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p21-activated protein kinase γ-PAK in pituitary secretory granules phosphorylates prolactin

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Abstract p21-activated protein kinase γ-PAK phosphorylates prolactin (PRL) in rat pituitary secretory granules on Ser-177 and on the equivalent site, Ser-179, in recombinant human PRL. This is shown by comparison of phosphopeptide maps with the human PRL mutant S179D. γ-PAK is present in rat and bovine granules as identified by in-gel phosphorylation of histone H4, and by immunoblotting. Thus, phosphorylation of PRL by γ-PAK in granules produces the PRL molecule that has been shown to antagonize the growth-promoting activity of unmodified PRL, and is consistent with the identified role of γ-PAK in the induction and maintenance of cytostasis.

Key words: Prolactin; Protein kinase; p21-activated protein kinase; Pituitary secretory granule; Phosphorylation; PAK

1. Introduction

γ-PAK (Pak2) is a ubiquitous member of the family of p21-activated protein kinases (PAKs) which are activated by autophosphorylation following binding of the GTP-bound form of the small G-protein Cdc42 (for reviews see [1–5]). γ-PAK is expressed ubiquitously, in contrast to α-PAK which is found mainly in brain, but is also found in other tissues, and β-PAK which is specifically expressed in brain [1–5]. γ-PAK, but not α-PAK or β-PAK, is activated by caspase cleavage during apoptosis [6,7]. Both α- and γ-PAK are autophosphorylated and activated in vivo and in vitro directly by sphingosine [8,9].

α- and γ-PAK are highly homologous but appear to have different functions. α-PAK is involved in growth, as it is activated by growth factors [10,11] and insulin [12], and is involved in the regulation of actin cytoskeletal reorganization [13–16]. γ-PAK is activated in response to a variety of stimuli that induce stress or lead to cytostasis or cell death, including hyperosmolarity [17], DNA damaging drugs and UV and ionizing radiation [18]. γ-PAK appears to be involved in maintaining cells in a non-dividing or cytostatic state [1,19].

γ-PAK phosphorylates a variety of proteins such as histones 4 and 2B, translation initiation factors, myosin light chain from smooth muscle, myosin light chain kinase, myelin basic protein, avian and Rous sarcoma virus nucleocapsid protein, and Abelson tyrosine kinase (c-Abl) (for review see [1]). The variety of substrates suggests the involvement of γ-PAK in the regulation and possible coordination of cellular processes. Regulation of biological functions by phosphorylation with γ-PAK has been shown for c-Abl [20], smooth muscle myosin light chain [21] and myosin light chain kinase [22], and Rous sarcoma virus nucleocapsid protein [23,24]. Using synthetic peptides as substrates, the recognition/phosphorylation determinants have been identified as K/RRXS where X is preferably an acidic amino acid, such as glutamate or aspartate [25].

Prolactin (PRL) is a polypeptide hormone (23 kDa) involved in maintaining normal reproductive functions as well as regulation of growth (for reviews, see [26,27]). PRL exists as multiple charged isoforms and is stored at high concentrations (50 mM) in pituitary secretory granules as osmotically inert structures [28,29]. Phosphorylation of PRL affects its biological functions [30,31]; in two different PRL-responsive cell lines, phosphorylated PRL has been shown to antagonize the growth-promoting effects of unmodified PRL [30,32]. The major phosphorylation site in vivo in rat PRL is serine 177 [33]; this corresponds to serine 179 in human and bovine PRL [34]. A mutant of human prolactin (S179D), where serine 179 is mutated to aspartate to mimic phosphorylated serine, is a highly effective antagonist to unmodified PRL [35]. In addition, it has been shown that phosphorylated PRL autoregulates PRL secretion in normal pituitary cells [36], and the phosphorylation states of the hormone released from the pituitary change during the estrous cycle [37] and during pregnancy and pseudopregnancy [38].

Previous studies show that purified rat PRL is phosphorylated in vitro by γ-PAK (originally known as PAK I) [39]. The in vivo phosphorylation site in rat PRL, serine 177/179 in the sequence RRDSHK [33], is in a highly conserved region among all PRLs [34] and contains the phosphorylation recognition determinant for γ-PAK. Here, we report that γ-PAK is present in rat and bovine pituitary secretory granules and phosphorylates Ser-177/179. The data suggest that γ-PAK regulates the growth-promoting activity of PRL in vivo.

2. Materials and methods

2.1. Materials

Histone 4 and histone IIAS were from Roche Biochemicals; cypermethrin was from LC Laboratories. Antibodies which react specifically with the regulatory domain of γ-PAK (N-19; SC 1872) or the catalytic domain of the PAK isoforms (C-19; SC 1519), peroxidase-

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Abbreviations: PAK, p21-activated protein kinase; PRL, prolactin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase

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coupled secondary antibodies and protein A/G agarose were from Santa Cruz Biotechnology. RR-1 anti-PAK antibody, which reacts preferentially with endogenously activated γ-PAK [18], was obtained as previously described [19]. Cellulose thin-layer chromatography sheets were from Selecto Scientific and silica gel thin-layer chromatography sheets were from EM Science.

Glutathione *S*-transferase (GST)-γ-PAK obtained by expression of the cDNA in insect cells (TN5B-4) was purified by binding to glutathione-Sepharose 4B and released with glutathione or by cleavage with thrombin as described previously [40]. GST-Cdc42 [41] and wild-type human PRL and the S179D mutant [35] were expressed in *Escherichia coli* and purified as described.

2.2. Isolation of prolactin secretory granules

Prolactin secretory granules from bovine and rat were obtained as previously described [42,43]. Briefly, the production of rat granules involved homogenization in 0.32 M sucrose, removal of debris and nuclei by low-speed centrifugation, pelleting of organelles by centrifugation for 30 min at $15\,000\times g$, and puromycin treatment of the pellet which resulted in the detachment of ribosomes from rough microsomes and lysis of contaminating growth hormone granules. The puromycin-treated material was loaded onto discontinuous sucrose gradients to yield a prolactin secretory granule fraction relatively free of contaminants, as indicated by ultrastructural and biochemical criteria [43]. The secretory granules were washed in high salt to remove proteins adsorbed on the cytoplasmic face of the granules, then pelleted in 0.32 M sucrose containing 5 mM $MgCl_2$ and 1.0 M KCl; the pellet was resuspended in 0.5 ml of 0.32 M sucrose solution. As described earlier, two-dimensional polyacrylamide gel electrophoresis and electron microscopic analyses of the subcellular fraction served as controls for purity [43]. Analysis of 5′ nucleotidase as a plasma membrane marker, and succinate cytochrome *c* reductase as a mitochondrial marker, demonstrated maximal contamination of 2.7% and 1.35%, respectively [44].

2.3. Phosphorylation of prolactin

In order to solubilize the limiting granule membrane and the proteins and to monomerize the multimeric structure of PRL, secretory granules (5–10 μg) were pre-incubated at 37°C for 50–60 min in 12 μl of buffer A (50 mM Tris–HCl, pH 7.5, 75 mM 2-mercaptoethanol, 7.5 mM ethylenediaminetetraacetic acid (EDTA), 0.02% Triton X-100) containing protease inhibitors, aprotinin and leupeptin (0.17 mg/ml) and the phosphatase inhibitor cypermethrin (0.4 μM). Phosphorylation of PRL (10–20 μg of granule) was carried out in 30 μl reaction mixtures containing 20 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 3 mM EDTA, 30 mM 2-mercaptoethanol, 0.008% Triton X-100, 0.2 mM [γ^{32}P]ATP (2000 cpm/pmol), 0.16 μM cypermethrin, 0.07 mg/ml of aprotinin and leupeptin, and GST-γ-PAK (0.2 μg) activated with Cdc42 (GTPγS) [40]. Incubation was for 30 min at 30°C. Reactions were terminated by addition of 10 μl of 100 mM ATP and sodium dodecyl sulfate (SDS) sample buffer. Following SDS polyacrylamide gel electrophoresis (SDS–PAGE), phosphorylated proteins were detected by autoradiography on X-ray film or with a phosphorimager.

Purified recombinant wild-type PRL and the mutant S179D (1.0 μg) were phosphorylated as described above, except that there was no preincubation and Triton X-100 was omitted from buffer A.

2.4. Tryptic phosphopeptide mapping and phosphoamino acid analysis

Phosphorylated PRL was excised from SDS polyacrylamide gels and extensively digested with trypsin and analyzed by two-dimensional phosphopeptide mapping and phosphoamino acid analysis [45].

2.5. Detection of γ-PAK activity by in-gel phosphorylation of histone

Proteins in PRL granules from rat (10 μg) and bovine (20 μg) were separated on 10% polyacrylamide gels cast with 0.2 mg/ml of histone IIAS; following electrophoresis, the proteins in the gel were denatured and renatured and the protein kinase activity was assayed in-gel as described [19].

2.6. Western blotting

Proteins in PRL granules from rat (10 μg) and bovine (20 μg) pituitary glands were resolved by SDS–PAGE on a 10% gel and were electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked using 5% milk in Tris-buffered saline containing 0.1% Tween 20 and probed with antibodies (N-19 or RR-1) which react specifically with the regulatory domain of γ-PAK, or C-19 which reacts preferentially with the catalytic domain of γ-PAK as described previously [18,19]. Antibody binding was detected using peroxidase-conjugated secondary antibodies and visualized with ECL chemiluminescence reaction reagents.

3. Results

3.1. γ-PAK phosphorylates PRL on serine 177/179

To determine whether PRL in secretory granules was phosphorylated by γ-PAK, rat granules were pre-incubated in the presence of Triton X-100, EDTA and reducing agent; this opened the granule membrane, allowing access to [γ-^{32}P]ATP, solubilizing the semicrystalline proteins and converting the multimeric structure of PRL into monomers. The solubilized proteins were analyzed as substrates for γ-PAK by incubation with [γ-^{32}P]ATP and Mg^{2+} in the presence or absence of active γ-PAK. A low level of phosphorylation of rat PRL was observed when the extract was incubated alone (Fig. 1A). Upon addition of exogenous γ-PAK activated by preincubation with Cdc42(GTPγS), phosphorylation of PRL was increased 15–20-fold over that observed with endogenous protein kinase(s).

Previously, Ser-179 was shown to be the phosphorylation site involved in conferring antagonistic properties to phosphorylated human PRL, as compared to non-phosphorylated PRL. To determine whether γ-PAK phosphorylated this site, purified wild-type human PRL and the mutant S179D were examined as substrates for γ-PAK; both were phosphorylated,

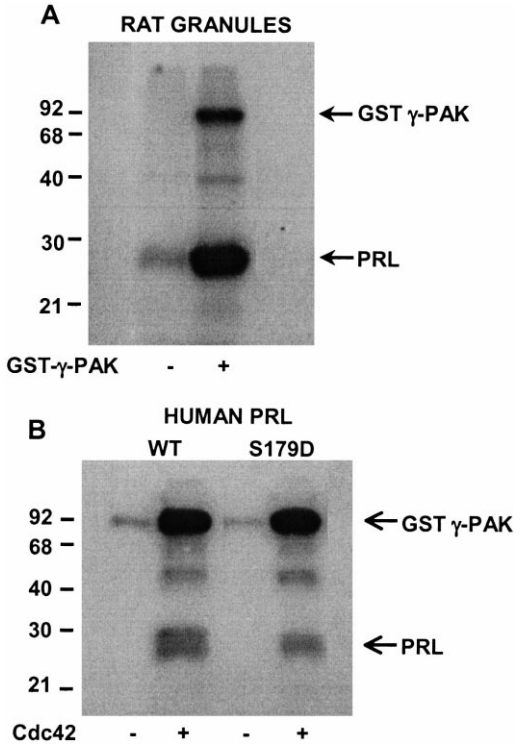

Fig. 1. Phosphorylation of PRL by γ-PAK. A: Solubilized protein from rat secretory granules (5 μg) was incubated with [γ-^{32}P]ATP in the presence or absence of activated GST-γ-PAK as described in Section 2. B: Purified recombinant human PRL or the mutant S179D (1.0 μg) phosphorylated with GST-γ-PAK (0.2 μg) in the presence or absence of Cdc42 (GTPγS). The samples were analyzed by SDS–PAGE and the radiolabeled proteins were detected by autoradiography.

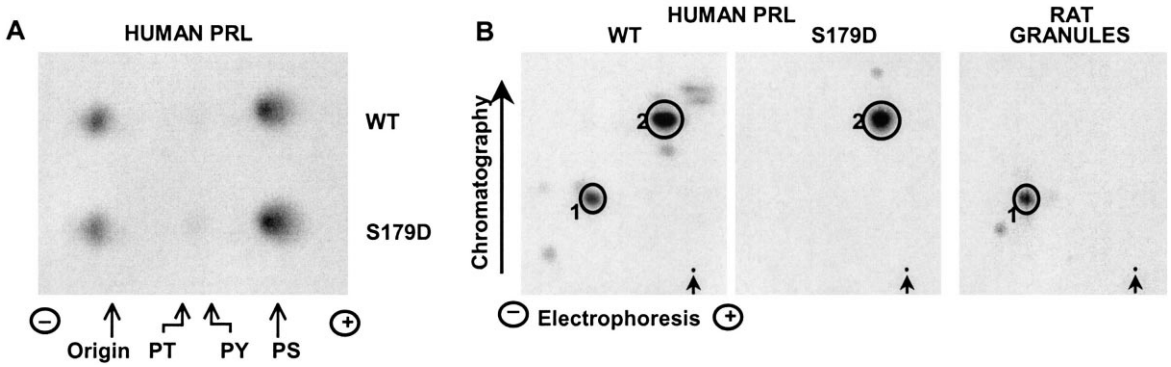

Fig. 2. Identification of a phosphorylation site for γ-PAK in PRL. A: Phosphoamino acid analysis of human PRL and S179D phosphorylated by γ-PAK. The positions of the phosphoamino acid standards are identified. B: Two-dimensional tryptic phosphopeptide mapping of wild-type human PRL (left), S179D (middle) and PRL from rat granules (right) phosphorylated by exogenous γ-PAK. The ^{32}P-labeled phosphoamino acids and phosphopeptides were detected using a phosphorimager system.

with 30–40% more phosphate incorporated into the wild-type protein, as compared to the mutant (Fig. 1B). Phosphoamino acid analysis showed only serine was phosphorylated in both wild-type PRL and S179D (Fig. 2A). Tryptic phosphopeptide mapping of wild-type PRL showed two major phosphopeptides. One of the phosphopeptides (spot 1) was missing in S179D, identifying serine 179 as a site phosphorylated by γ-PAK (Fig. 2B).

When solubilized PRL from rat granules was phosphorylated by exogenous γ-PAK, and the phosphorylation sites in PRL were analyzed by tryptic phosphopeptide mapping, only one tryptic phosphopeptide was observed (Fig. 2B). This phosphopeptide migrated at the same position as the tryptic phosphopeptide that contained phosphorylated Ser-179 (spot 1). Since the sequences of the tryptic phosphopeptide containing Ser-179 in human PRL and Ser-177 in rat PRL [34] are identical, we can conclude that γ-PAK phosphorylates Ser-177 in rat PRL.

3.2. γ-PAK is present in PRL secretory granules

To determine whether the secretory granules contained γ-PAK, solubilized granule proteins were assayed for γ-PAK activity using histone 4. Histone 4 has been shown to be a specific substrate for PAKs; they are the only serine/threonine protein kinases reported to phosphorylate this substrate. Histone 4 was phosphorylated by a protein kinase in both bovine and rat secretory granules (Fig. 3A). Addition of GST-γ-PAK resulted in increased phosphorylation of histone 4 and of rat PRL.

To determine the molecular weight of the histone 4 kinase, in situ phosphorylation of histone cast into SDS polyacrylamide gels was carried out using rat and bovine secretory granules, and purified recombinant γ-PAK. The protein kinase activity corresponded to the same molecular weight (58–60 kDa) as the recombinant γ-PAK standard (Fig. 3B). It also corresponded to the phosphoprotein bands in Fig. 3A which migrated at 58–60 kDa (see arrow to γ-PAK) in the absence of GST-γ-PAK. The in-gel protein kinase activity was stimulated 1.5-fold when the solubilized proteins from bovine granules were preincubated with Cdc42(GTPγS) and ATP prior to analysis by SDS–PAGE and autoradiography (Fig. 3C); activation by Cdc42 is a characteristic feature of PAKs. Thus,

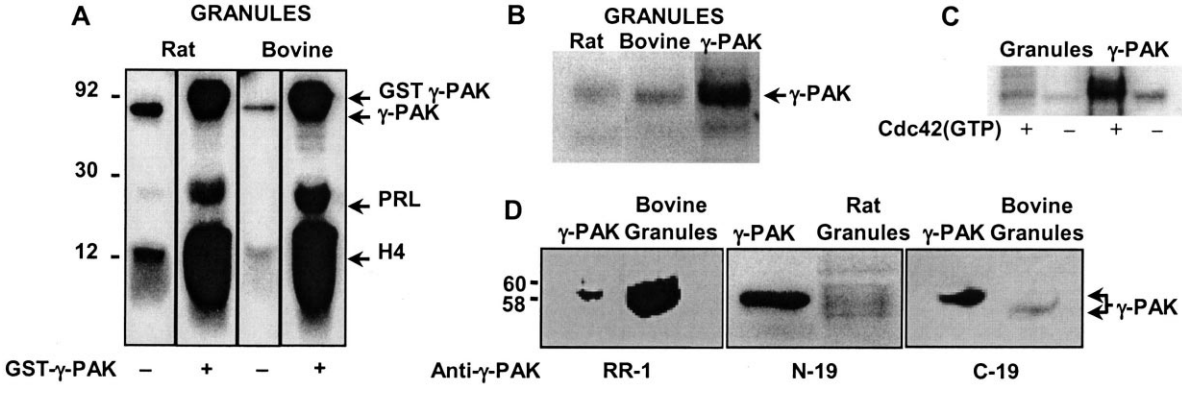

Fig. 3. Detection of γ-PAK in Secretory Granules. A: Proteins from secretory granules from rat (5 μg) or bovine (10 μg) were solubilized and assayed for γ-PAK activity using histone 4 as substrate. The samples were analyzed by SDS–PAGE on 15% gels, and the radiolabeled proteins were detected by autoradiography. B: Solubilized protein from rat granules (10 μg) or from bovine granules (20 μg), and recombinant γ-PAK (0.2 μg), were analyzed by in situ phosphorylation of histone following SDS–PAGE on 10% polyacrylamide, as described in Section 2. γ-PAK activity was detected by autoradiography. C: Solubilized protein from bovine granules (20 μg) and recombinant γ-PAK were incubated with Cdc42(GTPγS) and ATP prior to analysis by in situ phosphorylation of histone. D: Solubilized proteins from rat (10 μg) and bovine (20 μg) secretory granules were immunoblotted with antibodies N-19 and RR-1, which react specifically with the regulatory domain of γ-PAK, and C-19 which reacts with the highly conserved catalytic domain of the three PAK proteins. Detection was carried out with horseradish peroxidase-linked secondary antibodies and ECL reagents.

preactivation enhanced phosphorylation of histone in the gel, indicating both active and inactive forms of γ-PAK were present in the granule. Since α- and β-PAK migrate at 68 and 65 kDa on SDS–PAGE, respectively, these forms of PAK were not present in any significant amounts in the granules.

Further confirmation of the identity of the protein kinase in PRL granules was provided by immunoblotting studies with antibodies specific for γ-PAK. A protein migrating at the same molecular weight as γ-PAK (58–60 kDa) was immunoreactive with antibodies specific for the N-terminus of γ-PAK, N-19 and RR-1. With N-19 antibody, multiple bands were detected, reflecting the multiple phosphorylated forms of γ-PAK [18]. C-19, prepared to the conserved catalytic domain of the PAKs, reacted only with protein migrating at 58 kDa, supporting the finding in Fig. 3C that α- and β-PAK were not present in the granule preparation.

4. Discussion

The biological activity of PRL has previously been shown to be regulated, in part, by the state of phosphorylation [30,42]. Non-phosphorylated PRL promotes growth, while phosphorylated PRL induces cytostasis. The major phosphorylation site in PRL is Ser-177/179 [33,34]. Here we show that γ-PAK is present in secretory granules and both endogenous and exogenous γ-PAK phosphorylate PRL on Ser-177/179.

Phosphorylation of PRL from secretory granules is more effective when the membrane is disrupted with Triton X-100, metal ions are chelated by EDTA and intermolecular bridges are disrupted by reducing agents; these aid in the formation of monomers from the multimeric semi-crystalline PRL [25,46]. PRL is also phosphorylated in secretory granule extracts by the endogenous protein kinase that has been identified as γ-PAK using a variety of methods. These include phosphorylation of the PAK-specific substrate histone 4, migration of the protein kinase activity at 58–60 kDa by an in-gel kinase assay, Western blotting with antibody specific for γ- PAK and activation of the endogenous protein kinase by Cdc42.

Recombinant human PRL is phosphorylated by γ-PAK at two sites as determined by tryptic phosphopeptide mapping. One of the phosphorylation sites is Ser-179 in phosphopeptide 1, as deduced from the disappearance of phosphopeptide 1 when the mutant S179D is phosphorylated by γ-PAK. The identity of phosphopeptide 2 is not known at this time. Only one major phosphopeptide is observed when PRL from rat secretory granules is phosphorylated by γ- PAK. The migration of this phosphopeptide is identical to the phosphopeptide that contains Ser-179 in human PRL; thus it can be concluded that rat PRL is phosphorylated specifically on Ser-177 by γ-PAK. Ser-177/179 is in the sequence RRDSHK that contains the recognition/phosphorylation determinant for γ-PAK [27]. Protein kinase A has been shown to phosphorylate this same site in rat PRL [33], although large amounts of the enzyme are needed to effect significant phosphorylation.

The reported sequence for γ-PAK contains no signal sequence that would automatically place it in the secretory pathway and eventually into secretory granules. Since the vast majority of γ-PAK is cytosolic [17,18], this is to be expected. However, it is unlikely that the γ-PAK activity observed in the granules is the result of contamination by cytoplasmic γ-PAK for three reasons: (1) preparation of the granules involves a high salt wash with 1 M KCl; (2) previous studies have demonstrated that solubilization of the granule membrane, the material to which cytosolic γ-PAK could adhere during granule isolation, does not eliminate PRL kinase activity in the granules [43]; (3) there is a possibility that PAK in granules is *N*-glycosylated, since treatment with a deglycosylation enzyme, purified peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (PNGase F) [47], results in a decrease in molecular weight (data not shown). *N*-Glycosylation is a posttranslational modification which only occurs within the secretory pathway.

The question remains therefore as to how γ-PAK finds its way into the secretory granule to effect phosphorylation of PRL. There are several known mechanisms whereby proteins translated on free ribosomes can cross membranes after synthesis. Likely possibilities include the TAP protein mechanisms for cytosol to endoplasmic reticulum cisternal space import (for review see [48]) and the ABC-ATPase system, variants of which can move proteins from the cytosol into membrane-bound compartments in the cell, or can move them out of the cell through the plasma membrane [49]. Alternatively, it remains possible that a differentially spliced mRNA may be produced in the pituitary (as a minor species) which encodes a version with a signal sequence. Interestingly, protein kinase A and protein kinase C have been shown to be present in anterior pituitary granules [50], although they also do not contain a signal sequence.

The phosphorylated and non-phosphorylated forms of PRL have been shown to have different effects on cell growth. Phosphorylation at Ser-177/179 has a major effect on biological activity; it not only eliminates the growth-promoting qualities of PRL, but antagonizes the growth-promoting effect of unmodified PRL. For example, we recently showed that unmodified PRL can be an autocrine growth factor in human prostate cancer cells, and that the mimic of phosphorylated PRL (S179D) interrupts growth in vivo [51]. The data herein suggest that γ-PAK may retard growth in these and perhaps other cells via phosphorylation of PRL at Ser-177/179. γ-PAK has been shown to have cytostatic properties (as reviewed in [1]). It is interesting to note that apoptotic stimuli also activate γ-PAK and that S179D promotes apoptosis in the lung and thymus of exposed rat pups [52].

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