

Interaction of Estrogen Receptor Associated Protein (ERAP) 140 with ER β Decreases but its Expression Increases in Aging Mouse Cerebral Cortex

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Abstract Following binding to cognate ligand, estrogen receptor (ER) β interacts with specific responsive elements of the target genes and recruits a host of nuclear proteins for hormone dependent gene regulation. However, it is poorly known which proteins interact with ER β in mouse brain and whether their interaction and expression change with age. In this report, we have used his-tag mouse ER β for interaction with nuclear proteins of cerebral cortex of young (6 ± 1 weeks), adult (25 ± 2 weeks), and old (70 ± 5 weeks) female mice. We have identified estrogen receptor-associated protein (ERAP) 140 as one of the interacting proteins and studied its interaction by pull down immunoblotting, far-Western blotting and immunoprecipitation, and expression by western blotting. The data show that ERAP 140 interacts with ER β and its interaction decreases but its expression increases with age in mouse cerebral cortex, suggesting its role in estrogen-mediated brain functions during aging.

Keywords Estrogen signaling · Estrogen receptor β · Coregulators · ERAP 140 · Aging brain

Introduction

Estrogen mediates a plethora of functions through its two well-characterized estrogen receptors (ER) α and β by genomic and non-genomic pathways in almost all tissues including the brain. ER α and β are abundantly expressed

in different organs like ovary, prostate, testis, lung, thymus, spleen, and many areas of the brain (Evans 1988; Beato and Klug 2000; Koehler et al. 2005). ER β exerts a variety of actions in specific regions of the brain that influence cognition, learning, and mood (Mckenna and O'Malley 2002).

Transcriptional responses of estrogen are controlled by cell and gene-specific interactions, and a large number of coregulators. The understanding of coregulators is important because of the existence of receptor subtypes with differential tissue distribution, ligand responses, and cell-dependent functions. Over 30 coregulators have been identified to affect the ER-mediated transcriptional response and this number is increasing (McDonnell and Norris 2002; Lonard and O'Malley 2008; Thakur and Paramanik 2009). They bind to activation function (AF)-1 of transactivation domain and AF-2 of ligand binding domain of ER, resulting in either stimulation (coactivators) or suppression (corepressors) of expression of specific genes.

Unlike majority of coactivators, estrogen receptor-associated protein (ERAP) 140 was characterized as a conserved tissue specific nuclear receptor coactivator with abundant expression in the brain, exclusively in neurons (Greiner et al. 2000). It interacts with ER α , thyroid receptor β and retinoic acid receptor α , and shows no sequence similarity with other coactivators, though its homologs are present in both invertebrates as well as vertebrates including human (Shao et al. 2002). The role of ERAP 140 in estrogen signaling is crucial in mammalian brain where estrogen exerts multiple functions, some of which decline with age (Thakur and Sharma 2006). Here, we report that the interaction of ERAP 140 with ER β decreases but its expression increases in old mouse cerebral cortex, suggesting its role in estrogen-mediated brain functions during aging.

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Methods

Animals

Young (6 ± 1 weeks), diestrus adult (25 ± 2 weeks), and old (70 ± 5 weeks) female mice of AKR strain maintained under controlled laboratory conditions were used for the experiments. They were killed by cervical dislocation and the cerebral cortices were pooled from three mice of each age group. The tissue was washed to remove the adhering blood and used to prepare the nuclear extract.

Purification of Nuclei and Preparation of Nuclear Extract

Nuclei were purified from the cerebral cortices of young, adult, and old female mice according to Hewish and Burgoyne (1978). Briefly, the cerebral cortex was homogenized in solution A containing 0.34 M sucrose, 0.5 mM EGTA, 2 mM EDTA, and 0.5% Triton X-100 in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β -mercaptoethanol, and 15 mM HEPES, pH 7.5). The homogenate was kept on ice for 5 min, filtered and centrifuged at $5,000\times g$ for 15 min at 4°C . The resulting pellet was suspended in solution B containing 1.8 M sucrose, 0.5 mM EGTA, and 0.2 mM EDTA in buffer A and layered over cushion of solution B (half the volume of solution A used in the first step). The tube containing sample was centrifuged at $50,000\times g$ for 1 h at 4°C . The pellet containing clean nuclei was suspended in solution C containing 0.34 M sucrose in buffer A. For the preparation of nuclear extract, the method of Dignam et al. (1983) with some modifications was followed. Briefly, the nuclei were suspended in nuclear protein extraction buffer containing 20 mM HEPES (pH 7.6), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 0.4 mg/ml complete EDTA-free protease inhibitor (Roche Diagnostics, Germany) and stirred for 30 min on ice. Then, the suspension was centrifuged at $50,000\times g$ for 40 min at 4°C . The supernatant containing nuclear extract was checked quantitatively by Bradford (1976) method and qualitatively by silver staining.

Cloning and Expression of Mouse ER β

The mouse ER β cDNA (a kind gift from Prof V Giugure, Montreal, Canada) was subcloned in XhoI and EcoRI sites of the prokaryotic expression vector pRSETA, and used for transformation of *E. coli* DH5 α and subsequently BL21 (DE3) competent cells. Then, pRSETA ER β was induced with 0.05 mM IPTG for the production of recombinant mouse ER β protein. Subsequently, the bacterial cells were

collected after centrifugation at $3,000\times g$ for 10 min at 4°C followed by incubation in lysis buffer (20 mM Tris-HCl pH 8.0, 10 mM imidazole, 150 mM NaCl, 0.2% Triton X 100, and 2 mM β -mercaptoethanol). Then, the cell lysate was processed for purification of recombinant protein.

Purification of Recombinant Protein

Preparation of Sepharose Beads and Protein Loading

IDA-Sepharose beads were charged with 2 volumes of 0.1 M nickel sulfate in a microfuge tube followed by washing with water and equilibration in binding buffer (20 mM Tris-HCl pH 8.0, 10 mM imidazole, 150 mM NaCl, 0.2% Triton X 100, and 2 mM β -mercaptoethanol). Then, the cell lysate protein was loaded onto the charged IDA-Sepharose beads and incubated in ice for 1 h.

Washing and Elution of Recombinant Protein

The microfuge tube containing the charged beads with protein was centrifuged at $1,600\times g$ for 5 min at 4°C . The supernatant was collected and the pellet was repeatedly washed with buffer containing varying concentrations of imidazole (50, 100, 150, 200, and 250 mM). Finally, the recombinant protein was eluted with 300 mM imidazole.

Interaction of Purified Recombinant ER β with Nuclear Extract of Mouse Brain

Pull Down Assay and Immunoblotting

The purified recombinant mouse ER β protein bound to IDA-Sepharose beads was incubated with 50 μg of nuclear extract of cerebral cortex for 1 h in 500 μl NETN buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.5% NP-40, and 1 mM PMSF) in the presence of 10nM 17β -estradiol. Then, the beads were washed three times with NETN buffer and the bound proteins were eluted by boiling in 1X SDS-PAGE buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 0.2% SDS, 0.1% bromophenol blue, and 10% glycerol). The eluted proteins were separated by 7% SDS-PAGE and electroblotted. The blot was incubated first in blocking buffer and then in fresh blocking buffer containing anti-ERAP antibody (1:1,000) overnight at 4°C . After washing in PBS (3 \times 5 min), the blot was again incubated in blocking buffer containing anti-rabbit antibody HRPO conjugate (1:2,000) for 2 h at room temperature. The signal was finally detected by ECL method. After stripping the blot, same procedure was followed to detect β actin.

Immunoprecipitation

Nuclear extract (50 µg) of cerebral cortex from young, diestrus adult, and old female mice were incubated with protein A beads at 4°C for 4 h and then centrifuged at 10,000×*g* for 10 min at 4°C. The precleared supernatants were incubated overnight with beads and 5 µl of anti-ER β (Santa Cruz Biotechnology, USA) in 500 µl immunoprecipitation (IP) buffer (50 mM HEPES pH 7.6, 50 mM NaCl, 0.1% Triton-100, 10% glycerol, and 1 mM PMSF) in the presence of 10 nM 17β-estradiol. The unbound proteins were removed from the beads by washing with IP buffer. Finally, the bound proteins were eluted and denatured by boiling with SDS sample buffer. The interacting proteins were resolved by 10% SDS-PAGE, blotted onto PVDF membrane and probed with anti-ERAP 140 antibody as mentioned above. After stripping the blot, same procedure was followed to detect β actin.

Far-Western Blotting

The recombinant ER β was incubated with 30 µCi of ³⁵S (BRIT, India) using RTS kit (Roche Diagnostics, Germany) for 6 h at 30°C. The labeled protein was purified through spin column affinity chromatography. Nuclear extract (50 µg) of cerebral cortex from young, diestrus adult, and old mice were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane. Then, the blots containing the proteins were denatured with 6 M Guanidine-hydrochloride and renatured with decreasing concentrations of 3, 1, and 0.1 M in a buffer containing 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder, and 1 mM DTT. The ³⁵S labeled protein was used as a probe and hybridized to renatured proteins onto the membrane in the presence of 10 nM 17β-estradiol and exposed for autoradiography. The same blot was used to detect β actin.

Western Blotting

Nuclear extract (50 µg) of cerebral cortex from young, diestrus adult, and old female mice were resolved by 10% SDS-PAGE and transferred onto PVDF membrane using semi dry apparatus (Amersham Pharmacia, USA). Then, the blot was incubated in blocking buffer, followed by fresh blocking buffer containing anti-ERAP antibody (1:1,000) overnight at 4°C. After washing in PBS (3 × 5 min), the blot was again incubated in blocking buffer containing anti-rabbit antibody HRPO conjugate (1:2,000) for 2 h at room temperature. The signal was finally detected by ECL method. After stripping the blot, same procedure was followed to detect β actin.

Densitometric Scanning and Statistical Analysis

The signals detected on the autoradiogram by pull down immunoblotting, far-Western blotting, immunoprecipitation, and western blotting were scanned densitometrically and the relative density value (RDV) was analyzed using Alpha imager software V 3.1.2 2200. The mean and standard errors of RDV obtained from three independent experiments for each age group were calculated. The statistical analysis of the data was done using Sigma Stat 3.5 followed by all pairwise multiple comparison procedure (Student–Newmann–Keuls method). The data with *P* < 0.001 was considered significant.

Results

Detection of Mouse ER β Interacting Protein from Nuclear Extract and Identification of ERAP 140

Using pull down assay, we detected many nuclear proteins in the range of 30–203 kD that were retained by his-tag mouse ER β preloaded on IDA-Sepharose beads. These proteins were visible with coomassie blue staining. However, no interacting protein was observed when only beads were used as control (Fig. 1a). Pull down assay showed less number of interacting proteins in young and old as compared to adult mouse cerebral cortex (Fig. 1b). As pull down assay might show both direct and indirect interaction (Cavaillès et al. 1994), we used far-Western blotting to confirm the direct interaction of ERAP 140 with ER β (Fig. 2). The data from far-western blotting as well as immunoblotting following pull down assay and immunoprecipitation revealed highest interaction in case of young female mice as compared to adult and old (Fig. 3).

Age-Dependent Difference in the Interaction of Mouse ER β with ERAP 140

To study the effect of age on the interaction of ERAP 140 with ER β, we compared the extent of interaction (RDV) among young, adult, and old mice observed by pull down immunoblotting, immunoprecipitation, and far-western blotting. The interaction of ERAP 140 with ER β decreases significantly with age (*P* < 0.001) (Fig. 2).

Age-Dependent Difference in the Expression of ERAP 140

To determine the effect of age on the expression of ERAP 140, we compared the expression level of ERAP 140 (RDV) in young, adult, and old mice. The expression of

Fig. 1 Pull down assay showing interaction of ER β with nuclear extract of mouse cerebral cortex. **a** Purified recombinant mouse ER β protein was bound to IDA-Sepharose beads followed by incubation with 50 μ g of nuclear extract from adult cerebral cortex. Then, the beads were washed three times with NETN and bound proteins were eluted by boiling with 1X SDS-PAGE buffer. IDA-Sepharose beads were taken as control. The eluted proteins were finally resolved by 7% SDS-PAGE. **b** Interacting proteins from young, adult and old mouse cerebral cortex were resolved by 7% SDS-PAGE. *M* MW marker from Fermentas

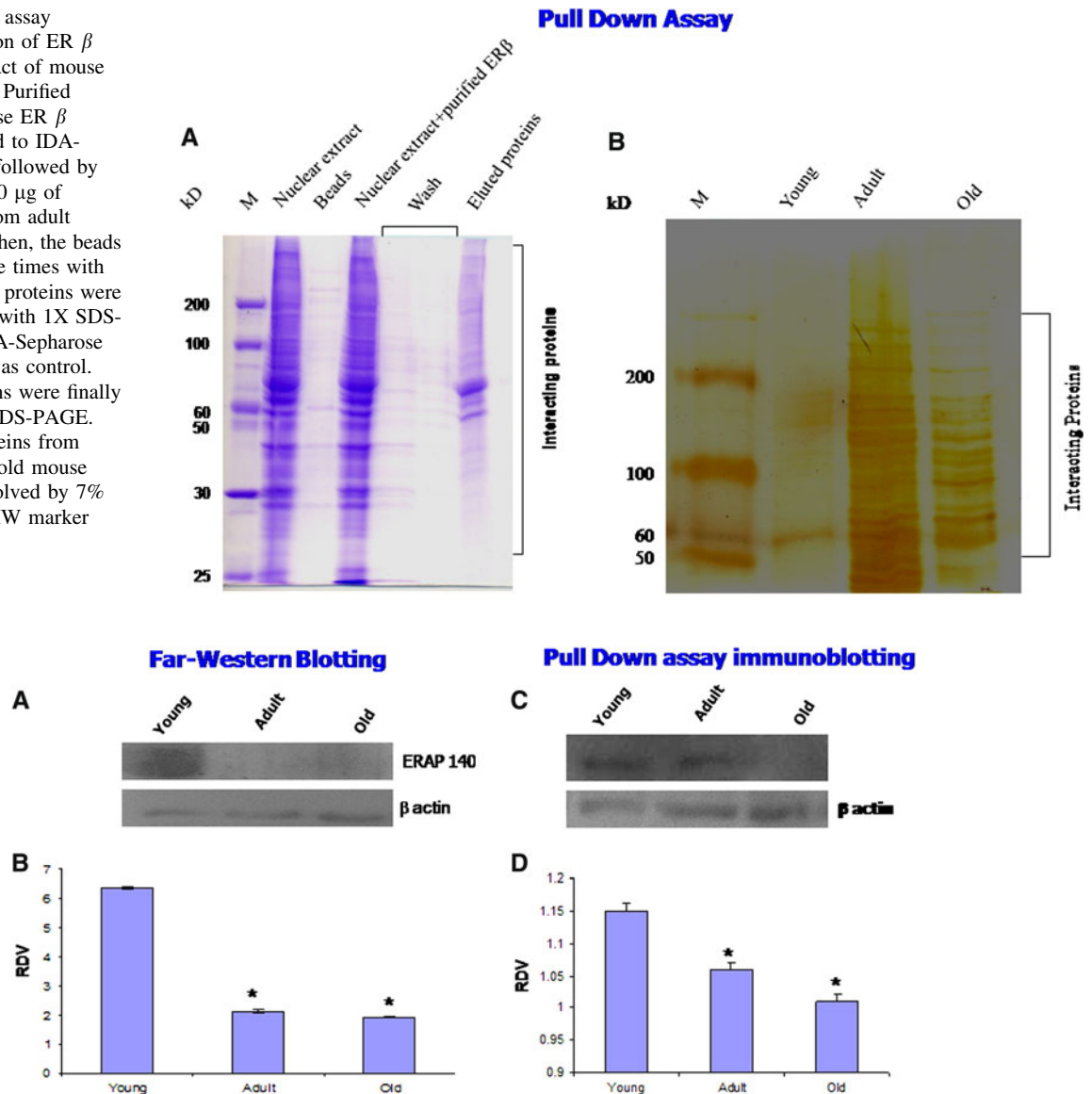


Fig. 2 **a** Far-western blot analysis showing ER β interaction with ERAP 140 of young, adult and old mouse cerebral cortex, **b** A graphical representation of densitometric analysis provided after normalization with β -actin (RDV). The results are representative of three separate experiments. One way analysis of variance was done using Sigma Stat 3.5 followed by all pairwise multiple comparison procedure (Student–Newmann–Keuls method). The data presented

here was found significant at (* $P < 0.001$). **c** Pull down immunoblotting of ERAP 140 with NE from young, adult and old mouse cerebral cortex **D**. A graphical representation of densitometric analysis provided after normalization with β -actin (RDV) as mentioned above (* indicates comparison of adult and old with young as control)

ERAP 140 increases significantly with age ($P < 0.001$, Fig. 4).

Discussion

Previous studies from our laboratory showed that ER α and β expression and interaction of ER α promoter with transcription factors decline in old mouse brain (Sharma and Thakur 2004, 2006). As estrogen plays a crucial role in

gene regulation during brain aging, it is important to identify the ER interacting proteins and examine their possible roles in brain aging. Recently, we reported that mouse ER α LBD interacts with PELP1, RIP140, PGC1 α , BAF60, and ADA3 (Ghosh and Thakur 2009a), and ER α TAD interacts with metastasis-associated protein (MTA) 1 and p68 RNA helicase (Ghosh and Thakur 2009a, b, c) and these coregulators vary in their levels of interaction and expression during aging of mouse brain. In-silico study using PIP, BIND, and STRING 8.0 suggests that ER β may

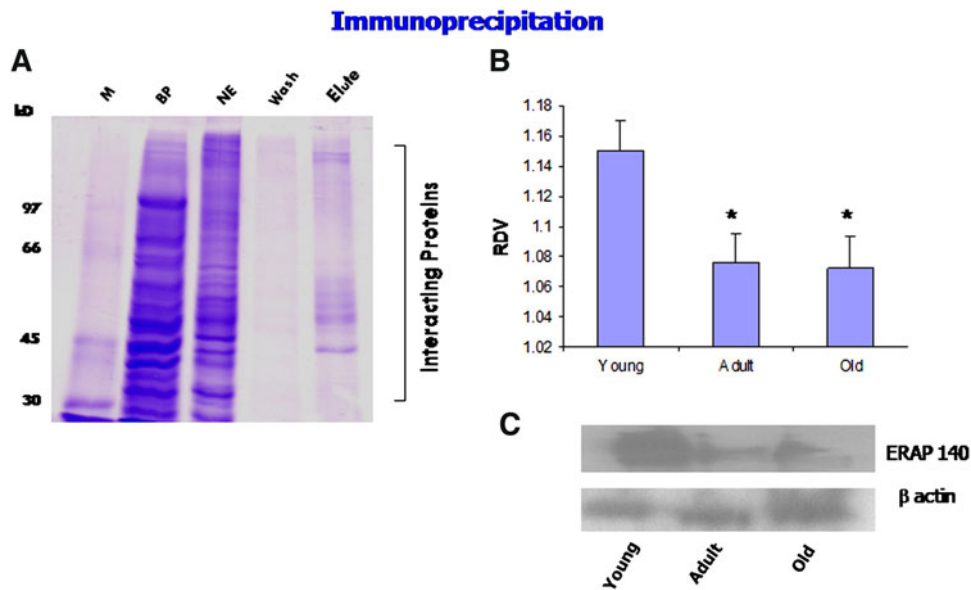


Fig. 3 a Immunoprecipitation shows ER β interacting proteins from adult mouse cerebral cortex. Fifty microgram of nuclear extract (NE) was incubated with protein A beads at 4°C for 4 h and then centrifuged at 10,000 \times g for 10 min at 4°C. The pellet was washed with immunoprecipitation buffer (wash). Finally, the bound proteins were eluted and denatured by boiling with SDS sample buffer. As negative control, a bacterial protein (BP) was taken in place of NE,

b A graphical representation of densitometric analysis provided after normalization with β -actin (RDV) as mentioned above. **c** Immunoprecipitation of NE (50 μ g from cerebral cortex of young, adult and old mice) with anti-ER β , followed by immunoblotting with anti-ERAP 140 (* indicates comparison of adult and old with young as control)

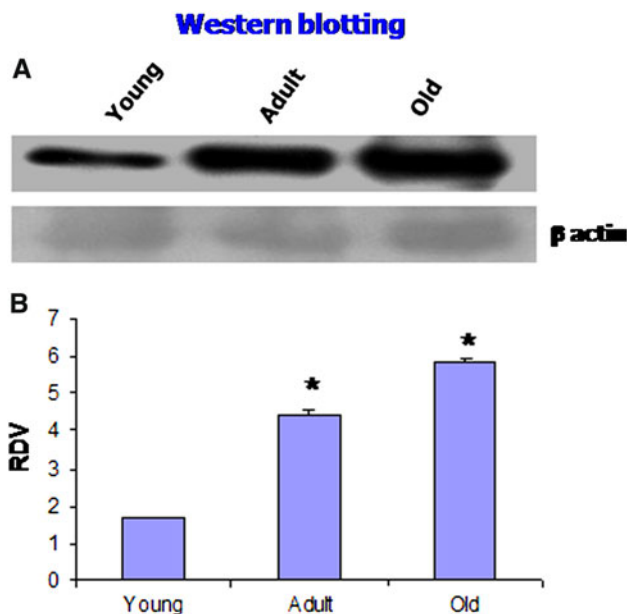


Fig. 4 a Western blotting of ERAP 140 A using the NE from the cerebral cortex of young, adult and old mice, **b** A graphical representation of densitometric analysis after normalization with β -actin (RDV) as mentioned above (* indicates comparison of adult and old with young as control)

interact with SRC1, Rnpc2, AIB3, Mad 2a, Msh3, ERAP 140, GnRh, Ahr, Shbg, Ta, Arom, TIF1, and CREB. The majority of coregulators are found in various cells and

tissues, but ERAP 140 is abundantly expressed in neurons of cerebral cortex, thalamus, hypothalamus, hippocampus, cerebellum, striatum, and choroid plexus (Shao et al. 2002). As these brain regions also show high expression of ER β , it is likely that ERAP 140 regulates estrogen functions through ER β in these regions. This has prompted us to study ERAP 140 interaction and expression with ER β as a function of age.

Although interaction of ERAP 140 with ER has been shown earlier (Lazennec et al. 1997), we report here that the interaction of ERAP 140 with ER β decreases but its expression increases in old mouse cerebral cortex as compared to young and adult. As ERAP 140 is involved in retinoic acid mediated neuronal differentiation in neuroblastoma-derived RTBM1 cells (Arai et al. 2008), high level of ERAP 140 interaction in young brain may be involved in neuronal growth and differentiation. Age-dependent decrease in the interaction of ERAP 140 may be due to reduced level of estrogen and change in its conformation similar to human lens protein (Harding 1972; Levine and Stadtman 2001). This may be correlated with reduced transcriptional activity in old age (Halachmi et al. 1994) and neurogenesis in old canine brain (Pekcec et al. 2008). The high similarity of NCOA7 gene product, a member of ERAP family, to human OXR1 protein which functions in response to oxidative resistance, suggests that the higher expression of ERAP 140 may be due to higher oxidative stress in old age (Durand et al. 2007).

A specific sequence located between 395 and 637 amino acids of ERAP 140 interacts with LXXLL motif of ER α (Shao et al. 2002). As this motif is conserved in both receptors, it is likely that the interaction of ER β with ERAP involves similar regions. Further studies may reveal whether manipulation of ERAP 140 interaction can regulate estrogen dependent brain functions during aging and neurodegenerative diseases.

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