitor specificity. Electron microscopy of negatively stained preparations revealed the TER ATPase to be a ring-shaped structure with sixfold rotational symmetry. A 19-amino acid sequence of TER ATPase having 84% identity with valosin-containing protein and 64% identity with a yeast cell-cycle control protein CDC48p was obtained. Anti-synthetic peptide antisera to a 15-amino acid portion of the sequence of TER ATPase recognized a 100-kD protein from TER. These antisera reduced the ATP-dependent cell-free formation of transition vesicles from isolated TER of rat liver. In a reconstituted membrane transfer system, TER ATPase antisera inhibited transfer of radiolabeled material from endoplasmic reticulum to Golgi apparatus, while preimmune sera did not. The results suggest that the TER ATPase is obligatorily involved in the ATP requirements for budding of transition vesicles from the TER. cDNA clones encoding TER ATPase were isolated by immunoscreening a rat liver cDNA library with the affinity-purified TER ATPase antibody. A computer search of deduced amino acid sequences revealed the cloned TER ATPase to be the rat equivalent of porcine valosin-containing protein, a member of a novel family of ATP binding, homo-oligomeric proteins including the \( N \)-ethylmaleimide-sensitive fusion protein.

Despite a rapidly growing understanding of the process of membrane transfer between endoplasmic reticulum and Golgi apparatus at the biochemical level (Novick and Brennwald, 1993), less progress has been made to identify the essential components involved. Transfer of membranes and other materials between endoplasmic reticulum and Golgi apparatus has long been assumed to involve small 50–70 nm vesicles (Morré et al., 1971). These vesicles, termed transition vesicles (Palade, 1975), are derived from part-rough, part-smooth transition elements of the endoplasmic reticulum.

Balch and Keller (1986) reported that transport of the vesicular stomatitis virus strain ts045G protein from endoplasmic reticulum to an early Golgi compartment containing \( \alpha \)-mannosidase I was ATP dependent. Subsequently, they located the ATP-dependent step in a very early stage between endoplasmic reticulum and Golgi apparatus using semi-intact cells (Beckers et al., 1990). Evidence for ATP-dependent formation of transition vesicles has been provided as well using a cell-free system from rat liver (Morré et al., 1986; Nowack et al., 1987). The formation of small blebbing profiles from isolated transitional endoplasmic reticulum (TER) fractions, similar to those associated with TER in situ (Morré et al., 1986), was observed in vitro. These vesicles, formed in a cell-free system, were separated from the bulk of the endoplasmic reticulum–derived elements by preparative free-flow electrophoresis and were characterized morphologically and functionally (Paulik et al., 1988). The isolated transition vesicles functioned in the cell-free transfer system of rat liver as an intermediate to transfer donor membrane to acceptor Golgi apparatus. Transfer was rapid, specific and apparently unidirectional in that the Golgi

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Abbreviations used in this paper: C\(_{12}\)E\(_8\), octaethyleneglycol monododecyl ether; KLH, keyhole limpet; SB, solubilizing buffer; TER, transitional endoplasmic reticulum.

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apparatus membranes were ineffective as donor membranes and endoplasmic reticulum membranes were ineffective as acceptor membranes (Paulik et al., 1988). The transfer was confirmed by fucosylation of transferrin dipeptidylaminopeptidase IV (Paulik et al., 1988) and alterations of VSV-G proteins which indicated α-mannosidase I processing (Morré et al., 1993).

Using the cell-free system from rat liver, the budding process from isolated TER was shown to be ATP dependent (Paulik et al., 1988). When isolated TER was incubated with cytosol only, formation of vesicles was decreased dramatically as compared with cytosol plus ATP and an ATP-regenerating system. When different nucleotide triphosphates were employed in the cell-free system to study the nucleotide triphosphate dependence, transition vesicle formation specifically required ATP (M. Paulik and D. J. Morré, unpublished results). Subsequently a Mg2+-ATPase activity was observed in the TER and was shown by ion-exchange chromatography to be distinct from proteins associated with IDPase and GTPase activities (Zhao et al., 1990). Here we report the characterization, isolation and amino acid sequence of the ATPase from the TER which appears to represent a functional component of the ATP-vesicle budding from the TER.

Materials and Methods

Animals

Rats were 150-180 g males of the Holtzman strain from Harlan Sprague Dawley (Indianapolis, IN) provided ad libitum with feed and drinking water. Sacrifice was by decapitation. Carcasses were drained of blood and the livers were removed.

Chemicals

[γ-32P]ATP was purchased from Amersham Corp. (Arlington Heights, IL) or ICN Biochemical (Irvine, CA). Unless specifically noted otherwise, chemicals were from Sigma Chemical Co. (St. Louis, MO).

Isolation of the Transitional Endoplasmic Reticulum

Homogenates were prepared in isotonic buffered sucrose as described (Morré et al., 1988). Briefly, livers were homogenated in two volumes of 37°C homogenization medium, pH 6.5, 0.5 M sucrose, 5 mM MgCl2, and 1% dextran for 45 s with a polytron 20ST operated at 6000 rpm. The homogenates were centrifuged for 15 min at 4,500 g and the pellets used as a source of Golgi apparatus (Morré, 1971). The supernatant was diluted 1:5 with the homogenation medium and, following a second centrifugation at 10,000 g to remove mitochondria, was layered onto a discontinuous sucrose gradient consisting of 2.0, 1.5, and 1.3 M sucrose layers (Morré, 1973). After centrifugation at 85,000 g for 90 min, membranes at the 1.3 M sucrose/sample interface were removed with a Pasteur pipette and pelleted for 20 min at 70,000 g as the TER.

ATPase Assay

TER ATPase was assayed in a reaction mixture of 50 mM Tris-MES, pH 8.0, 2 mM MgCl2 and 2 mM ATP (see Fig. 1). The reaction mixture also contained 0.1 mM NaN3 and 1.0 mM sodium molybdate to inhibit phosphatases. Release of inorganic phosphorous was measured using a colorimetric procedure (Berezi and Morré, 1993). Results were corrected for non-specific hydrolysis of substrate by subtracting appropriate substrate and zero-time controls.

Solubilization and Chromatography

TER was resuspended at 4°C for 1 h in solubilization buffer (SB) containing 250 mM sucrose, 25 mM KCl, 0.1 mM NaN3, 0.1 mM EDTA, 0.4 mM PMSF, 50 mM Tris-MES, pH 8.0, and 0.01% (wt/vol) octaethyleneglycol monododecyl ether (C12E8) (Calbiochem-Behring Corp., La Jolla, CA). Additional C12E8 was added to the solubilization mixture to a final concentration of 0.11% (wt/vol), (detergent/protein ratio of 1:51 [wt/vol]). Unsolubilized material was pelletted by centrifugation at 100,000 g for 1 h. The supernatant was collected and was loaded onto the DEAE-52 anion-exchange column equilibrated with SB buffer. ATPase was eluted with a linear salt gradient from 0 to 0.6 M NaCl (Fig. 2). Fractions were pooled, dialyzed against SB, and concentrated using Centricon-10 (Amicon Corp., Beverly, MA) microcentrifuges. The concentrated fractions were resolved by non-denaturing PAGE with detection of ATPase activity as described below. Fractions from DEAE-52 containing a majority of the ATPase activity were purified further by TSKG3000 SWXL gel filtration (30 x 78 mm ID; Supelco, Bellefonte, PA) pre-equilibrated with SB. Elution was with the same buffer at a flow rate of 0.5 ml/min. Fractions were collected, concentrated, and analyzed by SDS- or non-denaturing PAGE.

Enzyme Activity Staining

Non-denaturing PAGE was according to Harada et al. (1991) in slab gels in the absence of SDS. ATPase activity was detected by soaking the gel in a solution containing 50 mM Tris, 0.2 M glycine, pH 8.3, 2 mM ATP, 2 mM MgCl2, 0.1 mM sodium molybdate, 0.1 mM NaN3, and 0.05% lead acetate, for 4 to 8 h at 37°C. ATP hydrolysis was detected by the formation of a white lead phosphate precipitate. The ATPase activity band was then cut out, soaked in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 5% β-mercaptoethanol and subsequently analyzed on 8% SDS-PAGE. SDS-PAGE gels were stained with Coomassie brilliant blue 250.

Determination of Native Molecular Weight

The molecular weight of TER ATPase was determined from size exclusion chromatography (100 µl sample injection) on TSKG3000 SWXL gel filtration compared to standard thyroglobulin (669 kD, bovine), apoferritin (44.5 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), and bovine serum albumin (66 kD). Chromatography used a partially purified TER ATPase (fraction 2 of DEAE-52) (see Fig. 3, lane D). Column fractions were assayed by SDS-PAGE, silver staining and Western blot analysis with anti-TER ATPase antibody.

Protein Digestion, Isolation of Peptide Fragments, and Partial Amino Acid Sequence Analysis

For amino acid sequence analysis, the proteins separated by 8% SDS-PAGE were electroblotted to PVDF paper according to Towbin et al. (1979). Both native and SDS-PAGE were pre-electrophoresed with 0.1 mM diiodoglycine to block free radicals. Regions containing the ATPase on PVDF paper were cut into small pieces and placed in 10 mg/ml CNBr (dissolved in 70% formic acid) overnight in the dark at room temperature. The digested solutions were then collected and dried under nitrogen. The residues were rewetted with water and dried twice to reduce acid residues. The dried peptide fragments were resuspended in sample buffer and the pH of the samples was adjusted to neutrality with 1 M Tris. The digested fragments were separated on a discontinuous 16% acrylamide gel as described by Schlüger and Von Jagow (1987). Gels were then blotted to Problott PVDF paper for sequencing. The analysis of the amino acid sequence was with an automated pulsed–liquid peptide sequencer.

Peptide Antibody

Peptide synthesis and antibody production were by Immuno-Dynamics Inc. (La Jolla, CA). The undetermined amino acids were substituted by the corresponding amino acids at the homologous valosin-containing protein. Briefly, the peptides were purified over a C4 reversed phase column, and injected intradermally with complete Freund's adjuvant. The peptide conjugated to keyhole limpet hemocyanin (KLH) was used for immunization of rabbits, 2.5 mg of peptide–KLH per rabbit. The rabbits were boosted intramuscularly after 3 and 6 wk with incomplete Freund's adjuvant. Test bleeds were taken after 3, 4, 6, and 9 wk.

Peptide-linked Affinity Purification of TER ATPase Antibody

The TER-ATPase–derived peptide dissolved in 50 mM Tris, 5 mM
Proteins were separated on 8% SDS-PAGE and electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) at a 1:15,000-fold dilution in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) supplemented with 5% BSA and then incubated with primary antibody. The blot subsequently was incubated with alkaline phosphatase-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) at a 1:15,000-fold dilution in TBST for 2 h at 4°C. The reactive bands were visualized by color development with nitroblue tetrazolium chloride and 5-bromo-4 chloro-3 indolyl phosphate (Promega Biotec, Madison, WI).

**Cloning of the Rat Liver TER ATPase cDNA**

A rat liver cDNA library primed with oligo (dT) and packaged into a UNI-ZAP XR vector (Stratagene, La Jolla, CA) was screened with affinity-purified TER ATPase antibody. Briefly, dry nitrocellulose filters (Schleicher & Schuell, Inc.) were placed onto the top agarose containing 5 mM IPTG for 4 min. The filters were removed and rinsed in TBST and subsequently blocked by incubating with TBST plus 3% BSA for 1 to 2 h at room temperature. The membranes were then incubated with the affinity-purified TER ATPase antisera at 4°C overnight. Filters were washed with TBST and incubated with alkaline phosphatase-conjugated secondary antibody (1:10,000 dilution) (Jackson Immunoresearch Laboratories, Inc.). Positive clones were visualized by reacting the filter with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Promega Biotec) until positive clones of desired intensity were obtained. Positive phage clones were purified by three additional rounds of screening as described above. Excision of the plBluescript plasmid containing the cloned cDNA from the UNI-ZAP vector was by coinfection with R408 helper phage, according to the manufacturer's instructions. The Sequenase version 2.0 DNA sequencing kit from United States Biochemical Corp. (Cleveland, OH) was used to either partially or fully sequence the cDNA clones with universal primers or primers generated from determined sequences.

Alternatively, the rat liver Xgt 11 cDNA library, primed with a mixture of oligo (dT) and random hexamers (Clontech, Palo Alto, CA), was screened using a 30-mer oligonucleotide probe derived from the determined NH2-terminal sequence of the clone obtained by immunoscreening and synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA). The probe was labeled with [γ-32P]ATP (ICN Biochemical, Irvine, CA) to a specific activity of 10^10 cpm/μg using T4 DNA kinase according to standard procedure (Sambrook et al., 1989). Nitrocellulose filters were prehybridized in 6× SSC, 5× Denhardt's solution, 0.25% SDS, 100 μg/ml of denatured salmon sperm DNA, and 0.05% sodium pyrophosphate at 35°C for 4 h (compositions of SSC and Denhardt's solution were according to Sambrook et al., 1989). The filters were then hybridized in the same solution except Denhardt's solution was used at 1×. The concentration of [32P]-labeled probe in the hybridization solution was 10 μg/ml. Filters were washed at room temperature with 6× SSC plus 0.05% sodium pyrophosphate for 20 min followed by three additional washes for 15 min each at 55-65°C. The filters were examined by autoradiography for positive hybridization signals, and the corresponding plaques were picked. Positive phage clones were plaque purified and DNA was prepared by the plate lysis method (Sambrook et al., 1989). Phage DNA was digested with restriction enzyme EcoRI, and resulting fragments were subcloned into pUC19. Recombinant plasmid DNA, isolated by Wizard miniPreps (Promega Biotec) and associated with alkali, was subjected to DNA sequencing using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) and protocol and [α-35S]dATP (Amersham Corp.).

**Electron Microscopy of Thin Sections**

Membrane fractions pelleted onto nitrocellulose strips were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2, at room temperature followed by post-fixation in 1% osmium tetroxide in the same buffer. Dehydration was through an acetone series with embedment in Epon (Luft, 1961). Thin sections were examined and photographed with a Phillips EM 200 electron microscope.

**Negative Staining for Electron Microscopy**

Fractions from the DEAE-52 column, highly enriched in TER ATPase were dialyzed against water for 1 h at room temperature and mixed on an electron microscope grid with an equal volume of 2% aqueous phosphotungstic acid, pH 7.2. Preparations were examined and photographed with a Phillips EM 200 electron microscope. Rotational analysis was according to Markham et al. (1963).

**Phosphorylation with [γ-32P]ATP**

Phosphorylation was at 0°C for 6 s in a buffer containing 50 mM Tris-MES, 2 mM MgCl2, 10 mM [γ-32P]ATP, 0.3 mg/ml of C2Es, 1.0 mM Na2ATP, 1.0 mM sodium molybdate, pH 7.0. The reaction was initiated by addition of 10 nM [γ-32P]ATP (5,000 Ci/mmol) and stopped by addition of an equal volume of cold sample buffer containing 62.5 mM Tris-HCl, 4% SDS, and 5% β-mercaptoethanol, pH 6.8, and immersion in boiling water for 5 min. The samples were divided into two aliquots and both were resolved by SDS-PAGE (8%). One part was stained with Coomassie brilliant blue 250. The other part was exposed to X-ray film and phosphorylated proteins were detected by autoradiography.

**Transition Vesicle Formation**

Transition vesicle formation and isolation by free flow electrophoresis was according to Paulik et al. (1988). The isolated TER was resuspended at a final protein concentration of 1 mg/ml in 30 mM Hepes (pH 7.0) containing 2.5 mM magnesium acetate and 30 mM KCl (Hepes/Mg(OAc)2/KCl). To 1 ml of the resuspended TER was added 100 μl of preimmune serum, 200 μl of rat liver cytosol prepared according to Paulik et al. (1988) and 900 μl of Hepes/Mg(OAc)2/KCl with incubation for 30 min on ice with shaking. A parallel incubation containing 1 mg/ml of resuspended TER was preincubated with 100 μl anti-TER ATPase serum together with the same amount of cytosol and Hepes/Mg(OAc)2/KCl as above. At the end of the preincubation, 1 ml of ATP/ATP regenerating system (Balch and Keller, 1986) was added to both preincubation mixtures with further incubation at 37°C for 1 h with shaking. The incubated reaction mixtures were subjected to preparative free-flow electrophoresis as described (Paulik et al., 1988). The shoulder regions corresponding to the fractions were concentrated by centrifugation at 85,000 g for 30 min onto nitrocellulose. Portions of the nitrocellulose were prepared for electron microscopy. The remainder was used for determination of total protein by the bicinchoninic acid procedure (Smith et al., 1985).

**Reconstituted Cell-free Membrane Transfer**

Preparation of the [3H]acetate-labeled TER donor and unlabeled Golgi apparatus acceptor were according to Paulik et al. (1988) and Moreau et al. (1991), respectively. The reconstituted membrane transfer system was as described (Moreau et al., 1991). Briefly, the reconstituted cell-free system (1 ml final volume) contained 250 μl of an ATP-regenerating system (30 mM Hepes, 2.5 mM Mg(OAc)2, 30 mM KCl, 80 μM ATP, 300 μM UTP, 2 mM creatine phosphate, 10 μM of creatine phosphokinase [rabbit muscle/ml] and 250 μl of Hepes/Mg(OAc)2/KCl, final pH 7.0). All solutions were maintained at 4°C until initiation of the reaction by addition of ATP and the nitrocellulose strips, three strips per determination, and transfer to 37°C. At the end of the incubation, the strips were rinsed four times with Hepes/Mg(OAc)2/KCl, placed individually in scintillant counting vials and radioactivity was determined after addition of 5 ml of scintillant (ACS; Amersham Corp.). When antisera or preimmune sera were used in the reconstituted assay, donor membrane (TER fraction) was first incubated with 70 μl of anti-TER ATPase sera (about 1.4 mg protein) or preimmune sera (+5.4 mg protein) on ice for 30 min prior to their introduction into the transfer medium.

**Results**

**Ion and Inhibitor Sensitivity of the TER ATPase Activity**

The activity of TER ATPase was maximal at pH 8.3 (Fig.
The background of nonspecific hydrolysis of ATP was 1.5 μmol/mg/h. Optimum activity was observed with an ATP/Mg²⁺ ratio of 1:1 (Fig. 1 b). The Km for ATP was 0.55 mM as determined from a double reciprocal plot (Fig. 1 c). The activity was not affected by monovalent cations but 2 mM Ca²⁺ could replace Mg²⁺ (Table I). In the presence of 2 mM Mg²⁺, 10 mM Ca²⁺ inhibited the activity about 40% suggesting competition between these two cations.

The TER ATPase activity was not inhibited by classical ATPase inhibitors (Table I). Vanadate and KNO₃ had little effect on the TER ATPase. At 50 μM vanadate, the TER ATPase activity was 102% of the control. The activity was 92% of the control in the presence of 50 mM KNO₃. Azide, a potent inhibitor of mitochondrial ATPase, inhibited TER ATPase at 100-fold higher concentrations than was required for inhibition of the mitochondrial ATPase. At 50 μM ouabain or 10 μg/ml of oligomycin, the activity was about 80% of the control. The sulfhydryl reagent N-ethylmaleimide inhibited the activity by 40% at 1 mM. Adenosine-5′-O(3-thiophosphate) and cobalt chloride, inhibitors of transition vesicle formation in situ (Hammerschlag et al., 1976; Morrè et al., 1989; Melancon et al., 1987), also inhibited the TER ATPase activity. At 2 mM adenosine-5′-O(3-thiophosphate) and 10 mM CoCl₂, respectively, the activity was 65 and 93% inhibited.

**ATPase Activity Staining on Native PAGE and Determination of Native Molecular Mass by Gel Filtration**

The solubilized TER was centrifuged at 100,000 g, the supernatant was collected and fractionated on a DEAE-52 anion-exchange column equilibrated with SB at 4°C (Fig. 2). The absorbed protein was eluted by a linear gradient of 0 to 0.6 M NaCl. Pooled fractions indicated by arrows were subjected to enzyme activity staining on 6% native gels (Fig. 3).
A single band had a molecular weight of 100 kD by silver staining (Fig. 6). Because activity staining was carried out at 37°C for 4–8 h before the ATPase band was cut out, the several faint bands under the 100-kD protein may be proteolytic fragments of the 100-kD protein. These results, taken together with those of Fig. 5, provide evidence that the TER ATPase is a homo-oligomer composed of six 100-kD subunits.

A 100- and 55-kD Protein from Rat Liver Microsomes Were Phosphorylated by ATP

Addition of [γ-32P]ATP to isolated endoplasmic reticulum in the presence of C2E8 led to the hydrolysis and formation of phosphorylated intermediates which were detected on autoradiograms as γ-32P-labeled proteins (Fig. 7, A and B).

TER ATPase Is a Hexameric Complex Consisting of Six Single Subunits of M 100 kD

To identify the subunit of the ATPase, the ATPase band was cut from the activity stained PAGE and reelectrophoresed (8% SDS-PAGE). A single band had a molecular weight of 100 kD by silver staining (Fig. 6). Because activity staining was carried out at 37°C for 4–8 h before the ATPase band was cut out, the several faint bands under the 100-kD protein may be proteolytic fragments of the 100-kD protein. These results, taken together with those of Fig. 5, provide evidence that the TER ATPase is a homo-oligomer composed of six 100-kD subunits.

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Addition of [γ-32P]ATP to isolated endoplasmic reticulum in the presence of C2E8 led to the hydrolysis and formation of phosphorylated intermediates which were detected on autoradiograms as γ-32P-labeled proteins (Fig. 7, A and B).
Figure 5. Molecular weight determination of TER ATPase oligomer complex by HPLC gel filtration chromatography. Blue dextran was first applied to the column to determine the void volume ($V_0$). The elution volume ($V_e$) of each standard (100 μg) was determined by measuring the volume of effluent collected from the point of sample application to the center of the effluent peak. (1) Thyroglobulin (669 kD); (2) Apoferritin (443 kD); (3) β-Amylase (200 kD); (4) Alcohol dehydrogenase (150 kD); (5) Albumin, bovine serum (66 kD). Fraction 2 from the DEAE-52 column (50 μg) was applied to the column. The eluted TER ATPase was identified by silver staining and Western blot analysis with anti-TER ATPase antisera.

When ATPase, partially purified by P-11 fibrous cation exchange chromatography, was phosphorylated with [γ-32P] ATP, two bands with molecular weight of 100 and 55 kD were labeled with 32P (Fig. 7 A, lane 1). The 100-kD constituent also appeared in the microsome fraction but disappeared from the unsolubilized fraction when microsomes were extracted with C6E12 (Fig. 7 A, lane 3). The 55-kD constituent appeared in both the microsome and the unsolubilized fractions (Fig. 7 A, lane 2). The band underneath the 100-kD protein in lane 2 of Fig. 7 A was not consistently observed and may be a degradation product of the 100-kD protein. Vanadate (100 μM) did not inhibit the phosphorylation of the 100- and 55-kD proteins but p-fluorosulfonylbenzoyl adenosine (5'-FSBA), an ATP analog, inhibited the phosphorylation of both.

Figure 6. Analysis of TER ATPase by 8% SDS-PAGE. The ATPase band was cut from the activity gel, re-electrophoresed on a 8% SDS-PAGE, and stained with silver. A single band of a ca. molecular weight of 100 kD was found (right lane). The lane on the left contains high molecular weight standards.

Figure 7. Phosphorylation (A) and Coomassie blue staining (B) of a 100-kD protein from the microsome fraction. (Lane 1) Microsomes separated by a P-11 fibrous cation exchange column. The fraction containing the ATPase activity was eluted by a step NaCl gradient and used for phosphorylation. (Lane 2) Microsome fraction from rat liver. (Lane 3) Unsolubilized proteins after solubilization.

A 19-Amino Acid Sequence Obtained from One Peptide of the 100-kD Protein Digested by Cyanogen Bromide

With the 100-kD protein blotted on PVDF membrane after SDS-PAGE, we attempted to obtain sequence directly. No sequence was obtained indicating the possibility of an NH2-terminal block. Cyanogen bromide was used to digest the 100-kD polypeptide for determination of internal sequences. A sequence was obtained from one isolated peptide (Fig. 8). Methionine was added by inference. The six residues within this sequence length that could not be assigned unambiguously were indicated by an X. A computer search with this sequence using FASTA with the Swiss Prot database (Genetics Computer Group, Madison, WI, 1991) showed an 84% identity at positions 160-184 with porcine valosin-containing protein (VCP) of unknown function (Fig. 8) (Koller and Brownstein, 1987). Also from the Swiss Prot database, the TER ATPase-derived sequence shared 64% identity with a yeast cell division control protein CDC48p, at positions 169-193 (Fig. 8) (Fröhlich et al., 1991). CDC48p shared 70% identity to VCP. Antibody to CDC48p detected a 100-kD protein from a rat liver microsome fraction.

Antibody to the First of the 19-Amino Acids Specifically Recognized a 100-kD Protein from TER

To obtain antisera to TER ATPase, a peptide from the TER ATPase-derived sequence as underlined in Fig. 8 was synthesized with an NH2-terminal cysteine. The peptide conjugated to a protein carrier, keyhole limpet hemocyanin (KLH), was used for immunization of rabbits. The undetermined amino acids were substituted by the corresponding amino acids in the homolog protein VCP. Western blot analyses (Fig. 9 A, lane 1) demonstrated that the anti-ATPase peptide antibody recognized a major band of 100 kD from TER while preimmune sera did not (Fig. 9 A, lane 2). Two slower migrating faint bands of 200 and 300 kD also were recognized by the antisera. The 200- and the 300-kD species may represent residual dimers and trimers, respectively, of the 100-kD TER ATPase. The 100-kD constituent also was recognized by affinity-purified anti-TER ATPase antibody.
Figure 8. Amino acid sequence comparison of the TER ATPase-derived peptide and the corresponding region of VCP and CDC48p proteins. Amino acids not assigned were denoted by an X. The sequence underlined was used for generation of anti-synthetic peptide antiserum. The NH2-terminal methionine was added by inference since the peptide sequence was derived by cyanogen bromide cleavage of the purified protein.

In immunoprecipitation experiments, the anti-TER ATPase did not efficiently immunodeplete TER ATPase nor inhibit ATPase activity (10 μl of antiserum/500 μl reaction inhibited ≈15%). However, the anti-TER ATPase did recognize the native oligomeric TER ATPase when partially (Fig. 9, lane 3). Lane 4 in Fig. 9 was silver staining of 4 μg of TER proteins. Numerous proteins were observed, yet the anti-TER ATPase recognized specifically the 100-kD component demonstrating a high degree of monospecificity for the antiserum.

Figure 9. Immunological detection of the 100-kD ATPase from TER. (A) By Western blot analysis with anti-synthetic peptide antiserum (lane 1), preimmune sera (lane 2) and affinity-purified TER ATPase antibody (lane 3). Lanes 4–3 contained 30 μg of TER protein. Lane 4 was silver staining of 4 μg of TER protein. (B) Immunoblot of fraction 2 from DEAE-52 separated on non-denaturing PAGE using antibodies to the TER ATPase 100-kD subunit. The arrow denotes the position of the TER ATPase oligomeric complex.

Figure 10. Nucleotide and deduced amino acid sequence of rat liver TER ATPase cDNA. The nucleotide sequence of the combined 6B and clone IB cDNAs is shown. Amino acid differences between rat liver TER ATPase and murine VCP are indicated in bold and underlined. The 30-mer oligonucleotide used for screening the Agt 11 rat liver cDNA library is in bold and underlined. The underlined nucleotide sequence is the peptide sequence obtained from the purified protein. The adenylation signal, AATAAA is underlined and in bold. These sequences were amplified using the polymerase chain reaction and sequenced.

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Isolation of cDNA Clones Encoding Rat Liver TER ATPase

Three independent clones by immunoscreening were obtained from $\approx 5 \times 10^6$ recombinants: clone 6B (2.5 kb), clone 17A (1.2 kb), and clone 20A (1.2 kb). Clone 17A and clone 20A were identical and were contained in clone 6B. The longest clone, clone 6B, 2,560 bp, was sequenced in both strands. The sequence of clone 6B contained an open reading frame of 2,560-bp flanked by a 3'-untranslated region of about 214 bp. A putative polyadenylation signal, AATAAA (Proudfoot et al., 1976), was present 182-bases downstream (Fig. 10) from the stop codon. Since no 5'-untranslated region and no translation initiation consensus sequence as defined by Kozak (1989) were found, clone 6B was still missing the NH$_2$-terminal sequence. To identify cDNAs containing the NH$_2$-terminal coding sequence of TER ATPase that was missing from clone 6B, a Xgtll rat liver cDNA primed with a mixture of oligo (dT) and random hexamers was screened using a 30-mer oligonucleotide probe from the 5' end of clone 6B (nucleotide 336-365, see Fig. 10). A total of $5 \times 10^6$ independent recombinants were plated and eight independent clones were isolated. Three clones (1B, 8A, and 5A) contained a 5'-untranslated region of 303 bp and a portion of the coding region that overlapped with clone 6B. The methionine codon in position 1 was in a favorable context for initiation of translation since it was surrounded by a G at positions -3 and +4 (Kozak, 1989). In addition, the translation initiation site was preceded by an in-frame stop codon at nucleotide -48. The protein structure deduced from the cDNA was 806 amino acids in length of 89.2 kD.

Sequence Analysis

The deduced amino acid sequence from the open reading frame contained the peptide sequence used for production of anti-TER ATPase antibody (underlined in Fig. 10). Positions 249, 252, and 261 of this deduced amino acid sequence were different from the amino acid sequence in Fig. 8. The three amino acid differences were Thr (position 249) instead of Leu (cycle 11), Ser (position 252) instead of Asn (cycle 14), and Thr (position 261) instead of Ile (cycle 23). Sequencing was at high sensitivity due to limited material. At the sites where divergence was noted, we are unable to rule out the possibility the alternative residues may have been present. The predicted amino acids did appear in the respective cycles and the quantities of Ser and Thr are frequently much less than would be expected on the basis of their relative abundance.

Computer analysis of the amino acid sequence deduced from the cloned gene revealed a 99% identity at the amino acid level and 96% identity at nucleotide level with murine valosin-containing protein. The two amino acid differences were at residue 206 (Ile instead of Val) and at residue 794 (Asn instead of Ser) (Fig. 10). Also, TER ATPase shared 99% identity with porcine VCP. Amino acid differences were at residue 95 (Arg instead of His) and 794 (Asn instead of Ser). While the cDNA of TER ATPase, murine VCP and porcine VCP all exhibited a 5'-untranslated region with similar sequence, that of the TER ATPase was longer. The 3'-untranslated sequence was shorter than both murine VCP and porcine and showed less homology than the 5'-untranslated region and the coding regions.

Figure 11. Electron microscopy of negatively stained purified TER ATPase (fraction 2 from DEAE) (A) consisting of single particles and particle aggregates and subsequent rotational analysis of single unaggregated particles (B-M). (A) Survey view to show the overall appearance of the preparations. The particles, after detergent solubilization, release from the membranes and purification in detergent, tended to form aggregates in aqueous solution. The presence of aggregates accounts almost entirely for the heterogeneous appearance of the preparations. However, even within aggregates, the ringlike appearance with a central cavity can be seen. (B-M) Rotational analysis of unaggregated single particles revealed structures having sixfold symmetry. (B, F, and J, n = 0, C, G, and K, n = 5, D, H, and L, n = 6, E, I, and M, n = 7). Bars: (A) 0.06 μm; (B-M) 0.01 μm.
Negative Staining Electron Microscopy of the Highly Purified TER ATPase

When partially purified TER ATPase, e.g., fraction 2 from DEAE-52 (Fig. 4 B, lane 1), or highly purified TER ATPase, e.g., fraction 1 from gel filtration (Fig. 4 B, lane 2) were negatively stained with phosphotungstic acid, the fractions consisted predominantly of aggregates of particles with a ring-like structure. Individual particles not involved in aggregates had a diameter of about 10 nm and a central cavity (Fig. 11 A). Rotational analysis of such particles revealed a sixfold symmetry (Fig. 11, B–M). The latter, taken together with information from gel filtration would indicate that the TER ATPase was a hexamer consisting of six subunits of \(~100 \text{kD}\).

Anti-ATPase Peptide Antibody Reduced Transition Vesicle Formation from Rat Liver TER as Well as Cell-free Transfer to Golgi Apparatus Acceptor

When TER from rat liver was incubated with ATP and cytosol, the trailing shoulder of the least electrophoretically mobile portion of the separation by free-flow electrophoresis contained the transition vesicles as demonstrated by electron microscopy (Paulik et al., 1988). The anti-TER ATPase inhibited by 75% this ATP-dependent formation of transition vesicles (Table II). Inhibition by Fab fragments prepared as described by Harlow and Lane (1988) was similar. Preimmune sera or antisera to a calreticulin-like protein isolated from rat endoplasmic reticulum or to rat CTP: choline phosphate cytidylyltransferase, a protein located predominantly in ER of rat liver (Jelsema and Moore, 1978) (Table II), or antisera to a p38\(^\text{cis-Golgi apparatus cisterna}\) of rat liver (Brightman et al., 1993) did not inhibit.

Transition vesicles were quantitated by total protein (Fig. 12) and analyzed by electron microscopy (Fig. 13). Based on total protein, the transition vesicle-enriched fraction represented \(\sim5\%\) of the total TER (Table II). When treated with pre-immune serum, the amounts of transition vesicles were essentially unchanged. However, anti-TER ATPase, either crude or affinity purified, reduced numbers of transition vesicles nearly to base-line levels of about 2% measured in the absence of ATP (Table II).

To examine the effect of anti-ATPase antibody on the cell-free transfer process, \(^3\text{H}\)-labeled acetate-labeled TER was preincubated with anti-ATPase and transfer of radiolabeled material from ER to Golgi apparatus was determined. Transfer was initiated by the addition of ATP plus an ATP-regenerating system. ATPase antisera inhibited the transfer up to 90% over 15 min as compared to preimmune sera (Table III). The transfers represented several hundred cpm per \(1 \text{cm}^2\) strip and, with the addition of multiple strips, transfers of 3 to 5% of the starting radioactivity were achieved. These total transfers corresponded approximately to the total ATP-dependent production of transition vesicles by these particular TER preparations (Table III).

Two Antisera Directed to Motif A and B Regions of Protein VCP Recognize the 100-kD Protein from the TER ATPase

Two synthetic peptide antisera directed to the conserved ATP binding sequences of motif A (GILLYGPGTGTKTL) and motif B (ESNLRKAFFEAEKN) of VCP (Koller et al.,

**Table II.**

| Additions                        | Transition vesicles, percent of total TER |
|---------------------------------|------------------------------------------|
| Complete                        | 4.6 ± 1.1                                 |
| + Preimmune sera                | 4.1 ± 1.1                                 |
| + Affinity-purified anti-ATPase  | 2.6 ± 0.4                                 |
| + Anti-ATPase Fab                | 2.7 ± 1.1                                 |
| + Anti-calreticulin-like protein | 4.4                                       |
| + Anti-CTP: choline phosphate cytidylyltransferase | 5.0                                      |
| − ATP + hexokinase              | 2.0 ± 0.4                                 |

Inhibition by affinity purified TER ATPase antibodies of transition vesicle formation by part-rough, part-smooth TER from rat liver compared to preimmune and other ER-specific antisera. The complete system contained both ATP and cytosol. Transition vesicles were separated from the bulk transitional endoplasmic reticulum by preparative free-flow electrophoresis.

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Table III. Cell-free Transfer (Rat Liver) Over 15 min Was Inhibited by the ATPase Antisera

| Treatment          | Transfer (15 min) (%)/strip |
|--------------------|----------------------------|
| No addition + ATP  | 0.42 ± 0.07                |
| − ATP              | 0.22 ± 0.035               |
| + Preimmune sera + ATP | 0.44 ± 0.04            |
| − ATP              | 0.13 ± 0.04                |
| Antisera + ATP     | 0.04 ± 0.02                |
| − ATP              | 0.08 ± 0.08                |

Experiments were as described in Materials and Methods. + ATP, ATP plus an ATP-regenerating system (Balch and Keller, 1986); − ATP, 30 U hexokinase plus 30 μmol glucose only. Values were corrected for a t = 0 blank equivalent to ca. 0.1% transfer (acceptor and ATP-dependent transfer).

Discussion

Vesicle budding, both in vivo and in vitro, is dependent upon ATP (Balch and Keller, 1986; Morré et al., 1986; Beckers et al., 1990). The present study suggests that this ATP requirement is somehow mediated through the ATPase we describe here. One line of evidence is that antisera raised against the 100-kD ATPase monomer blocks cell-free transfer in a system derived from rat liver. Additionally, the presence of the antibody prevents the ATP-induced cell-free formation of transition vesicles. This was shown from experiments in which transition vesicles were induced to form by the addition of ATP in the presence and absence of ATPase antisera. In the presence of antisera, the numbers of ATP-induced transition vesicles were reduced considerably.

The unique characteristic of the transitional endoplasmic reticulum ATPase was first indicated from inhibitor studies where the transitional endoplasmic reticulum ATPase responded to inhibitors differently from ATPases found in other parts of the cell. TER ATPase did not respond to classical inhibitors of mitochondrial, vacuolar or plasma membrane ATPases (Nelson and Taiz, 1989), but was NEM sensitive. The ATPase was inhibited neither by ouabain nor vanadate to distinguish it from the classical plasma mem-

Figure 13. Electron micrographs of the isolated transition vesicle fractions pelleted on nitrocellulose and sectioned at right angles to the nitrocellulose. The arrows indicate the position of the underlying nitrocellulose. The number of small (50-70 nm) vesicles representative of transition vesicles was reduced in proportion to the protein content of the fractions upon incubation with the anti-TER ATPase sera. (A) Preimmune sera; (B) anti-TER ATPase sera. Bar, 0.5 μm.

Figure 14. Immunochemical specificity of anti-motif A and anti-motif B antisera. (Lane 1) Anti-TER ATPase Western blot of partially purified TER ATPase (fraction 2 from DEAE-52, Fig. 2). (Lane 2) Anti-motif A Western blot of the same sample as in lane 1. (Lane 3) Anti-motif B Western blot of the same sample as in lane 1. The arrow denotes the 100-kD protein recognized by all three antisera.
brane ATPases. Nor was it inhibited by nitrate as were the vacuolar ATPases. Much more oligomycin and azide were required to inhibit the endoplasmic reticulum ATPase than were normally required, for example, to inhibit the $f_0/f_1$ ATPase of mitochondria. Another characteristic of the transitional endoplasmic reticulum ATPase was its extreme sensitivity to inhibition by cobalt ions. Cobalt ions have been used as inhibitors of vesicle formation in axonal transport (Hammerschlag et al., 1976) and to inhibit formation of transition vesicles in cultured cells (Morré et al., 1989). Using size exclusion chromatography, we have estimated the relative molecular weight of the native TER ATPase to be ca. 600 kD. Enzyme activity staining and subsequent re-electrophoresis on SDS-PAGE revealed a single polypeptide of 100 kD.

Two lines of evidence connect the ATPase with a 100-kD polypeptide. The first is that the 100-kD peptide quantitatively copurified with ATPase activity through chromatographic separations based on ion exchange and size. The second is that when the band containing the enzyme activity was cut out from the native gel and reelectrophoresed on SDS-PAGE, only a single protein of 100 kD was revealed. Taken together, these data demonstrate that the 100-kD constituent is the polypeptide responsible for ATPase activity but that the native protein consists of a homo-oligomeric complex of 100-kD subunits.

Electron microscopy revealed that TER ATPase was a ring-shaped particle with a central cavity superficially resembling the homo-oligomeric ring-shaped ATPase particles from *Xenopus* oocytes described by Peters et al. (1990). While our preparations did not permit the high degree of structural resolution provided by the cytosolic hexameric ATPase, rotational analysis of single particles stripped from TER did show characteristics consistent with a sixfold symmetry. The isolated particles were detergent solubilized from TER and tended to aggregate in aqueous solution. The formation of aggregate structures by the detergent-solubilized particles necessitated that rotational analyses be carried out only on occasional particles (ca. <10%) not present in aggregates. The survey preparations may appear non-homogeneous as a result of the presence of such aggregates rather than as a result of particles of heterogeneous origins.

Based on the biochemical and structural similarity between TER ATPase and porcine p97, the TER ATPase appears to be the rat equivalent of porcine VCP. This is confirmed from the cloning and sequencing of cDNA of the TER ATPase. Although the function of porcine VCP remains unknown, amino acid sequence analysis of VCP has shown that VCP is a member of a family of proteins which shares one or two copies of a highly conserved domain of about 200–amino acid residues, including a consensus motif for an ATP-binding site (Fröhlich et al., 1991; Peters et al., 1990; Erdmann et al., 1991). Some members have ATPase activity (Erdmann et al., 1991).

A VCP analogue, p97-ATPase, has been isolated from *Xenopus laevis* oocytes (Peters et al., 1990). It displayed an ATPase activity optimum at pH 9 which depended on the presence of Mg$^{2+}$. The native form of the p97-ATPase had a molecular mass of 612 kD and by analytical ultracentrifugation acted as a homo-oligomer complex composed of six single subunits of 97 kD. Electron microscopy of negatively-stained specimens showed that the p97-ATPase complex consisted of ring-shaped particles of 12.5-nm diam with a central cavity (Peters et al., 1992).

Murine VCP was identified recently as an early substrate for tyrosine phosphorylation following T cell receptor activation (Egerton et al., 1992). VCP, its analog p97-ATPase and CDC48p displayed high homology to the N-ethylmaleimide-sensitive (NSF) protein from Chinese hamster ovary cells and Sec18p from yeast, proteins required for protein transport between ER and Golgi apparatus and intra Golgi cisternae (Wilson et al., 1989). It was proposed that all these proteins belong to the same, novel family of ATPases (Erdmann et al., 1991). Two members of this family, Sec18p and its homologous mammalian NSF protein are essential for transport of proteins from the endoplasmic reticulum to the Golgi apparatus and intra-Golgi transport (Clary et al., 1990; Kaiser and Schekman, 1990; Wilson et al., 1989; Weidmann et al., 1989). NSF requires additional cytosolic proteins termed soluble NSF proteins (SNAPS) to associate with Golgi apparatus membranes (Weidmann et al., 1989) and ATP hydrolysis to disassemble from its NSF/membrane complex (Beckers et al., 1989). Recent studies have shown that ATP hydrolysis by NSF is required for fusion. Mutation of either ATP site eliminated the fusion activity of NSF and ATPase activity (Tagaya et al., 1993).

Through a combination of genetic studies in the yeast *Saccharomyces cerevisiae* and biochemical in vitro cell-free assays, a set of interacting proteins has been identified that were required to produce functional transport vesicles from the ER membrane such as Sec12p, Sec13p/150, Sec23p, Sec24p, and Sarlp (Novick et al., 1980; Kaiser and Schekman, 1990; Nakano and Muramatsu, 1989; d'Enfert et al., 1991; Salama et al., 1993; Barlow and Schekman, 1993). Sec12p, a resident ER protein, was a GDP/GTP exchange protein and was found to promote the association of Sarlp with the ER (Barlowe and Schekman, 1993). Sec23p was shown to act as a GTPase-activating protein specific for Sarlp and formed a complex with another protein required for vesicle budding, Sec24p (Yoshihisa et al., 1993). Most recently, Sarlp, a ras-like GTP-binding protein, has been demonstrated to be required for vesicle budding from the ER in mammalian cells, but was not required for vesicle traffic between Golgi apparatus compartments (Kuge et al., 1994). Although all of the reconstitution systems, including those for vesicle budding, required energy in the form of ATP, it was not at all clear how the ATP was being used. The ATP requirement was assumed to indicate a need for the energy released by ATP hydrolysis. In this study we have shown that anti-TER ATPase antibody diminished the ATP-dependent budding process of transitional ER both from a direct measurement of transition vesicle formation and from measurements of the transport between the ER and the Golgi apparatus. These observations suggested that TER ATPase may serve as an ATPase to hydrolyze ATP for vesicle budding from the TER.

The free energy derived from ATP hydrolysis of TER ATPase may be required not only to drive the process of budding but may also modulate the affinity of the TER ATPase and the proteins it may be associated with in a manner similar to the type of function ascribed to NSF.

The findings are expected to serve as the basis for addi-
tional studies to further elucidate the role of the TER ATPase in vesicle budding. Doubtless the TER ATPase will emerge as only one of a complex of proteins that may cooperate in the ATP-dependent formation of transition vesicles from the TER. We are hopeful that cell-free systems can continue to be profitably used to probe details of the mechanism whereby the chemical energy of ATP hydrolysis is translated into the lateral membrane displacements of vesicle budding.

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