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IDENTIFICATION OF THE MULTICATALYTIC ENZYME AS A POSSIBLE γ-SECRETASE FOR THE AMYLOID PRECURSOR PROTEIN

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Summary. One of the main components of the senile plaques in brain tissue from patients with Alzheimer's disease is the ß-amyloid peptide. This peptide is proteolytically cleaved from the amyloid precursor protein by the action of at least two proteases , a ß-secretase which generates the N-terminus and a γ-secretase which generates the C-terminus. Neither of these proteases have been identified. To identify proteases that are candidates for the γ-secretase we synthesized a small fluorescent peptide substrate containing the amino acids comprising the C-terminus of the longest ß-amyloid peptide identified. This substrate is hydrolyzed by a single activity in homogenates from both cells and brain tissue and we have demonstrated that this activity is the multicatalytic enzyme or proteasome. Furthermore, using specific inhibitors, we have demonstrated that the fluorescent substrate is hydrolyzed by the chymotrypsin-like activity of the multicatalytic enzyme.

Alzheimer's disease (AD), the most common human neurodegenerative disorder, is characterized by the formation of senile plaques containing deposits of the ß amyloid peptide (Aß) in selected regions of the brain (1, 2). The Aß peptide is a 39-43 amino acid proteolytic fragment of a larger, integral membrane protein that spans the membrane once, has a large extracytoplasmic N-terminus and is both N and O-glycosylated and tyrosine sulfated (3,4,5). Aß is comprised of the 28 amino acids immediately before the transmembrane domain plus 11-15 hydrophobic residues that are predicted to be buried in the membrane in the intact molecule. The amyloid precursor proteins (APP's) (MW 100-140 kDa's) are a group of protein isoforms that arise from alternative splicing of the 19 exons encoded by a single gene, located on the long arm of chromosome 21. It is currently not known whether all the isoforms or only some give rise to the Aß peptide.

The intracellular processing of APP and the pathways by which Aß could be generated or degraded have become an important focus for AD research. During transport through the secretory

Abbreviations: AD, Alzheimer's diease; Aβ, 4kDa β-amyloid peptide; APP, amyloid precursor protein; AMC, amino methyl coumarin; ER, endoplasmic reticulum; pHMPS, p-hydroxymercuriphenyl sulfonic acid.

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pathway the APP's are cleaved by an as yet unidentified protease, designated the α-secretase (Figure 1), to create a large secreted form of APP and a 10kDa C-terminal fragment that remains membrane bound (5,6,7,8). Cleavage occurs between Lys 687 and Leu 688 (numbered according to APP770-amino acids 16 and 17 of Aß) which lie just outside the transmembrane region. Because this cleavage occurs within the Aß domain, this processing pathway prevents the formation of the Aß peptide. Although cleavage at this site seems to occur in virtually all cells that express the precursor, the proportion of APP molecules that are secreted varies considerably and at most seems to be only 30% of the total amount synthesized (5). This finding and the recent demonstration that the Aß peptide is continuously produced and secreted during normal cellular metabolism (9,10,11) indicates that alternative pathways for the processing and removal of APP exist. One such pathway involves the reinternalization of APP from the plasma membrane and subsequent delivery to the endosomal/lysosomal system (12). Several studies have demonstrated that lysosomes contain carboxy terminal fragments of APP, some of which are potentially amyloidogenic since they contain the intact Aß region (13,14). However, experiments using toxins and agents that inhibit lysosomal function suggest that the constitutively produced Aß is not produced by the lysosome (15,16).

A second site of cleavage , known as the ß-secretase site (Figure 1) occurs at Met 671 and generates the amino terminus of Aß, Asp 672. Cleavage at this site releases a truncated secreted form of ßAPP which has been detected in conditioned media of human mixed brain cultures (17). The enzyme involved in this cleavage is also unknown. The importance of this cleavage to the production of the Aß peptide is suggested by the finding that one of the APP mutations found in a Swedish family with early onset AD, is a double mutation at amino acids 670 and 671. The substitution of the normal Met-Asp by an apparently highly cleavable Leu-Asp bond results in a 5-8 fold increase in the production of the Aß peptide in transfected cells (18,19). The generation and release of Aß also requires cleavage at the carboxy terminus or γ-secretase site (Figure 1). The precise peptide bond that is cleaved by the γ-secretase is not known and again the protease(s) that may hydrolyze this site have not been identified. Hydrolysis of APP at this site can occur independent of hydrolysis at the α- or ß-secretase site to generate a secreted form of APP that contains the complete Aß peptide (20). This fragment is therefore potentially amyloidogenic. Hydrolysis at both the ß- and the γ-secretase sites would generate the Aß peptide.

Significant heterogeneity has been observed in both the N- and C-terminus of the Aß peptide derived either from cerebrospinal fluid (21), the brains of AD patients (22) or produced by transfected cells (23). Although the major species begins at Asp (residue 1 of Aß) minor species begin at Ile(-6), Val(-3), Phe(-4) and Glu(-11). At the C-terminus the major species ends at Val(40) but both longer and shorter peptides are present in culture medium , plaques and cerebrospinal fluid. The largest Aß species that has been identified is the 1-43 derivative and it seems likely that the heterogeneity is determined by amino- and carboxy-peptidase action, although the possibility remains that they are derived from differential endoproteolytic cleavage of APP. In order to identify proteases that might serve as the γ-secretase we have designed a fluorescent peptide substrate which incorporates the amino acids at the C-terminus of this longest derivative. We show here that this substrate is cleaved by a single protease which we have identified as the multicatalytic enzyme or proteasome.

METHODS

Subcellular fractionation: PC12 cells were maintained as monolayers in a 5% CO_2 atmosphere in DMEM containing 10% horse serum, 5% fetal calf serum ,100 units/ml penicillin/ streptomycin and 10 mM HEPES. Ten 150 cm^2 flasks of PC12 cells were harvested with Hank's balanced salt solution and washed once with homogenization buffer (HB: 0.25 M sucrose in 20mM Hepes pH 7.4 and 1mM magnesium acetate). The cell pellet was diluted with 2-3 volumes of HB and the cells broken by 10 passes through a ball bearing homogenizer with a clearance of 0.008 in. (24). Post nuclear supernatants (PNS) were prepared by centrifugation at 800xg for 5 min at 4°C. Two mls of the PNS was layered on a sucrose gradient consisting of 1.5 ml of 50%, 45%, 40%, 35%, 30%, and 25% and 2 mls each of 20% and 15% sucrose (w/w) in 20 mM HEPES and centrifuged at 25,000 rpm for 16-18 hrs in an SW 28.1 rotor at 4°C. Fractions of approximately 0.6 ml were collected from the top using a gradient fractionator (Isco). The fractions were analyzed for the following marker activities: galactosyl transferase for the Golgi apparatus (25); α-glucosidase for the ER (26); alkaline phosphodiesterase for plasma membrane; ß-hexosaminidase for lysosomes; LDH for cytosol (27) and succinic dehydrogenase for mitochondria (28). For some experiments crude cytosol was separated from membranes by centrifugation at 100,000 x g for 90 min or by layering 3 mls of the homogenate onto a step gradient consisting of 6 mls 0.33M sucrose and 1 ml of 2M sucrose in 20 mM HEPES pH 7.0. These gradients were centrifuged for 1 hr at 35,000 rpm in a SW 40 rotor. The membranes form a tight band at the 2M sucrose cushion and the cytosol remains at the top. The purified cytosol was then layered on the sucrose gradient and centrifuged as described for the PNS.

To analyze the fractions for the C9 subunit of the proteasome, 45 µl of each fraction was mixed with 15 µl of a 4 times concentrated Laemmli solution and 25 µl were separated on 10% SDS-PAGE. The electrophoretically separated proteins were transferred to Immobilon P (Millipore) in 10 mM CAPS, pH 11 containing 10% methanol. Membranes were blocked with 5% nonfat dry milk in TTS (20 mM Tris pH 7.5, 0.05% Tween-20, 150 mM NaCl) and then incubated several hours at 25°C with a 1:200 dilution of a rabbit antibody (29). The membranes were washed with TTS 3 times and incubated with 0.55 µCi ^{125}I-protein A for 1 hr. The membranes were again washed with TS, dried and exposed to Kodak XOMAT film at -80°C usually for 24 hrs.

Enzyme assays: The TVI hydrolyzing activity was assayed in a total volume of 50 µl containing HB and 0.2mM substrate (diluted from a 4mM stock dissolved in DMSO) and incubated for 16-20 hrs at 37°C. Samples were boiled , diluted with 1 ml of water and read at ex 380nm , em 460nm (slit widths ex 10 nm, em 5 nm). The assay's for GGF-AMC; GGR-AMC and LLE-2NA hydrolysis were exactly as described in McDermott et al., (30).

RESULTS

To assay for protease activities that may hydrolyze APP to produce the C-terminus of the ßA4 peptide, we synthesized a substrate that contains the amino acids MeOsucc-Thr-Val-Ile coupled to a fluorescent reporter group, amino methyl coumarin (AMC) (TVI-AMC). This fluorescent substrate contains the amino acid that comprises the extreme C-terminus of the longest Aß peptide that has been identified (see Figure 1). Subcellular fractions of PC12 cells were assayed for protease activities which hydrolyze TVI-AMC. Post nuclear supernatants of PC12 cells were layered on a 15-50% sucrose gradient and separated by centrifugation. A single peak of protease activity hydrolyzing TVI-AMC was detected in fractions 11-16 (Figure 2). This TVI hydrolyzing activity was cytosolic since most of the hydrolytic activity remained soluble after removing membranes from the post nuclear supernatants before centrifugation on the sucrose gradient (data not shown). However, the bulk of the cytosolic proteins (determined by measuring lactate dehydrogenase (LDH) activity) remained at the top of the gradient (fractions 5-8) while the TVI hydrolyzing activity partially enters the gradient. This suggested that the activity was part of a large

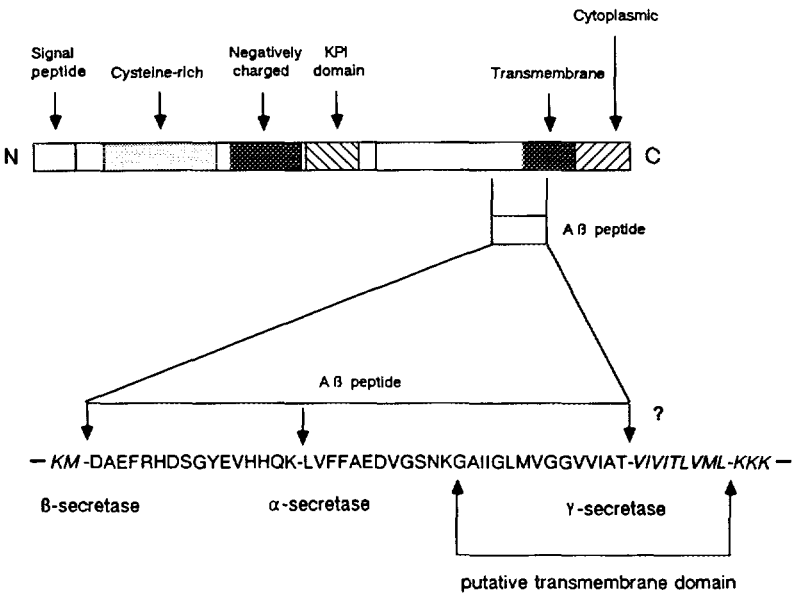

Figure 1. APP and the sites cleaved by the α-, ß- and γ-secretases.

cytosolic complex. Next, we tested a variety of protease inhibitors and demonstrated that the TVI hydrolyzing activity was completely inhibited by p-hydroxymercuriphenyl sulfonic acid (pHMPS) (Figure 3A). Further studies showed that this activity is also inhibited by calpain inhibitor I , chymostatin and 150 mM NaCl (Figure 3B). The enzyme activity was not inhibited by antipain (Figure 3B), aprotinin, leupeptin or phosphoramidon (data not shown). These characteristics, particularly the sensitivity to salt and the fact that the TVI hydrolyzing activity exists as a large cytosolic complex, suggested that this enzyme was the multicatalytic enzyme or proteasome (31). To establish this further we immunoblotted the sucrose gradient fractions with an antibody recognizing the C9 subunit of the multicatalytic enzyme (29). The C9 subunit co-fractionated with the TVI hydrolyzing activity (Figure 2B).

The multicatalytic enzyme contains at least three proteolytic activities that hydrolyze three distinct fluorescent peptide substrates. A chymotrypsin like activity hydrolyzes the fluorescent substrate glutaryl-gly-gly-phe-AMC (GGF-AMC); a trypsin-like activity hydrolyzes benzyloxycarboxyl-gly-gly-arg-AMC (GGR-AMC) and a cucumisin-like activity hydrolyzes benzyloxycarboxyl-leu-leu-glu-2-naphthylamide (LLE-2NA) (30). Each fraction from the sucrose gradient was examined for its ability to hydrolyze these three peptide substrates. The protease activities hydrolyzing these three substrates, and TVI-AMC were all contained in the same sucrose fractions (Figure 4) indicating that the TVI hydrolyzing activity is the multicatalytic enzyme.

The three protease activities of the multicatalytic enzyme can be distinguished from each other by differential inhibition by protease inhibitors (Table 1). All three protease activities are inhibited by salt and pHMPS. Both chymostatin and calpain inhibitor I strongly inhibit GGF-AMC hydrolysis , partially inhibit GGR-AMC hydrolysis and have no effect on LLE-2NA hydrolysis.

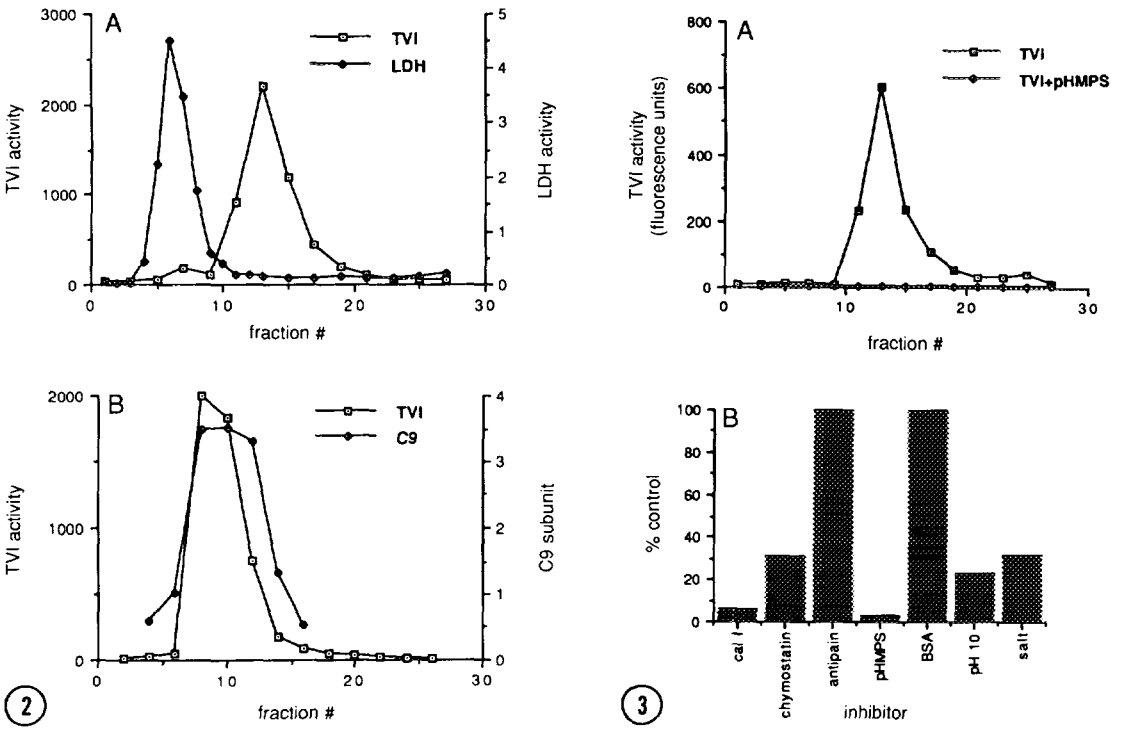

Figure 2. The TVI hydrolyzing activity in PC 12 cells is separated from cytosolic markers on sucrose gradients and co-fractionates with a subunit of the multicatalytic enzyme. A. 2 mls of a postnuclear supernatant of PC 12 cells were loaded onto a 15-50% linear sucrose gradient and centrifuged for 16 hours at 25,000 rpm in a SW 28.1 rotor (Beckman). 600 µl fractions were collected from the top of the gradient. TVI and LDH activities were assayed as described in Methods. B. The amount of the C9 subunit in each fraction was assessed by immunoblotting with a polyclonal rabbit antibody and quantitated by densitometry. The TVI hydrolyzing activity in each fraction was also determined.

Figure 3. The TVI hydrolyzing activity is inhibited by pHMPS, calpain inhibitor I and salt (150 mM NaCl). A. Fractions from the sucrose gradient were assayed for TVI hydrolyzing activity in the presence or absence of 0.1 mM pHMPS. B. Fractions containing the TVI activity were pooled from the sucrose gradient and 10 µl aliquots were assayed for activity in the presence of the indicated inhibitors. The concentrations are : calpain inhibitor I , 20 µg/ml; chymostatin, 0.1 mM ; antipain, 0.1 mM ; pHMPS, 0.1 mM; BSA, 1 mg/ml. Activity at pH 10 was assayed in 50 mM glycine. To assay activity in the presence of salt the buffer was 50 mM Tris pH 7.4; 150 mM NaCl. The TVI hydrolyzing activity in 50 mM Tris, pH 7.4,without salt was equivalent to the activity in HB. The data are combined from several experiments where the total activity ranged between 400-600 fluorescence units and the buffer backgrounds were 10-15 fluorescence units.

Antipain inhibits the GGR activity specifically and has no effect on the other two activities. In addition, the GGR hydrolyzing activity is maximal at pH 10 while the other two proteases exhibit no activity at this pH. BSA is a potent inhibitor of the LLE activity but has no effect on the GGR or GGF activities. We found that TVI-AMC hydrolysis is inhibited by calpain inhibitor I and chymostatin but not by antipain or BSA. No hydrolysis of the TVI fluorescent substrate is observed at pH 10. By comparing the inhibitor profile of these various protease activities with that for TVI hydrolysis, we conclude that the TVI-AMC substrate is hydrolyzed by the chymotrypsin-like activity of the multicatalytic enzyme (Table 1 and Figure 3B).

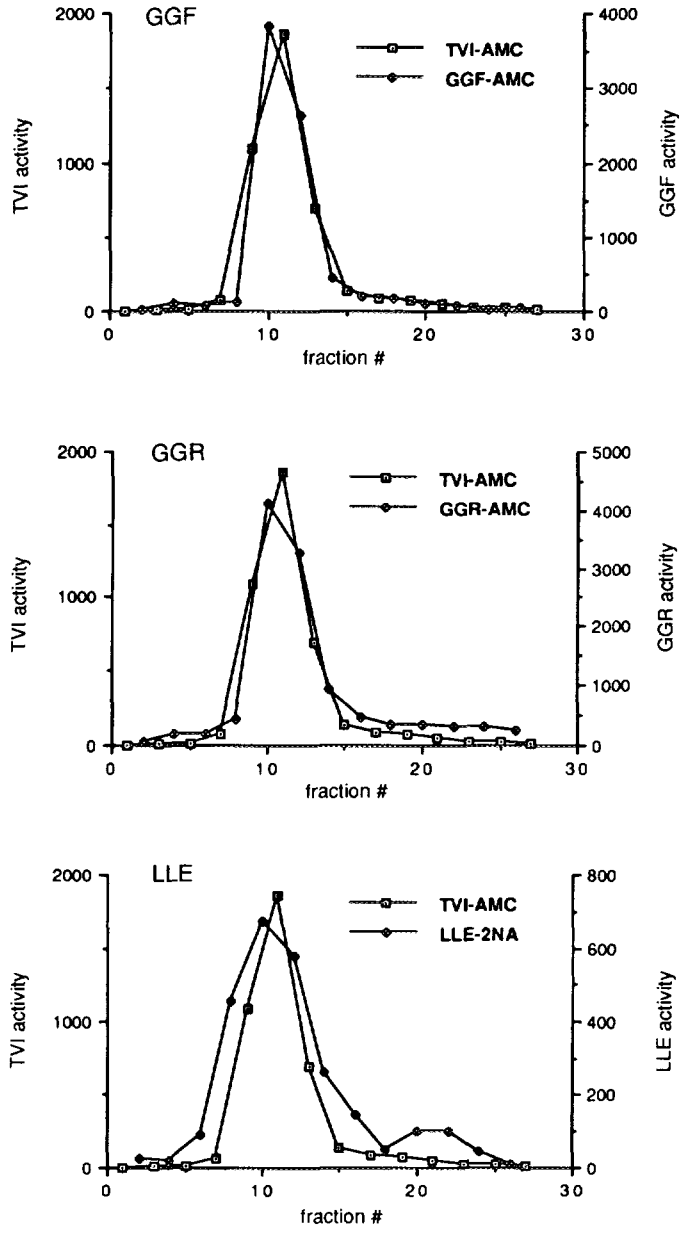

Figure 4. The TVI hydrolyzing activity cofractionates with the multicatalytic proteolytic activities. 20 µl of each fraction was assayed for TVI hydrolyzing activity in a total volume of 50 µl. The fractions were also assayed for activities that hydrolyzed each of the indicated substrates in a total volume of 100 µl containing 40µl of each fraction.

DISCUSSION

We are interested in identifying proteases capable of hydrolyzing the APP at the C-terminus of the Aß peptide which are therefore candidates for the γ-secretase. Using a small fluorescent substrate containing the amino acids TVI which contains the hydrolysis site of the longest Aß

Table 1. PERCENT INHIBITION

	pHMPS	Calpain inh I	antipain	Chymostatin	BSA
TVI	97%	94%	0%	69%	0%
GGF	98%	97%	7%	78%	10%
GGR	79%	67%	84%	39%	2%
LLE	88%	3%	0%	5%	82%

peptide described (1-43), we identified a single protease activity in PC 12 cells. Furthermore, we have demonstrated that this TVI hydrolyzing activity is the multicatalytic enzyme or proteasome. The multicatalytic enzyme is a 19S particle found in the cytosol of all eukaryotic cells that is incorporated into a 26S particle in an ATP dependent manner where it provides the catalytic core of the ubiquitin-dependent degradation pathway (32). This enzyme complex also participates in the regulation of mitosis where it hydrolyzes cyclins and other proteins, to allow exit from M phase into G1 (33,34,35). The evidence that the TVI hydrolyzing activity identified here is the multicatalytic enzyme is 1) the TVI hydrolyzing activity is located in the cytosol and behaves as if it is associated with a large particle, 2) the TVI hydrolyzing activity co-fractionates with the three enzymatic activities that are associated with the multicatalytic enzyme , 3) the same sucrose gradient fractions that contain these proteases also contain the C9 subunit of the multicatalytic enzyme and 4) the TVI hydrolyzing activity is inhibited by the same inhibitors that block the chymotrypsin-like activity of the multicatalytic enzyme.

Other reports have suggested that the multicatalytic enzyme may also cleave the APP's but these have focused on sites at the amino terminus of the Aß peptide (36,37,38). How might the multicatalytic enzyme, a cytosolic enzyme, act as a γ-secretase since the C-terminus of the Aß peptide is within the putative membrane spanning domain? One interesting possibility is suggested by the recent report that the membrane spanning domain anchoring proteins in the Golgi apparatus is much shorter (15 amino acids) than the domain anchoring proteins in the plasma membrane (20 amino acids) (39). This difference seems to arise because of the increasing cholesterol content, which increases the thickness of the membrane, as you progress from the ER to the plasma membrane. In eukaryotes, the ER has at most a few percent cholesterol, while the Golgi apparatus contains significantly more until at the plasma membrane the cholesterol content is approximately equimolar to the sum of all other lipids. Therefore, as a protein traverses the secretory pathway amino acids which are outside the membrane in the ER and the Golgi apparatus may become buried as the protein reaches the plasma membrane. The predicted transmembrane domain of APP is 24 amino acids (see Figure 1). If we allow 15 amino acids in order to traverse the membrane when the APP is in the ER or the Golgi apparatus then the TVI sequence is in the cytoplasmic, not

the membrane spanning domain and would be accessible to cytosolic proteases. Interestingly, both immunofluorescence and subcellular fractionation studies show that a significant amount of the intracellular APP accumulates in the Golgi apparatus, which may be an important factor in processing by the γ-secretase.

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