Mutation of yeast Eug1p CXXS active sites to CXXC results in a dramatic increase in protein disulphide isomerase activity

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Protein disulphide isomerase (PDI) is an essential protein which is localized to the endoplasmic reticulum of eukaryotic cells. It catalyses the formation and isomerization of disulphide bonds during the folding of secretory proteins. PDI is composed of domains with structural homology to thioredoxin and with CXXC catalytic motifs. *EUG1* encodes a yeast protein, Eug1p, that is highly homologous to PDI. However, Eug1p contains CXXS motifs instead of CXXC. In the current model for PDI function both cysteines in this motif are required for PDI-catalysed oxidase activity. To gain more insight into the biochemical properties of this unusual variant of PDI we have purified and characterized the protein. We have furthermore generated a number of mutant forms of Eug1p in which either or both of the active sites have been mutated to a CXXC sequence.

To determine the catalytic capacity of the wild-type and mutant forms we assayed activity in oxidative refolding of reduced and denatured procarboxypeptidase Y as well as refolding of bovine pancreatic trypsin inhibitor. The wild-type protein showed very little activity, not only in oxidative refolding but also in assays where only isomerase activity was required. This was surprising, in particular since mutant forms of Eug1p containing a CXXC motif displayed activity close to that of genuine PDI. These results lead us to propose that general disulphide isomerization is not the main function of Eug1p *in vivo*.

Key words: carboxypeptidase Y, protein folding, *Saccharomyces cerevisiae*, thiol oxidation.

INTRODUCTION

The endoplasmic reticulum (ER) constitutes the folding environment for secretory proteins in eukaryotic cells. A secretory protein can undergo various post-translational modifications, including formation of disulphide bonds, that are often vital for the folding and stability of the protein. The formation and isomerization of disulphide bonds is catalysed by protein disulphide isomerase (PDI) [1], which is an abundant protein in the ER.

PDI is composed of four domains in an a-b-b'-a' arrangement [2]. The thioredoxin-like a and a' domains contain the two active sites, each with the sequence motif CGHC. The ability of PDI to form a disulphide bond between the proximal cysteines in this motif enables the protein to carry oxidizing equivalents and transfer them to a protein substrate. The *EUG1* gene product is highly homologous to PDI from yeast and mammals and appears to have the same domain organization [3]. It is, however, unique among PDI-like proteins as it contains CXXS active-site sequences. Mutant PDI with the active-site sequences CXXS has almost no oxidase activity, while isomerase activity is much less affected [4]. Whereas Eug1p is therefore not expected to be able to directly introduce disulphide bonds, it should, in principle, be able to catalyse rearrangement of non-native disulphide bonds.

With its unique active-site sequences, Eug1p is interesting in terms of enzymology, evolution and cell biology. Based on the logic summarized above it has been suggested that Eug1p acts as a dedicated isomerase in the yeast ER. To investigate this possibility further, we have chosen to prepare Eug1p in *Escherichia coli* with a C-terminal His₆ tag and to characterize

the purified enzyme. From these studies it appears that Eug1p is an extremely inefficient PDI. This is surprising as mutation of either of the active sites in Eug1p to a CXXC motif improves the PDI-like function *in vitro*. We have characterized various mutant forms of Eug1p, using an *in vitro* assay based on oxidative refolding of denatured and reduced procarboxypeptidase Y (proCPY). These forms containing one or two CXXC active-site sequences all exhibit significantly increased foldase activity as compared with the wild-type protein. This is consistent with previous results showing that mutation of the Eug1p active sites to a CXXC sequence motif improved its ability to complement a *PDI1* deletion [5].

MATERIALS AND METHODS

Media and materials

E. coli was grown in Luria–Bertani (LB) medium supplemented with the appropriate antibiotic [6]. Restriction enzymes, T4 DNA polymerase, T4 polynucleotide kinase and T4 DNA ligase were from Promega (Madison, WI, U.S.A.). Oligonucleotides were synthesized by DNA Technology (Aarhus, Denmark). Sequencing was performed using a *Taq* Dye Deoxy[®] terminator cycle sequencing kit on an ABI 373A DNA sequencer, both from Applied Biosystems (Foster City, CA, U.S.A.). 1,4-Dithiothreitol and *o*-phthalaldehyde were from Merck (Darmstadt, Germany). *N*-(3-[2-furyl]acryloyl)-Phe-Phe, tris(carboxyethyl)phosphine and PMSF were from Sigma (St. Louis, MO, U.S.A.). 5',5'-Dithiobis(2-nitrobenzoic acid) was from ICN Biomedicals Inc

Abbreviations used: proCPY, procarboxypeptidase Y; ER, endoplasmic reticulum; GS-proCPY, glutathionylated proCPY; LB, Luria–Bertani; PDI, protein disulphide isomerase.

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Table 1 Plasmids used for expression in *E. coli*

Plasmid	Active sites
pCT65	CXXS-CXXS
pPN335	CXXS-CXXC
pPN342	CXXC-CXXC
pPN365	CXXC-CXXS
pPN398	CXXC-SXXS
pPN406	SXXS-CXXC

(Aurora, OH, U.S.A.). Isopropyl β -D-thiogalactoside was from US Biological (Swampscott, MA, U.S.A.).

Strains and plasmids

pBH1865 is described by Nørgaard et al. [7]. pCT58 is described by Holst et al. [5]. *EUG1* was mutated using conventional techniques [8,9] and subcloned into pBH1865, giving pCT56 (containing *EUG1*_{CXXS-CXXC}) and pCT61 (containing *EUG1*_{CXXC-CXXS}). For construction of the expression plasmid pCT65, the coding sequence without signal sequence was amplified by PCR using the primers oCT10 (5'-CCC GGG CAT ATG GCT AGC ACG CCG GGA TCA GAT TTA CTC-3') and oCT11 (5'-CCC GGG GCG GCC GCC TCG AGT ACG GTA TCT TCA GTA GAC-3'). The PCR product was digested with *NheI* and *XhoI* and cloned into the corresponding sites of pET-24a(+) (Novagen). pPN335, pPN342 and pPN365 were made by subcloning the *NcoI/BstBI* fragments from pCT56, pCT58 and pCT61, respectively, into the corresponding sites of pCT65. pPN398 and pPN406 were made by mutagenesis of pPN335 and pPN365. The sequence of the *EUG1* open reading frame was in all cases verified.

Purification of Eug1p

E. coli BL21(DE3) carrying the relevant expression plasmid (see Table 1) was grown in LB medium containing kanamycin (30 μ g/ml). The cells were grown at 30 °C until a D_{600} of 0.2–0.3, when the production of Eug1p was induced by addition of isopropyl β -D-thiogalactoside at a final concentration of 1 mM. The culture was harvested after 4–5 h at 30 °C, and cells were resuspended in $\approx$ 5 ml of binding buffer (20 mM Tris/HCl, pH 8.0/0.5 M NaCl/5 mM imidazole) per litre of culture. PMSF was added to a concentration of 1 mM, and the cells were sonicated for 6 $\times$ 30 s. Cell debris was removed by centrifugation, and the lysate was added to a pre-equilibrated Ni²⁺-chelating column (Novagen), which was eluted as described by the manufacturer. The eluate was dialysed against 25 mM Tris/HCl, pH 8.0, and the dialysate was applied to a MonoQ ion-exchange column (Amersham Pharmacia Biotech) equilibrated with the same buffer. Eug1p was eluted at $\approx$ 0.3 M NaCl. Relevant fractions were pooled and dialysed against 25 mM Tris/HCl, pH 8.0, and frozen for storage. The purity of Eug1p was analysed by SDS/PAGE and estimated to > 90 %.

Determination of disulphide-bond formation in proCPY during oxidative refolding

Refolding was carried out in a glutathione redox buffer as described by Westphal et al. [10]. Samples of 500 μ l were removed from the refolding mixture at various times. The refolding reaction was quenched by addition of 10 μ l of 1 M HCl to give a final pH of $\approx$ 2. Glutathione was removed by applying the sample to an NAPTM-5 (Amersham Pharmacia Biotech) gel-

filtration column equilibrated with 50 mM acetate buffer, pH 5.0. An eluate of 600 μ l was collected. To 300 μ l of the eluate, 200 μ l of 0.1 M potassium phosphate buffer, pH 7.0, and 500 μ l of 8 M guanidinium chloride in 0.1 M phosphate buffer, pH 7.0, were added. The proCPY concentration was measured using fluorescence at excitation and emission wavelengths of 295 and 335 nm, respectively, and quantified using a standard curve. To 250 μ l of the NAP-5 eluate 10 μ l of 10 mM 5',5'-dithiobis(2-nitrobenzoic acid) were added, incubated for a minimum of 1 h, and the peak corresponding to thio-nitro-benzoate was detected using reversed-phase HPLC. The concentration of free thiols was determined by relating the area of the peak to a standard curve. The number of free cysteines residues per proCPY molecule was subsequently calculated.

Glutathionylation of proCPY

GSSG was added at a concentration of 30 mM to a solution containing denatured and reduced $\approx$ 12 μ M proCPY in 6 M guanidinium chloride/0.1 M Tris/HCl, pH 7.4. This represents an $\approx$ 250-fold molar excess of GSSG over proCPY thiols. The mixture was incubated for 5 h. The subsequent 60-fold dilution of this mixture with 2 mM GSH, which initiates the refolding, resulted in a GSSG concentration of 0.5 mM and a GSH concentration of 2 mM.

To determine the degree of glutathionylation, the glutathionylated proCPY (GS-proCPY) was separated from non-bound glutathione by applying the sample to a HiTrap (Amersham Pharmacia Biotech) desalting column equilibrated with 50 mM acetate buffer, pH 5.0. Of the 1–1.5 ml eluate pool, 300 μ l were used to determine the proCPY concentration as described above. The eluate fraction (100 μ l) was mixed with 2 μ l of 1 mM tris(carboxyethyl)phosphine, which reduces all the disulphide bonds. After incubation for 30 min, the amount of liberated GSH was determined fluorometrically after derivatization with *o*-phthalaldehyde as described by Senft et al. [11].

By relating the GSH concentration to the proCPY concentration, the GS-proCPY preparations were found to contain 9.4 ± 1 mol of GSH/mol of proCPY, corresponding to $\approx$ 85 % glutathionylation.

Assay of insulin reduction

The assay mixture contained 0.1 M potassium phosphate, pH 7.0, 2 mM EDTA, $\approx$ 0.7 mg/ml human insulin and 0.2 M NaCl in a final volume of 200 μ l. The assays were performed in the presence or absence of 0.5 μ M yeast PDI or Eug1p. The reaction was started by addition of 10 μ l of 0.2 M 2-mercaptoethanol. The reactions were carried out in microtitre plates, and the turbidity (630 nm) of the reaction mixtures was read, at 2 min intervals, using a plate reader.

RESULTS

Oxidative refolding of reduced and denatured proCPY is only marginally enhanced by the presence of wild-type Eug1p

A prerequisite for the ability of PDI to function as an isomerase is that its active site CXXC motif is in the reduced state. In the oxidation and isomerization of protein thiols in the ER, this prerequisite puts the cell in a dilemma. On the one hand a high capacity for oxidation is essential for efficient production of disulphide-bonded secretory proteins. Much evidence indeed suggests that protein oxidation is mediated by oxidized PDI [12]. On the other hand, the more efficient the oxidative machinery is, the more likely it is that incorrect disulphide bonds are formed.

Figure 1 Oxidative refolding of reduced and denatured proCPY

Reduced and denatured proCPY (0.15 μ M) was refolded in the absence or presence of 1 μ M Eug1p. The refolding was carried out in 0.1 M Tris/HCl, pH 7.4/0.2 M KCl/0.5 mM GSSG/2 mM GSH at 25 °C. (A) Refolding in the absence of Eug1p (●) and in the presence of Eug1p_{CXXS-CXXS} (■) and Eug1p_{CXXC-CXXC} (◆). The dotted line shows refolding in the presence of 1 μ M yeast PDI [10]. (B) Refolding in the absence of Eug1p (●) and in the presence of Eug1p_{CXXC-CXXS} (■) and Eug1p_{CXXC-SXXS} (◆). (C) Refolding in the absence of Eug1p (●) and in the presence of Eug1p_{CXXS-CXXC} (■) and Eug1p_{SXXS-CXXC} (◆).

A highly oxidizing environment inherently disfavors the presence of those free thiols of PDI that are necessary for maintenance of its isomerizing activity. Yeast is probably the eukaryotic organism in which the disulphide-bond-forming apparatus has been most extensively characterized. Here an obvious role for Eug1p could be to maintain an isomerase activity under hyper-oxidizing conditions. We tested this hypothesis by assaying the properties of purified Eug1p.

In vivo, *EUG1* is a high-copy suppressor of *PDII* deletion [3,7]. Whereas this suppression is rather poor, the ability of *EUG1* to complement a *PDII* deletion is highly improved by changing its

active sites to the PDI-like CXXC motifs [5,7]. To obtain a more quantitative measure for the activity of the wild-type and mutant forms we assayed the activity of the proteins produced in *E. coli* using an assay based on oxidative refolding of unfolded and reduced proCPY [10].

proCPY is an *in vivo* PDI substrate in yeast. In its native form, proCPY contains five disulphide bonds. The folding and maturation of this protein can be followed *in vitro* as well as *in vivo*. *In vitro*, the oxidative refolding of proCPY is studied by dilution of the denatured and reduced protein in a redox buffer, which includes reduced and oxidized glutathione. During refolding, samples are withdrawn and treated with proteinase K, which activates proCPY by removal of the propeptide [13]. Proteinase K also degrades unfolded or partially folded proCPY. The activity of carboxypeptidase Y is subsequently measured and compared with the activity of a non-denatured sample.

The spontaneous oxidative refolding of proCPY leads to $\approx 40\%$ recovery of activity after 5 h (Figure 1A). The progress curve appears sigmoidal, with a lag phase of ≈ 1 h. Addition of wild-type Eug1p has a surprisingly small effect. When catalysed by wild-type Eug1p, the lag phase is the same as the spontaneous refolding, but the recovery of folded proCPY is higher ($\approx 55\%$ after 5 h, Figure 1A). Conversion of the two active-site sequences to CXXC, on the other hand, dramatically increases the efficiency of the enzyme. The lag phase is significantly shorter, and the recovery reaches $\approx 90\%$ after 5 h (Figure 1A). These data are consistent with the effect observed *in vivo*, where the CXXS-to-CXXC mutations cause a markedly improved growth rate in an *EUG1*-complemented strain [5]. It should be noted, however, that *in vitro* the oxidative refolding still proceeds significantly more efficiently with yeast PDI than with Eug1p_{CXXC-CXXC} under the same conditions and concentrations of enzyme (Figure 1A).

To compare the relative importance of the two active sites, the mutant forms Eug1p_{SXXS-CXXC} and Eug1p_{CXXC-SXXS} were compared. SXXS active sites are catalytically inert and PDI_{SXXS-SXXS} does not catalyse proCPY refolding [10]. The α' domain by far plays the most important role, as seen from a decreased lag phase, as well as a higher recovery in the Eug1p_{SXXS-CXXC}-catalysed refolding reaction (Figure 2). This is particularly evident from the observation that the progress curves for the CXXC-CXXC and the SXXS-CXXC mutant forms are very similar (Figures 1A and 1C). This is in spite of the fact that the CXXC-CXXC mutant has double the concentration of functional sites as compared with the SXXS-CXXC mutant form.

Instead of an inert SXXS motif, if the mutant forms of Eug1p investigated above had the wild-type motif CXXS, would that single active-site cysteine contribute to the oxidative refolding of proCPY, or would the observed effect solely be due to the CXXC active site? To answer this question, we compared refolding in the presence of Eug1p mutants with one CXXC motif plus either a CXXS or a SXXS motif. Assay of Eug1p_{CXXC-CXXS} and Eug1p_{CXXC-SXXS} mutant forms showed $\approx 75\%$ recovery after 5 h. The two progress curves arising from the presence of either of the two Eug1p mutant forms are superimposable, indicating that the presence of a single cysteine in the α' site does not contribute significantly to the activity (Figure 1B). On the other hand, comparison of the two mutant forms with Eug1p_{CXXS-CXXC} and Eug1p_{SXXS-CXXC} shows that the presence of a single cysteine in the α site has a small effect, leading to a slightly reduced lag phase (Figure 1C). However, the main contribution to the foldase activity again appears to stem from the CXXC active-site sequence.

PDI has previously been associated with a disulphide-independent chaperone activity [14]. To gauge this activity we asked whether Eug1p would facilitate refolding of denatured

Figure 2 Recovery of activity after 1 and 5 h of oxidative refolding of proCPY

Reduced and denatured proCPY was refolded in the absence or presence of various mutant forms of Eug1p. Carboxypeptidase Y activity was measured after 1 (closed bars) and 5 (open bars) h. Refolding for 1 h in the presence of any of the mutant forms with CXXS-to-CXXC mutations shows a dramatically higher activity compared with refolding catalysed by wild-type Eug1p. Also, the recovery after 5 h is higher. S.E.M. based on 2–7 independent measurements are shown.

Figure 3 Refolding of non-reduced proCPY

Refolding of denatured proCPY, in the absence or presence of Eug1p. Oxidative refolding in the absence of Eug1p (for comparison; ●); non-oxidative refolding in the absence of Eug1p (◆) and non-oxidative refolding in the presence of Eug1p_{CXXC-CXXC} (■).

proCPY possessing all native disulphide bonds. As seen in Figure 3, the refolding of denatured proCPY containing all native disulphide bonds is much faster than oxidative refolding. However, the presence of Eug1p has no effect on the refolding rate or yield under these conditions, demonstrating that only

proCPY forms with no or incorrect disulphide bonds are substrates for Eug1p.

Unfolded oxidized proCPY as a substrate for Eug1p

The experiments described above investigated the potential of Eug1p for oxidative folding. However, wild-type Eug1p might reasonably be expected to be more suited to carrying out disulphide shuffling reactions, as only the N-proximal cysteine of the CXXC motif of a PDI should be necessary here. Denatured and glutathionylated proteins have previously been exploited as model substrates for PDI [15,16]. Such substrates are in an oxidized form, but reshuffling is necessary for the protein to obtain the native disulphide configuration. We wanted to explore GS-proCPY as a uniformly oxidized substrate. Under the experimental conditions used, spontaneous oxidation of proCPY must occur through formation of a mixed disulphide bond between a protein cysteine and glutathione. The lag phase observed in the progress curve for spontaneous oxidative refolding could represent this initial oxidation of cysteine residues. Firstly, to estimate the time scale of this reaction, we followed the disappearance of free thiol groups in proCPY during the spontaneous oxidative refolding by taking samples at various time points. proCPY was then separated from free glutathione, and the number of free thiol groups per proCPY molecule was determined. The experiment showed that the level of free protein thiol decreases rapidly within the first ≈ 20 min (Figure 4). Under these conditions, essentially no folded proCPY

Figure 4 Disappearance of free thiol groups during spontaneous oxidative refolding of proCPY

The number of free protein thiol groups at various time points during spontaneous oxidative refolding of proCPY was measured and related to the protein concentration (the curve was fitted to an exponential equation). Fully reduced proCPY has 11 free thiol groups. The time course during the oxidative refolding shows establishment of a plateau of 1.5–2 thiol groups per proCPY molecule after 20–30 min.

Figure 5 Refolding of GS-proCPY

The free thiol groups on fully reduced proCPY were oxidized by formation of mixed disulphide bonds with GSH prior to initiating refolding. Refolding of proCPY in the absence of Eug1p (●) and in the presence of Eug1p_{CXXC-CXXC} (■). Refolding of GS-proCPY in the absence of Eug1p (▲), and in the presence of Eug1p_{CXXC-CXXC} (◆) and Eug1p_{CXXS-CXXS} (×).

appears within this time period (Figure 1A). Therefore, the lag phase most likely represents the initial formation of disulphide bonds, both with glutathione and intra-molecularly, whereas the subsequent phase showing regain of activity mainly consists of disulphide rearrangement and/or final folding to the native proCPY structure. Consistent with this interpretation, the lag phase of the spontaneous refolding reaction of GS-proCPY is significantly shorter than that of the fully reduced proCPY (Figure 5). Surprisingly, the progress curves for refolding of GS-proCPY with and without wild-type Eug1p are very similar,

Figure 6 Insulin-reduction assay with yeast PDI and wild-type Eug1p

The precipitation of the insulin B-chain, caused by reduction of insulin, was followed spectrophotometrically at 630 nm. The assay was performed in the absence of PDI or Eug1p (■) and in the presence of Eug1p_{CXXS-CXXS} (●) and yeast PDI (▼).

suggesting that Eug1p is a poor isomerase (Figure 5). As this could be a consequence of the specific substrate used, we wanted to test the isomerase activity of wild-type Eug1p using a different substrate. Here, we chose the folding intermediate of bovine pancreatic trypsin inhibitor, called N*, as yeast PDI previously has been shown to catalyse rearrangement of this particular substrate [10]. However, consistent with the results obtained using GS-proCPY as substrate, wild-type Eug1p had no detectable activity in this assay (results not shown). This lack of isomerase activity could be a consequence of non-reactive active-site cysteines. To test this, we used wild-type Eug1p in an insulin-reduction assay [17], where precipitation of the B-chain caused by reduction of the disulphide bonds between the two chains can be followed. Wild-type Eug1p had a small but significant activity in this assay, suggesting that the cysteines are indeed reactive (Figure 6). The activity is, however, extremely small compared with the activity of a similar concentration of yeast PDI.

The presence of Eug1p_{CXXC-CXXC} during refolding of GS-proCPY gives rise to a progress curve almost identical with the one obtained from refolding of fully reduced proCPY (Figure 5). This suggests that the shuffling reaction is the rate-limiting step in the catalysed refolding of proCPY.

DISCUSSION

Contrary to what is generally believed, Eug1p does not appear to be a good PDI. It may be argued that substrates other than the ones chosen for the present study are more suitable. However, proCPY is in principle a native yeast PDI substrate, and the fact that Eug1p can be converted into an efficient PDI by simply changing the active-site motifs from CXXS to CXXC argues against proCPY being an inherently poor substrate for Eug1p.

In vitro, we found the impact of wild-type Eug1p on the oxidative refolding of proCPY to be very small (Figure 1A). However, CXXS-to-CXXC mutations result in a dramatically increased effect on oxidative refolding of proCPY. Spontaneous oxidative refolding of proCPY progresses via a long lag phase (Figure 1). Under these conditions, oxidation of proCPY must occur through the reaction of protein thiol groups with GSSG, which in this assay functions as a donor of oxidizing equivalents. This lag phase represents mainly oxidation, as seen by disappearance of free proCPY thiol groups (Figure 4). The lag

phase is markedly shortened when the refolding is catalysed by an Eug1p mutant with one or two CXXC active sites. This suggests a higher oxidase activity of these mutants compared with wild-type Eug1p. This is also consistent with the *in vivo* observation that *EUG1*_{CXXC-CXXC}, unlike the wild-type gene, is able to complement a yeast strain with no auxiliary PDI-like proteins present [7].

Lyles and Gilbert [18] found that, in the case of PDI, the N- and C-terminal domains are functionally different. At saturating RNase concentrations, a PDI mutant with an inactivated C-terminal active site retains near wild-type activity. However, a mutant with an inactivated N-terminal site shows only $\approx 30\%$ activity as compared with the wild-type. A non-equivalence has also been shown for yeast PDI, but here the a' site is the more active one [10]. We here show that oxidative refolding of proCPY in the presence of Eug1p with a CXXC active-site sequence in the a' site, similar to yeast PDI, is more efficient than the presence of Eug1p with a CXXC active site.

The rate-limiting step in the Eug1p_{CXXC-CXXC}-catalysed folding appears to be reshuffling of incorrectly formed disulphide bonds. This is supported by the notion that the progress curve from refolding of GS-proCPY is identical with the curve arising from refolding of fully reduced proCPY (Figure 5). GS-proCPY has here been used as a homogeneously oxidized substrate useful for assay of isomerase activity. Surprisingly, wild-type Eug1p did not show any activity during refolding of GS-proCPY (Figure 5). This suggests that the CXXC enzymes are not only better oxidases, but also better isomerases.

It is remarkable, both from an evolutionary and a biochemical point of view, that the activity of the Eug1p_{CXXC-CXXC} mutant form is around 30–50% of what is found for native yeast PDI. It seems reasonable to assume that Eug1p has evolved to perform a highly specialized function in the yeast ER and that its high-copy suppression of *PDI1* deletion does not directly bear on what it normally does in the ER. In this light, the possibility that such a specialized function might relate to retrograde transport of proteins out of the ER is tantalizing (M. Zhou and R. Schekman, personal communication).

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