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Ser-Thr PHOSPHATASES IN THE RAT BRAIN THAT DEPHOSPHORYLATE PHOSPHO-Ser^{1291}-GluN2A SUBUNIT OF GLUTAMATE RECEPTOR

R. R. PRABHU[1,2]

[1]*P. G. Department of Biotechnology, Government Arts College, Thycaud P. O, Trivandrum, India;*
[2]*Rajiv Gandhi Centre for Biotechnology, Poojappura, Thycaud P. O, Trivandrum, India;*
e-mail: ramyarprabhu@gmail.com

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N-methyl-D-aspartate receptors (NMDARs), are one of the major ionotropic glutamate receptors found in excitatory synapses which play a key role in glutamatergic synaptic transmission. The receptors are regulated by post translational modifications such as phosphorylation. One of the major receptor subunits is GluN2A which is likely to get phosphorylated in vitro at a putative site Ser^{1291}. However, the regulation of phosphorylation of this site by kinases and phosphatases is not yet completely understood. In the present study, we have used the fusion constructs of GluN2A tagged with glutathione S-transferase (GST) as substrate for phosphorylation, purified calcium/calmodulin dependent protein kinase type II (CaMKII) and radioactive P^{32}. We demonstrated that the site phosphorylated by αCaMKII on GluN2A was Ser^{1291} and that protein phosphatases 1, 2A and 2C were able to dephosphorylate this phospho-GST-GluN2A-Ser^{1291} in vitro. In the rat brain tissue post synaptic density and cytosolic fraction the major phosphatase responsible for dephosphorylating phospho-GluN2A-Ser^{1291} was protein phosphatase 1.

K e y w o r d s: N-methyl-D-aspartate receptors, GluN2A-Ser^{1291} subunite, CaMKII, protein phosphatase 1, rat brain, cytosol.

N-methyl-D-aspartate receptors (NMDARs) are key molecules involved in excitatory synaptic transmission. They play significant roles in synaptic plasticity and excitotoxicity by mediating Ca^{2+} signaling. NMDARs are heterotetramers composed of two obligatory GluN1 subunits and two GluN2 subunits [1]. There are four types of GluN2 subunits viz, GluN2A, GluN2B, GluN2C and GluN2D. GluN2A and GluN2B are found to be enriched in forebrain when compared to other regions [2] and therefore are widely studied. GluN2 subunits are very important because the type of GluN2 subunit determines the pharmacological and electrophysiological properties of NMDARs like channel conductance, sensitivity to polyamines and Zn^{2+}, the open probability of the channel and the deactivation kinetics [3]. The GluN2 subunits are homologous and the predicted homology between GluN2A and GluN2B is 70% [4]. The fine regulation of these receptor subtypes happens in the brain during plasticity regulated by activity and experience [5].

NMDAR function is regulated by many mechanisms, including phosphorylation [6] mediated by kinases like calcium/calmodulin dependent protein kinase type II (CaMKII) and protein kinase C (PKC), as well as dephosphorylation mediated by phosphatases like protein phosphatase 1 (PP1) [7] and could be implicated in the pathophysiology of various neurodegenerative disorders [8]. The memory molecule in the brain, CaMKII can bind to both GluN2A and GluN2B subunits of NMDAR at distinct sites at the C terminal region [9]. The GluN2B subunit of NMDAR has a phosphorylation site at Ser^{1303} which gets phosphorylated *in vivo*. It is well known that this phosphorylation regulates the binding of CaMKII to GluN2B as phosphorylation inhibits binding of autophosphorylated CaMKII [10] and promotes slow dissociation of preformed CaMKII–GluN2B complexes *in vitro*. The major phosphatase responsible for regulation of GluN2B-Ser^{1303} in the postsynaptic density (PSD) is PP1 [11]. But in the case of GluN2A, the binding of unphosphoryla-

ted αCaMKII at the C-terminal region of GluN2A occurs at a site different from the phosphorylation site. Data also suggests that CaMKII phosphorylates GluN2A possibly at $Ser^{1289/1291}$ [12]. Apart from CaMKII, the site Ser^{1291} is known to be phosphorylated by protein kinase C [13]. However, the physiological role of phosphorylation of GluN2A at Ser^{1291} is not known apart from certain studies which report that it can potentiate current through NMDA channel [14] and NMDA C terminal signaling could play a significant role in neurodegenerative disorders [15].

We have used the fusion constructs of GluN2A tagged with Glutathione S-Transferase (GST) as substrate for phosphorylation. We show that the fusion protein GST-GluN2A (amino acid residues 1265 to 1301) is phosphorylated at Ser^{1291} by αCaMKII. It is dephosphorylated by phosphatases present in both PSD and cytosol and the main phosphatase responsible for dephosphorylation is PP1. The aim of this work is to understand the regulation of phosphorylation at the putative site Ser^{1291} of GluN2A as there are not many studies which have focused on the same. We also aim to identify the phosphatases, recombinant ones as well as those present in subcellular brain fractions which are involved in the regulation of phosphorylation of GST-GluN2A- Ser^{1291} *in vitro.*

Materials and Methods

ATP disodium salt, Okadaic acid, Cyclosporin A, and Cyclophilin A were from Sigma Chemicals, USA. Sanguinarine chloride was from Tocris Biosciences, PP1α was from New England Biolabs, USA, PP2A was from Promega, USA, PP2B was from Calbiochem, USA and PP2C was from R&D Biosystems, USA. γ32P-ATP was obtained from Bhabha Atomic Research Centre, Mumbai, India.

Expression of GST fusion proteins. The fusion protein GST-GluN2A with the Ser^{1291} phosphorylation site on GluN2A and the phosphorylation deficient mutant GST-GluN2A-S1291A were expressed using prokaryotic expression vectors pGEX-GluN2A and has been characterized as described previously [16]. αCaMKII was prepared and purified as described before [16].

Ethical statement. The animals used in the study were albino Wistar male rats aged 40–45 days weighing an average of about 100 g. The animals were maintained at the animal house of Rajiv Gandhi Centre for Biotechnology and the experiments conducted were in conformity with the guidelines of Institutional Animal Ethics Committee.

Preparation of postsynaptic density (PSD) and cytosol from rat brain. The rat brains were collected from animals mentioned above after euthanizing them for the preparation of PSD and cytosol. PSD is a subcellular fraction which is highly enriched in neuronal receptors and signaling molecules. The PSD fraction was prepared as described before [17]. The cytosolic fraction is another subcellular fraction, possibly known to contain phosphatases which could play a role in signaling. For cytosol preparation, the forebrains were dissected out and were homogenized with homogenization buffer containing 20 mM Tris (pH 7.4), 0.1 mM PMSF, 1 mM DTT and 1X Protease inhibitor cocktail. 6 ml of buffer was used per gram weight of brain and was homogenized. The homogenate was subjected to centrifugation at 1,50,000 g for 45 min. The pellet was discarded, and the supernatant was used as the cytosolic fraction for further experiments. The cytosol was characterized by the presence of various proteins of interest, especially the Ser-Thr phosphatases; PPI, PP2A, PP2B and PP2C.

Phosphorylation of WT-GST-GluN2A and the mutant GST-GluN2A-S1291A. WT-GST-GluN2A and the mutant GST-GluN2A-S1291A were subjected to phosphorylation by αCaMKII *in vitro* using 300 μM γ^{32}P-ATP (1000-3000 cpm/mole) at 30°C for 10 min. Initially the reaction mix without enzyme was incubated for 1 minute at 30°C followed by the addition of enzyme. The reaction mixture consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM EGTA, 1.3 mM $CaCl_2$, 17 μM CaM and 0.2 mg/ml BSA. The phosphorylation was stopped using 1 μM staurosporine, a general kinase inhibitor. The phosphorylation status was monitored by subjecting the samples to SDS–PAGE followed by autoradiography by exposing the dried gel to activated phosphor screen.

Dephosphorylation of phospho-GST-GluN2A by phosphatases in vitro. WT-GST-GluN2A was phosphorylated by αCaMKII as mentioned in section 2.4. To this phosphorylation reaction mix, different phosphatases which were obtained commercially, such as PP1α, PP2A, PP2B and PP2C were added, and the reaction mixes were incubated for one hour at 30°C. The dephosphorylation using PP1, PP2A and PP2B was done as described previously [17], whereas PP2C was added in the presence of buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mg/ml BSA and 0.02% Brij 35. The phosphorylation status was monitored by subjecting the sam-

ples to SDS–PAGE followed by autoradiogram by exposing the dried gel to activated phosphor screen.

Dephosphorylation of phospho-GluN2A-Ser^{1291} by PSD and cytosol in vitro. GST-GluN2A, which was phosphorylated at Ser^{1291} as mentioned in section 2.4, was subjected to treatment with PSD or cytosol with and without various phosphatase inhibitors. Dephosphorylation was done at 30°C for one hour. The various inhibitors used were cyclosporine A (CsA) 1 μM with cyclophilin A (CyPA) 120 nM for PP2B, okadaic acid (OA) at 5 nM for PP2A, OA at 10 μM for PP1 and sanguinarine chloride (SgC) at 1 μM for PP2C. The phosphorylation status was monitored as described above.

Results and Discussion

GST-GluN2A is phosphorylated by αCaMKII specifically at Ser^{1291}. Previous studies have shown that Ser^{1291} is a possible site on GluN2A which could be phosphorylated by αCaMKII [12]. To confirm the site of phosphorylation, we used a non-phosphorylatable mutant of GluN2A viz., GST-GluN2A-S1291A and subjected it to phosphorylation using αCaMKII and γ^{32}P-ATP. Upon autoradiography, it was found that only WT-GST-GluN2A showed incorporation of γ^{32}P-phosphate indicating that the site of phosphorylation is Ser^{1291} (Fig. 1).

Dephosphorylation of phospho-GST-GluN2A-Ser^{1291} by phosphatases. Phosphorylated GST-GluN2A-Ser^{1291} was found to be dephosphorylated by commercial phosphatases like PP1, PP2A and PP2C but not by PP2B (Fig. 2).

Dephosphorylation of phospho-GluN2A-Ser^{1291} by PSD and cytosol *in vitro.* In order to see whether phosphatases in PSD and cytosol can dephosphorylate phospho-Ser^{1291}-GST-GluN2A, the latter was treated with either PSD or cytosol in the presence of various phosphatase inhibitors. The PSD and cytosol were characterized for the presence of various phosphatases of interest by western blots (data not shown). Phosphatases like PP1, PP2A and PP2B have previously been shown to be present in PSD [11], whereas PP2A and PP2C are mostly found in the cytosol. Both PSD (Fig. 3) and cytosol (Fig. 5) can dephosphorylate phospho-GST-GluN2A-Ser^{1291} and this dephosphorylation was inhibited only in the presence of 10 μM OA, a concentration sufficient to inhibit PP1. OA in low concentrations (5 nM) (Fig. 3) or CsA-CyP or SgC (Fig 4) did not inhibit dephosphorylation showing that PP2A, PP2B or PP2C respectively do not have any significant roles

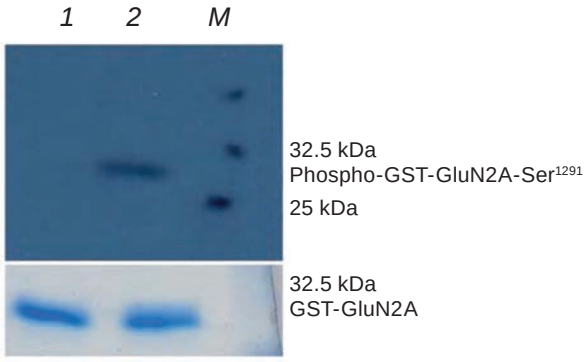

Fig. 1. Phosphorylation of WT-GST-GluN2A and GST-GluN2A-S1291A by αCaMKII. Each lane had 3 μg of substrate and 0.04 μg of αCaMKII. The phosphorylation was visualized by autoradiography. Lanes: 1 – GST-GluN2A-S1291A; 2 – phospho-GST-GluN2A-WT; 3 – Marker. The band of GST-GluN2A-Ser^{1291} in lane 2 corresponds to size of 30.4 kDa. The lower panel shows the picture of the gel containing WT-GST-GluN2A and GST-GluN2A-S1291A substrates stained with Coomassie brilliant blue

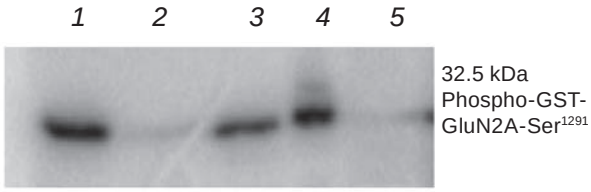

Fig. 2. Dephosphorylation of phospho-Ser^{1291}-GST-GluN2A by commercial phosphatases. GST-GluN2A phosphorylated using γ^{32}P-ATP was treated with the indicated phosphatases. Each lane had 1 μg of GST-GluN2A. The phospho-GST-GluN2A was visualized by autoradiography. The phosphatases added were: lanes: 1 – none; 2 – PP1; 3 – PP2A; 4 – PP2B; 5 – PP2C. The band of GST-GluN2A-Ser1291 corresponds to size of 30.4 kDa

in dephosphorylating phospho-GST-GluN2A-Ser^{1291}. PP2C is a phosphatase known to be present in cytosol and can dephosphorylate phospho-Thr^{286}-CaMKII along with PP1 although majority of the Thr^{286} dephosphorylation in cytosol is done by PP2A. To see if PP2C or PP2A in the cytosol has any role in GluN2A dephosphorylation or is it only mediated by PP1, various phosphatase inhibitors were also given along with cytosol. OA at 10 μM concentration inhibited dephosphorylation of phospho-GST-GluN2A-Ser^{1291} (Fig. 5). This shows that in PSD as

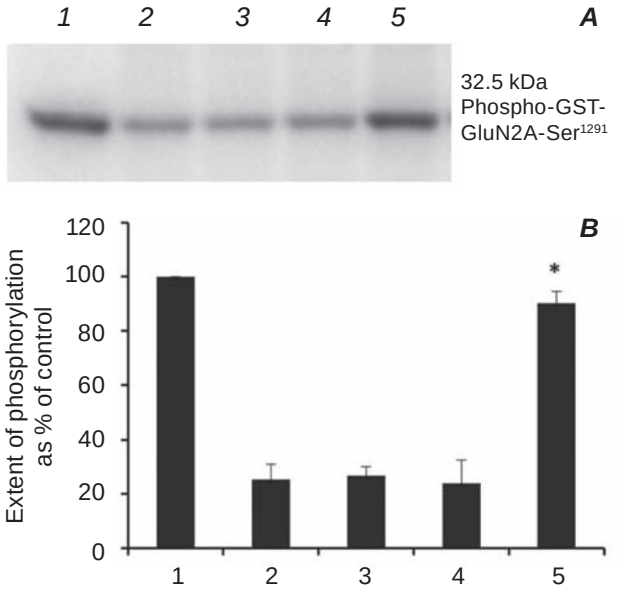

Fig. 3. ***A*** *– Dephosphorylation of phospho-Ser-GST-GluN2A by PSD in the presence of phosphatase inhibitors. GST-GluN2A phosphorylated using γ^{32}P-ATP was treated with the indicated phosphatase inhibitors. Each lane had about 1 μg of GST-GluN2A. Lanes 1-5 had PSD with respective inhibitors. The autoradiogram (****A****) shows the effect of all inhibitors except SgC which is shown in Fig. 5. Phospho-GST-GluN2A was visualized by autoradiography. Treatment given for the samples were: lanes: 1 – none; 2 – PSD (15 μg); 3 – PSD + CsA; 4 – PSD + OA (5 nM); 5 – PSD + OA (10 μM).* ***B*** *– The graph represents quantitation of the autoradiogram for mean ± SD of three experiments. The significance was calculated using One way ANOVA followed by Tukey's post hoc analysis and * represents P < 0.01 when compared to lane 2, the dephosphorylated state. The band GST-GluN2A-Ser1291 corresponds to size of 30.4 kDa*

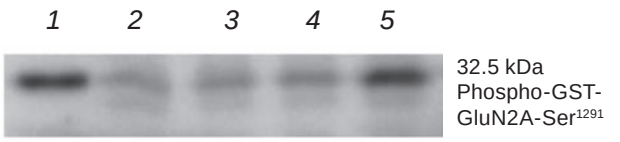

Fig. 4. Effect of PSD on phosphorylation status of GST-GluN2A-Ser1291 in the presence of SgC, the PP2C inhibitor. Lanes: 1 – none; 2 – PSD (15 μg); 3 – PSD + SgC (1 μM); 4 – PSD + SgC (5 μM); 5 – PSD + OA (10 μM). Each lane has about 1 μg of GST-GluN2A. The band GST-GluN2A-Ser1291 corresponds to size of 30.4 kDa

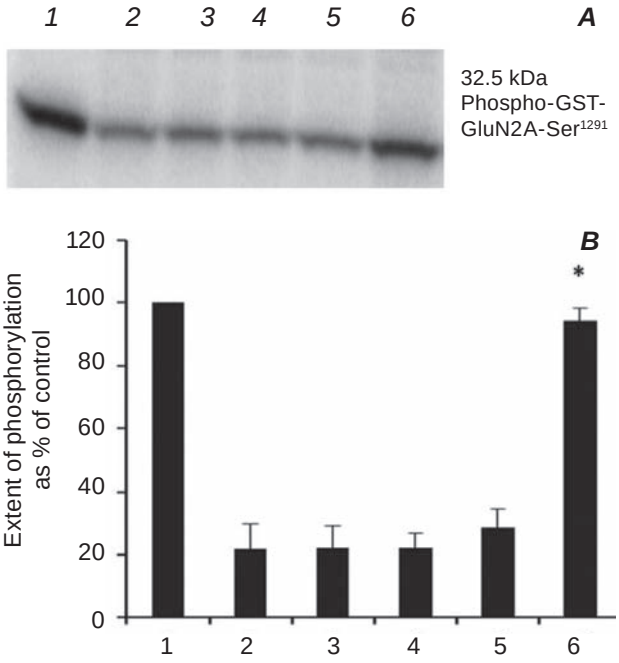

Fig. 5. Dephosphorylation of phospho-Ser1291-GST-GluN2A in presence of cytosol. GST-GluN2A phosphorylated using γ^{32}P-ATP was treated with cytosol along with the indicated phosphatase inhibitors. Each lane had about 1 μg of GST-GluN2A. Lanes 2-6 had cytosol with respective inhibitors. ***A*** *– The phospho-GST-GluN2A was visualized by autoradiography. Lanes: 1 – phospho-GST-GluN2A-Ser1291; 2 – no inhibitor; 3 – CsA; 4 – SgC; 5 – OA (5 nM); 6 – OA (10 μM).* ***B*** *– shows the quantitation of the autoradiogram. The significance was calculated using one way ANOVA followed by Tukey's post hoc analysis and * represents P < 0.01 in the marked lanes as compared to lane 2, the dephosphorylated state. The band of GST-GluN2A-Ser1291 corresponds to size of 30.4 kDa*

well as in cytosol, the main phosphatase responsible for dephosphorylating phospho-GST-GluN2A-Ser1291 is PP1.

Calcium is an important secondary messenger in neurons and Ca^{2+} influx brings about several activity-dependent changes in post-synaptic neurons, one of them being regulation of phosphorylation. GST-GluN2A gets phosphorylated by αCaMKII *in vitro* indicating the possibility that it could also get phosphorylated at the same site *in vivo.* Among the phosphatases, the Ca^{2+} sensing phosphatase, PP2B is known to activate PP1 upon Ca^{2+} influx which has an important role in long term depression in the hip-

pocampus [18]. Our studies show that the regulation of phosphorylation of GluN2A is mediated by PP1 which is enriched in PSD. But we could also see that PP1 in the cytosol was responsible for regulation of the same phosphorylation, although cytosol is rich in other phosphatases like PP2A and PP2C. Although purified preparations of PP1, PP2A and PP2C could dephosphorylate GST-GluN2A-Ser^{1291} *in vitro*, when PSD or cytosol was used, only PP1 was responsible for dephosphorylation activity. This pattern looks similar to that of GST-GluN2B where it is known to be dephosphorylated by PP1, PP2A but not by PP2B [17]. In PSD and cytosol, PP2A and PP2C could be having restricted accessibility towards the substrate and hence are not effective in dephosphorylating phospho-GST-GluN2A-Ser^{1291}.

Both GluN2A and GluN2B are found to have highly similar amino acid sequences, but still possess distinct pharmacological and electrophysiological properties [2]. They also show distinct spatial and temporal expression patterns and hence may mediate different signaling pathways. In mature synapses, it is found that GluN2B becomes extra synaptic whereas GluN2A is present at synapse [9]. It is also known that GluN2B is neonatal and is surpassed by GluN2A in postnatal stage [19]. There have been contradictory reports explaining the role of these subunits in LTP and LTD [20], and certain studies suggest that NMDA receptor subunit composition might not be necessary for plasticity, especially LTD [21]. But still regulation of phosphorylation in both subunits at GluN2A-Ser^{1291} or GluN2B-Ser^{1303} seems to be mediated by PP1.

The phosphorylation of receptors could also have functional roles as reported in the case of GluN2B-Ser^{1303} [16]. In the case of GluN2A-Ser^{1291} such physiological roles are not known, yet.

Conclusion. A previous study observed that GST-GluN2A is getting phosphorylated by αCaMKII at the site which is possibly Ser^{1289}/Ser^{1291} [12]. Our results are consistent with this observation and it is clearly confirmed from our study that αCaMKII phosphorylates GST-GluN2A at Ser^{1291} itself. The regulation of phosphorylation at this site, either *in vitro* or *in vivo*, was not known hitherto. Our study has also addressed the regulation of phosphorylation of GluN2A-Ser^{1291} *in vitro* in the presence of PSD and cytosol and has shown that PP1 is the main phosphatase involved in its dephosphorylation. This study is a preliminary investigation towards exploring the role of GluN2A phosphorylation at Ser^{1291} *in vivo*. It would be interesting to know that physiological relevance and status of GluN2A-Ser^{1291} phosphorylation and its regulation by the molecules CaMKII and if PP1, if any, *in vivo*.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ФОСФАТАЗИ Ser-Thr У МОЗКУ ЩУРІВ, ЯКІ ДЕФОСФОРИЛЮЮТЬ СУБОДИНИЦЮ ФОСФО-Ser^{1291}-GluN2A РЕЦЕПТОРА ГЛУТАМАТУ

R. R. Prabhu[1,2]

[1]P. G. Department of Biotechnology, Government Arts College, Thycaud P. O, Trivandrum, India;
[2]Rajiv Gandhi Centre for Biotechnology, Poojappura, Thycaud P. O, Trivandrum, India;
e-mail: ramyarprabhu@gmail.com

Рецептори N-метил-D-аспартату (NMDARs) є одними з основних іонотропних рецепторів глутамату, знайдених у збуджуючих синапсах, які відіграють ключову роль у глутаматергічній синаптичній передачі. Рецептори регулюються посттрансляційними модифікаціями, такими як фосфорилювання. Однією з головних субодиниць рецептора є GluN2A, який, ймовірно, фосфорилюється *in vitro* в передбачуваному місці Ser^{1291}. Однак регуляція фосфорилювання цього сайту кіназами та фосфатазами ще не достатньо вивчена. У цьому дослідженні ми використовували химерні конструкції GluN2A, помічені глутатіон-S-трансферазою (GST) як субстрат для фосфорилювання, очищену кальцій/кальмодулін-залежну протеїнкіназу типу II (CaMKII) і радіоактивний P^{32}. Ми продемонстрували, що сайт на GluN2A, що фосфорилюється αCaMKII, був Ser^{1291} і що протеїнові фосфатази 1, 2A і 2C здат-

ні дефосфорилювати цей фосфо-GST-GluN2A-Ser^{1291} *in vitro*. У постсинаптичному ущільненні та цитозольній фракції тканини головного мозку щурів основною фосфатазою, відповідальною за дефосфорилювання фосфо-GluN2A-Ser^{1291}, була протеїнова фосфатаза 1.

Ключові слова: рецептори N-метил-D-аспартату, субодиниця GluN2A-Ser^{1291}, CaMKII, протеїн-фосфатаза 1, мозок щура, цитозоль.

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