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A functional calcium-transporting ATPase encoded by chlorella viruses

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Calcium-transporting ATPases (Ca²⁺ pumps) are major players in maintaining calcium homeostasis in the cell and have been detected in all cellular organisms. Here, we report the identification of two putative Ca²⁺ pumps, M535L and C785L, encoded by chlorella viruses MT325 and AR158, respectively, and the functional characterization of M535L. Phylogenetic and sequence analyses place the viral proteins in group IIB of P-type ATPases even though they lack a typical feature of this class, a calmodulin-binding domain. A Ca²⁺ pump gene is present in 45 of 47 viruses tested and is transcribed during virus infection. Complementation analysis of the triple yeast mutant K616 confirmed that M535L transports calcium ions and, unusually for group IIB pumps, also manganese ions. *In vitro* assays show basal ATPase activity. This activity is inhibited by vanadate, but, unlike that of other Ca²⁺ pumps, is not significantly stimulated by either calcium or manganese. The enzyme forms a ³²P-phosphorylated intermediate, which is inhibited by vanadate and not stimulated by the transported substrate Ca²⁺, thus confirming the peculiar properties of this viral pump. To our knowledge this is the first report of a functional P-type Ca²⁺-transporting ATPase encoded by a virus.

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INTRODUCTION

P-type ion-transporting ATPases are essential molecules in eukaryotes and in most eubacteria and archaea. These ATP-hydrolysing enzymes are responsible for the primary transport of charged substrates, generally cations, across membranes. Typical of the P-type ATPase superfamily is the temporary conservation of ATP energy in the form of a phosphorylated enzyme intermediate (hence P-type) formed between the γ -phosphate of hydrolysed ATP and an invariant Asp-residue in a highly conserved sequence: SDKTGT[L/I/V/M][T/I/S] (Brini & Carafoli, 2009). This large family of primary transporters is divided into five major groups (I–V) on the basis of phylogenetic analyses and substrate specificity (Axelsen & Palmgren, 1998, 2001). All Ca²⁺ pumps described to date belong to group II, subgroups A and B. Type IIA Ca²⁺ pumps are localized primarily in endomembranes, have short N- and C-cytosolic termini and are not stimulated by calmodulin (CaM). They are found in eubacteria, archaea and

eukaryotes (Axelsen & Palmgren, 2001). Type IIB Ca²⁺ pumps are localized both in the plasma membrane and in endomembranes and are stimulated by CaM binding to C- or N-cytosolic domains. They only exist in eukaryotes.

This manuscript describes two Ca²⁺-ATPase IIB members encoded by chlorella viruses, a group of viruses belonging to the family *Phycodnaviridae*. Chlorella viruses are large, icosahedral, plaque-forming, dsDNA viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae. They contain an internal lipid bilayered membrane surrounded by an outer glycoprotein capsid (Van Etten, 2003; Wilson *et al.*, 2009). The chlorella viruses have genomes as large as 370 kb that contain as many as 400 protein-encoding and 16 tRNA-encoding genes. Six chlorella virus genomes have been sequenced and about 80% of the genes are common to all six viruses (Li *et al.*, 1997; Fitzgerald *et al.*, 2007a, b, c). Three of the sequenced viruses, PBCV-1, NY-2A and AR158, infect *Chlorella* NC64A; two, MT325 and FR483, infect *Chlorella* Pbi; and one, ATCV-1, infects *Chlorella* SAG 3.83 (Fitzgerald *et al.*, 2007a, b, c). Annotation of the six genomes revealed that

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two viruses, MT325 and AR158, encode genes for putative Ca^{2+} -ATPases. This manuscript describes the functional characterization of the Ca^{2+} pump encoded by virus MT325.

RESULTS

Sequence analysis

Annotation of six chlorella virus genomes revealed that two of them, MT325 and AR158, code for putative proteins that belong to the P-type superfamily subgroup IIB Ca^{2+} -ATPases. Assignment to the P-type superfamily of ATPases is based on predicted membrane topology and sequence alignment of the two viral proteins with the well-known calcium pumps ACA8 from *Arabidopsis thaliana* (Bonza *et al.*, 2000) and PMCA4b from *Homo sapiens* (James *et al.*, 1988). The two viral proteins (Fig. 1a) are predicted to have 10 transmembrane domains (TM), a small loop between TM2 and TM3 and a large loop between TM4 and TM5 (Palmgren & Axelsen, 1998; Bonza *et al.*, 2004). Sequence alignments of M535L and C785L with prototype Ca^{2+} pumps (Fig. 1b) highlight the conserved domain of the P-type superfamily, DKTGT, containing the aspartic acid residue that becomes phosphorylated during the catalytic cycle. Furthermore, M535L and C785L have several additional motifs characteristic of subgroup II (A and B) P-type ATPases. These motifs (in grey in Fig. 1b) include the PEGL sequence that plays a central role in energy transduction and the KGAPE sequence implicated in nucleotide binding (Møller, *et al.*, 1996; Palmgren & Axelsen, 1998). Finally, a feature only found in type IIB ATPases exists in the viral proteins: one conserved putative-binding site for calcium, formed by residues E³⁰² in TM4, N⁷⁰³ and D⁷⁰⁷ in TM6 (numbers refer to the M535L amino acid sequence; the residues are marked with asterisks in Fig. 1b).

M535L and C785L sequences are 64% identical and 82% similar to each other, 37 and 33% identical, 56 and 50% similar to ACA8 and PMCA4b, respectively. The cytosolic termini of the viral proteins are short (27 aa at the N terminus and 4–6 aa at the C terminus) and lack a CaM-binding domain typically located at the N terminus of plant members and at the C terminus of animal members.

Molecular phylogenetic analyses of the AR158_C785L and MT325_M535L gene products also support the relatedness of these molecules to members of the P-type IIB Ca^{2+} -transporting ATPases (Drummond *et al.*, 2008) (Fig. 1c). IIB Ca^{2+} pumps fall into two clades, one in higher plants and the other in animals (Axelsen & Palmgren, 1998; Bonza *et al.*, 2004; Marchler-Bauer *et al.*, 2009). The viral pumps clearly reside in the algal clade containing *Chlorella* and *Chlamydomonas*, more closely related to the animal than to the plant clade. Given the high similarity of the two viral proteins, further analysis was conducted only on M535L.

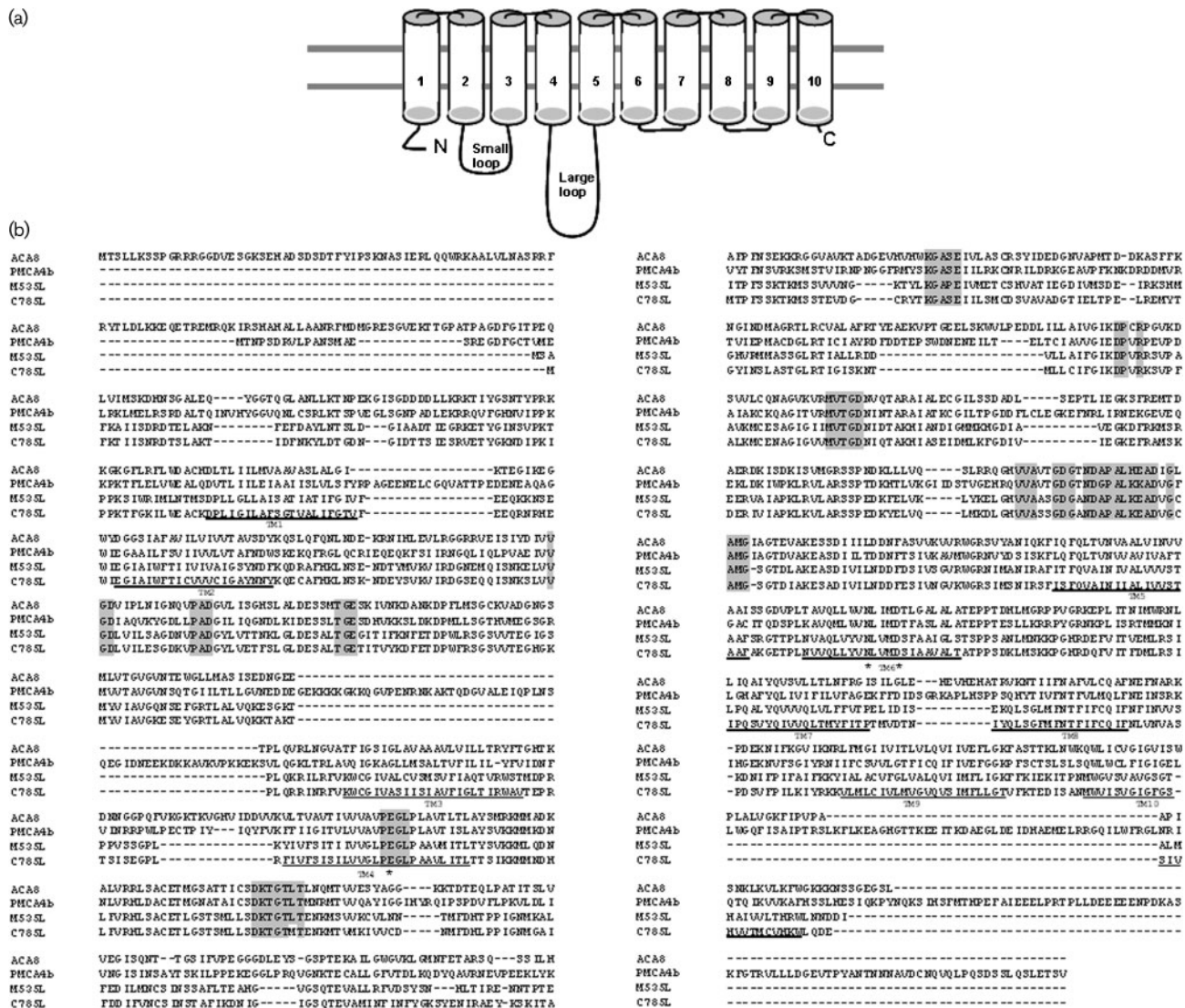
Presence of the *m535l* gene in other viruses and its expression during host infection

To determine if the Ca^{2+} pump gene is common among the chlorella viruses, genomic DNAs from 47 *Chlorella* Pbi viruses from diverse geographical regions were hybridized with an *m535l* probe (Fig. 2a). The probe hybridized strongly to 45 of 47 viruses. Virus Nw655.2 hybridized poorly with the probe and no hybridization occurred with virus Fr483. Virus Fr483 has been sequenced (Fitzgerald *et al.*, 2007a) and it lacks a Ca^{2+} -transporting ATPase homologue. The strength of the hybridization signal differs among the 45 positive viruses (Fig. 2a), suggesting there are significant nucleotide substitutions among these genes. The *m535l* probe did not hybridize to the virus MT325 host, *Chlorella* Pbi DNA.

To determine if the *m535l* gene is expressed during viral infection and at which stage, the probe was hybridized to total RNA extracted from MT325-infected cells. The probe hybridizes to a single transcript of ~3.6 kb that is an appropriate size for a 871 aa protein (Fig. 2b). Hybridization is strongest at 15 min post-infection (p.i.) and decreases slowly with time (Fig. 2b). Assuming the replication cycle of virus MT325 resembles that of the prototype chlorella virus PBCV-1 (Van Etten, 2003), *m535l* is an early gene, i.e. it is expressed prior to virus DNA synthesis. Proteomic analyses did not detect M535L or C785 in their respective virions (D. D. Dunigan and others, unpublished data). This result is consistent with the finding that *m535l* is an early gene because proteins packaged in nascent virions are usually transcribed at later stages of infection. The fact that the calcium transporter gene is present in most of the Pbi viruses and that it is transcribed during virus infection suggests the protein might serve a function in virus replication. Consequently, we tested the gene product for functional activity.

Heterologous expression of *m535l* in *Saccharomyces cerevisiae* triple mutant K616

The viral *m535l* gene was expressed in *S. cerevisiae* mutant K616 that lacks all endogenous Ca^{2+} -ATPases (Cunningham & Fink, 1994). Consequently, K616 does not grow in Ca^{2+} -depleted medium unless it is transformed with a gene encoding a fully active Ca^{2+} pump (Geisler *et al.*, 2000; Bonza *et al.*, 2004; Baekgaard *et al.*, 2006). The *m535l* gene was cloned into yeast vector pYES2-NTC, which adds an N-terminal His tag to the recombinant protein. Protein expression in high calcium, a non-selective condition, was evaluated by Coomassie staining and Western blot and compared to that of control yeast transformed with the empty vector. Coomassie staining of the proteins in the microsomal fraction reveals a strong band with the expected molecular mass of the M535L polypeptide (96.3 kDa) (data not shown). Western blot analysis with antiserum against the His-tag clearly identifies this band as the *m535l* gene product (data not shown).



Complementation of the K616 phenotype

To determine if the expressed protein was functional, we incubated K616 transformed with *m53l* in low external calcium concentrations. When the test was performed on solid medium (Fig. 3a), yeast growth occurred at calcium concentrations as low as 200 μM, a non-permissive condition for the negative control (K616 transformed with the empty vector). As a positive control, we used yeast K601, which grows in low (nM) external calcium concentrations. Even though *m53l* clearly supports K616 growth in micromolar calcium concentrations, we never observed complementation at nanomolar concentrations, a result expected for high-affinity calcium pumps (Geisler *et al.*, 2000; Kabala & Klobus, 2005). Therefore, we used a different complementation test in which yeast growth was evaluated in liquid culture that provides better aeration of the medium. Protein expression was induced in mid-exponential cultures by substituting fresh medium containing either

10 mM CaCl₂ or 10 mM EGTA; the latter medium contains nominal nanomolar concentrations of free calcium. Yeast growth was evaluated by OD₆₀₀ measurements 24 h after induction and the results plotted as the ratio of OD₆₀₀ in EGTA over CaCl₂ (Fig. 3b). The M53L protein clearly supports growth in nanomolar calcium, allowing yeast cells to grow almost as fast as in millimolar calcium (OD₆₀₀ ratio EGTA/CaCl₂=0.75 ± 0.07). In contrast, poor growth occurs in the negative control at low calcium concentrations (OD₆₀₀ ratio EGTA/CaCl₂=0.37 ± 0.02). From these experiments we conclude that M53L forms a functional high-affinity calcium transporter in *S. cerevisiae*.

Selectivity of M53L

To determine the ion selectivity of M53L, we tested the ability of the protein to rescue the K616 phenotype on solid medium supplemented with either 0.5, 1.0 or 2.0 mM Mn²⁺; at these concentrations this ion is toxic to K616 that

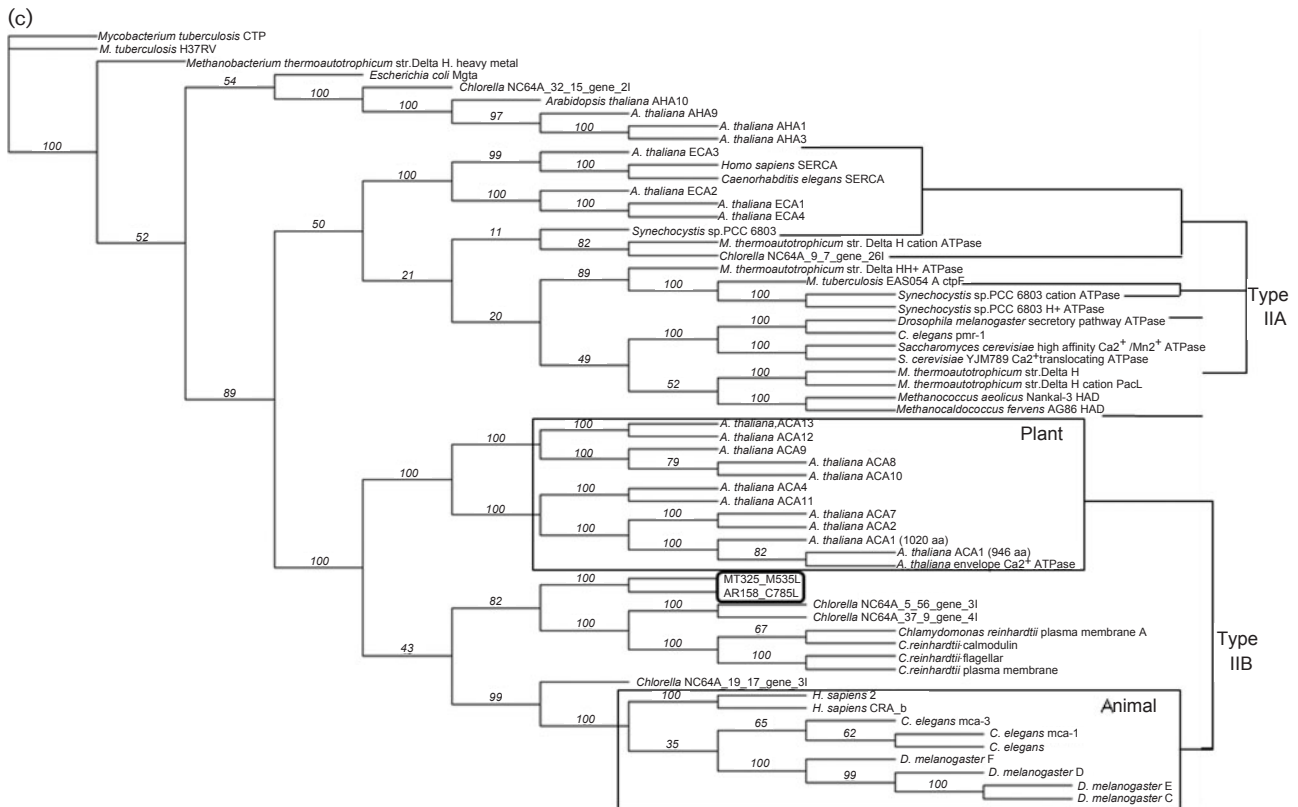


Fig. 1. Predicted membrane topology, comparative alignment and phylogeny of chlorella virus Ca^{2+} -transporting ATPases. (a) Hypothetical membrane topology of the viral Ca^{2+} pump. (b) Multiple sequence alignments performed with CLUSTAL W (1.82) of the deduced amino acid sequences of chlorella virus MT325 ORF M535L (NCBI reference no. ABT14089.1), chlorella virus AR181 ORF C785L (NCBI reference no. YP_001498866.1), ACA8 (NCBI reference no. NP_200521.3) and PMCA4b (GenBank accession no. NM_001684) from *A. thaliana* and *H. sapiens*, respectively. Conserved residues characteristic for P-type ATPases type II are highlighted in grey. Residues corresponding to the single binding site for calcium found in type IIB Ca^{2+} -ATPases are highlighted with asterisks. Bold lines indicate the ten transmembrane helices, TM1–TM10, predicted by the TMHMM program (www.cbs.dtu.dk/services/TMHMM/). (c) Maximum-likelihood tree of 60 P-type ATPase protein sequences. The phylogenetic tree was generated using the MUSCLE alignment and PHYLIP tree building programs within the Geneious Pro 4.7.5 software program. The Whelan and Goldman (WAG) amino acid substitution model was used to derive 100 bootstrap datasets (the transition/transversion ratio for DNA models and the gamma distribution parameter were estimated, proportion of invariable sites was zero and four substitution rate categories produced the illustrated unrooted tree; bootstrap values are shown).

lacks the $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump Pmr1, which removes excess Mn^{2+} from the cytoplasm (Cunningham & Fink, 1994). While K616 cells transformed with the empty vector barely grew on 1 mM Mn^{2+} and died on 2 mM Mn^{2+} , cells expressing M535L survived in Mn^{2+} concentrations as high as 2 mM and their growth was indistinguishable from the control strain K601 (Fig. 4). The finding that M535L protects K616 from excess Mn^{2+} suggests that the viral protein transports manganese in addition to calcium. This behaviour differs from other IIB type Ca^{2+} pumps and resembles IIA Ca^{2+} pumps ECA1 or ECA3 from *A. thaliana* or LCA1 from tomato that confer tolerance to Mn^{2+} (Wu *et al.*, 2002; Mills *et al.*, 2008; Johnson *et al.*, 2009). However, in contrast to ECA1 and ECA3, M535L does not reverse the toxic effect of Zn^{2+} (results not shown).

ATPase activity

ATPase activity was assayed by monitoring Ca^{2+} -dependent ATP hydrolysis in yeast membrane fractions (Bonza *et al.*, 2004). We initially measured M535L Ca^{2+} -dependent ATPase activity in crude microsomal membranes but, under these conditions, activity was barely detectable. The low Ca^{2+} -dependent ATPase activity of the viral transporter did not increase by systematically modifying the assay conditions including: substrate concentrations, pH and addition of CaM (results not shown). To improve the specific activity of M535L, microsomes from K616 cells expressing M535L were fractionated on a sucrose density gradient to separate the viral protein from endogenous ATPases, such as the yeast plasma membrane proton pump. A strong 95 kDa band

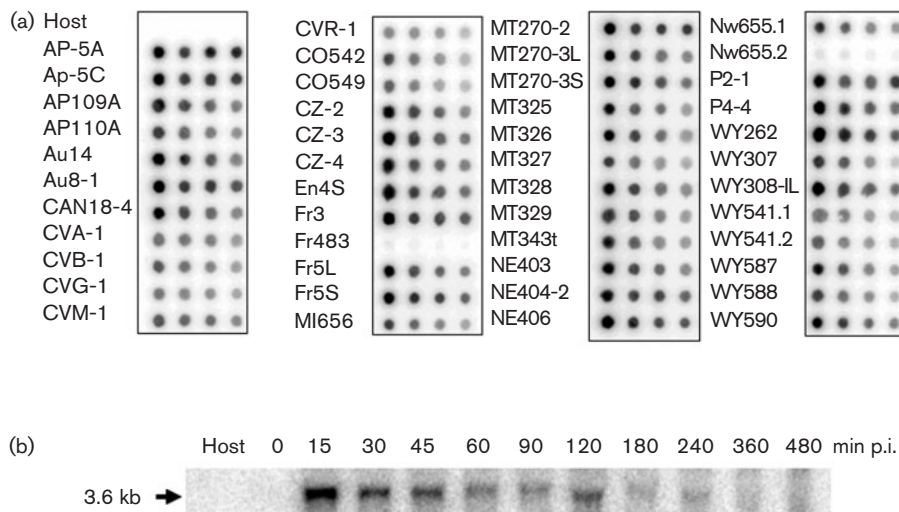


Fig. 2. Presence of the Ca^{2+} ATPase gene in other chlorella viruses and transcription pattern of *m535l* during virus MT325 infection of its host *Chlorella Pbi*. (a) Dot blot hybridization of *m535L* to DNA isolated from 47 viruses that infect *Chlorella Pbi*. The spots contain 1.0, 0.5, 0.25 and 0.125 μg DNA (left to right). The radioactive probe includes ~ 300 bp of the large loop preceding the TM5 of the *m535l* gene (Fig. 1a). Host: *Chlorella Pbi*. No hybridization signal was detected with viruses Fr483 and Nw655.2. The virus Fr483 genome has been sequenced and it lacks a Ca^{2+} -ATPase homologous gene (Fitzgerald *et al.*, 2007a). (b) Transcription of *m535l* in the host *Chlorella Pbi* infected with virus MT325. Total RNA isolated from uninfected (Host) and infected *Chlorella Pbi* sampled at the indicated times (min p.i.) were hybridized with the *m535l* probe used in Fig. 2(a).

corresponding to the viral protein was detected at the 18–33% sucrose interface (Fig. 5a), which corresponds to the ER-enriched fraction. In contrast, the endogenous yeast plasma membrane H^{+} -ATPase was located primarily in a heavier fraction (33–45%) corresponding to the plasma membrane (Fig. 5b). P-type ATPases overexpressed in yeast and in particular Ca^{2+} -ATPases overexpressed in K616 cells are typically located in the endoplasmic reticulum (ER) fraction (Geisler *et al.*, 2000; Sze *et al.*, 2000; Bonza *et al.*, 2004; Fusca *et al.*, 2009). The ER-enriched fraction was then assayed for ATPase activity. Although, no Ca^{2+} - or Mn^{2+} -dependent activity was detected (results not shown), a 70% increase in ATPase-specific activity occurred between the ER fraction purified from cells expressing M535L versus microsomes (Fig. 6). This enrichment parallels the strong M535L accumulation detected by Western blotting (Fig. 5a). In contrast, ATPase activity in the ER fraction from yeast transformed with the empty vector decreased by 30%, a result that can be explained by the separation of the plasma membrane, containing the H^{+} -ATPase, from the ER membranes. The ATPase activity of ER-enriched membranes expressing *m535l* was inhibited by vanadate about three times more than that from control membranes purified from cells transformed with the empty vector (Fig. 7).

Since vanadate specifically inhibits formation of the phosphorylated intermediate during the catalytic cycle of P-type ATPases (Møller *et al.*, 1996), we looked for such an intermediate in M535L-expressing cells. ER-enriched membranes from control and M535L-expressing yeast cells were exposed to $[^{32}\text{P}]\text{ATP}$ in the presence and in the absence of

vanadate. The predicted M535L phosphorylated intermediate was detected on an acidic SDS-PAGE (Fig. 8). Two bands are clearly visible: a band, at about 100 kDa, which most likely is the plasma membrane yeast proton pump and is also present in control membranes (Fig. 8, lane 4). A second band, migrating at about 95 kDa, is about the expected size of the viral protein; this band only appears in membranes expressing M535L (Fig. 8, lanes 1–3). The three lanes show the level of phosphorylation obtained with $[^{32}\text{P}]\text{ATP}$ in the presence of Mg^{2+} and Ca^{2+} (Fig. 8, lane 1), Mg^{2+} , Ca^{2+} and vanadate (Fig. 8, lane 2), and Mg^{2+} alone (Fig. 8, lane 3). The formation of the phosphorylated intermediate is strongly inhibited by vanadate; surprisingly, the formation of the phosphorylated intermediate does not require Ca^{2+} (Fig. 8, compare lanes 1 and 3). This result is unique among Ca^{2+} -transporting ATPases and agrees with our inability to measure a Ca^{2+} -dependent ATPase activity *in vitro*.

DISCUSSION

In this manuscript we provide evidence that some chlorella viruses encode P-type ATPases that are expressed during virus replication and we show that one of them, M535L from virus MT325, is functional. Phylogenetic analysis indicates that the viral proteins belong to the IIB subgroup of Ca^{2+} -transporting ATPases. Sequence comparison with plant and animal proteins identified several conserved motifs and only one putative Ca^{2+} -binding site, characteristic of IIB Ca^{2+} pumps (Brini & Carafoli, 2009). The two viral proteins have short cytosolic termini and lack the

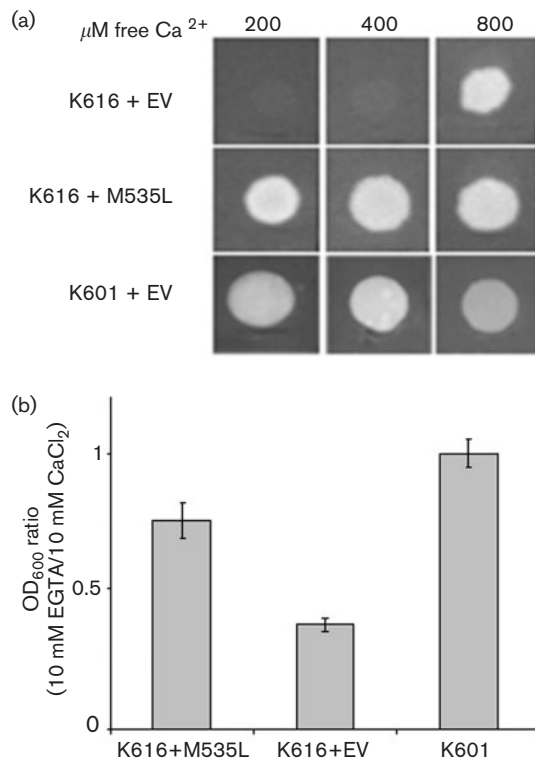


Fig. 3. Complementation of the K616 phenotype by *m535l*. (a) Complementation on solid medium. The Ca²⁺ ATPase-deficient yeast strain K616 transformed with pYES2-*m535l* and with the empty pYES2 vector (EV) were grown at increasing free Ca²⁺ concentrations (200–800 μM). The K601 strain transformed with empty pYES2 vector served as a positive control. Results are from one of three independent experiments. (b) Complementation in liquid culture. Cells were grown 24 h in solutions containing 10 mM Mn²⁺, the presence of a pump in the ER that transports Mn²⁺ ions efficiently allows the yeast cells to survive because it removes the toxic Mn²⁺ ions from the cytosol and accumulates them in the internal stores. Results are expressed as the ratio of OD₆₀₀ of the two cultures (10 mM EGTA/10 mM CaCl₂) and are the mean of four independent experiments; bars represent the standard error.

regulatory domains present at the N- or C-termini of plant and animal IIB Ca²⁺-transporting ATPases (Kabala & Klobus 2005; Di Leva *et al.*, 2008). The M535L protein is functional because it complements mutant yeast K616 growth on Ca²⁺-depleted medium. Complementation of the K616 phenotype clearly indicates that M535L is a fully active Ca²⁺ pump. In fact, only expression of non-autoinhibited plant Ca²⁺-ATPases support K616 growth on Ca²⁺-depleted medium; expression of functional, but autoinhibited IIB pumps do not (Geisler *et al.*, 2000; Sze *et al.*, 2000; Fusca *et al.*, 2009). M535L also complements K616 growth in toxic concentrations of Mn²⁺, indicating that the protein transports both calcium and manganese. These apparently conflicting observations can be reconciled because transformation of yeast cells with the functional viral pump allows cell survival in low external Ca²⁺ and high external Mn²⁺ for different reasons. Expression of a functional calcium pump, presumably in the ER, allows the

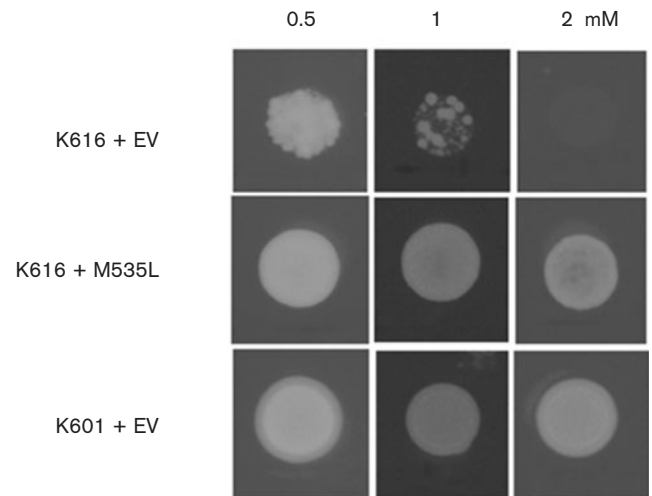


Fig. 4. M535L expression restores growth of yeast strain K616 on Mn²⁺-supplemented medium. K616 cells transformed with pYES2-*m535l* and with the empty pYES2 vector (EV) were grown on solid media containing increasing concentrations of MnCl₂ (0.5–2 mM). Strain K601 transformed with empty pYES2 vector served as a positive control. Results are from one of four independent experiments.

yeast mutant to survive low external Ca²⁺ because it provides a high-affinity mechanism for pumping the (very low) Ca²⁺ ions present in the cytosol into its internal stores. Replenishing stores with Ca²⁺ is essential for yeast cell survival and is related to signal transduction. On the other hand, when the yeast cells are grown in high external Mn²⁺, the presence of a pump in the ER that transports Mn²⁺ ions efficiently allows the yeast cells to survive because it removes the toxic Mn²⁺ ions from the cytosol and accumulates them in the internal stores.

The ability to transport Mn²⁺, in addition to Ca²⁺, is a property of pumps in the secretory pathway (SPCA) (Brini & Carafoli, 2009). In plants that lack SPCA pumps, only Ca²⁺-ATPases belonging to type IIA subgroup, such as ECA1 and ECA3 from *A. thaliana* and LCA1 from tomato, function as Ca²⁺/Mn²⁺ pumps (Wu *et al.*, 2002; Mills *et al.*, 2008; Li *et al.*, 2008; Johnson *et al.*, 2009). The best characterized Ca²⁺/Mn²⁺-transporting pump is PMR1, a yeast protein localized in the Golgi apparatus. In PMR1, the Mn²⁺ selectivity is defined by residues Q⁷⁸³ in TM6 and V³³⁵ in TM4 (Brini & Carafoli, 2009). These residues are not conserved in M535L; furthermore, the viral protein has slightly less resemblance to PMR1 (30% aa identity, 49% similarity) than to plant and animal IIB Ca²⁺-ATPases (see results). Therefore, to our knowledge, this is the first report of a type IIB pump that transports both calcium and manganese.

No convincing Ca²⁺-dependent or Mn²⁺-dependent ATPase activity was detected in the *in vitro* experiments. However, vanadate-sensitive ATPase activity was enriched

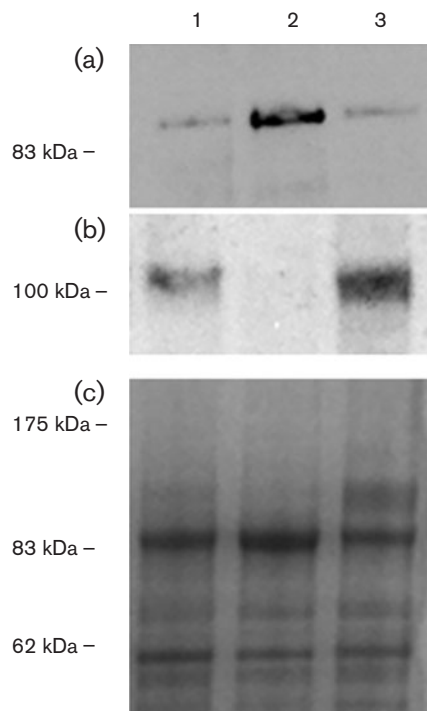


Fig. 5. M535L protein enrichment in the ER fraction. Western blot analysis of different membrane fractions obtained from K616 cells expressing M535L. Microsomes isolated from K616 cells expressing M535L (lane 1) were loaded onto a sucrose step gradient. After overnight centrifugation, fractions corresponding to the 18–33% interface (lane 2) and 33–45% interface (lane 3) were collected and subjected to SDS-PAGE. Western analysis was performed with antisera against the His-tag (panel a, 4 µg proteins) or against the PM H⁺-ATPase of *Neurospora crassa* (panel b, 2 µg proteins). (c) Coomassie blue staining of the proteins.

about 70% in the ER fraction purified from yeast cells expressing *m535l* and this enrichment paralleled M535L accumulation in the ER, the typical cellular location of plant Ca²⁺-ATPases expressed in yeast K616 (Bonza *et al.*, 2004). Moreover, M535L forms a vanadate-sensitive phosphorylated intermediate. The formation of the phosphorylated intermediate also occurs in the presence of MgCl₂ alone, suggesting that M535L also transports magnesium. Since the presence of Mg²⁺ is unavoidable in the ATPase assay, this could explain the independence of M535L ATPase activity from exogenous Ca²⁺ or Mn²⁺. The presumption that M535L is a functional protein is supported by the finding that the gene is expressed during viral replication. In addition, the gene is present in 45 of 47 viruses that infect *Chlorella* Pbi. The common, but not universal, presence of the gene in the chlorella viruses suggests that the function of the protein is auxiliary, but not essential for virus infection/replication. The conclusion of an auxiliary function is supported by the fact that the gene is not present in all chlorella viruses that infect

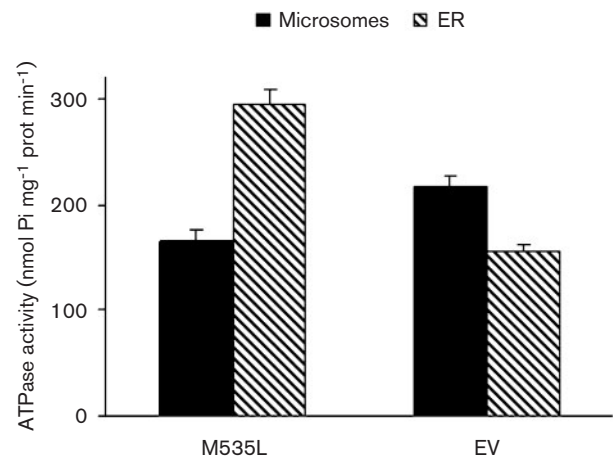


Fig. 6. Total ATPase activity in microsomes (black bars) and in ER fractions purified from cells expressing *m535l* or from cells transformed with the empty vector (dashed bars). Results (\pm SEM) are from three gradients performed on different yeast cultures.

different hosts. Out of four other sequenced viruses that infect either *Chlorella* NC64A (three viruses) or *Chlorella* SAG 3.83 (one virus), only one contained the gene (virus AR158 that infects *Chlorella* NC64A). Again these data suggest that the activity of the protein is not essential; presumably the predecessor chlorella virus had the gene and some of the viruses lost the gene with time without losing their infectivity. However, we cannot eliminate the

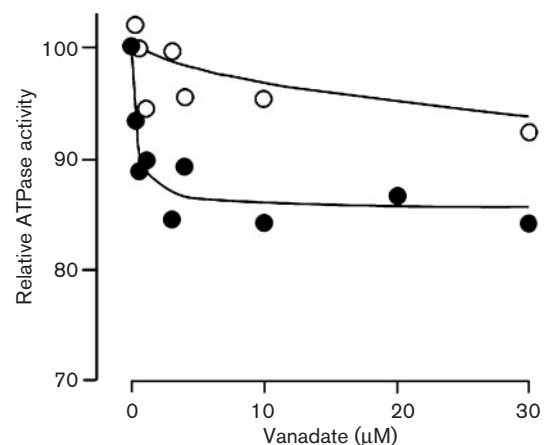


Fig. 7. Vanadate inhibition of ATPase activity from an ER-enriched fraction purified from K616 cells either expressing M535L (closed symbols) or transformed with the empty pYES2 vector (open symbols). ATPase activity, assayed in the presence of increasing concentrations of vanadate, is expressed as a per cent of the activity measured in the absence of vanadate (350 nmol Pi mg⁻¹ protein min⁻¹ for M535L and 160 nmol Pi mg⁻¹ protein min⁻¹ for empty vector).

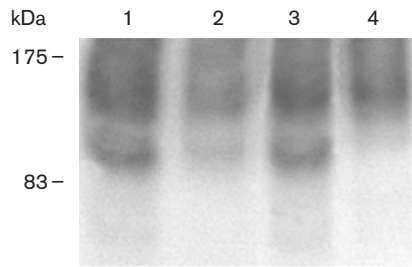


Fig. 8. M535L forms a phosphorylated intermediate during its catalytic cycle. Phosphorylation with [γ - 32 P]ATP of ER-enriched membranes from K616 transformed with *m535l* was performed in the presence of either MgCl₂ and CaCl₂ (lane 1), MgCl₂, CaCl₂ and vanadate (lane 2) or MgCl₂ alone (lane 3). As a negative control, ER membrane proteins from K616 transformed with the empty vector were used (lane 4). Acidic SDS-PAGE, all lanes were loaded with 40 μ g protein. Numbers on the left indicate the size of the molecular mass marker in kDa.

possibility that the gene was acquired independently by the viruses on two occasions.

The evidence that M535L is functional together with its transcription during infection suggests that the protein functions as an ATP-driven ion pump during the virus infection/replication cycle. Altering ion conducting membrane proteins during virus replication is a common mechanism to change the ion milieu in host cells or change the electrical properties of membranes. Several genes encoding membrane transport proteins have been described in the chlorella viruses, including ion channels, aquaglyceroporins and transporters (Plugge *et al.*, 2000; Kang *et al.*, 2003; Gazzarrini *et al.*, 2004, 2006). While the role(s) of the aquaglyceroporin and of the Ca²⁺-transporting ATPase are still unknown, there is circumstantial evidence that the potassium ion channel is required early during virus infection. The present study does not define the role of the virus ATPase. However, expression studies indicate that the gene is transcribed early during virus replication, suggesting the protein fulfils an important function in Ca²⁺ homeostasis during infection. This speculation is not unreasonable because Ca²⁺ is an important messenger in cells and many enzymes in eukaryotes are sensitive to Ca²⁺. M535L function could be important under the non-physiological conditions which probably occur during infection, e.g. altered ion concentrations, an alkaline pH, etc.

The presence of a Ca²⁺-ATPase in some chlorella viruses is also interesting from an evolutionary point of view. To date only a few green algae have been sequenced and annotated, including one of the virus hosts, *Chlorella* NC64A. *Chlorella* NC64A and another green alga, *Chlamydomonas reinhardtii*, as well as others not listed in Fig. 1(c), encode several IIB type Ca²⁺ pumps and all of them, like the viral ones, group with the animal clade. This is surprising because green algae are considered to be

ancestors of higher plants. One possible explanation for this unexpected finding is that higher plants have lost these genes through evolution. Another explanation is that algae acquired these genes after separation from higher plants. The latter hypothesis is not unreasonable because presumably the family *Phycodnaviridae* of viruses exclusively infect algae; higher plants are not hosts. Hence, the phycodnaviruses or their ancestors may have shuttled genes between the animal and plant kingdoms. This is reasonable because the phycodnaviruses have a common ancestor with several other large DNA viruses including poxviruses, iridoviruses, asfarviruses, ascoviruses and mimiviruses, referred to as nuclear, cytoplasmic and large DNA viruses (NCLDV). Accumulating evidence indicates that the NCLDVs have a long evolutionary history, possible dating from the time eukaryotes diverged from prokaryotes (2–3 billion years ago) (Yutin *et al.*, 2009).

METHODS

Phylogenetic analyses. A BLASTP search with the MT325_M535L (ABT14089.1) amino acid sequence was conducted using the NCBI non-redundant protein sequence database with the default settings. In addition, the bait sequence was blasted against genomes of *A. thaliana* (taxid: 3701), *Escherichia coli* (taxid: 5620), *Mycobacterium tuberculosis* (taxid: 1773), *Synechocystis* PCC6803 (taxid: 1148), *Methanobacterium thermoautotrophicum* str. Delta H (taxid: 187420), *Methanococci* (taxid: 183939), *S. cerevisiae* (taxid: 4932), *Caenorhabditis elegans* (taxid: 6239), *Drosophila melanogaster* (taxid: 7227) and *H. sapiens* (taxid: 9606). Similar analyses were carried out using the homologous sequence from AR158_C785L (YP_001498866.1). An unrooted maximum-likelihood tree of 60 P-type ATPase amino acid sequences from the above organisms was generated based on sequence alignment by using MUSCLE and PHYML in the Geneious Pro 4.7.5 software program (Drummond *et al.*, 2008). The Whelan and Goldman (WAG) amino acid substitution model was used to derive 100 bootstrap datasets [the transition/transversion ratio for DNA models and the gamma distribution parameter were estimated, proportion of invariable sites was zero and four substitution rate categories produced the tree in Fig. 1(c); bootstrap values are shown].

Dot blot hybridization. DNA was isolated from *Chlorella* Pbi and 47 viruses that infect *Chlorella* Pbi, transferred to nylon membrane (Osmonics) and cross-linked by UV light as described previously (Graves *et al.*, 2001). A 281 bp highly conserved domain in *m535l*, located from 1744 to 2024 bp, was amplified by PCR as a hybridization probe. The probe was labelled with Random Primers DNA labelling kit (Invitrogen). The dot blot membrane was pre-hybridized in 6 \times SSC (standard sodium citrate), 5 \times Denhardt's reagent, 0.5% SDS and denatured salmon sperm DNA at 68 $^{\circ}$ C for 1 h. The denatured dsDNA probe, labelled with [32 P]dATP was added to the membrane, and hybridized at 68 $^{\circ}$ C for 1 h. The membrane was washed in 2 \times SSC, 0.5% SDS at room temperature for 5 min, 2 \times SSC, 0.1% SDS at room temperature for 15 min, twice, and 0.1 \times SSC, 0.5% SDS at 65 $^{\circ}$ C for 2 h, and finally subjected to signal detection with a Storm Phosphorimager and ImageQuant software (Molecular Dynamics).

Northern hybridization. Three $\times 10^9$ *Chlorella* Pbi cells were collected at various times after virus MT325 infection (m.o.i. of 5), frozen in liquid nitrogen, and stored at -80 $^{\circ}$ C. RNA was extracted with TRIzol reagent (Invitrogen), denatured with formaldehyde, separated on a 1.5% agarose gel, and then transferred to a nylon membrane. [32 P]dATP labelled probe was prepared as in the dot blot

hybridization experiment. The membrane was pre-hybridized in 20 ml Church's buffer (1 mM EDTA, pH 8.0, 0.5 M NaPO₄, 7% SDS) for 1 h at 65 °C, hybridized with fresh Church's buffer and denatured probe for 16 h. After hybridization, the membrane was washed twice with 0.1 × SSC, 0.1% SDS, first time for 30 min, second time for 15 min. The signal detection was the same as in the dot blot hybridization.

Cloning. Amplification of the *m535l* gene from virus MT325 DNA and addition of *XhoI* and *XbaI* restriction sites were done by standard PCR methods. Forward primer: 5'-CAACTCGAGTAAAAGATGTC-CGCGTTTAAAGC-3', reverse primer: 5'-CAATCTAGATTATTAGATGTCATCATTGTTGA-3'. The 2643 bp PCR product was cloned into *XhoI* and *XbaI* sites in a modified version of the yeast expression vector pYES2-NTC (Invitrogen). This vector has a shorter version (MGHHHHHH) of the original N-terminal tag and contains a uracil gene for selection and a galactose inducible promoter upstream of the multiple cloning site for induction of protein expression in *S. cerevisiae*.

Yeast transformation and growth media. *S. cerevisiae* strain K616 [MAT α pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, *ade2*, *ura3* (Cunningham & Fink, 1994)] was used to express M535L (Bonza *et al.*, 2004). K616, transformed with an empty pYES2 vector, served as a negative control. Yeast strain K601/W3031A (MAT α leu2, *his3*, *ade2* and *ura3*) transformed with the empty pYES2 vector served as a positive control. The transformants were selected for uracil prototrophy on a synthetic medium lacking uracil (SC-URA) supplemented with 2% (w/v) glucose. For complementation studies, single URA-positive colonies were grown in SC-URA medium containing 2% (w/v) glucose and 10 mM CaCl₂, pelleted and washed twice with sterile water prior to protein induction in selective conditions. All media were supplemented with 50 mM succinic acid/Tris-Cl pH 5.5 and 0.7% (w/v) yeast nitrogen base.

Complementation in liquid culture at nM Ca²⁺ concentrations. Cells were diluted fourfold with either SC-URA medium, 2% (w/v) galactose, 1% (w/v) raffinose, 10 mM EGTA or SC-URA medium, 2% (w/v) galactose, 1% (w/v) raffinose, 10 mM CaCl₂ (selective and non-selective medium, respectively) and then grown for 24 h by shaking at 30 °C.

Complementation on solid medium. Five microlitre drops of yeast glucose culture (*A*₆₀₀=1) was spotted on solid SC-URA plates containing either 2% (w/v) galactose, 1% (w/v) raffinose and 5 mM EGTA with increasing free Ca²⁺ concentrations (200–800 μ M) or without EGTA but supplemented with increasing concentrations of MnCl₂ (0.5, 1 or 2 mM) and incubated at 30 °C for 3–5 days. The free Ca²⁺ concentrations were calculated using the *K*_d Ca²⁺/EGTA at pH 5.5 (7.12 × 10⁻⁴).

Isolation of microsomes and ER-enriched fraction. Pelleted yeast cells were homogenized and microsomes were harvested as reported previously (Bonza *et al.*, 2004). Yeast membranes were purified by sucrose gradient centrifugation as described previously (Meneghelli *et al.*, 2008). The membrane fraction was frozen at -80 °C until use. Protein concentration was determined using the Bio-Rad assay with γ -globulin as a standard.

Electrophoresis and immunoblotting analysis. SDS-PAGE was performed as described previously (Bonza *et al.*, 1998). Immunodetection was performed with a monoclonal anti-polyhistidine antibody (Sigma-Aldrich) or with antiserum against the PM H⁺-ATPase of *Neurospora crassa* as described previously (Hager *et al.*, 1986).

ATPase assays. ATPase activity was measured as MgATP hydrolysis. Samples (4 μ g membrane proteins) were incubated at 25 °C for 1 h,

during which the reaction proceeds linearly, in a reaction buffer containing 40 mM BTP-HEPES pH 7, 5 mM ATP, 7 mM MgSO₄. Sensitivity to vanadate was tested in a reaction buffer containing 50 mM KCl and increasing concentrations of Na₃VO₄ (0–100 μ M). Released inorganic phosphate was determined colourimetrically (De Michelis & Spanswick, 1986). Assays were performed at least three times with three replicates.

Phosphorylated intermediate formation. The formation of ³²P-phosphorylated intermediate was performed as reported in Rasi-Caldogno *et al.* (1995) with minor changes. The reaction mixture (0.1 ml final volume) contained 50 mM KCl, 10 mM BTP-HEPES pH 7, 0.2 μ M [γ -³²P]ATP [250 μ Ci (9.25 MBq) nmol⁻¹], 12 μ M MgSO₄, 100 μ M CaCl₂ or 100 μ M Na₃VO₄ were included as indicated in the text. The reaction was started by adding 100 μ g ER-membrane proteins from K616-expressing M535L or transformed with empty pYES2 vector. After 60 s the reaction was stopped by adding 1.7 ml ice-cold 12% trichloroacetic acid, 1 mM ATP and 50 mM NaH₂PO₄, incubated for 1 h at 0 °C and centrifuged for 1 h at 4 °C at 20 000 g. Pellets were resuspended with protease inhibitors, solubilized and separated by acidic SDS-PAGE on a 5.6% polyacrylamide gel (40 μ g protein) as described in Rasi-Caldogno *et al.* (1995). For ³²P autoradiography, the dried gel was exposed to Kodak Biomax MS film for 3 days at -80 °C.

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