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Cloning and tissue distribution of the cyclic AMP generating peptide of the grey flesh fly *Neobellieria bullata* (Diptera : Sarcophagidae)

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ABSTRACT. Originally, two forms of cyclic AMP Generating Peptide (cGP) were identified in whole body extracts of the flesh fly *Neobellieria bullata*. The long form, a 48-mer, stimulated cAMP production by Malpighian tubules of *Manduca sexta*. The short form, which had been discovered previously with an ovarian bioassay, turned out to be the 1-15 aa sequence of the 48-mer. We here report on the cloning, sequencing and expression profile of the cGP-gene in *N. bullata*. The full-length cDNA sequence, obtained by RT-PCR in combination with 5´ and 3´ RACE, encodes a single copy of the cGP precursor. No signal peptide is present in this precursor, which, compared to the mature peptide, is only extended at the N-terminus with an extra methionine residue. RT-PCR revealed that Neb-cGP is expressed in both larval and adult brain, testis and ovaries, flight muscles, Malpighian tubules, midgut, and fat body.

KEY WORDS : insect endocrinology, reproduction, neuropeptide, gonadotropin, testis.

INTRODUCTION

In an attempt to identify new hypothalamic hormones regulating anterior pituitary hormone secretion, MIYATA & collaborators (1989) discovered a novel member of the secretin/glucagon/VIP peptide family. This peptide was called Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), reflecting its ability to potently stimulate cAMP accumulation in cultured adenohypophyseal cells. PACAP occurs as two variants, PACAP38 (a 38 amino-acid polypeptide) and the C-terminally truncated form PACAP27. The evolutionary origin and history of PACAP in Chordates, from Tunicates to mammals and birds, are well documented (VAUDRY et al., 2000).

In line with the PACAP research in vertebrates, SCHOOFS et al. (1994) engaged in the search for cAMP-stimulating peptides in insects, in particular in the flesh fly *Neobellieria* (=*Sarcophaga) bullata* Parker, 1916. First, a 15-mer peptide was isolated that potently stimulated cAMP production by young vitellogenic ovaries. Next, a 48-mer was isolated by using by Malpighian tubules of *Manduca sexta* Linnaeus, 1763 (SPITTAELS et al., 1996) as a bioassay. It turned out that the 15-mer peptide is the N-terminal sequence of the 48-mer. Neither the 15-mer nor the 48-mer displays any substantial structural similarity to PACAP. However, a ubiquitous 58-mer protein displaying pronounced sequence similarity with cGP has recently been identified in rats, treated with Streptozotocin, a glucose analogue that induces type-1 diabetes by destroying the pancreatic β-cells. This protein was named DAPIT (diabetes-1 associated protein in insulin sensitive tissues) (PAIVARINNE & KAINULAINEN, 2001). Genes coding for analogue proteins have been described in several vertebrate species, but in all of them their function remains enigmatic.

In this paper we report the cloning and the expression pattern of the 48-mer cGP gene and its similarity to the as yet functionally uncharacterized *Drosophila* gene CG15304. In addition we also measured the effect of both the 1-15 mer and full-length fly cGP on cAMP production by several fly tissues.

MATERIAL AND METHODS

Breeding and tissue collection

Neobellieria bullata individuals were reared as described by HUYBRECHTS & DE LOOF (1982). Staging of ovarian development was done according to PAPPAS & FRAENKEL (1978) BYLEMANS et al. (1997). Ovaries are staged as 4A, 4B, 4C and M when the terminal oocytes occupy 25, 50, 75 and 100%, respectively, of the total length of the ovarian follicle. As long as the larvae feed on liver, they are referred to as liver-stage larvae (LsL). After they stop feeding they start searching for a dry environment [wandering stage (ws)] and finally pupate 3 days later.

Female flies in the stages of 4B and 4C and males of corresponding age were collected, lightly anaesthetized with CO_2 and dissected in *Neobellieria* Ringer (121.5 mM NaCl, 10 mM KCl, 1.0 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.7 mM $MgCl_2$, 2.2 mM $CaCl_2$, pH 6.8 (BYLEMANS et al., 1998)).

To study the expression of Neb-cGP at the level of mRNA, the following tissues were collected and immediately transferred to liquid nitrogen. From adults of both sexes : batches of 20 gonads, 100 Malpighian tubules, 20 midguts, 20 abdominal fat body masses and 10 flight muscle masses. From larvae (unsexed) in the wandering stage : 100 brains.

For cAMP measurements, the following individual organs were dissected and transferred to the wells of a micro-titre plate containing 100 µl of IBMX (100 µM) in *Neobellieria* Ringer : ovary (4A and 4B stage), testis, brain, a piece of flight muscle, fat body and Malphigian tubule.

RT-PCR, Cloning and Sequencing

mRNA was purified from brains of vitellogenic females using the Quick prep® micro mRNA purification kit (Amersham, Pharmacia Biotech) according to the manufacturer's instructions. Single stranded cDNA was synthesized according to the protocol of the Marathon™ cDNA amplification Kit (Clontech Laboratories Inc) : 1 µg of mRNA was reverse transcribed using 20 units of AMV reverse transcriptase and the provided cDNA synthesis primer.

Based on the known sequence of Neb-cGP 48–mer (Spittaels et al., 1996) two degenerated primers, spanning a theoretical cGP cDNA internal fragment of 120 bp, were designed and obtained from Eurogentec, Belgium.

1. cAMPGRP F :
   5′GCNGARAARYTNWSNGGNYTN3′
2. cAMPGRP R :
   5′GGYTTCATNACRTTRTANGCDA3′

PCR reaction mixtures contained 2 µl (<0.1µg) of brain cDNA template, 1 µl of 50X Advantage® 2 Polymerase Mix, 5 µl of 10X Advantage® 2 PCR Buffer, 1 µl of each dNTPs (10 mM), 5 µl of each primers (10 µM) and H_2O added up to a 50 µl reaction volume. Hot start PCR was performed in a Thermotrio-Block TB-1 Thermocycler (Biometra). Thermal cycling consisted of 30 cycles with a denaturation for 1 min at 94˚C, annealing step for 1 min at 35˚C, extension for 1 min at 68˚C and an extra elongation step for 5 min at 68˚C. A second PCR amplification, using 1 µl of the above PCR result and identical PCR conditions was carried out.

For cloning, the PCR products of the second round were first electrophoretically separated in a 3.5% Nusieve® GTG® (low melting) agarose (Sanvertech) gel in 1X TAE buffer. The band of the expected size was excised and directly ligated into PCR® 2.1-Topo vector (low melt agarose method). This ligation product was used to transform competent *E. coli* Lacz(cells according to the protocol of the Topo TA cloning ® Kit (version I, Invitrogen, The Netherlands).

Recombinant plasmid DNA was isolated from the obtained white colonies according to the instructions of the manufacturer of the High pure plasmid Isolation Kit (Roche Molecular Biochemicals, Germany). Automated sequencing was performed on an ABI Prism(R) sequencer 310 (Applied Biosystems) using the ABI PRISM(R) Big Dye™ Terminator Cycle sequencing Ready Reaction Kit version 2.0 (PE Biosynthesis).

Rapid amplification of cDNA ends and identification of full length nucleotide sequence of Neb-cGP

In order to obtain a complete nucleotide sequence, Rapid Amplification of cDNA Ends (RACE) was employed (Frohman et al., 1998; Janssen et al., 2001). 5′ and 3′ ends RACE PCR, and 3′-nested RACE PCR was performed according to the protocol of the Marathon ™ cDNA amplification kit (Clontech, Westburg, The Netherlands). Adaptor-ligated double stranded cDNA served as template for these RACE PCR. Single stranded cDNA (as described previously) was now synthesized from total RNA that was isolated from 100 mg of brain tissue of young vitellogenic females using Trizol Reagent (GIBCO-BRL) (Chomczynski & Saachi, 1987).

5′ and 3′ RACE reactions were performed using Marathon Adaptor Primer (AP_1, Clontech) in combination with one of the respective gene specific primers, which were constructed according to the previously obtained sequence information : 5′-ATG-CGA-TGA-TCA-AAC-CAA-TG-3′ for 5' RACE and 5′- CAT-GGC-TGG-ACG-TGC-TAA-TGT-TGC-3′ for 3' RACE.

In the case of 3′ end RACE reaction, nested PCR were performed using nested AP_2 primer (Clontech) and nested gene specific primer 5′CATCGCATACAACGTAATGAAGCC3′. RACE products were analysed by 1.2% agarose gel electrophoresis and purified using Qiaex II DNA extraction kit (Qiagen). Purified DNA fragments were cloned into PCR® 2.1 Lacz α vector employing the TOPO TA Cloning kit (Invitrogen). Further downstream processing, up to sequencing of the inserts, was done as described above.

Sequence analysis

Blast programs were used to search databases for sequence homologies (Altschul et al., 1997). Multiple sequence alignment results were obtained by employing the Clustal W program (European Bioinformatics Institute). For prediction of the presence of a signal peptide, the sequence was analysed with Prot param tool and Signal P V2.O web site.

RT-PCR : Tissue specific expression profile

The expression of Neb-cGP in different organs/tissues of both sexes (brain, flight muscle, midgut, Malpighian tubule, fat body, testes and ovaries) was examined using reverse transcriptase PCR (Peeters et al., 1999). Single stranded cDNAs were prepared using total RNA as described before. Neb-cGP specific primers (5′CGGTGCTGAAGCT-GAAAAAT-3′, 5′GGCAATGATCAAACCATGA-3′, Eurogentec, Belgium) were used for the subsequent PCR. Hot start PCR was performed for 30 cycles with a denaturation step for 1 min at 94˚C, annealing for 1 min at 45˚C and elongation for 30 sec at 68˚C. As a positive control for cDNA quality actin primers were used. The amplified PCR products were visualized under UV transillumination following 1.5% agarose gel electrophoresis.

cAMP stimulation assay

Synthetic 1-15 mer (Invitrogen) and full-length peptide (a kind gift from Dr. Hui, Tian, Tularik, San Francisco) of Neb-cGP were tested for their ability to stimulate cAMP production in the different organs/tissues of *Neobellieria*.

Following dissection, the organs/tissues were incubated in wells of a microtitre plate for 1h at 35˚C in 100 µl of Ringer containing 100 µM IBMX. After 1h, 25 µl of the incubation medium was removed for quantification of basal release level of cAMP and replaced by 25 µl of test peptide dissolved in IBMX-Ringer. Incubation at 35˚C continued for 1h. Next, a 25 µl aliquot of incubation medium was taken from each well for cAMP measurement according to the manufacturers instructions (Amersham ^{3}H cAMP assay kit).

RESULTS

Cloning of full–length Neb-cGP cDNA sequence

According to the information provided in the Materials and Methods section, two subsequent PCR reactions at a rather low annealing temperature of 35˚C and using a degenerated primer combination were needed for obtaining an internal Neb-cGP cDNA fragment starting from brain mRNA. The resulting fragment provided sequence information for designing the gene specific primers used in the subsequent RACE reactions. Combination of the sequence information obtained with 5′ and 3′ RACE allowed the reconstitution of the full-length cDNA sequence (Fig. 1) (GenBank accession number AY141181). *In silico* translation of the cDNA revealed a singly open reading frame corresponding to the Neb-cGP precursor, which lacks a signal peptide and, when compared to the purified mature peptide, only has an extra N-terminal methionine. Although no conserved polyadenylation motif can be traced in the cDNA sequence, a 3′ poly A stretch is clearly present in addition to a short 5′ leader and 3′ trailer sequence. The entire cDNA sequence up to the poly A tail is only 188 bp long.

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                  M  A  G  A  E  A  E  K  L  S  K  L  S  K  Y
5′- taataacaaca**ATG**GCCGGTGCTGAAGCTGAAAAATTAAGTGGTTTATCCAAATAT
 F  N  G  T  T  M  A  G  R  A  N  V  A  K  A  T  Y  A  V  I
TTCAATGGTACAACCATGGCTGGACGTGCTAATGTTGCTAAAGCCACTTATGCTGTCATTG
 G L  I  I  A  Y  N  V  M  K  P  K  K  K
GTTTGATCATTGCCTACAATGTCATGAAACCCAAAAAAAAAG**taa**attttgttattgttgtt
acaattcgca*AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*-3′
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Fig. 1. – Complete cDNA sequence with deduced amino acid sequence of *N. bullata* cyclic AMP Generating Peptide. Start (ATG) and stop (taa) codon marked in bold, Poly-A tail shown in italics.

Blast searches for conserved genes in other species pointed towards the occurrence of an homologous gene (AE003450, CG15304) in *Drosophila* showing 67% sequence identity at cDNA level (Fig. 2A) and 81% at the amino acid level. Both Neb-cGP and the corresponding *Drosophila* cDNA share 46% identity with *Rattus norvegicus* DAPIT cDNA. At the amino acid sequence level (Fig.2B) Neb-cGP shows 43% identity and 62% similarity to this ubiquitously expressed 58-mer DAPIT protein originally found in diabetic rats. There are homologs of DAPIT in all studied mammals as well as in *Xenopus* (Paivarinne & Kainulainen, 2001).

RT-PCR

Tissue specific expression of Neb-cGP in a variety of tissues was examined by RT-PCR (Fig. 3 A and B). The cDNA quality of all samples was confirmed by generating an actin fragment (data not shown). A large amount of mRNA encoding Neb-cGP was detected in all tissues tested, namely brain, flight muscle, midgut, Malpighian tubules, fat body, testis and 4A ovaries. Not only the adult brain but the larval one as well expresses Neb-cGP gene.

cAMP stimulation by Neb-cGP

Because only small amounts of the synthetic full-length 48 mer fly peptide were available, only a few experiments with ovaries and testis but not with other tissues could be done. The 1-15 mer peptide of Neb-cGP could be tested more extensively, namely on 4A and 4B stage ovaries, testis, flight muscle Malphigian tubules, brain and fat body. In all tissues tested the basic rate of cAMP release into the incubation medium during 1 h incubation varied from 0.7 to 1.4 (±0.1-0.6 SD) pmol. Substantial stimulation of cAMP release could only be obtained in testis and 4A ovaries. The strongest stimulation, namely sevenfold, was obtained for testis with the 1-15 mer peptide at a final concentration of 17.5 µM (9.8 (3.3 pmol/h versus 1.4 (0.6 pmol/h in the control). At the same dose the 1-15 mer less potently stimulated the ovaries (1.7±0.4 pmol/h versus 1.3 ±0.1 pmol in the control). Three replications were used for each dose.

TABLE 1

Activity of 1-15 mer cGP in different tissues (data in parenthesis indicate only single replication for 1-48 mer peptide)

Tissues	Pre incubation Mean ± SD (Picomol/h)	After incubation Mean ± SD (Picomol/h)
Testis	1.40 ± 0.58 (0.59)	9.84 ± 3.26 (15.37)
Ovary	1.32 ± 0.08 (0.71)	1.70 ± 0.40 (3.64)
Flight muscle	1.08 ± 0.03	1.28 ± 0.05
Malphigian tubules	0.91± 0.03	1.04 ± 0.04
Fat body	1.40 ± 0.07	1.39 ± 0.23
Brain	0.7 ± 0.05	0.7 ± 0.01

At a concentration of 27 µM full-length peptide provoked a very strong cAMP production in testis (15.4 pmol/h versus 0.6 pmol/h in the control). Ovaries were less stimulated than testis (3.6 pmol/h versus 0.7 pmol/ h in the control at the same concentration).

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Neobellieria    ------------------------------------------------------------
Drosophila      ATCGAAGACCGATAGACCTCGATAGACTCAGTTGTATAACCTCTGCTCGCAAATATTTTT 60
Rattus          ------------------------------------------------------------

Neobellieria    ------------------------------TAATAA--CAACAATGGCCGGTGCTGAAGC 28
Drosophila      TACAGGCAACTAGCAGCTCGAATTCCGTGCAAATAAACCTTTAAAAATGGCTGGCGAAGG 120
Rattus          -----GTGATCGGACGAAGAAGATTGAAGTCATGGCTGGCCCAGAAAGTGATGGCCAATT 55
                                          *          *      * **   **

Neobellieria    TGAAAAATTAAGTGGTTTATCCAAATATTTCAATGGTACAACCATGGCTGGACGTGCTAA 88
Drosophila      CGAGAAACTGACCGGCCTGTCGAAAATCTTCAATGGCACCACCATGAGCGGCCGTGCAAA 180
Rattus          CCAG---TTCACTGGTATTAAAAAATATTTCAACTCTTATACCCTCACAGGTAGAATGAA 112
                  *     * *  **  *    ***   *****       *** *    **  *    **

Neobellieria    TGTTGCTAAAGCCACTTATGCTGTCATTGGTTTGATCATTGCCTACAATGTCATGAAACC 148
Dosophila       TGTGGCCAAGGCCACGTACGCCGTGATGGGCCTGCTGATCGCCTACCAGGTGCTGAAGCC 240
Rattus          TTGTGTCCTGGCCACATATGGAGGCATTGCTTTGTTGGTCCTATACTTTAAGTTAAGGCC 172
                *   *     ***** ** *  *  ** *   ** *  *    ***       * *  **

Neobellieria    CAAAAAAAAGTA---------AATTTTGTTATTGTTGTTACAATTCGCAAAAAAAAAAAA 199
Drosophila      CAAGAAGAAGTAGAGGGATGCAACTGCTCCAACGCGGCTACAACTCATCCGCCACAATCG 300
Rattus          TAAAAAAACCCCAGCTGTGAAAGCAACATAAATGGATTTTGAAATGTCTGGCCTTATCTG 232
                 ** ** *             *        *  *    *  ** *          *

Neobellieria    --AAAAAAAAAAAAAAAAAA---------------------------------------- 218
Drosophila      --ATGTCTTGGATCCGGGGCCATCGCTG-GACTGCGGATCAGTTTATTTTATGTGTAGTG 357
Rattus          TTAAGTCCCACGCCTGAAGAAGCTGATGTGAACTCATCATGTAATACTCAATTTGTACAA 292
                  *

Neobellieria    ------------------------------------------------------------
Drosophila      CAAGACCTTGAAGGAATAAATAAGCTGAGAATGTAACACACTGCAAAAAAAAAAAAAAAA 417
Rattus          TAA-ATTATGAACCTGGAAAAAAAAAAAAAAA---------------------------- 322
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Fig. 2. – Nucleotide and protein sequence alignment.

2A. Nucleotide sequence alignment-showing comparison between Neb-cGP, the *Drosophila* homolog and the *Rattus norvegicus* DAPIT. Residues that are identical in all precursors are indicated with an asterisk (*).

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Neobellieria      -AGAEAE---KLSGLSKYFNGTTMAGRANVAKATYAVIGLIIAYNVMKPKKK------ 48
Drosophila        -MAGEGE---KLTGLSKIFNGTTMSGRANVAKATYAVMGLLIAYQVLKPKKK------ 48
Rattus            MAGPESDGQFQFTGIKKYFNSYTLTGRMNCVLATYGGIALLVLYFKLRPKKTPAVKAT 58
                    . *.:   :::*:.* **. *::** * . ***. :.*:: *  ::***.
```

2B. Sequence alignment for the cGP protein of *Neobellieria*, *Drosophila,* and *Rattus norvegicus* DAPIT. Symbols under each set of lines indicate identity (*), highly conserved substitutions (:) and conserved substitutions (.).

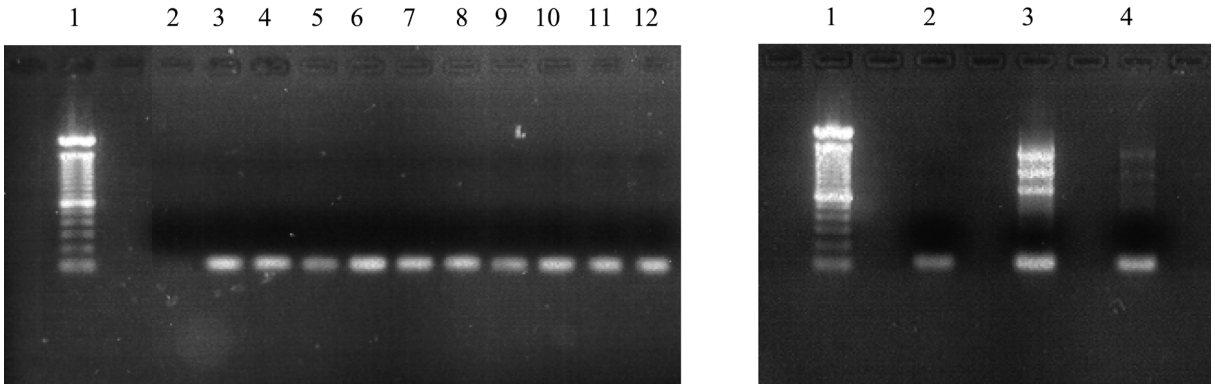

Fig. 3. – Tissue distribution of Neb-cGP as revealed by RT-PCR.

3A (left). Lane 1 : 100 bp DNA ladder. Lane 2 : Water control, Lane 3 and 4 : Flight muscle from female and male flies. Lane 5 and 6 : Midgut from female and male flies. Lane 7 and 8 : Malpighian tubules from female and male flies. Lane 9 and 10 : Fat bodies from female and male flies. Lane 11 and 12 : ovary and testis.

3B (right). Lane 1 : DNA size marker. Lane 2 and 3 : Brain from female and male flies. Lane 4 : Larval brain.

DISCUSSION

The aa-sequence of 48-mer cGP as originally reported by SPITTAELS et al. (1996) was confirmed by our sequencing of the cGP cDNA with its single open reading frame. The 1-15 mer is probably a proteolytic cleavage product of the 48-mer but the aa sequence at position 15-16, namely phenylalanine-asparagine, is not a common cleavage site. In a mass spectrometrical analysis of the corpus cardiacum of *Neobellieria*, BAGGERMAN et al. (2002) could only detect the 48-mer, not the 1-15 mer. Obviously the 1-15 mer was a degradation product that was formed during the extraction procedure as applied by SCHOOFS et al. (1994). Using immunocytochemistry these authors identified cGP immunopositive cells in the pars lateralis of the brain but unfortunately they did not test other tissues. In combination with their mass spectrometric detection of the 48-mer in extracts of fleshfly corpora cardiaca they suggested synthesis of cGP in the brain followed by storage in and subsequent release from the corpora cardiaca. Accordingly cGP is assumed to be a neurohormone. The results obtained in the bio-assay, using distinct tissues and major response to exogenously applied cGP being observed with testis and to a minor extend with ovaries as well, make it tempting to speculate about cGP being a true gonadotropin.

Our cDNA sequencing data are in conflict with this assumption. The usual situation is that the precursor of secreted proteins contains a signal peptide that is cleaved off when the peptide is secreted. However, there is no signal peptide encoded in the cGP cDNA, neither in *Neobellieria*, nor in *Drosophila*. Until the opposite will be proven we have to assume that the mature cGP peptide is not released from the cells where it is produced. Anyhow, in receptive tissues there should be a signalling system that is activated by exogenously applied cGP. Either cGP interacts with an as yet unidentified plasma membrane receptor or the peptide can surpass the plasma membrane and directly interact with the cAMP generating pathway intracellularly. This last possibility has been suggested for the related DAPIT protein by PAIVARINNE AND KAINULAINEN (2001). They used several software programmes such as HMMTOP, TMpred, DAS and SPLIT to obtain conformational information about DAPIT. Their results suggest the possibility of an outside-inside oriented transmembrane helix at the C-terminus while the N-terminus should be directed outwards in both DAPIT protein and Neb-cGP. If this prediction is correct, direct signal transduction activity of this externally applied protein could be realistic. On the other hand our own observation that the 1-15-mer N-terminal sequence suffices for stimulating cAMP production does not support this assumption.

The wide tissue distribution of cGP in flies, and the occurrence of similar proteins throughout the animal Kingdom where the coding mRNA also have such an ubiquitous tissue distribution, suggest that this family of proteins somehow plays a key role in some physiological processes. It will be a challenge to uncover its exact role(s).

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