Inactivation of Integrin-linked Kinase Induces Aberrant Tau Phosphorylation via Sustained Activation of Glycogen Synthase Kinase 3β in N1E-115 Neuroblastoma Cells*

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Integrin-linked kinase (ILK) is a focal adhesion serine/threonine protein kinase with an important role in integrin and growth factor signaling pathways. Recently, we demonstrated that ILK is expressed in N1E-115 neuroblastoma cells and controls integrin-dependent neurite outgrowth in serum-starved cells grown on laminin (Ishii, T., Sato, E., and Nishimura, M. (2001) J. Biol. Chem. 276, 42994–43003). Here we report that ILK controls tau phosphorylation via regulation of glycogen synthase kinase-3β (GSK-3β) activity in N1E-115 cells. Stable transfection of a kinase-deficient ILK mutant (DN-ILK) resulted in aberrant tau phosphorylation in N1E-115 cells at sites recognized by the Tau-1 antibody that are identical to some of the phosphorylation sites in paired helical filaments, PHP-tau, in brains of patients with Alzheimer’s disease. The tau phosphorylation levels in the DN-ILK-expressing cells are constant under normal and differentiating conditions. On the other hand, aberrant tau phosphorylation was not observed in the parental control cells. ILK inactivation resulted in an increase in the active form but a decrease in the inactive form of GSK-3β, which is a candidate kinase involved in PHP-tau formation. Moreover, inhibition of GSK-3β with lithium prevented aberrant tau phosphorylation in the DN-ILK-expressing cells. These results suggest that ILK inactivation results in aberrant tau phosphorylation via sustained activation of GSK-3β in N1E-115 cells. This paper is available on line at http://www.jbc.org

Reagents—LY294002 was obtained from Sigma. Rabbit polyclonal anti-ILK IgG (UB 06–550 and UB 06–592) and myelin basic protein were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-GSK-3β antibody for immunoprecipitation was obtained from Roche Applied Science. Anti-Tau, anti-phospho(Ser199,Ser202)-GSK-3β, and anti-GSK-3β were obtained from Calbiochem (La Jolla, CA). Anti-phospho(Ser216)/GSK-3β and anti-phospho(Tyr279/216)-GSK-3α/β were obtained from ABA (Golden, CO). AlexaFluor (R488 goat anti-rabbit IgG was obtained from Molecular Probes (Eugene, OR). GSK-3β substrate (2B-SP) was obtained from Takara (Custom Synthesis Service, Tokyo, Japan). All other chemicals were of analytical grade and were obtained from Sigma or Wako Pure Chemical Co. (Osaka, Japan) unless otherwise specified.

Construction and Transfection of cDNA Vectors and Cell Culture—Mouse N1E-115 neuroblastoma cells were maintained in Dulbecco’s modified Eagle’s medium containing 50% fetal bovine serum (FBS; Hyclone, Logan, UT). The kinase-deficient ILK (DN-ILK) was generated by site-directed mutagenesis (Glu to Lys) at amino acid residue 359 within the kinase domain of wild type ILK (GenBankTM accession number AF256520) using PCR as described previously (11). Wild-type ILK and DN-ILK cDNAs were ligated into the polylinkers in two different mammalian expression vectors, pTracerTM-CMV2 (V855–01; Invitrogen) and pRe-CMV (V750–20; Invitrogen). The DN-ILK cDNA was transfected into N1E-115 cells (5 × 10^4 cells/100-mm culture dish) using the calcium phosphate precipitation method as described by Graham and van der Eb (12) and 48 individual Zeoic-resistant cell
lines were isolated over the next 4 to 5 weeks. Among them, three different cell lines were selected based on the detection of green fluorescent protein fluorescence and confirmation of gene transcription using reverse transcriptase PCR. The cloned cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 20% FBS and Zeocin (0.5 mg/ml).

ILK Assay—ILK assay was performed as described by Delemonenene et al. (13). Cells were lysed by boiling in 50 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 µg/ml leupeptin, 2.5 µg/ml aprotinin, 1 µM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, and 1 mM sodium orthovanadate. The lysates were incubated with anti-ILK antibody (UB 06–592) for 4 h at 12°C. After incubation, the lysates were precleared, and immune complexes were collected with protein A-Sepharose. After washing twice with lysis buffer and once with 50 mM HEPS (pH 7.0) buffer containing 1 mM EDTA, the immunoprecipitated ILK was incubated for 20 min at 30°C in the presence or absence of 10 µg of the exogenous substrate myelin basic protein in a total volume of 50 µl kinase reaction buffer (50 mM HEPS (pH 7.0), 10 mM MnCl₂, 10 mM MgCl₂, 2 mM NaF, 1 mM Na₃VO₄) containing 6 µM [γ-32P]ATP (10 µCi; NEG-502Z, PerkinElmer Life Sciences). The reaction was stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer. The kinase reaction products were analyzed using SDS-PAGE (5–20% polyacrylamide) and autoradiography. For detection of the immunoprecipitated ILK and DN-ILK proteins, the precipitated proteins were released from the immunoprecipitates by boiling in 80% SDS-PAGE sample buffer for 5 min. Equal volumes of the samples were loaded onto SDS-PAGE. Total ILK and DN-ILK proteins were detected by immunoblotting with an anti-ILK antibody (UB 06–550) that recognized both ILK and DN-ILK proteins.

GSK-3β—GSK-3β assay was performed essentially as described by Cross (14). Cells were lysed in 50 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EDTA, 100 mM okadaic acid, and protease inhibitors (Complete, Roche Diagnostics). The lysates were incubated with anti-GSK-3β antibody (Transduction Laboratories, Lexington, KY) at 4°C for 1 h followed by overnight incubation with protein G-Sepharose. After washing twice with lysis buffer of 80% SDS-PAGE sample buffer, the immunoprecipitated GSK-3β was incubated for 30 min at 30°C in the presence or absence of 4 µM specific substrate peptide 2B-SP (Ac-RRAEEELDSRAGS(p)PQL) in a total volume of 50 µl kinase reaction buffer (50 mM HEPS (pH 7.0), 10 mM MnCl₂, 10 mM MgCl₂, 2 mM NaF, 1 mM Na₃VO₄) containing 10 µM [γ-32P]ATP (0.5 µCi; NEG-502Z, PerkinElmer Life Sciences). The reaction was stopped by placing the samples on ice. After brief centrifugation, 25 µl of the reaction supernatant was spotted onto P81 phosphocellulose paper filters (Whatman, Maidstone, UK), washed with 75 mM phosphoric acid, and rinsed with acetone. The amount of radioactive phosphate incorporated into substrate peptides was determined by scintillation counting.

Immunofluorescent Staining—Cells grown on Lab-Tek® chamber slides (Nunc, Tokyo, Japan) were fixed in 1% neutral buffered formaldehyde solution for 10 min and then permeabilized with 0.25% saponin in Hanks’ balanced salt solution for 20 min. Permeabilized cells were incubated for 1 h in rabbit anti-phosphoSer999, Ser1038-Tau antibody (Calbiochem; final dilution 1:100 in phosphate-buffered saline). After rinsing in phosphate-buffered saline, the cells were incubated for 1 h in AlexaFluor (R)488 goat anti-rabbit IgG (1:100). Images were obtained by fluorescence microscopy (Olympus, Tokyo, Japan) and confocal laser scanning microscopy (Nikon, Tokyo, Japan).

Antibodies—Anti-Tau (recognizes both native and phosphorylated forms of tau) anti-Tau-1 (recognizes tau dephosphorylated at Ser195, Ser202, and Thr205 (15, 16)), and anti-GSK-3β antibody for immunoprecipitation (Transduction Laboratories) were mouse monoclonal antibodies. All the other antibodies, antiphospho (Ser199, Ser202)-Tau (recognizes tau phosphorylated at Ser199 and Ser202), anti-GSK-3β (recognizes both native and phosphorylated forms of GSK-3β), anti-phospho/Tyr122-GSK-3β (Affinity Bioreagents; recognizes phosphorylated at Ser199, anti-phospho/Tyr192, Thr196 and Thr216), anti-Affinity Bioreagents; recognizes GSK-3α and GSK-3β phosphorylated at Tyr279 and Tyr216, respectively), and anti-ILK (Upstate Biotechnology, Inc.; UB 06–550 for immunoblotting and UB 06–593 for immunoprecipitation), were rabbit polyclonal antibodies.

Western Blot Analysis—Cells were solubilized in 100 µl of sample buffer containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue, and 62.5 mM Tris-HCl (pH 6.8) after washing once with phosphate-buffered saline. For detection of ILK expression, cells were solubilized in 5 volumes of buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, and 2 µM phenylmethylsulfonyl fluoride at 4°C. The solubilized materials were subjected to SDS-PAGE (5–20% gradient, 6.5 or 10% polyacrylamide) and transferred onto nitrocellulose membranes at 4°C for 25 min. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20, followed by goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase. The final protein/IgG complexes were visualized following the reaction to 3,3'-diaminobenzidine tetrahydrochloride.

RESULTS

DN-ILK Inhibits Basal ILK Activity and Prevents Stimulation of ILK Activity after Cell Adhesion on Laminin under Serum-Free Conditions—Mouse NIE-115 neuroblastoma cells grown on a laminin matrix exhibit neurite outgrowth in response to serum deprivation (11, 17). We have demonstrated previously (11) that ILK is expressed in NIE-115 neuroblastoma cells and controls integrin-dependent neurite outgrowth in serum-starved cells grown on laminin. To inactivate endogenous ILK, cells were stably transfected with DN-ILK, which behaves as a dominant negative (11, 13). Based on the results obtained from immunoblotting (Fig. 1), the expression level of DN-ILK protein in DN-ILK-transfected cells was estimated to be at least twice that of endogenous ILK protein, and neither the ILK nor DN-ILK expression level changed under differentiating conditions. The ILK activity in the parental cells under serum-free conditions was transiently activated after seeding on the laminin matrix, whereas in the DN-ILK-transfected cells was not. Also, weak basal ILK activity was detected only in the parental cells under non-differentiating conditions (Fig. 1). These findings are consistent with our previous observations (11). Thus, DN-ILK inactivates endogenous ILK under both differentiating and non-differentiating conditions.

![Fig. 1](http://www.jbc.org/content/26971/1/1.full)
Inactivation of Endogenous ILK Induces Aberrant Tau Phosphorylation—To examine whether tau phosphorylation is involved in neurite outgrowth in N1E-115 cells, we analyzed the tau phosphorylation level in parental and DN-ILK-transfected cells using phosphorylation- and dephosphorylation-dependent anti-Tau antibodies. Western blots of tau from both parental and DN-ILK-transfected cells were probed with three different tau antibodies, anti-Tau-1, which recognizes an epitope of tau only when it is not phosphorylated, antiphospho(Ser199,Ser202)-Tau, which recognizes tau phosphorylated at Ser199 and Ser202, and anti-Tau, which recognizes both native and phosphorylated forms of tau. In non-transfected parental cells, tau was recognized by anti-Tau-1 but not antiphospho(Ser199,Ser202)-Tau antibody, under both normal and differentiating conditions. Thus, tau in the parental cells was not phosphorylated at sites recognized by those antibodies in either condition. On the other hand, cells stably transfected with a DN-ILK to inactivate the endogenous ILK had dramatically decreased anti-Tau-1 immunoreactivity but an increased antiphospho(Ser199,Ser202)-Tau immunoreactivity (Fig. 1). Thus, tau was phosphorylated in DN-ILK-transfected cells, and the tau phosphorylation level did not change even under differentiating conditions. Total tau detected with anti-Tau antibody migrated as several bands in the 40- to 70-kDa range, but some of the protein bands, which migrate relatively slower on SDS-PAGE, were weaker when tau was phosphorylated (Fig. 1). These results suggest that inactivation of endogenous ILK results in aberrant hyperphosphorylation of tau, at least at Ser199 and Ser202.

Immunofluorescent Staining of Aberrantly Phosphorylated Tau—To examine the intracellular localization of aberrantly phosphorylated tau, cells were stained with antibody against phosphorylated tau. Immunofluorescent staining of cell monolayers with antiphospho(Ser199,Ser202)-Tau antibody is shown in Fig. 2. DN-ILK-transfected cells were strongly stained with the antibody against phosphorylated tau under normal and differentiating conditions. On the other hand, parental cells were not significantly stained under differentiating conditions, but very weak dot-like structures were observed only in the non-differentiated cells, suggesting that a small minority of the tau, which could not be detected by the immunoblotting analysis, might be phosphorylated under non-differentiating conditions. In the DN-ILK-transfected cells, intracellular cytoplasm, except the nucleus, was stained with strong immunofluorescence intensity, and microtubule-like structures were observed. Further analysis of the DN-ILK-transfected cells using confocal laser scanning microscopy indicated that microtubule-like structures spread and cover right under the whole plasma membrane of the cells and form basket-like structures (top, left and right).

Active Form of GSK-3β Increases in DN-ILK-transfected Cells—To examine signal pathways involved in tau phosphorylation, we analyzed the activation status of GSK-3β, because GSK-3β is one of the candidate kinases that can phosphorylate tau at both Ser199 and Ser202 (18) and also has an important GSK-3β phosphorylation, we analyzed the activation status of GSK-3β by guest on July 18, 2018http://www.jbc.org/Downloaded from
of DN-ILK-transfected cells with LiCl reduced anti-phospho(Ser\(^{199}\),Ser\(^{202}\))-Tau immunoreactivity but increased anti-Tau-1 immunoreactivity in a dose-dependent manner (Fig. 4). Moreover, reaction of slowly migrated tau bands with anti-Tau, which were supposed to be non-phosphorylated tau, was recovered after treatment with LiCl in a dose-dependent manner. The same results were obtained in DN-ILK-transfected cells under differentiating conditions (data not shown). These results suggest that LiCl inhibited tau phosphorylation at Ser\(^{199}\) and Ser\(^{202}\) and at the sites recognized by anti-Tau-1 in DN-ILK-transfected cells but not in parental cells, 2) ILK inactivation increased the active form but decreased the inactive form of GSK-3\(\beta\), leading to increased GSK-3\(\beta\) activity, and 3) treatment of the DN-ILK-transfected cells with LiCl partially recovered neurite outgrowth, in the presence or absence of SB203580, to the levels of that in the SB203580-treated parental cells (Fig. 5). On the other hand, treatment of the parental cells with LiCl did not affect neurite outgrowth in either the presence or absence of SB203580 (Fig. 5). These results suggest that aberrant tau phosphorylation is partly involved in the inhibition of neurite outgrowth in DN-ILK-transfected cells.

**DISCUSSION**

The present study demonstrates that ILK inactivation induces aberrant tau phosphorylation via GSK-3\(\beta\) activation in N1E-115 cells, which is partly involved in the inhibition of neurite outgrowth, based on the following observations: 1) tau was phosphorylated at Ser\(^{199}\) and Ser\(^{202}\) and at the sites recognized by anti-Tau-1. On the other hand, both the same levels of parental cells with 25 mM LiCl did not affect either anti-Tau-1 or anti-phospho(Ser\(^{199}\),Ser\(^{202}\))-Tau immunoreactivity. These results suggest that GSK-3\(\beta\) activation induced by ILK inactivation is directly involved in tau phosphorylation at Ser\(^{199}\) and Ser\(^{202}\) and also at the sites recognized by anti-Tau-1.

**Aberrant Tau Phosphorylation Is Partly Involved in Inhibition of Neurite Outgrowth in DN-ILK-transfected Cells**—We demonstrated previously (11) that activation of p38 mitogen-activated protein (MAP) kinase is involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth in N1E-115 cells. In our previous report (11), we suggested that signaling pathways other than p38 MAP kinase, which is also activated via ILK activation, might be involved in aberrant tau phosphorylation. On the other hand, the aberrant tau phosphorylation was not observed in the parental cells under either normal or differentiating conditions. ILK activity in the parental cells was increased after seeding on the laminin matrix under serum-free conditions, whereas that in the DN-ILK-
transfected cells was not. Thus, both cell adhesion to laminin and serum deprivation were necessary for full activation of ILK. On the other hand, weak basal ILK activity was detected in the parental cells under non-differentiating normal conditions (Fig. 1). It remains unknown how basal ILK activity is maintained under non-differentiation. These results suggest that endogenous ILK prevents aberrant tau phosphorylation. Moreover, aberrant tau phosphorylation did not significantly occur in the parental cells under normal conditions, suggesting that weak basal ILK activity is sufficient to protect tau from aberrant phosphorylation.

To examine whether aberrantly phosphorylated tau participates in microtubule formation, we stained cells with antibody against phosphorylated tau (Fig. 2). Intracellular cytoplasm, except for the nucleus, was stained with strong immunofluorescent intensity, and microtubule-like structures were observed only in DN-ILK-transfected cells. Moreover, analysis of the DN-ILK-transfected cells using confocal laser scanning microscopy indicated that microtubule-like structures spread and cover right under the whole plasma membrane of the cells and form basket-like structures (Fig. 2, top, left and right). Thus, aberrantly phosphorylated tau participates in microtubule-like structures but is located only immediately under the plasma membrane in the cytosol without being able to form neurites in DN-ILK-transfected cells. On the other hand, parental control cells under non-differentiating conditions were very weakly stained with antibodies against phosphorylated tau, which could not be detected by the immunoblotting analysis, but neurite-bearing control cells under differentiating conditions were not (Fig. 2, a and c). These results suggest that tau phosphorylation at Ser199 and Ser202 might negatively control microtubule rearrangement necessary for neurite outgrowth via microtubule instability.

Tau is phosphorylated at Ser199 and Ser202 and at the sites recognized by anti-Tau-1, which correspond to Ser195, Ser198, Ser200, and Thr205. Ser199 and Ser202 are phosphorylated by GSK-3β (18), which is a candidate kinase involved in PHF-tau formation (26, 27). Moreover, GSK-3β has an important role in the ILK-mediated signal pathway (10, 19). We therefore examined the involvement of GSK-3β in aberrant tau phosphorylation in DN-ILK-transfected cells. Activation of GSK-3β is dependent upon the Tyr216 phosphorylation (21). On the other hand, GSK-3β activity is inhibited by direct Ser3 phosphorylation by ILK (19, 22) and by protein kinase B/Akt whose activity is also activated via ILK (13, 23). Tyr216 in GSK-3β was highly phosphorylated in DN-ILK-transfected cells but was very weakly phosphorylated in parental cells. In contrast, Ser9 in GSK-3β was highly phosphorylated in parental cells but not in DN-ILK-transfected cells. These phosphorylation levels were not significantly different between non-differentiating and differentiating conditions (Fig. 3A). Thus, GSK-3β was phosphorylated at both Ser9 and Tyr216 in parental cells, whereas Tyr216 phosphorylation was considerably lower (Fig. 3A). Recently, Bhat et al. (28) suggested that Ser9 phosphorylation is sufficient to override the Tyr216 phosphorylation-induced activation of GSK-3β. Therefore, the level of GSK-3β activity seems to maintain lower via Ser9 phosphorylation in parental cells. These results suggest that ILK inactivates GSK-3β via phosphorylation at Ser9 and prevents activation. In contrast, ILK inactivation results in Ser9 dephosphorylation and increased Tyr216 phosphorylation in GSK-3β, thereby activating the enzyme. Indeed, the level of GSK-3β activity in DN-ILK-transfected cells was significantly higher than that in parental cells (Fig. 3B).

Lithium, a selective competitive inhibitor of GSK-3β (24), inhibited tau phosphorylation at Ser199 and Ser202 and also at the sites recognized by anti-Tau-1 in a dose-dependent manner (Fig. 4). These results suggest that GSK-3β activity induced by ILK inactivation is directly involved in tau phosphorylation at Ser199 and Ser202 and also at the sites recognized by anti-Tau-1. As both Ser199 and Ser202 are phosphorylated by GSK-3β (18), GSK-3β at least directly phosphorylates these Ser residues in the DN-ILK-transfected cells. Although a specific tyrosine kinase, which should be activated by ILK inactivation, is probably involved in the Tyr216 phosphorylation in GSK-3β, we have not yet determined the kinase involved in this study. To understand the ILK-mediated regulatory mechanisms of GSK-3β, the specific tyrosine kinase that is activated by ILK inactivation and phosphorylates Tyr216 in GSK-3β must be determined.
reorganization. Thus, ILK is not only involved in p38 MAP kinase activation but might also control microtubule dynamics via regulation of GSK-3β activity during neurite outgrowth in N1E-115 cells (Fig. 6). A recent study (33) of nerve growth factor-induced neurite outgrowth using pheochromocytoma (PC12) cells demonstrated that ILK is involved in nerve growth factor-induced neurite outgrowth via inhibition of tau hyperphosphorylation. This recent report using PC12 cells and our results obtained using N1E-115 cells suggest that ILK is an important regulator of both integrin- and growth factor-mediated signaling in neurons and controls neurite outgrowth. Moreover, ILK might be critical for the regulation of microtubule stability and rearrangement necessary for integrin- and growth factor-mediated neurite outgrowth.

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**Fig. 6. Schematic model of aberrant tau phosphorylation in DN-ILK-transfected N1E-115 cells.** ILK is activated after cell adhesion, followed by serum-free conditions in phosphatidylinositol 3-kinase-dependent manner. Stimulation of ILK activity results in activation of p38 MAP kinase activity, which is important for ILK-dependent neurite outgrowth in N1E-115 cells (11). ILK phosphorylates GSK-3β at Ser9, leading to GSK-3β inactivation. GSK-3β inactivation results in inhibition of aberrant tau phosphorylation, an increase in microtubule stability, and induces neurite outgrowth in combination with p38 MAP kinase action. On the other hand, ILK inactivation by DN-ILK induces GSK-3β phosphorylation at Tyr176 via activation of an unidentified tyrosine kinase, leading to GSK-3β activation. GSK-3β activation results in aberrant tau phosphorylation, microtubule instability, and decreased neurite outgrowth.

We demonstrated previously (11) that p38 MAP kinase in the ILK-mediated signal pathway has an important role in integrin-dependent neurite outgrowth in N1E-115 cells. On the other hand, a p38 MAP kinase inhibitor, SB203580, blocked ~80% of the ILK-dependent neurite outgrowth but not to the levels of the DN-ILK-transfected cells (see Fig. 5). In the previous study (11), we suggested that signaling pathways other than p38 MAP kinase, which are also activated via ILK activation, are involved in integrin-dependent neurite outgrowth. We therefore examined whether aberrant tau phosphorylation is involved in the inhibition of neurite outgrowth in DN-ILK-transfected cells. Treatment of the DN-ILK-transfected cells with 10 mM LiCl completely prevented aberrant tau phosphorylation but only partially recovered neurite outgrowth to the levels of that in the SB20358-treated parental cells. These results suggest that aberrant tau phosphorylation is partly involved in the inhibition of neurite outgrowth in DN-ILK-transfected cells. Furthermore, the results also suggest that a p38 MAP kinase pathway is the sole downstream pathway in ILK-dependent neurite outgrowth.

Tau hyperphosphorylation decreases the association of tau with microtubules (29) and inhibits total neurite number (30–32). The inhibitory effect of aberrant tau phosphorylation on neurite outgrowth was as expected. The effect of aberrant tau phosphorylation, however, is considered to depend on the tau phosphorylation level and also on the number of phosphorylated tau molecules. Although we could not estimate how many molecules in total tau are phosphorylated in the DN-ILK-transfected cells in this study, it is possible that the aberrant tau phosphorylation induced by ILK inactivation at least affects microtubule stability or dynamics and leads to inhibition of neurite outgrowth. On the other hand, endogenous ILK protects tau from aberrant phosphorylation and probably maintains a kind of equilibrium status responsible for microtubule formation and stability.
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