Five mutants of protein kinase CK2,α subunit in which altogether 14 basic residues were singly to quadruply replaced by alanines (K74A,K75A,K76A,K77A; K79A, R80A,K83A; R191A,R195A,K198A; R228A; and R278A, K279A,R280A) have been purified to near homogeneity either as such or after addition of the recombinant β subunit. By this latter procedure five mutated tetrameric holoenzymes were obtained as judged from their subunit composition, sedimentation coefficient on sucrose gradient ultracentrifugation, and increased activity toward a specific peptide substrate as compared with the isolated α subunits. The kinetic constants and the phosphorylation efficiencies ($V_{\text{max}}/K_m$) of all the mutants with the parent peptide RRADDSDDDDD and a series of derivatives, in which individual aspartic acids were replaced by alanines, have been determined. Three mutants, namely K74A,K75A,K76A,K77A; K79A,R80A, K83A; and R191A,R195A,K198A display dramatically reduced activity toward the peptide substrate as compared with CK2 wild type. Such differences either disappear or are attenuated if the mutants R191A,R195A, K198A; K79A,R80A,K83A; and K74A,K75A,K76A,K77A are assayed with the peptides RRADDSDDDDDD, RRA-DDSDDDD, and RRRADDSDDDAA, respectively. In contrast, the phosphorylation efficiencies of the other substituted peptides decrease more markedly with these mutants than with CK2 wild type. These data show that one or more of the basic residues clustered in the 191–198, 79–83, and 74–77 sequences are implicated in the recognition of the acidic determinants at positions +1, +3, and +4/5, respectively, and that if these residues are mutated, the relevance of the other acidic residues surrounding serine is increased. In contrast the other two mutants, namely R228A and R278A,K279A, R280A, display with all the peptides $V_{\text{max}}$ values higher than CK2 wild type, counterbalanced however by some what higher $K_m$ values. It can be concluded from these data that all the five mutations performed are compatible with the reconstitution of tetrameric holoenzyme, but all of them influence the enzymatic efficiency of CK2 to different extents. Although the basic residues mutated in the 74–77, 79–83, and 191–198 sequences are clearly implicated in substrate recognition by interacting with acidic determinants at variable positions downstream from serine, the other basic residues seem to play a more elusive and/or indirect role in catalysis.

Protein kinase CK2, formerly termed casein kinase-2 (or -11), is a ubiquitous Ser/Thr protein kinase normally composed by the tight association of two catalytic (α and α′) and two non catalytic β subunits that appears to play a central albeit still enigmatic role in cell regulation (1, 2). The presence among the myriad of its substrates of many proteins implicated in gene expression and signal transduction (3), the increase of CK2 activity in transformed and proliferating tissues (4), and development of leukemias in transgenic mice transfected with CK2α subunits (5) suggest the involvement of CK2 in both normal and uncontrolled cell proliferation. Though CK2 is endowed with basal catalytic activity toward most of its substrates and by contrast to previous reports, it seems not to be subjected to any kind of direct regulation by growth factors (6), its activity can be modulated by polycationic effectors acting through its β subunit, which has been shown to exert a dual function of positive as well as negative regulation over the catalytic subunit (7–9). The negative effect of the β subunit, especially evident with some substrates exemplified by calmodulin, is mediated by an acidic cluster located in the N-terminal part of the molecule. This would imply the interaction with basic residues of the catalytic subunit. Other properties of CK2 would imply the presence of crucially relevant basic residues in the catalytic subunit, namely inhibition by heparin (10) and other polyanionic compounds, like poly(Glu, Tyr)4:1 (11) and substrate specificity. This latter is invariably determined by multiple acidic residues located at positions between −2 and +5 (and probably farther) relative to the target amino acid (mostly Ser and rarely Thr) (12). Heparin inhibition is reduced but not abolished by mutations affecting lysyl residues 74/75 (13) and 75/76 (14). On the other hand the substitution of Asp for His160 homologous to PKA Glu170 (interacting with Arg at position −2 in PKA substrates) (15–17), affects the phosphorylation of peptide substrates whose recognition is partially dependent on an acidic residue at position −2 (18). In contrast the most powerful determinants of CK2 specificity normally are acidic residues located downstream from serine, the ones at positions...
In order to identify the basic residues responsible for substrate recognition, inhibition, and intrinsic down-regulation, we have applied the "charged-to-alanine" scanning mutagenesis strategy (21) to a number of basic residues of the human α subunit that are conserved across various species but divergent from the homologous residues of other protein kinases. Six such mutants in which collectively 16 residues have been singly or quadruply mutated to alanines have been obtained, and three of them have been shown to be seriously defective in catalytic activity (18). Here we describe the purification of five of these mutants, either as such or combined with the β subunit to give heterotetrameric holoenzymes, and we analyze their kinetic properties with a set of peptide substrates varying for the replacement of individual aspartyl residues between positions -2 and +5 within the structure of the reference peptide RRRADDSDDDDD.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptide substrates were prepared as described in Ref. 22. [γ-32P]ATP (2 mCi/ml) was from Amersham Corp. P11 phosphocellulose was from Whatman. MonoQ HR 5/5 column was from Pharmacia Biotech Inc. Antiserum against CK2 α subunit was raised using the peptide CVVKILPVKKKIKREKIKLE reproducing the sequence 66–86. The peptide was coupled to keyhole limpet hemocyanin using m-maleimidobenzenz-N-hydroxysuccinimide ester (Pierce). Nitrocellulose membrane (0.2 μm) was from Bio-Rad. All the other reagents were of the highest purity available. Recombinant human CK2 α and β were prepared as described in Refs. 22–24.

Expression and Purification of CK2 α Mutants—Expression of the mutants in Escherichia coli was performed as previously described (22–24). For subsequent purification, 2 g of bacteria pellets were resuspended in 30 ml of buffer A (25 mM Tris-HCl, pH 8.5, and 7 mM 2-mercaptoethanol) and sonicated (6 × 20 s) in ice. After sonication the bacterial extract was centrifuged for 15 min at 80,000 χ g. The supernatant was adjusted at the salt concentration of 0.2 M NaCl and loaded on a P11 phosphocellulose column (20 ml) previously equilibrated with buffer A (+ 0.2 M NaCl). The column was eluted with a linear gradient of 2 × 100 ml 0.2–1.5 M NaCl. 2-ml fractions were collected. 15-μl aliquots were analyzed by 12% SDS-PAGE to identify the presence of the protein. The fractions with α subunits were collected, dialyzed against 25 mM Tris containing 0.1 M NaCl, and stored in small aliquots at -20 °C.

In order to obtain CK2 holoenzymes with mutated α subunits, 1.5 g of bacteria pellets expressing mutated α subunits were resuspended and sonicated together with 1.5 g of bacteria expressing the wild type β subunit. A same purification procedure was applied as for α subunit alone, but after the phosphocellulose column a further purification step was performed by pooling all the fractions containing both the α and β the subunits (as judged by SDS-PAGE) and subjecting them to MonoQ fast protein liquid chromatography. The column was eluted with a linear gradient from 0.1 to 1 M NaCl, and the eluent was analyzed by OD monitor. The fractions in the OD peaks were assayed for CK2 activity, and the presence of the holoenzyme was assessed by 12% SDS-PAGE showing both the α (mutated) and the β subunits. The reconstituted enzyme was generally eluted from the column at a salt concentration of 0.5–0.6 M (corresponding to the prominent peak of both OD and catalytic activity). The fractions containing the purified holoenzyme were pooled and dialyzed for 4–5 h against 5 mM Tris-HCl, pH 7.5, 50 μM phenylmethylsulfonyl fluoride, and 50% glycerol and stored at -20 °C. The specific activities of CK2 holoenzymes were: CK2 wild type, 300 units/mg; K74A,K75A,K76A,K77A; 270 units/mg; K79A,R80A,K83A, 93 units/mg; R228A; lane 2, R282A; lane 3, R191A, R195A,K198A; lane 4, R278A,K279A,R280A; lane 5, K74A,K75A, K76A,K77A; lane 6, K79A,R80A,K83A; B, CK2 holoenzymes reconstituted with mutants of the α subunit and wild type β subunit and purified by phosphocellulose chromatography and MonoQ fast protein liquid chromatography (see "Experimental Procedures"). The arrows denote the positions of wild type α and β subunits. Lane 1, wild type α subunit; lane 2, R228A; lane 3, R191A, R195A,K198A; lane 4, R278A,K279A,R280A; lane 5, K74A,K75A, K76A,K77A; lane 6, K79A,R80A,K83A.

Kinetic Analysis of CK2 Mutants

Materials—Synthetic peptide substrates were prepared as described in Ref. 22. [γ-32P]ATP (2 mCi/ml) was from Amersham Corp. P11 phosphocellulose was from Whatman. MonoQ HR 5/5 column was from Pharmacia Biotech Inc. Antiserum against CK2 α subunit was raised using the peptide CVVKILPVKKKIKREKIKLE reproducing the sequence 66–86. The peptide was coupled to keyhole limpet hemocyanin using m-maleimidobenzenz-N-hydroxysuccinimide ester (Pierce). Nitrocellulose membrane (0.2 μm) was from Bio-Rad. All the other reagents were of the highest purity available. Recombinant human CK2 α and β were prepared as described in Refs. 22–24.

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RESULTS

Five mutants of CK2 α subunit (22) have been purified by submitting to phosphocellulose chromatography the extracts of bacteria expressing the mutated α subunits (see "Experimental Procedures"). The Coomassie-stained SDS-PAGE gels of the final preparations are shown in Fig. 1A. All mutants display a prominent 44-kDa band with the same mobility as wild type. In several cases a doublet rather than a single band is visible, probably indicative of limited proteolysis occurring during the isolation and purification procedure. This conclusion is corroborated by the finding that anti-α antibodies (see "Experimental Procedures") recognize not only the 44-kDa band but also the paper and washed as described (25). 32P incorporation into peptide substrates was evaluated by the phosphocellulose paper procedure (26). Kinetic constants were determined by double reciprocal plots constructed from initial rate measurements fitted to the Michaelis-Menten equation.

Gel Electrophoresis and Immunodetection of CK2 α Mutants—Aliquots (1 and 2 μg, respectively) of either mutated α subunits or CK2 holoenzymes reconstituted with mutated α subunits purified as described above were subjected to 12% SDS-PAGE according to Laemmli (27). The gels were either stained with Coomassie Blue or transblotted to nitrocellulose in a Haefer apparatus at 250 mA for 2.30 h. The filters were blocked for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, containing 0.15 mM NaCl and treated with anti CK2 α antisera (diluted 1:2000). Immunoreactive proteins were incubated with donkey anti-rabbit Ig, biotinylated, and detected by incubation with streptavidin alkaline phosphatase conjugate.

Other Methods—Protein concentration was determined by the method of Bradford (28) using bovine serum albumin as standard. Sucrose density gradient ultracentrifugation was performed as described previously (29).
ones with lower molecular masses (not shown).

The phosphotransferase activity of the purified mutants, either as such or after addition of equimolar amounts of pure β subunit, was determined using the peptide substrate RRAADSDDDDDDD (29). As shown in Table I two mutants, R228A and R278A,K279A,R280A, display an activity significantly higher to that of α w.t., whereas the phosphorylation rate by the other mutants is much lower, being too low for a reliable measurement in the case of mutant K79A,R80A,K83A. Upon addition of equimolar amounts of β subunit, the catalytic activity of α w.t. increases, as already observed (30). A similar or even higher increment of activity is observable adding β subunit to all the mutants. This also causes the appearance of significant activity with the mutant whose activity is undetectable in the absence of the β subunit.

These data would indicate that all the mutants are still capable of associating with the β subunit to give the heterotetrameric holoenzyme. This conclusion was corroborated by sucrose gradient ultracentrifugation experiments showing that the addition of the β subunit causes a change in the sedimentation coefficient similar to that induced by α w.t., consistent with the reconstitution of αβtetramers (Fig. 2).

Once established that all the mutants are still capable of associating with the β subunit, a strategy was developed for preparing mutated holoenzymes for sake of comparison with CK2 w.t., either recombinant or native. The most successful approach was to mix together the bacteria expressing the mutated subunits in approximately equal proportions and to apply the normal purification procedure of CK2 (see “Experimental Procedures”). In such a way all the five mutants could be purified to near homogeneity as heterotetrameric holoenzymes, as judged from both their SDS-PAGE Coomassie patterns, showing the α and the β subunits in approximately the same ratio as CK2 w.t. (Fig. 1B) and sucrose gradient ultracentrifugation revealing peaks of activity with the same sedimentation coefficient as CK2 w.t. (see Fig. 2). Heat denaturation curves, another criterion for judging the reconstitution of normal CK2 holoenzyme that is much more heat stable than the isolated α subunit (30), are shown in Fig. 3. Four mutants exhibited heat stability comparable with that of CK2 w.t. holoenzyme; the mutant K79A,R80A,K83A, however, exhibited a reduced heat stability, suggesting that susceptibility to protection by the β subunit is partially compromised in it. All mutants displayed Km values for ATP comparable with that of CK2 w.t. (17 μM) ranging between 10 and 25 μM.

The kinetic constants of all the CK2 mutants with the optimal peptide substrate RRAADSDDDDDDD and with a series of six peptide derivatives in which individual aspartic acids have been replaced by alanines were determined and compared with CK2 w.t. The Vmax and Km values as well as the overall phosphorylation efficiencies expressed by the Vmax/Km ratios are summarized in Table II.

![Figure 2](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3014883&tool=pmcentrez&rendertype=html)

**Figure 2.** Sucrose density gradient ultracentrifugation of CK2 holoenzymes reconstituted with variably mutated α subunits. Equimolar amounts of β subunit were combined with 18 μg of α subunits prior to sucrose gradient ultracentrifugation. Analysis of CK2 activity was done as described in Ref. 20 using casein (1 mg/ml) as substrate. CK2 α w.t. ( ), K74A,K75A,K76A,K77A ( ), K79A,R80A,K83A ( ), R191A,R195A,K198A ( ), R228A ( ), R278A,K279A,R280A ( ). The arrows indicate the positions of wild type α subunit alone and reconstituted CK2 holoenzyme (n2p2).

![Figure 3](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3014883&tool=pmcentrez&rendertype=html)

**Figure 3.** Thermal stability of CK2 holoenzymes. The catalytic activities of CK2 w.t. ( ), K74A,K75A,K76A,K77A ( ), K79A,R80A,K83A ( ), R191A,R195A,K198A ( ), R228A ( ), R278A,K279A,R280A ( ) were determined after preincubation of 0.1 μg of each enzyme at 40 °C for the time indicated. The samples were immediately ice-cooled, and the residual activity was determined as described under “Experimental Procedures.”

All the Asp→Ala substitutions, with only the exception of the one at position +2, are more or less detrimental to the phosphorylation efficiency of the peptide substrates by CK2 w.t.; two substitutions, however, are especially deleterious, namely the ones at positions +3 and +1, both causing a 10-fold drop in phosphorylation efficiency, accounted for by both a raise of Km and a decrease of Vmax.

An overall examination of the data of Table I allows a rough subdivision of the mutants into two categories: (i) mutants whose affinity for the parent peptide (expressed by Km) is only slightly decreased (whereas the Vmax is actually increased) and whose phosphorylation efficiency is altered by the structure of the peptide substrate in a manner similar to that of CK2 w.t. (R228A and R278A,K279A,R280A) and (ii) mutants whose affinity for the parent peptide is substantially decreased and whose phosphorylation efficiency is altered by modifications of the peptide substrate in a sharply different manner as compared with CK2 w.t. (K74A,K75A,K76A,K77A; K79A,R80A,K83A; and R191A,R195A,K198A).

In order to facilitate a comparative analysis, the relative efficiencies of CK2 w.t. and the mutants are represented in Fig. 4A as histograms normalized to the phosphorylation efficiencies of the parent peptide conventionally set equal to 1 for each
## Kinetic Analysis of CK2 Mutants

### Table II

| Substrate | $K_m$ (M) | $V_{max}$ (mmol/min) | $K_m/V_{max}$ | Michaelis-Menten constant (M⁻¹·mmol⁻¹/min) |
|-----------|----------|----------------------|---------------|------------------------------------------|
| Wild type | 0.535    | 267.5                | 0.829         | 0.011                                    |
| R228A     | 2.0     | 22.2                | 0.21          | 0.016                                    |
| R278A,K279A | 2.2 | 20.670               | 0.020         | 0.016                                    |

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mutant. It can be seen that although the profiles of mutants (control) (31) are superimposable to that of CK2 w.t., the histograms of mutants (control) (31) are dramatically altered. With the last mutant, e.g., the phosphorylation efficiency of the peptide lacking the acidic residue at position +1 (which is normally negligible as compared with the parent peptide) is actually the highest, surpassing by 3-fold that of the parent peptide. In contrast the relative phosphorylation efficiencies of the peptides with acidic gaps at all positions other than +1 are drastically reduced as compared with CK2 w.t. In the case of mutant K74A,K75A,K76A,K77A, the relative phosphorylation efficiencies of three peptides, with Ala for Asp substitutions at positions +3, +4, +5, and +6 are increased, whereas those of the other three peptide derivatives are decreased. The mutations occurring in K79A,R80A,K83A and R191A,R195A, K198A are those referring to the peptides having acidic gaps at positions +1, +2, +3, +4, +5 being represented by bars that surpass that of the parent peptide.

A summary of the mutations examined in this paper and of their effects on holoenzyme reconstitution, catalytic activity, substrate recognition, and susceptibility to polyanionic inhibitors (31) is reported in Table III.

### DISCUSSION

This paper describes the reconstitution, purification, and kinetic characterization of five CK2 mutants in which the $\alpha$ subunit underwent substitution of basic residues with alanines. Charged-to-alanine mutagenesis has been successfully used to identify residues implicated in substrate recognition by other protein kinases, namely PKA (15, 17, 21), myosin light chain kinase (32), and phosphorylase kinase (33). In all these cases such residues were found to be acidic in nature consistent with the knowledge that these kinases recognize basic specificity determinants. These specificity determinants moreover are located upstream from the phosphorylatable amino acid, notably at positions –2 and –3. In contrast, the main specificity determinants for CK2 are acidic residues located on the C-terminal side of the target amino acid. Our strategy therefore was to mutate basic residues that are conserved in CK2 from different species but are replaced by nonbasic residues in other protein kinases with special reference to the basophilic ones. Consequently the basic residues mutated by us (listed in Table III) are not homologous to residues (either acidic or basic) mutated in previous studies and in particular in the pioneering study of Gibbs and Zoller (21) in which all charged residues of yeast PKA were mutated to alanine. Two of the residues mutated in Ref. 21, Cys$^{116}$ and Glu$^{135}$, were found to be implicated in ATP binding. Both residues are highly conserved throughout the protein kinase family, CK2 included, and therefore were not modified in our study. On the other hand none of our mutations significantly modifies the $K_m$ for ATP nor prevents the association with the $\beta$ subunit to give tetrameric holoenzyme. In one case, however, where Lys$^{79}$, Arg$^{89}$, and Lys$^{83}$ were mutated into alanines, the resulting holoenzyme displays a reduced heat stability as compared with CK2 w.t.. This suggests that the interactions of this mutated $\alpha$ subunit with the $\beta$
subunit, which is responsible for thermostability (24), are weakened. Also with this mutant, however, the association with the \( b \) subunit promotes a severalfold increase of basal activity with peptide substrate, apparently even higher than that observed with CK2 w.t., although a precise evaluation is hindered by the extremely low activity in the absence of the \( b \) subunit (see Table I).

The kinetic constants of all the mutants with a set of seven peptides including the optimal substrate RRRADDSDDDDD (29) and its derivatives in which the aspartyl residues acting as specificity determinants have been variably replaced by alanine were calculated and analyzed. The main outcome of this study is that one or more of the basic residues replaced in three mutants, namely K74, K75, K76, K77; K79, R80, K83; and R191, R195, K198, are directly implicated in substrate recognition by interacting with definite acidic determinants of the peptide substrate.

In particular it is clear that one or more of the basic residues substituted by Ala in the mutant R191, R195, K198 are responsible for the recognition of the acidic determinant at position +1. The substitution of Asp (+1) with Ala in the peptide RRRADDSDDDDD in fact, which is one of the most detrimental substitutions with CK2 w.t., does not decrease but actually increases the phosphorylation efficiency by mutant R191, R195, K198. This conclusion is also in agreement with the knowledge that the basic residues Arg, Arg, and Lys are homologous to the PKA hydrophobic residues Leu, Pro, and Leu that interact with the hydrophobic residue found in many PKA substrates at position +1 (34, 35).

By similar arguments it can be concluded that one or more of...
The sequence of human PKA is used as reference in which the invariant residues Lys\textsuperscript{39} and Glu\textsuperscript{36}, characteristic of subdomains II and III, respectively, are in bold type, whereas Lys\textsuperscript{33} representing a hinge between helices B and C and facing Asp\textsuperscript{24} (n + 3) of PKI bound to PKA (38) is underlined. CK2 is aligned either beyond PKA according to the multiple alignment program of Hanks and Quinn (37) or below it manually based on local higher similarity and functional criteria (see text). PKC and Cdc2 are aligned to PKA according to Hanks and Quinn (37). The basic residues of CK2 implicated in the recognition of determinants at position +3 (Lys\textsuperscript{39}) and +4/+5/+6 (Lys\textsuperscript{34}, Lys\textsuperscript{36}, Lys\textsuperscript{37}) are underlined. Acidic residues of protein kinase C and p34\textsuperscript{cdc2} matching these basic residues of CK2 according to the manual alignment are underlined as well.

| II | III |
|----|-----|
| CK2 (37) | KILKPV------ | KKKRKFREEK |
| PKA | KLIDQKVKVL E\textsuperscript{39} | QIENTHKKR |
| CK2 (manual) | KILKPVKKRI E\textsuperscript{39} | R------EIK |
| Protein kinase C | KILKDVQVGD | DVECTHEKRR |
| Cdc2 | KIIRLEEEG | VSTSAIREIS |

The basic residues Lys\textsuperscript{79}, Arg\textsuperscript{80}, and Lys\textsuperscript{83} are specifically implicated in the recognition of another crucial specificity determinant, namely the acidic residue at position +3, because the replacement of this residue is almost ineffective with mutant K79A,R80A,K83A, whereas it is dramatically detrimental with CK2 w.t.

The case of the first part of the basic cluster 74–83 is more complicated because the mutation of the four lysyl residues 74–77 gives rise to a mutant whose low phosphorylation efficiency as compared with CK2 wild type can be improved in the recognition of another crucial specificity determinant located upstream from serine at position +1 and, less dramatically, –2; both these positions, which are relatively unimportant with CK2 w.t., become crucial (especially the former) with mutants R191A,R195A,K198A; K79A,R80A,K83A; and K74A,K75A,K76A,K77A. These observations may also provide the structural basis accounting for the efficient phosphorylation of “atypical” CK2 sites lacking the acidic determinant at position +3 and in which the presence of acidic residues at position –2 and even more at position –1 is essential (42). Using these atypical peptide substrates, it was possible to show that CK2 His\textsuperscript{166} contributes to the recognition of the acidic determinant at position –2 (18).

It should finally be noted that even the two mutants that are almost indistinguishable from CK2 w.t. reveal significant differences by the kinetic scrutiny of this work. Their almost unchanged phosphorylation efficiency in fact results from significantly and reproducibly higher V\textsubscript{max} values counterbalanced by higher K\textsubscript{m} values. The behavior of mutant R228A is especially intriguing because this mutation also dramatically increases CK2 sensitivity to inhibition by heparin (see Table III). A possible interpretation is that heparin, besides inhibiting CK2 by competing with some of the substrate binding elements (namely the Lys\textsuperscript{74}–Lys\textsuperscript{75}–Lys\textsuperscript{76}–Lys\textsuperscript{77} basic cluster and the p+1 loop) might also stimulate CK2 activity by interacting with Arg\textsuperscript{228}. This would be consistent with the finding that the mutation of the Lys\textsuperscript{74}–Lys\textsuperscript{75}–Lys\textsuperscript{76}–Lys\textsuperscript{77} cluster not only suppresses inhibition by heparin but even induces a stimulation by it (31), as expected assuming the existence of an up-regulating heparin binding site in CK2 α subunit. Additional mutations are in progress in order to check these possibilities and to identify the residues responsible for the recognition of the determinants located upstream from serine and downstream from position +5.

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