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EVIDENCE FOR A CONSERVED 95–120 kDa SUBUNIT ASSOCIATED WITH AND ESSENTIAL FOR ACTIVITY OF V-ATPases

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Summary

Vacuoles purified from *Saccharomyces cerevisiae* bearing the *vph1-1* mutation had no detectable bafilomycin-sensitive ATPase activity or ATP-dependent proton pumping. Furthermore, the vacuolar H^+-ATPase (V-ATPase) nucleotide binding subunits were no longer associated with vacuolar membranes yet were present at wild-type levels in yeast whole-cell extracts. The *VPH1* gene was cloned by screening a λgt11 expression library with antibodies directed against a 95 kDa vacuolar integral membrane protein and independently cloned by complementation of the *vph1-1* mutation. Deletion disruption of the *VPH1* gene revealed that the *VPH1* gene is required for vacuolar H^+-ATPase assembly and vacuolar acidification but is not essential for cell viability or for targeting and maturation of vacuolar proteases. *VPH1* encodes a predicted polypeptide of 840 amino acid residues (95.6 kDa) with putative membrane-spanning regions. Cell fractionation and immunodetection demonstrate that Vph1p is a vacuolar integral membrane protein that co-purifies with V-ATPase activity. Vph1p has 42 % identity to the 116 kDa polypeptide of the rat clathrin-coated vesicles/synaptic vesicle proton pump, 42 % identity to the TJ6 mouse immune suppressor factor, 42 % identity to the *Caenorhabditis elegans* proton pump homologue and 54 % identity to the predicted polypeptide encoded by the yeast gene *STV1* (Similar To *VPH1*, identified as an open reading frame next to the *BUB2* gene).

Introduction

The first accounts of V-ATPase structure did not report any associated subunits larger than the 70 kDa nucleotide binding subunit. Susceptibility of the 95–120 kDa subunit to proteolysis and to aggregation may be in part attributed to its absence in these early reports. More recently, the 95–120 kDa subunit has been shown to co-purify with V-ATPases from several sources and to be essential to V-ATPase activity in both biochemical and genetic experiments. Here we review the recent evidence that a conserved 95–120 kDa integral membrane polypeptide is associated with V-ATPases from various organelles and is essential to the assembly and activity of the enzyme.

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Possible explanations for the absence of the 95–120 kDa subunit from purified V-ATPases from specific sources

The first reports of V-ATPase structure (Uchida *et al.* 1985; Marin *et al.* 1985; Manolson *et al.* 1985) were generally one-step purifications on glycerol gradients or Sepharose columns resulting in low purifications and complex polypeptide mixtures. The subunits reported to be associated with these V-ATPases were conservatively limited to the polypeptides that could be specifically labeled with radioactive probes such as the 70 and 60 kDa nucleotide binding subunits and the 16 kDa *N,N'*-dicyclohexylcarbodiimide binding subunit. If one re-examines some of the protein gels from these first reports, a subunit of approximately $100\times10^3 M_r$ can indeed be seen in the lanes containing V-ATPase activity (Manolson *et al.* 1985, Fig. 8). Nevertheless, there are more recent reports on bovine renal tubules (Gluck and Caldwell, 1987), rat Golgi apparatus (Moriyama and Nelson, 1989*a*) and rat liver lysosomes (Moriyama and Nelson, 1989*b*) in which purified V-ATPases from these sources did not contain a subunit migrating between 95 and $120\times10^3 M_r$. For rat liver lysosome and Golgi apparatus V-ATPases, the absence of a $95–120\times10^3 M_r$ polypeptide may reflect a genuine difference in the composition of V-ATPases from various sources. Gillespie *et al.* (1991) have since presented immunological evidence of a $115\times10^3 M_r$ subunit associated with bovine renal tubule V-ATPase, suggesting that the absence of this subunit, as reported by Gluck and Caldwell (1987), may have resulted from the difficulty of visualizing the $95–120\times10^3 M_r$ subunit by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Susceptibility of the 95–120 kDa subunit to proteolysis during V-ATPase purification has been reported by Gillespie *et al.* (1991) and Kane *et al.* (1992). Adachi *et al.* (1990*a*) have shown that the $100\times10^3 M_r$ subunit from purified bovine coated vesicle V-ATPase is quickly degraded to an $80\times10^3 M_r$ and a $21\times10^3 M_r$ species by low concentrations of trypsin. To avoid the proteolysis problem when purifying the *Saccharomyces cerevisiae* vacuolar H^+-ATPase, we have used a yeast strain (BJ926, *MATα/MAT***a**, *trp1-1/+ +/his1, prc1-126/prc1-126 pep4-3/pep4-3, prb1-1122/prb1-1122 can1/can1 gal2/gal2*) in which the structural genes encoding the predominant yeast vacuolar hydrolases (protease A, protease B and carboxypeptidase Y) have been mutated. Furthermore, to reduce other cytosolic protease activities, we included $1\,mol\,l^{-1}$ EDTA, $2\,mmol\,l^{-1}$ phenylmethylsulfonyl fluoride and $1\,\mu g\,ml^{-1}$ pepstatin A during the V-ATPase purification procedure (Manolson *et al.* 1992).

A second potential problem for visualizing the $95–120\times10^3 M_r$ V-ATPase subunit on SDS–PAGE is its tendency to aggregate when heated in protein-denaturing sample buffer (Gillespie *et al.* 1991; Manolson *et al.* 1992). Denaturing whole-cell extracts in the presence of 1 % SDS and $0.6\,mmol\,l^{-1}$ β-mercaptoethanol at temperatures over 62 °C, or at 62 °C for longer than 15 min, resulted in the failure to detect any signal on an immunoblot from the yeast 95 kDa V-ATPase subunit, Vph1p (Fig. 1). Although 45 °C for 15 min and 62 °C for 2.5 min appear to result in equal signals (Fig. 1), the latter treatment was found to be empirically more reproducible.

Evidence that a 95–120 kDa subunit is associated with V-ATPases

Purifications of V-ATPases that have resulted in an associated 95–120×10^3 M_r subunit are listed in Table 1. Monoclonal (Kane *et al.* 1992) and polyclonal (Manolson *et al.* 1992) antibodies to a yeast 95–100×10^3 M_r polypeptide show co-purification of the

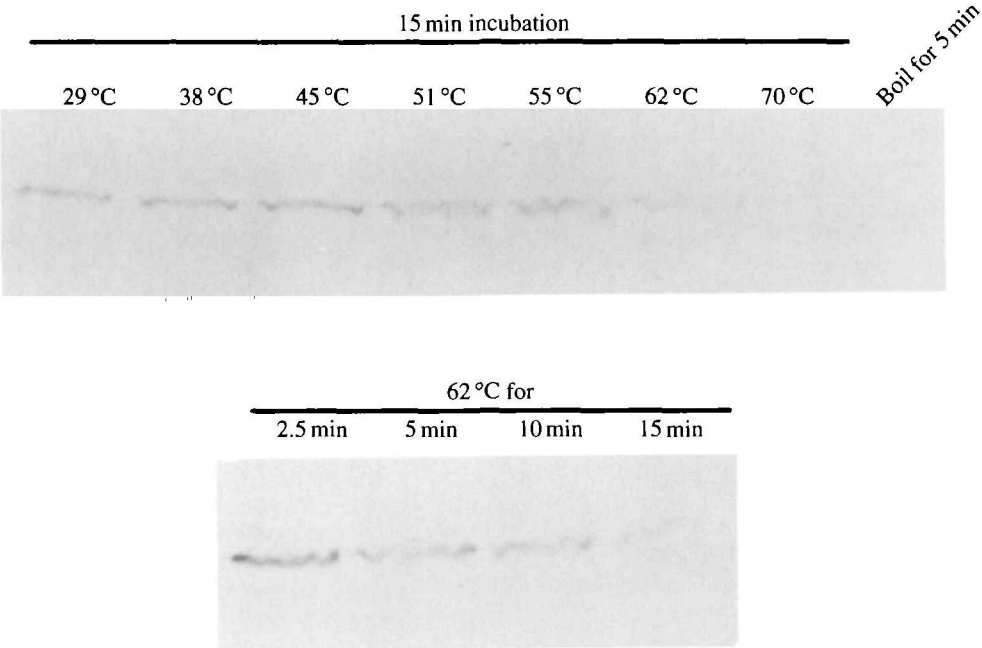

Fig. 1. The 95 kDa yeast V-ATPase subunit, Vph1p, requires brief low-temperature incubations for solubilization in SDS–PAGE denaturation buffer. 150 μg of whole-cell extracts (prepared as described in Manolson *et al.* 1992) were denatured for SDS–PAGE in 63 mmol l^{-1} Tris–HCl pH 6.8, 1 % SDS, 0.6 mmol l^{-1} β-mercaptoethanol and 5 % glycerol for 15 min at 29, 38, 45, 51, 55, 62 or 70 °C (top panel), boiling for 5 min (top panel, right-hand lane), or at 62 °C for 2.5, 5, 10 or 15 min (bottom panel). Samples were subjected to SDS–PAGE, transferred to nitrocellulose and then subjected to immunoblot detection using a 1:350 dilution of affinity-purified anti-Vph1p.

Table 1. *V-ATPases with associated 95–120 kDa subunits*

Organelle	Source	Molecular mass×10^{-3}	Reference
Clathrin-coated vesicles	Bovine brain	100–116	Xie and Stone (1986); Arai *et al.* (1987); Perin *et al.* (1991)
Chromaffin granule	Bovine adrenal medulla	140 115	Percy *et al.* (1985); Moriyama and Nelson (1987)
Chromaffin granule	Human phaeochromocytoma	115	Gillespie *et al.* (1991)
Renal tubule	Bovine renal medulla	115	Gillespie *et al.* (1991)
Vacuole	Red beet storage tissue	100	Parry *et al.* (1989)
Vacuole	Yeast	95–100	Kane *et al.* (1992); Manolson *et al.* (1992)

Fig. 2

antigen with V-ATPase activity whereas immunofluorescence microscopy (Kane *et al.* 1992) and immunogold electron microscopy (Manolson *et al.* 1992) have demonstrated the specificity of the antigen for the yeast vacuolar membrane. The V-ATPase nucleotide binding subunits appear to block epitope sites *in vivo* on the yeast $95–100\times10^3 M_r$ polypeptide (Kane *et al.* 1992), further supporting the association of this polypeptide with the yeast vacuolar H^+-ATPase.

Evidence that a 95–120 kDa subunit is required for assembly and activity of V-ATPases

Specific removal of the 116 and $38\times10^3 M_r$ subunits from the purified clathrin-coated V-ATPase did not affect Ca^{2+}-activated ATP hydrolysis yet eliminated Mg^{2+}-supported ATP hydrolysis and proton pumping activity (Xie and Stone, 1988). Mutations in the structural gene encoding the yeast 95 kDa subunit, *VPH1*, result in a pH of 6.9 in the vacuolar lumen instead of the wild-type pH of 6.2 (Preston *et al.* 1989). Vacuoles purified from strains bearing the *vph1-1* mutation had no measurable bafilomycin-sensitive ATP hydrolysis or proton-pumping activity (A. K. Bachhawat, M. F. Manolson, D. G. Murdock, D. Garmen and E. W. Jones, in preparation) and had barely detectable levels of the 60 and 69 kDa peripherally bound nucleotide-binding V-ATPase subunits (Manolson *et al.* 1992). In contrast, when either of the structural genes encoding the 60 and 69 kDa V-ATPase (*VAT2*, *TFP1*) is disrupted, the 95 kDa subunit is correctly targeted and stably inserted into the vacuolar membrane as long as the 16 kDa subunit is present (Kane *et al.* 1992). Taken together, these results suggest that the 95 and 16 kDa subunits are epistatic to the 60 and 69 kDa subunits for V-ATPase assembly.

The 95–120 kDa V-ATPase subunit is an integral membrane protein

The $100\times10^3 M_r$ subunit of the coated vesicle proton pump was labeled by the hydrophobic reagent 3-(trifluoro-methyl)-3-(*m*-iodophenyl)diazirine, implying that a portion of the polypeptide is buried within the membrane bilayer (Arai *et al.* 1988). Failure of chaotropic agents such as alkaline Na_2CO_3 (Kane *et al.* 1992; Manolson *et al.* 1992) or 0.3 mol l^{-1} KI (Adachi *et al.* 1990*b*) to strip the $95–100\times10^3 M_r$ subunit from membranes further suggests that this subunit is an integral membrane protein. This biochemical evidence is supported by sequence analysis of the 116 kDa subunit of the clathrin-coated vesicle/synaptic vesicle H^+-ATPase (Perin *et al.* 1991) and the 95 kDa subunit of the yeast vacuolar H^+-ATPase (Manolson *et al.* 1992). In both cases, the carboxyl terminal half of the protein contains at least six regions of sufficient length and hydrophobicity to be considered as membrane-spanning regions.

Fig. 2. Multiple sequence alignment. The entire lengths of the yeast *VPH1* gene product (Vph1p), the yeast *STV1* gene product (Stv1p), the 116 kDa polypeptide of the rat clathrin-coated vesicle H^+-ATPase (Rat), the mouse TJ6 mouse immune suppressor factor (Mouse) and the *Caenorhabditis elegans* (Cel) proton pump homologue have been aligned using the PILEUP and PRETTY programs (Genetics Computer Group Sequence Analysis Software Package). The alignment and consensus sequence were created using the default settings. Amino acid members of the winning coalition are in upper case and underlined; the consensus sequence is shown directly below the alignment (Consen).

The 95–120 kDa V-ATPase subunit is evolutionarily conserved amongst V-ATPases from a variety of sources

As shown is Table 1, a 95–120 kDa subunit has been found in V-ATPases from a variety of sources. Antibodies directed against the $120\times10^3 M_r$ subunit of the chromaffin-granule V-ATPase recognized a $115\times10^3 M_r$ polypeptide in the renal tubule V-ATPase, implying shared epitopes between the two proteins (Gillespie *et al.* 1991). There are now five sequences that appear to encode this V-ATPase subunit; the 116 kDa polypeptide of the rat clathrin-coated vesicle/synaptic vesicle proton pump (Perin *et al.* 1991, accession number M58758), the 95 kDa subunit (Vph1p) of the yeast vacuolar H^+-ATPase (Manolson *et al.* 1992, accession number M89778), the predicted polypeptide encoded by the Similar To *VPH1* yeast gene *STV1* (M. F. Manolson, D. Proteau, R. A. Preston, B. T. Roberts, M. A. Hoyt and E. W. Jones, in preparation), the TJ6 mouse immune suppressor factor (Lee *et al.* 1990, accession number M31226) and the *Caenorhabditis elegans* proton pump homologue (Sulston *et al.* 1992, accession number Z11115). Multiple sequence alignments demonstrate the extensive homology over the entire open reading frames of these five sequences (Fig. 2). Of the five sequences, only the rat 116 kDa polypeptide and the yeast 95 kDa subunit (Vph1p) have been directly linked to their respective V-ATPase enzyme. The TJ6 mouse immune suppressor factor and the *Caenorhabditis elegans* proton pump homologue are thought to be V-ATPase subunits solely on the basis of sequence homology. Although the Similar To *VPH1* yeast gene product, Stv1p, was initially identified merely on the basis of sequence homology, there is now genetic evidence that it is a functional homolog of Vph1p (M. F. Manolson, D. Proteau, R. A. Preston, B. T. Roberts, M. A. Hoyt and E. W. Jones, in preparation).

Possible function of the 95–120 kDa V-ATPase subunit

Although there is considerable evidence that a 95–120 kDa subunit is associated with a variety of V-ATPase, its specific function is unknown. Data showing that this one subunit contains domains exposed to the cytoplasm (Adachi *et al.* 1990*a*) and to the membrane bilayer (Adachi *et al.* 1990*b*; Kane *et al.* 1992; Manolson *et al.* 1992) have led Perin *et al.* (1991) to speculate that its function is in coupling ATP hydrolysis (carried out by the cytoplasmic subunits) to proton translocation (carried out by the intramembranous subunits). Yeast contain several membrane-bound organelles that require electrochemical gradients across their membranes (vacuole, clathrin-coated vesicles, peroxisomes and Golgi complex). The presence of at least two functional homologues of the 95–100 kDa V-ATPase subunit in yeast (Vph1p and Stv1p) has led Manolson *et al.* (1992) to hypothesize that this subunit may be involved in targeting or regulating V-APases for specific endomembrane organelles. In *Saccharomyces cerevisiae*, the 95 kDa subunit, Vph1p, is essential for the *in vivo* assembly and activity of the vacuolar H^+-ATPase.

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References

ADACHI, I., ARIA, H., PIMENTAL, R. AND FORGAC, M. (1990*a*). Proteolysis and orientation on reconstitution of the coated vesicle proton pump. *J. biol. Chem.* **265**, 960–966.

ADACHI, I., PUOPOLO, K., MARQUEZ-STERLING, N., ARAI, H. AND FORGAC, M. (1990*b*). Dissociation, cross-linking and glycosylation of the coated vesicle proton pump. *J. biol Chem.* **265**, 967–973.

ARAI, H., BERNE, M., TERRES, G., PUOPOLO, K. AND FORGAC, M. (1987). Subunit composition and ATP site labeling of the coated vesicle proton-translocating adenosinetriphosphatase. *Biochemistry* **26**, 6632–6638.

ARAI, H., TERRES, G., PINK, S. AND FORGAC, M. (1988). Topography and subunit stoichiometry of the coated vesicle proton pump. *J. biol. Chem.* **263**, 8796–8802.

GILLESPIE, J., OZANNE, S., PERCY, J., WARREN, M., HAYWOOD, J. AND APPS, D. (1991). The vacuolar H^+-translocating ATPase of renal tubules contains a 115-kDa glycosylated subunit. *FEBS Lett.* **282**, 9–72.

GLUCK, S. AND CALDWELL, J. (1987). Immunoaffinity purification and characterization of vacuolar proton-ATPase from bovine kidney. *J. biol Chem.* **262**, 15780–15789.

KANE, P. M., KUEHN, M. C., HOWALD, I. AND STEVENS, T. H. (1992). Assembly and targeting of peripheral and integral membrane subunits of the yeast vacuolar H^+-ATPase. *J. biol. Chem.* **267**, 447–454.

LEE, C.-K., GHOSHAL, K. AND BEAMAN, K. D. (1990). Cloning of a cDNA for a T cell produced molecule with a putative immune regulatory role. *Molec. Immun.* **27**, 1137–1144.

MANOLSON, M. F., PROTEAU, D., PRESTON, R. A., STENBIT, A., ROBERTS, B. T., HOYT, M. A., PREUSS, D., MULHOLLAND, J., BOTSTEIN, D. AND JONES, E. W. (1992). The *VPH1* gene encodes a 95-kDa integral membrane polypeptide required for *in vivo* assembly and activity of the yeast vacuolar H^+-ATPase. *J. biol. Chem.* **267**, 14294–14303.

MANOLSON, M. F., REA, P. A. AND POOLE, R. J. (1985). Identification of 3-O-(4-benzoyl)benzoyladenosine 5′-triphosphate- and *N,N′*-dicyclohexylcarbodiimide-binding subunits of a higher plant proton-translocating tonoplast ATPase. *J. biol. Chem.* **260**, 12273–12279.

MARIN, B., PREISSER, J. AND KOMOR, E. (1985). Solubilization and purification of the ATPase from the tonoplast of Hevea. *Eur. J. Biochem.* **151**, 131–140.

MORIYAMA, Y. AND NELSON, N. (1987). Nucleotide binding sites and chemical modification of the chromaffin granule proton ATPase. *J. biol. Chem.* **262**, 14723–14729.

MORIYAMA, Y. AND NELSON, N. (1989*a*). H^+-Translocation ATPase in Golgi apparatus. *J. biol Chem.* **264**, 18445–18450.

MORIYAMA, Y. AND NELSON, N. (1989*b*). Lysosomal H^+-translocating ATPase has a similar subunit structure to chromaffin granule H^+-ATPase complex. *Biochim. biophys. Acta* **980**, 241–247.

PARRY, R. V., TURNER, J. C. AND REA, P. A. (1989). High purity preparations of higher plant vacuolar H^+-ATPase reveal additional subunits. *J. biol. Chem.* **264**, 20025–20032.

PERCY, J. M., PRYDE, J. G. AND APPS, D. K. (1985). Isolation of ATPase I, the proton pump of chromaffin-granule membranes. *Biochem. J.* **231**, 557–564.

PERIN, M. S., FRIED, V. A., STONE, D. K., XIE, X.-S. AND SUDHOF, T. C. (1991). Structure of the 116-kDa polypeptide of the clathrin-coated vesicle/synaptic vesicle proton pump. *J. biol. Chem.* **266**, 3877–3881.

PRESTON, R. A., MURPHY, R. F. AND JONES, E. W. (1989). Assay of vacuolar pH in yeast and identification of acidification-defective mutants. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7027–7031.

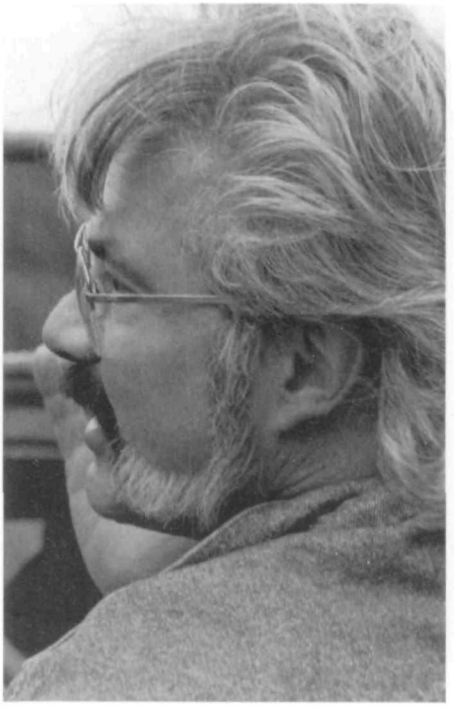
SULSTON, J., DU, Z., THOMAS, K., WILSON, R., HILLIER, L., STADEN, R., HALLORAN, N., GREEN, P., THIERRY-MIEG, J., QIU, L., DEAR, S., COULSON, A., CRAXTON, M., DURBIN, R., BERKS, M., METZSTEIN, M., HAWKINS, T., AINSCOUGH, R. AND WATERSTON, R. (1992). The *C. elegans* genome sequencing project: a beginning. *Nature* **356**, 37–41.

UCHIDA, E., OHSUMI, Y. AND ANRAKU, Y. (1985). Purification and properties of proton-translocating Mg^{2+}-adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. biol. Chem.* **260**, 1090–1095.

XIE, X.-S. AND STONE, D. K. (1986). Isolation and reconstitution of the clathrin-coated vesicle proton translocating complex. *J. biol. Chem.* **261**, 2492–2495.

XIE, X.-S. AND STONE, D. K. (1988). Partial resolution and reconstitution of the subunits of the clathrin-coated vesicle proton ATPase responsible for Ca^{2+}-activated ATP hydrolysis. *J. biol. Chem.* **263**, 9859-9867.

CHAPTER 3. Biochemistry of plant tonoplasts



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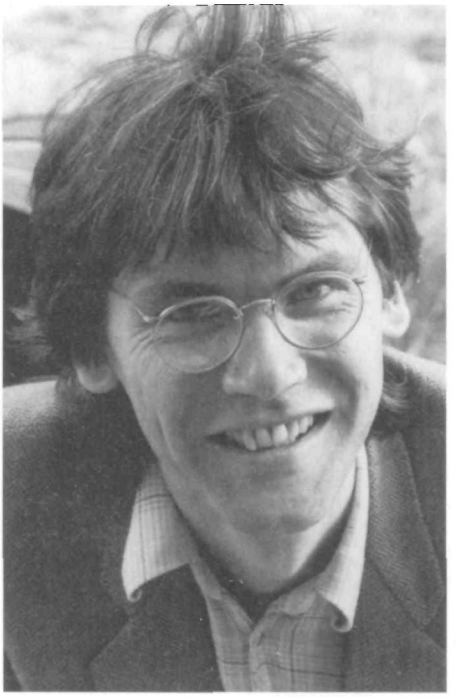

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