

Regulation of the *Drosophila melanogaster* Protein, Enhancer of Rudimentary, by Casein Kinase II

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ABSTRACT

The *Drosophila melanogaster* gene *enhancer of rudimentary*, *e(r)*, encodes a conserved protein, ER. Most ER homologs share two casein kinase II (CKII) target sites. In *D. melanogaster*, these sites are T18 and S24. A third CKII site, T63, has been seen only in drosophilids. The conservation of these CKII sites, particularly T18 and S24, suggests a role for these residues in the function of the protein. To test this hypothesis, these positions were mutated either to alanine as a nonphosphorylated mimic or to glutamic acid as a phosphorylated mimic. The mutations were tested individually or in double or triple combinations for their ability to rescue either a wing truncation characteristic of the genotype *e(r)^{pl} r^{hd1-12}* or the synthetic lethal interaction between *e(r)^{pl2}* and the *Notch* allele *N^{ind-p}*. All of the substitutions as single mutations rescued both mutant phenotypes, arguing that individually the phosphorylation of the three residues does not affect ER activity. The double mutants T18A-S24A and T18E-S24E and the triple mutants T18A-S24A-T63A and T18E-S24E-T63E failed to rescue. Together the data support the following model for the regulation of ER by CKII. ER that is unphosphorylated at both T18A and S24 is inactive. CKII activates ER by phosphorylating either T18 or S24. Further phosphorylation to produce the doubly phosphorylated protein inactivates ER.

THE *enhancer of rudimentary* gene, *e(r)*, was originally isolated in a *Drosophila melanogaster* screen for novel *rudimentary* alleles (TSUBOTA and SCHEDL 1986). The *rudimentary* gene, *r*, encodes the first three enzymatic activities of the *de novo* pyrimidine biosynthesis pathway (NORBY 1973; JARRY and FALK 1974; RAWLS and FRISTROM 1975). Localization studies mapped the *e(r)* gene to region 8B of the X chromosome. The *e(r)* gene acts as a recessive enhancer of a weak *r* mutant wing phenotype (WOJCIK *et al.* 1994). The *e(r)* gene encodes a protein, ER, which is highly conserved throughout eukaryotes (GELSTHORPE *et al.* 1997). The fruit fly and human homologs share 76% amino-acid identity (ISOMURA *et al.* 1996; GELSTHORPE *et al.* 1997). The *Xenopus laevis* homolog, termed XERH, was shown to be an interaction partner of dimerization cofactor of HNF1/pterin-4 α -carbinolamine dehydratase (DCoH/PCD), a multifunctional factor identified as a coactivator of the HNF1 homeobox transcription factors (POGGE VON STRANDMANN *et al.* 2001). A fusion of XERH-GAL4 binding domain was able to repress ex-

pression of a luciferase reporter in HeLa cells, but not in NIH3T3 cells, suggesting that XERH has a cell-type-specific, transcriptional repressor function (POGGE VON STRANDMANN *et al.* 2001). Recent publications have reported the structure of two *enhancer of rudimentary* homologs (ARAI *et al.* 2005; LI *et al.* 2005; WAN *et al.* 2005). Both proteins encoded by the human and murine *e(r)* contain three α -helices and four β -sheets, which constitute a novel fold (ARAI *et al.* 2005; LI *et al.* 2005; WAN *et al.* 2005). The β -sheets mediate dimerization of two monomeric ER proteins (WAN *et al.* 2005). The protein also contains hydrophobic pockets, suggesting that protein-protein interactions play a role in the function of the protein (WAN *et al.* 2005).

Three CKII target sites exist in ER. While the use of these sites has not been demonstrated *in vivo*, *in vitro* studies have shown that CKII can phosphorylate ER (GELSTHORPE *et al.* 1997). This investigation examines the role of the CKII target sites T18, S24, and T63 in the activity of ER. Of these sites, T18 and S24 are highly conserved, being found in groups as diverse as vertebrates, insects, and nematodes. In addition, T18 is found in higher plants, mosses, and slime molds. The high evolutionary conservation of these two CKII target sites argues for their importance in the regulation of ER. The third site, T63, has been seen only in fruit flies and slime molds, casting some doubt on the importance of this site in the regulation of ER. To test the role of

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TABLE 1
DNA sequences of the mutagenic primers used to generate the mutations in this study

Primer name	Primer sequence
Mutagenic primer T18A	5'-CTCGTAGTCACAGTAGGCGCGGGTCTCTGG-3'
Mutagenic primer T18E	5'-CTCGTAGTCACAGT <u>ACTCGCGGGTCTCTGG</u> ACG-3'
Mutagenic primer S24E	5'-CATGCACTCGTTGAC <u>CTCCTCGTAGTCACAGTA</u> -3'
Mutagenic primer E27Q	5'-CACGCCCTCCATGC <u>ACTGGTTGACGCTCTC</u> -3'
Mutagenic primer T63A	5'-GCTAATGTCCACCAT <u>CGCGTTCGATGAAATC</u> -3'
Mutagenic primer T18A S24A	5'-CTCCATGCACTCGTTGAC <u>GGCCTCGTAGTCACAGTAGGCG</u> -3'
Mutagenic primer T18E S24E	5'-CTCCATGCACTCGTTGAC <u>CTCCTCGTAGTCACAGTACTCG</u> -3'

The sequences are in the antisense orientation. The antisense sequences for the amino-acid substitutions are underlined.

phosphorylation of these sites on ER activity, a panel of mutants mimicking the phosphorylated state (with glutamic acid replacing serine or threonine) or the nonphosphorylated state (with alanine replacing serine or threonine) at any or all of these positions was generated. Mutations were constructed within a genomic DNA fragment that was previously shown to contain all of the sequences necessary for wild-type *e(r)* expression (WOJCİK *et al.* 1994). The mutated DNA fragments were cloned into pCaSpeR-4 and transformed into *D. melanogaster*. Mutant constructs were tested for their ability to rescue known mutant *e(r)* phenotypes. The results of these genetic studies are reported.

MATERIALS AND METHODS

Drosophila strains and transgenes: *Drosophila* strains, mutations, and balancers used in this study have been described at FlyBase (DRYSDALE *et al.* 2005; <http://flybase.bio.indiana.edu>). All transgenes used in this study were cloned into pCaSpeR-4 (THUMMEL and PIRROTTA 1992).

Drosophila culture: Fruit flies were reared using cornmeal-molasses-yeast-agar fly food, made using standard methods (WIRTZ and SEMEY 1982). Ingredients were obtained from Sigma Chemical (St. Louis). Fly food was poured into polystyrene vials or polyethylene bottles obtained from Applied Scientific (South San Francisco, CA) via Fisher Scientific (Pittsburgh). All fly vials were plugged using large rayon balls or Buzz Plugs for vials or bottles from Fisher Scientific.

Mutagenesis and mutant construct development: Mutations at positions T18, S24, E27, and T63 in the *e(r)* gene were generated using a template consisting of a 6.1-kb *SalI* genomic fragment that contained the *e(r)* coding sequence cloned into the *SalI* site of pBluescript KS- from Stratagene (La Jolla, CA). Mutagenesis reactions were performed via the Transformer Site-Directed Mutagenesis kit from BD Biosciences Clontech (Mountain View, CA), using primers obtained from Life Technologies (Carlsbad, CA) or Integrated DNA Technologies (Coralville, IA). Primer sequences used in mutagenesis are listed in Table 1. Blue Sky Biotechnology (Worcester, MA) generated mutant T63E. Mutant sequences were cloned as *EcoRI*-*XhoI* fragments into pCaSpeR-4 and transformed via standard methods (SPRADLING 1986) or by Genetic Services (Sudbury, MA). Double and triple mutants containing T63 mutations were generated via subcloning, according to the following strategy. Position T63 is flanked by two *BglII* sites in

the *e(r)* genomic sequence. Combination mutants containing T63 mutations were generated by isolating a 168-bp fragment containing the T63 mutant of interest and ligating it into the appropriate pCaSpeR-4 construct lacking the *BglII* fragment. After PCR confirmation of the correct orientation and DNA sequencing to confirm gene integrity, double and triple mutants were transformed into *D. melanogaster*. Transgenic flies were maintained in a *white*⁻ background to enable tracking of the mutant transgenes.

Plasmid purification: Plasmids were purified using one of two methods: via standard protocols (FELICIELLO and CHINALI 1993; SAMBROOK and RUSSELL 2001) using reagents from Sigma Chemical and Fisher Scientific or utilizing QIAGEN (Valencia, CA) kits.

DNA sequence analysis of *e(r)* mutants: The coding sequences of mutated *e(r)* genes were PCR amplified using primers that flanked the *e(r)* open reading frame. Mutants were sequenced in both directions. Mutants were sequenced from plasmids prior to transformation and from genomic DNA isolates after transformation. Fragments for DNA sequencing were amplified by PCR and purified using a QIAGEN PCR Preps kit and sequenced with the Big Dye sequencing kit from Applied Biosystems (Foster City, CA). Reactions were analyzed at the Edward A. Doisy Department of Biochemistry and Molecular Biology DNA Sequencing Center, part of the Saint Louis University School of Medicine.

Drosophila crosses: All fly crosses were performed in a *white*⁻ background to enable tracking of the transgene being studied. Mutated *e(r)* constructs were tested for their ability to rescue phenotypes characteristic of previously described *e(r)* mutants (WOJCİK *et al.* 1994; CLOSE and TSUBOTA 2005; S. I. TSUBOTA, unpublished results). The first phenotype tested was a wing phenotype shift from a score of 3 or 4 to a score of 2 (Figure 1; GREEN 1963; TSUBOTA and SCHEDL 1986; WOJCİK *et al.* 1994). The second phenotype tested was that of a lethal interaction between *e(r)*^{ps2} and the *Notch* allele *N^{ind-p}* (S. I. TSUBOTA, unpublished results).

Due to the fact that *N*, *r*, and *e(r)* reside on the X chromosome, scoring crosses required using only transgenes that were located on autosomes. Scoring crosses were set up using heterozygous flies. Females were heterozygous for the tester chromosome [*e(r)*^{ps1} *r^{hd1-12}* or *N^{ind-p}* *e(r)*^{ps2}] and the balancer chromosome *FM7c*. Males were heterozygous for the transgene being tested. Scoring was performed in male flies to isolate the tester chromosome with each transgene. Wing phenotypes and lethality could be scored in transgenic flies and compared with wing phenotypes and lethality in non-transgenic brothers. This strategy also provided a scoring standard for the lethality rescue in the form of balancer chromosome tracking.

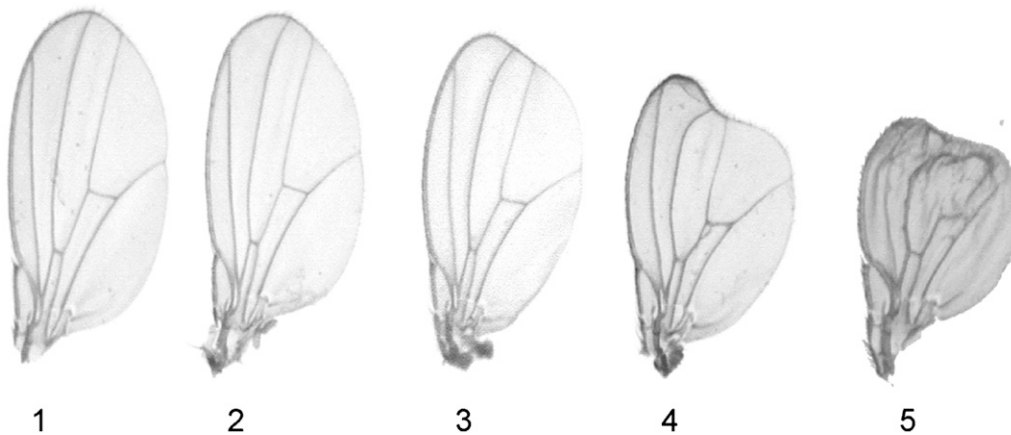


FIGURE 1.—Wing phenotypes characteristic of *rudimentary* and *enhancer of rudimentary* mutants. Mutants show a disruption of the regular array of bristles on the posterior edge of the wing and a distal truncation of the wing (GREEN 1963). A wild-type wing is given a value of 1, whereas a wing characteristic of a *rudimentary* deficiency is given a value of 5. Wings of the hypomorphic mutant r^{hd1-12} typically have a phenotype with a value of 2. Wings of the double mutant $e(r)^{p1} r^{hd1-12}$ have a phenotype with a value of 3–4.

RESULTS

Rescue of the mutant wing phenotype of $e(r)^{p1} r^{hd1-12}$.

The mutageneses were performed to answer the following question: Are the three CKII phosphorylation sites in ER involved in the regulation of its activity? This was performed by producing combinations of nonphosphorylated mimics (serine or threonine replaced with alanine) and phosphorylated mimics (serine or threonine replaced with glutamic acid) at each of the three CKII phosphorylation sites, T18, S24, and T63. For the nonphosphorylation mimic of S24, E27Q was used instead of S24A. This mutation destroys the CKII recognition sequence while maintaining the serine at position 24. This mutation was used because serine is conserved at this position even in cases in which the CKII consensus sequence is absent (GELSTHORPE *et al.* 1997; recent BLAST analyses), and thus the activity of ER may require a serine at residue 24 for a purpose other than as a phosphorylation site. The mutant transgenes were then tested for their abilities to rescue the mutant wing phenotype of the mutant $e(r)^{p1} r^{hd1-12}$. The wing phenotypes were scored using a previously published scale (GREEN 1963; Figure 1). The *rudimentary* mutant r^{hd1-12} in an $e(r)^+$ background has a mutant wing phenotype characterized by a modal value of 2 (Figure 1). The double mutant $e(r)^{p1} r^{hd1-12}$ has a mutant wing phenotype characterized by a modal value of 3 (Figure 1). Therefore, we defined rescue of the mutant wing phenotype as a shift from a modal value of 3 to a modal value of 2. The results of these experiments are summarized in Table 2.

All of the transgenes with single mutations rescued the mutant wing phenotype. These data argue that phosphorylation or the lack of phosphorylation at each of these sites individually does not affect ER activity. The S24E mutation also argues that S24, although strictly conserved, is not necessary for ER activity. Preliminarily, these results would indicate that CKII does not have a role in the regulation of ER activity; however, a different

story is seen with the double mutants. Two of the double mutants failed to rescue the mutant wing phenotype. The first was T18A-S24A. Since both the single mutations T18A and S24A rescued the mutant wing phenotype, the failure of the double mutant to rescue indicates that either T18 or S24 must be phosphorylated for ER to be active. Failure to phosphorylate both sites results in an inactive ER. Therefore CKII may activate ER by phosphorylating either T18 or S24. This interpretation is complicated by the failure of the double-mutant T18E-S24E to rescue. Apparently, phosphorylation at both T18 and S24 results in an inactive protein. This adds a twist to the proposed CKII regulation of ER. While CKII can activate ER by phosphorylating either T18 or S24, it can also inactivate ER by phosphorylating both sites. These results are supported by the results from the triple mutants. Both of the triple mutants T18A-S24A-T63A and T18E-S24E-T63E fail to rescue the mutant wing phenotype. The failure of both these mutants to rescue can be explained solely by the double mutations at T18 and S24. Finally, all of the constructs that did not rescue the mutant wing phenotype were represented by multiple insertions, making it unlikely that the lack of ER activity was caused by a position effect.

While phosphorylation at T18 and S24 is important for the regulation of ER activity, phosphorylation or lack of phosphorylation of the third CKII site, T63, has no effect on ER activity. All of the T63 mutants in single- and double-mutant configurations rescued the mutant wing phenotype. The behavior of T63 mutations in the triple-mutant combinations can be explained solely by the mutations at T18 and S24. Therefore, the phosphorylation of T63 has no apparent role in the regulation of ER activity. In hindsight, this is not too surprising, since T63 is by far the least conserved of the three CKII sites (GELSTHORPE *et al.* 1997). It has been seen only in the genus *Drosophila*.

Rescue of the synthetic lethality of $N^{md-p} e(r)^{p2}$: Similar results were seen with the rescue of the synthetic

TABLE 2
Rescue of the mutant wing phenotype of $e(r)^{\beta 1} r^{hd1-12}$ males

Transgene ^a	Male genotype	Wing phenotypes			
		1	2	3	4
$e(r)^+$	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	36	1135	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	28	8	1065	58
T18A	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	16	401	24	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	8	28	322	26
T18E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	27	119	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	32	12	102	11
S24E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	19	615	2	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	9	6	685	29
E27Q	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	27	92	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	22	2	83	0
T63A	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	14	187	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	9	0	183	0
T63E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	9	113	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	12	2	125	6
T18A-S24A	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	3	0	46	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	1	0	39	0
T18E-S24E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	54	47	517	6
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	51	35	511	10
T18A-T63A	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	27	222	5	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	24	6	200	0
T18E-T63E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	1	76	0	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	2	0	34	0
S24E-T63E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	13	104	6	0
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	8	0	94	2
T18A-S24A-T63A	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	8	2	106	1
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	7	0	134	0
T18E-S24E-T63E	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; transgene/+$	30	1	180	6
	$y^1 w^{67c23} e(r)^{\beta 1} r^{hd1-12}; +/+$	33	0	219	8

The crosses used to generate these flies are explained in MATERIALS AND METHODS. Wing phenotypes were determined using the scale shown in Figure 1.

^aData collected from one insert per construct: $e(r)^+$, T18A, T18E, S24E, T18E-T63E; data collected from two inserts per construct: E27Q, T63A, T63E, T18A-S24A, T18A-S24A-T63A; data collected from three inserts per construct: S24E-T63E, T18E-S24E-T63E; data collected from four inserts per construct: T18E-S24E, T18A-T63A.

lethality created by the $N^{nd-p} e(r)^{\beta 2}$ combination (Table 3). It should be noted that the number of $N^{nd-p} e(r)^{\beta 2}$ male escapers varied from cross to cross, so that rescue was measured in two ways to correct for the variability in the number of escapers. The first method took the fold increase in survival of the transgene-containing males *vs.* their nontransgenic brothers (transgenic/nontransgenic; Table 3, column 5). Rescue was defined as a value ≥ 4.0 . The second method subtracted the number of nontransgenic escapers from the number of transgenic males. This corrected value was then normalized by dividing it by the number of *FM7c* balancer males that the cross produced (Table 3, column 6). With this method, rescue was defined as ≥ 0.2 . These two measures clearly divide the transgenes into a group that rescues the lethality and a group that does not rescue. The only mutant combinations that did not rescue were the same combinations that did not rescue the mutant

wing phenotype: the double mutants T18A-S24A and T18E-S24E and the two triple combinations T18A-S24A-T63A and T18E-S24E-T63E. As with the rescue of the mutant wing phenotype, the nonrescuing transgenes were represented by multiple insertions. These data are consistent with wing-rescue data and support the hypothesis that the activity of ER can be regulated through the phosphorylation of T18 and S24. The lack of phosphorylation at both sites or the simultaneous phosphorylation at both sites inactivates ER.

DISCUSSION

CKII is a ubiquitous kinase with an extensive list of targets, many of which are transcription factors and regulators of the cell cycle (for review, see MEGGIO and PINNA 2003). While earlier work has suggested a role for CKII regulation of ER (GELSTHORPE *et al.* 1997), the

TABLE 3
Rescue of the synthetic lethality of $N^{nd-p} e(r)^{p2}$ males

Transgene ^a	$N^{nd-p} e(r)^{p2}$, transgene/+	$N^{nd-p} e(r)^{p2}$, +/+	<i>FM7c</i>	Transgenic/ nontransgenic	(Transgenic/nontransgenic) <i>FM7c</i>
<i>e(r)</i> ⁺	83*	7	237	11.9	0.32
T18A	112*	0	256	—	0.44
T18E	59*	2	104	29.5	0.55
S24E	32*	1	127	32.0	0.24
E27Q	180*	0	330	—	0.55
T63A	155*	0	138	—	1.12
T63E	106*	9	123	11.8	0.79
T18A-T63A	311*	74	641	4.2	0.37
T18E-T63E	74*	5	77	14.8	0.90
S24E-T63E	195*	18	257	10.8	0.69
T18A-S24A	39***	21	152	1.9	0.12
T18E-S24E	7****	9	290	0.8	0.0
T18A-S24A-T63A	22**	7	169	3.1	0.09
T18E-S24E-T63E	94*	53	406	1.8	0.10

The crosses used to generate these flies are explained in MATERIALS AND METHODS. *Corrected chi-square *P*-value <0.001; ***P*-value <0.01; ****P*-value <0.05; ****insufficient data to calculate *P*-value.

^aData collected from one insert per construct: *e(r)*⁺, T18A, T18E, S24E, T18E-T63E; data collected from two inserts per construct: E27Q, T63A, T63E, T18A-S24A, T18A-S24A-T63A; data collected from three inserts per construct: S24E-T63E, T18E-S24E-T63E; data collected from four inserts per construct: T18E-S24E, T18A-T63A.

data in this study are the first to tie changes brought about by CKII regulation with changes in measurable phenotypes. The data argue that CKII can regulate the activity of ER in a two-step manner. Initially the unphosphorylated ER is inactive. Phosphorylation by CKII at either T18 or S24 activates the protein. This active, singly phosphorylated form of ER can then be inactivated by phosphorylating the other site. Both the rescue experiments involving the mutant wing phenotype of *e(r)*^{p1 r^{hd1-12} and the synthetic lethality of $N^{nd-p} e(r)^{p2}$ support this conclusion.}

The crystal structures of human and murine ER have been solved (ARAI *et al.* 2005; LI *et al.* 2005; WAN *et al.* 2005). The structures reveal that T18 and S24 are solvent exposed, which permits access for phosphorylation by CKII. The protein forms a dimer in which both T18 and S24 are apposed to their counterparts on the other monomer. The authors suggest that phosphorylation of these residues would change the charge distribution on the surface of the dimer and possibly disrupt both dimerization and binding of ER to its partners (ARAI *et al.* 2005; LI *et al.* 2005; WAN *et al.* 2005). This possibility suggests a mechanism by which CKII may regulate ER. The unphosphorylated monomers dimerize, but form an inactive protein. Phosphorylation at either T18 or S24 does not disrupt the dimerization, but does enhance the binding of ER to its partners, thus activating it. The further phosphorylation at the second CKII site destabilizes the dimer, resulting in the inactivation of the protein. Support for this hypothesis awaits structural data on mutant forms of ER.

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