On Complex I and Other NADH:Ubiquinone Reductases of *Neurospora crassa* Mitochondria

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The mitochondrial complex I is the first component of the respiratory chain coupling electron transfer from NADH to ubiquinone to proton translocation across the inner membrane of the organelle. The enzyme from the fungus *Neurospora crassa* is similar to that of other organisms in terms of protein and prosthetic group composition, structure, and function. It contains a high number of polypeptide subunits of dual genetic origin. Most of its subunits were cloned, including those binding redox groups. Extensive gene disruption experiments were conducted, revealing many aspects of the structure, function, and biogenesis of complex I. Complex I is essential for the sexual phase of the life cycle of *N. crassa*, but not for the asexual stage. In addition to complex I, the fungal mitochondria contain at least three nonproton-pumping alternative NAD(P)H dehydrogenases feeding electrons to the respiratory chain from either matrix or cytosolic substrates.

KEY WORDS: Mitochondria; complex I; NADH:ubiquinone reductases; alternative NADH dehydrogenase; gene disruption; mutants; *Neurospora crassa*.

INTRODUCTION

The filamentous fungus *Neurospora crassa* has been used for decades as a very useful model organism for the study of many biological processes (Davis, 2000). It allies various advantageous characteristics, which are usually searched for in other organisms, both in the prokaryotic and eukaryotic worlds. Several contributions to the current understanding of bioenergetics and mitochondrial biogenesis came from research with *N. crassa*. In particular, the fungus has been employed in investigations on the respiratory chain complex I and alternative NADH dehydrogenases (Weiss *et al.*, 1991; Videira, 1998).

Complex I or the proton-pumping NADH dehydrogenase is a main component of the mitochondrial respiratory chain, located in the inner membrane of the organelle (Hatefi, 1985; Walker, 1992). Special difficulties in char-

acterizing complex I arise from the fact that it is a huge complex of proteins and other molecules, with a molecular mass of about 1 MDa. At least 42 (possibly 43) polypeptide subunits, with a dual genetic origin, were identified as constituents of the mammalian complex I (Skehel *et al.*, 1998). Most of them are encoded by nuclear genes, synthesized in the cytoplasm, and imported into mitochondria. Some of the subunits, seven in humans, are hydrophobic proteins encoded by mitochondrial DNA (Chomyn *et al.*, 1986). The well-known function of complex I is the electron transfer from the substrate NADH to ubiquinone, through a number of protein-bound prosthetic groups, FMN, 6–8 iron–sulfur clusters, and quinones. This electron transfer reaction is coupled to the translocation of protons across the inner mitochondrial membrane, which contributes to the maintenance of the proton-motive force that eventually results in ATP synthesis (Hatefi, 1985; Walker, 1992). Among many others, rotenone and piericidin A are well-known inhibitors of complex I activity (Degli Esposti, 1998). Several lines of evidence indicate that complex I is involved in other cellular functions, although this has not yet been convincingly proved.

Bacteria (like *Escherichia coli* and *Paracoccus denitrificans*) contain proton-pumping enzymes equivalents

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of mitochondrial complex I, also named NDH1 (Weidner *et al.*, 1993; Yagi *et al.*, 1998). The *E. coli* complex is also capable of pumping sodium (Steuber *et al.*, 2000). Although possessing a similar constitution of redox groups, these bacterial enzymes display a simpler polypeptide composition. They are composed of 14 polypeptide subunits and considered as the "minimal" structure required to perform electron transfer coupled to proton translocation: seven homologs of the hydrophobic subunits coded by the complex I genes of mitochondrial DNA, ND1-6, and ND4L, and seven homologs of the mammalian 75−, 51-, 49-, 30-, 24-, 23- (TYKY), and 20-kDa (PSST) proteins (Walker, 1992; Weidner *et al.*, 1993). Enzymes equivalent to mitochondrial complex I were also identified in chloroplasts and, more recently, in archaea. Besides this, several complex I subunits display similarity with other proteins, in particular, with different types of hydrogenases. Comparisons between all these proteins brought considerable insights about the origin, evolution, and structure of complex I (Friedrich and Scheide, 2000).

Structurally, complex I appears as two major domains, the peripheral and membrane arms, arranged perpendicularly to each other in the form of an L. The membrane arm is mainly embedded in the mitochondrial inner membrane, while the majority of the peripheral arm is located in the matrix (Hofhaus *et al.*, 1991). The latter contains most, if not all, prosthetic groups of complex I and is thus responsible for the electron-transfer reactions. It is believed that the membrane arm is engaged in proton translocation (Friedrich *et al.*, 2000). Each arm is assembled independently of the other and then the two arms are joined together (Tuschen *et al.*, 1990). Additional proteins not present in the final complex (chaperones) are involved in the process of complex I assembly (Kuffner *et al.*, 1998).

Deficiencies of complex I have been implicated in human mitochondrial diseases, plant and fungal development, and several abnormal phenotypes in different microorganisms (Rasmusson *et al.*, 1998; Smeitink *et al.*, 1998; Videira, 1998). In addition, despite the fact that some fermentative yeasts lack complex I (Overkamp *et al.*, 2000), the enzyme is essential for the survival of organisms like *P. denitrificans* (Finel, 1996) and, presumably, humans.

In addition to mitochondrial complex I, there are nonproton-pumping enzymes, the so-called alternative NADH dehydrogenases, that oxidize NADH and feed electrons into the respiratory chains of different organisms. These are single polypeptide enzymes that may use NAD(P)H from either the cytosol (external enzymes) or the mitochondrial matrix (internal enzymes). Their number and specificity vary among different organisms: from none in humans up to four proteins in plants have been described (reviewed by Kerscher, 2000).

COMPOSITION OF FUNGAL COMPLEX I

Complex I of *N. crassa* was estimated to contain around 35 polypeptide subunits (Weiss *et al.*, 1991). At least seven of them are encoded by mitochondrial DNA and synthesized in the organelle (Ise *et al.*, 1985; Videira and Werner, 1989). It is still possible that unidentified reading frames of the fungal mitochondrial genome (Davis, 2000) also code for complex I subunits. Twenty of the subunits encoded by the nuclear genome have been cloned and an additional 9.8-kDa subunit, homologous to bovine MWFE, was identified from the sequencing of *Neurospora* chromosomes by a German group (Table I). Among them, all protein homologs of the 14 subunits that constitute bacterial complex I were identified (Sousa *et al.*, 1999). It is now hoped that most other subunits, if not all, can be found in the course of ongoing projects for sequencing the entire genome of the fungus. The similarity between the fungal enzyme and that of higher eukaryotes is quite clear. Only three proteins (out of 28) appear to be fungal specific. With data from classical genetic crosses, analysis of restriction fragment-length polymorphisms (Ferreirinha *et al.*, 1998) and again the German sequencing project (www.uni-duesseldorf.de/WWW/MathNat/biochem/genome.shtml), 14 genes could be mapped in specific chromosomes of the *N. crassa* genome, which contains seven chromosomes or linkage groups.

Although several models have been put forward (Brandt, 1997; Dutton *et al.*, 1998), the mechanism of proton pumping by complex I remains unknown and its elucidation might require a refined crystallographic characterization of the enzyme. On the other hand, much more is known about the electron transfer processes (Ohnishi, 1998; Ohnishi *et al.*, 1998). In *N. crassa*, only four Fe–S clusters were observed by EPR spectroscopy, a binuclear center N1, and the tetranuclear centers N2, N3, and N4 (Wang *et al.*, 1991). However, the conservation of amino acid motifs suggests that the fungus also contains the centers detected in other organisms, namely, the bovine clusters N1a and N5. Cluster N5 was detected in the fungus *Yarrowia lipolytica* (Djafarzadeh *et al.*, 2000). The assignment of FMN and the Fe–S clusters to specific polypeptide subunits is more or less established (Videira, 1998). Still some uncertainty surrounds the assignment to either the 21.3c− or the 19.3-kDa proteins (TYKY and PSST homologs, respectively) of cluster N2. This assignment is particularly important since it is believed that a proton translocation step occurs concomitant to the oxidation

Table I. Characteristics of Subunits of Complex I from *Neurospora crassa*[a]

N. crassa protein kDa (LG)	Bovine/*E. coli* homologs	Predicted function	Relationships
78 (IIR)	75/NuoG	$2 \times$ [4Fe–4S]; [2Fe–2S]	CI; NH; FDH
51 (II)	51/NuoF	NADH binding; FMN; [4Fe–4S]	CI; NH; FDH
49	49/NuoD	Quinone interaction?	CI; $F_{420}H_2MO$; HD
40 (V)	39	NADPH binding	CI; reductases/isomerases
30.4 (VIL)	30/NuoC		CI; $F_{420}H_2MO$; HD
29.9 (IVR)	B13		CI
24 (VR)	24/NuoE	[2Fe–2S]	CI; NH; FDH
21.3a (VR)	—		—
21.3b	—		—
21.3c (VIR)	TYKY/NuoI	$2 \times$ [4Fe–4S]	CI; $F_{420}H_2MO$; HD
21 (IVR)	AQDQ	Phosphorylated	CI
20.9	—		CI
20.8 (IL)	PGIV		CI
19.3 (VIL)	PSST/NuoB	[4Fe–4S]; quinone interaction?	CI; $F_{420}H_2MO$; HD
17.8 (V)	—		—
14.8	B14		CI
12.3 (IR)	PDSW		CI
10.5	B8		CI
9.3	B9	Quinone interaction?	CI
ACP	SDAP	Pantothenate group	CI; ACPs
9.8 (II)	MWFE		CI
ND1	ND1/NuoH	Quinone interaction?	CI; $F_{420}H_2MO$; HD
ND2	ND2/NuoN		CI; $F_{420}H_2MO$
ND3	ND3/NuoA		CI; $F_{420}H_2MO$
ND4	ND4/NuoM	Quinone interaction?	CI; $F_{420}H_2MO$; K^+/H^+ antiporter
ND4L	ND4L/NuoK		CI; $F_{420}H_2MO$
ND5	ND5/NuoL	Myristoylated	CI; $F_{420}H_2MO$; HD; Na^+/H^+ antiporter
ND6	ND6/NuoJ		CI; $F_{420}H_2MO$

[a]The references for most proteins have been cited (Videira, 1998). See also Darrouzet *et al.* (1998), Schuler *et al.* (1999), Baumer *et al.* (2000), Davis (2000), Friedrich and Scheide (2000), and Plesofsky *et al.* (2000). LG, linkage group; CI, complex I of mitochondria, chloroplasts and/or bacteria; NH, NAD^+-reducing hydrogenase; HD, membrane-bound [NiFe] hydrogenases; $F_{420}H_2MO$, $F_{420}H_2$:methanophenazine oxidoreductase; FDH, some formate dehydrogenases like that of *Moorella thermoacetica* (AAB18330).

of cluster N2, possibly the direct reducer of ubiquinone. However, a novel redox group, of still unidentified nature, might mediate electron transfer between cluster N2 and ubiquinone (Friedrich *et al.*, 2000). We presented evidence that the PSST homolog is the ligand of N2, based on the findings that both the protein and the center are loosely associated with the unassembled peripheral arm of complex I (Sousa *et al.*, 1999). In agreement with this, site-directed mutants of the bacterial PSST homolog lack cluster N2 (Friedrich, 1998) and, correlating with the inhibition of enzyme activity, the subunit was specifically labeled with complex I inhibitors (Schuler *et al.*, 1999). Further support of the location of cluster N2 in PSST comes from the analysis of site-directed mutants of the homologous 19.3-kDa protein of *N. crassa* (unpublished results). The TYKY protein binds two tetranuclear iron–sulfur clusters, still unidentified (Yano *et al.*, 1999; Friedrich *et al.*, 2000). Different approaches identified several putative proteins that interact with ubiquinone (Table I).

In addition to the "minimal" subunits required for oxidative phosphorylation, mitochondrial complex I contains a large number of polypeptides, sometimes referred to as "accessory" proteins, some of which may be organism specific. A specific function could not yet be ascribed to most of them. It was proposed that some proteins serve to insulate complex I, preventing electrons from escaping and generating oxygen radicals (Robinson, 1998), others may be involved in NADH-channeling reactions (Fearnley and Walker, 1992), or the binding of other respiratory complexes (Budde *et al.*, 2000) and proteins. At least two subunits of complex I were suggested to be involved in biosynthetic pathways: an acyl carrier protein (Schneider *et al.*, 1995) and a 40-kDa subunit, which is similar to several reductases/isomerases (Fearnley and Walker, 1992) and carries a tightly bound NADPH molecule (Schulte *et al.*, 1999). Other proteins may work as modulators of complex I activity. For instance, phosphorylation of the AQDQ protein enhances enzyme activity (Scacco *et al.*, 2000) and the

MWFE protein is required for activity of complex I (Au *et al.*, 1999). These and other possible roles of complex I await further investigation.

Neurospora crassa MUTANTS IN SPECIFIC COMPLEX I GENES

The targeted manipulation of the mitochondrial genome is not possible, hampering detailed alterations of the ND proteins in *N. crassa*. Nevertheless, we have recently analyzed the mitochondrial E35 stopper mutant of the fungus with deleted ND2 and ND3 genes. We found that lack of these proteins prevents assembly of the membrane arm of complex I, without effect on formation of the peripheral arm (Alves and Videira, 1998). A similar requirement for membrane arm assembly was described for the ND4 protein in humans (Hofhaus and Attardi, 1993) and plants (Karpova and Newton, 1999) and for the mouse ND6 protein (Bai and Attardi, 1998). In contrast, lack of the ND5 protein had no influence in complex I assembly (Hofhaus and Attardi, 1995) consistent with the location of the protein at one extremity of the membrane arm of the enzyme (Sazanov and Walker, 2000).

The generation of knockouts in the nuclear-coded subunits of complex I have been quite successful in *N. crassa*. Indeed, the fungus is the only organism where an exhaustive characterization of the role of individual subunits of complex I through gene-disruption experiments has been carried out. Gene disruption has been obtained either by the replacement of the endogenous genes with defective copies by homologous recombination or by the generation of repeat-induced point mutations in the endogenous genes (Videira, 1998). These approaches yielded 16 mutants in independent nuclear-coded subunits of complex I, 12 in subunits of the peripheral arm, and 4 in subunits of the membrane arm of the enzyme (Table II). All mutants are viable in the assexual stage of *N. crassa*, although their growth rate may be somewhat slower than that of the wild-type strain. Disruption of specific subunits disturbs the assembly of complex I more or less drastically, leading to the accumulation of subcomplexes of the enzyme. Since the lack of various proteins has a pleiotropic effect in the assembly of others, their specific function has been difficult to characterize. The expression of (nonfunctional) proteins altered by mutagenesis that allow the assembly of the others will probably circumvent this problem in the future.

Assembly studies conducted in these mutants led to some interesting observations. Consistent with the independent assembly of the peripheral and membrane arms of complex I (Tuschen *et al.*, 1990), the lack of subunits belonging to one of the arms does not interfere with formation of the other arm. No mutant in membrane arm subunits has been found that completely lacks this hydrophobic domain of complex I. All four mutants in nuclear-coded subunits of the membrane arm assemble intermediate complexes. An exception is represented by the E35 stopper mutant described above, lacking detectable intermediates of the membrane arm, but this strain displays a severe phenotype of irregular stop–start growth that can be due to the complex I phenotype. The

Table II. Characteristics of Null Mutants of Complex I from *Neurospora crassa*[a]

N. crassa mutant	Disruption method	Assembly phenotype (location of disrupted protein)	References
nuo78	RIP	Membrane arm (p)	Harkness *et al.*, 1995
nuo51	HR	"Complex I" (p)	Fecke *et al.*, 1994
nuo49	HR	Membrane arm (p)	Schulte and Weiss, 1995
nuo40	HR	"Complex I" (p)	Schulte *et al.*, 1999
nuo30.4	RIP	Membrane arm (p)	Duarte *et al.*, 1998
nuo29.9	RIP	Membrane arm; "complex I"? (p)	Duarte *et al.*, 1995
nuo24	RIP	"Complex I" (p)	Almeida *et al.*, 1999
nuo21.3a	RIP	"Complex I" (p)	Alves and Videira, 1994
nuo21.3b	HR	Peripheral arm; membrane arm intermediates (m)	Nehls *et al.*, 1992
nuo21.3c	RIP	Membrane arm (p)	Duarte and Videira, 2000
nuo21	RIP	"Complex I" (p)	Ferreirinha *et al.*, 1999
nuo20.9	HR	Peripheral arm; small intermediate (m)	Schulte and Weiss, 1995
nuo20.8	RIP	Peripheral arm; membrane arm intermediates (m)	da Silva *et al.*, 1996
nuo19.3	RIP	Membrane arm (p)	Unpublished
nuo12.3	RIP	Peripheral arm; membrane arm (m)	Duarte *et al.*, 1995
nuoACP	HR	Membrane arm? (p)	Schneider *et al.*, 1995

[a] "Complex I," complex I without the specifically disrupted protein; HR, homologous recombination; RIP, repeat-induced point mutations; p, peripheral arm; m, membrane arm.

absence of specific subunits of the peripheral arm of complex I either results in the formation of a "complex I" just lacking the protein or prevents assembly of the hydrophilic domain of the enzyme. We may define a "core enzyme" containing proteins that are homologous to other protein complexes and are required for the assembly of other complex I subunits. In the peripheral arm, we have the 49-, 30.4-, 21.3c- (TYKY), and 19.3-kDa (PSST) subunits. All are required for peripheral arm assembly, have counterparts in bacterial complex I, and are the only ones present both in an archaeal $F_{420}H_2$ dehydrogenase and in a family of multisubunit membrane-bound [NiFe] hydrogenase (Table I). Concerning the conserved ND subunits of the membrane arm, two groups may be considered. While all of them have homologs in the $F_{420}H_2$ dehydrogenase, only the ND1 and ND5 proteins are present in the membrane-bound [NiFe] hydrogenases. Thus, ND1 and ND5 constitute one group and ND2, ND3, ND4, ND4L, and ND6 constitute another group. We would like to speculate that the "core enzyme" contains proteins of the latter group. At least the ND2, ND3, ND4, and ND6 proteins are required for membrane arm assembly and, interestingly, ND5 is not. The effects of other subunits in the complex assembly remains to be seen.

As already mentioned, some mutations in subunits of the peripheral arm of complex I prevent assembly of this domain of complex I, while the membrane arm accumulates. No special activity has been attributed to the membrane arm alone. In other cases, like in mutants nuo51, nuo40, nuo24, nuo21.3a, and nuo21, an almost intact complex is formed allowing investigations on the roles of these proteins for enzyme activity. Neither the 21.3a- nor the 21-kDa proteins were found essential for the rotenone-sensitive NADH:ubiquinone reductase activity of complex I, although complex I lacking the 21-kDa subunit differs from wild-type complex I in electron transfer to artificial acceptors (Ferreirinha *et al*., 1999). Both of the iron–sulfur 51 and 24-kDa polypeptides are required for the NADH:ubiquinone reductase activity of complex I (Fecke *et al*., 1994; Almeida *et al*., 1999). It is not clear how the iron–sulfur cluster of the 24-kDa polypeptide participates in electron transfer from NADH to ubiquinone, but requirement of the protein may result from an indirect effect on the assembly of the 51-kDa polypeptide. More surprising is why the 40-kDa and, after research in hamster cells (Au *et al*., 1999), the MWFE protein are essential to complex I activity. The involvement of the 40-kDa protein in the biosynthesis of an unknown redox group required for electron transfer to ubiquinone was proposed (Schulte *et al*., 1999).

Site-directed mutagenesis of nuclear-coded subunits was also initiated in *N. crassa*, by expressing altered cDNAs in the respective knockout mutants, under the control of an heterologous promoter. The analysis of strains in which selected cysteine residues of the 24- and 21.3c-kDa proteins were substituted with other amino acids revealed that the binding of iron–sulfur clusters to the ligand–proteins occurs before and is required for their assembly into complex I (Almeida *et al*., 1999; Duarte and Videira, 2000). This may also be true for other cofactors. The finding that the NADPH bound to the 40-kDa protein is shielded in complex I (Schulte *et al*., 1999), suggests that it is added to the protein before assembly. We also simulated specific mutations in the PSST and TYKY homologs of *N. crassa*, equivalents to those causing human disease. The homozygous V122M substitution in PSST and the compound heterozygous P79L and R102H in TYKY were identified in patients presenting Leigh syndrome (Loeffen *et al*., 1998; Triepels *et al*., 1999, respectively). We found only a slight, not significant, reduction of NADH:decylubiquinone reductase activity in isolated complex I carrying either of the three mutations, although the activity in mitochondrial membranes of the mutants was lower than in the wild-type strain (unpublished results). It is possible that a major effect of the mutations regards the levels of the proteins by interfering with its expression and/or its mitochondrial import/assembly. However, similar mutations were reported to reduce complex I activity by about 50% in mitochondrial membranes of the fungus *Y. lipolytica*, as well as affecting the affinity of the enzyme to ubiquinone and to complex I inhibitors (Ahlers *et al*., 2000).

PHYSIOLOGICAL ROLE OF NAD(P)H DEHYDROGENASES

In addition to complex I, mitochondria from fungi and plants contain alternative NADH dehydrogenases (Kerscher, 2000). The NDH2 represents a similar protein that can be found in prokaryotes (Yagi *et al*., 1998). These enzymes use NAD(P)H from either the cytosol (external enzymes) or the mitochondrial matrix (internal enzymes). They do not pump protons and may be useful as a system that keeps reducing equivalents at physiological levels, but their precise role is not completely clear. The variation in number and specificity among species suggests that they fulfill specific needs of different organisms. A maximum of four alternative NADH dehydrogenases was described in plants (Kerscher, 2000; Rasmusson *et al*., 1998). These types of proteins were best characterized in yeast, which possesses the internal NDI1 and the external NDE1 and NDE2 enzymes, but lacks complex I (De Vries *et al*., 1992; Overkamp *et al*., 2000).

A similar situation seems to occur in mitochondria of *N. crassa*, where both internal and external rotenone-insensitive alternative NADH dehydrogenases have been described (Weiss *et al.*, 1970). It was further reported that the internal enzyme is particularly active in the early exponential phase of fungal growth and that external NAD(P)H activity was sensitive to calcium (Moller *et al.*, 1982; Schwitzguebel and Palmer, 1982). We have recently characterized a fungal mutant lacking the NDE1 external enzyme, which works as a calcium-dependent NADPH dehydrogenase. Our results also indicated the presence of a second external alternative NADH dehydrogenase (Melo *et al.*, 2001). It is, therefore, likely that at least three nonproton-pumping NADH dehydrogenases are present in the inner membrane of *N. crassa* mitochondria.

The proton-pumping complex I and the alternative NADH dehydrogenases have overlapping roles and both activities are probably required for the optimal functioning of the cells. In *N. crassa*, it seems that complex I and the NDE1 protein are constitutively expressed throughout the fungal life cycle (Melo *et al.*, 2001). Recently, we reported the requirement of complex I for the process of sexual development in *N. crassa* (Duarte and Videira, 2000). The complexity of the enzyme, its involvement in several biological processes, such as fungal and plant development (Heiser *et al.*, 1997; Rasmusson *et al.*, 1998; Videira, 1998), and different metabolic pathways in microorganisms (Archer *et al.*, 1993; Zambrano and Kolter, 1993; Laval-Favre *et al.*, 1997; Dupuis *et al.*, 1998; Claas *et al.*, 2000) and, namely, its contribution to energy conservation argues against the possibility of the complete substitution of complex I with alternative enzymes. On the other hand, it is interesting that the alternative NADH dehydrogenases can be used to substitute for complex I in certain situations. For instance, the introduction of the *E. coli NDH-2* gene in *P. denitrificans* allowed the disruption of complex I genes in the latter organism (Finel, 1996). The use of the *NDI1* gene of *S. cerevisiae* to complement complex I defects in mammalian cells (Seo *et al.*, 1998, 1999, 2000) was a very important work toward opening a way for gene therapy in human mitochondrial diseases. A thorough characterization of the mitochondrial NAD(P)H dehydrogenases of different species will certainly add to our understanding of the biology of NAD(P)H and its importance for cellular metabolism.

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NOTE: In addition to those described, we found three complex I subunits (homologues of the bovine B22, B17.2 and 13 kDa polypeptides) and three putative mitochondrial alternative NADH dehydrogenases by searching data released from the Neurospora Sequencing Project of the Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu) and the *Neurospora crassa* cDNA Project at the University of Oklahoma (www.genome.ou.edu/fungal.html).

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