Association between v-Src and Protein Kinase C δ in v-Src-transformed Fibroblasts

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In response to the kinase activity of v-Src there is an increase in the membrane association of the novel protein kinase C (PKC) isoform PKC δ (Zang, Q., Frankel, P., and Foster, D. A. (1995) Cell Growth Differ., 6, 1367–1373). We report here that in v-Src-transformed cells PKC δ co-immunoprecipitates with v-Src and is phosphorylated on tyrosine. The tyrosine-phosphorylated PKC δ had reduced enzymatic activity relative to the non-tyrosine-phosphorylated PKC δ from v-Src-transformed cells. The association between Src and PKC δ was dependent upon both an active Src kinase and membrane association. The association between c-Src Y527F and PKC δ was substantially enhanced by mutating a PKC phosphorylation site at Ser-12 in Src to Ala indicating that PKC δ phosphorylation of Src at Ser-12 destabilizes the interaction, possibly in a negative feedback loop. These data demonstrate that upon recruitment of PKC δ to the membrane in v-Src-transformed cells there is the formation of a Src:PKC δ complex in which PKC δ becomes phosphorylated on tyrosine and down-regulated.

Protein kinase C (PKC) has been implicated in a wide variety of signaling mechanisms (1, 2). There are several isoforms of PKC that fall into three major categories based on differential Ca²⁺ and lipid requirements. The α, δ, ε, and ζ PKC isoforms are predominant in fibroblasts (3, 4). The conventional α PKC isoform requires both Ca²⁺ and diacylglycerol (DG). The novel δ and ε isoforms require DG but not Ca²⁺, and the atypical ζ isoform is insensitive to both DG and Ca²⁺. The activation of several transcriptional promoters by the oncogenic tyrosine kinase v-Src is dependent upon PKC (5–7). We recently reported that in both murine and rat fibroblast transformed by the oncogenic tyrosine kinase v-Src there is an increased membrane association of the α and δ but not the ε or ζ PKC isoforms (4). Since the δ and ε PKC isoforms both belong to the Ca²⁺-independent class of PKC, the preferential increase in membrane association of the δ over the ε isoform could not be explained by Ca²⁺ and suggested that regulation of this class of PKC isoform involved more than simply elevating DG levels.

The selective increase in membrane association of the δ over the ε isoform of PKC in v-Src-transformed cells was also surprising because of previous reports that overexpression of PKC δ inhibits cell proliferation and that overexpression of PKC ε enhances cell growth (8, 9). These observations suggested the possibility that PKC δ might have a different effect in v-Src-transformed cells than in the non-transformed parental cells. Alternatively, membrane association of PKC δ in v-Src-transformed cells may not correlate with an activation of its kinase activity since it has been demonstrated that PKC isoforms α and ε can affect phospholipase D (10, 11) and phosphatidate phosphohydrolase (12) activity independent of the kinase activity of the α and ε isoforms respectively.

Tyrosine phosphorylation of PKC δ in response to several different stimuli has recently been reported (13–16). The biological significance of the tyrosine phosphorylation of PKC δ is unclear. It has been reported that tyrosine-phosphorylated PKC δ has a reduced kinase activity in Ras-transformed cells (13). Similarly, epidermal growth factor receptor activation also resulted in a decrease in the kinase activity of tyrosine-phosphorylated PKC δ (16). In contrast, PKC δ that was phosphorylated on tyrosine by either Fyn or the insulin receptor in vitro had elevated kinase activity (14). In response to antigen activation of the IgE receptor, PKC δ becomes tyrosine-phosphorylated, and phosphorylation apparently alters its substrate specificity (15). Thus, the effect of tyrosine phosphorylation on PKC δ activity is apparently complex and may involve other cellular factors.

The tyrosine kinase(s) responsible for PKC δ phosphorylation are not known. In vitro studies have shown that PKC δ can be phosphorylated by Src family and receptor tyrosine kinases (14, 17). In this report, we describe a functional interaction between Src and PKC δ in cells transformed by v-Src.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Conditions—3Y1 and v-Src-transformed 3Y1 rat fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, Inc.) as described previously (4). In some cases, 12-O-tetradecanoylphorbol-13-acetate (TPA) was added at 200 nM for 30 min to activate PKC or 800 nM for 24 h to deplete cells of PKC.

Transfections and Plasmid Vectors—3Y1 cells were plated at a density of 10⁶ cells/100-mm dish 18 h prior to transfection. Transfections were performed using LipofectAMINE reagent (Life Technologies, Inc.) according to the vendor's instructions. The plasmid expression vectors contained the G418 resistance marker, and transfected cultures were selected in 400 ng/ml G418 for 8–10 days at 37 °C. At that time colonies were examined for morphology, picked, and expanded for additional analysis. The c-Src mutants transfected into 3Y1 cells are as follows: c-Src Y527F has a mutation of Tyr to Phe at position 527 (18); c-Src

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Association between Src and PKC δ

**Fig. 1. PKC δ is tyrosine-phosphorylated in v-Src-transformed 3Y1 cells.** A, cell lysates were generated from either 3Y1 cells or 3Y1 cells transformed by v-Src (3Y1-v-Src). Cell lysates (containing 1.5 mg of total protein) were immunoprecipitated (IP) with a control mouse serum or antibodies raised against Tyr(P) (P-Tyr) or PKC δ, and immune complexes were recovered with protein A-agarose and subjected to Western blot (WB) analysis using antibodies against either Tyr(P) (P-Tyr) or PKC δ as shown. 3Y1-vSrc – PKC, cells were depleted of PKC by prolonged treatment with TPA (800 nM, 24 h). 3Y1-vSrc + δ pep, the peptide against which the PKC δ antibody had been raised was included in the immunoprecipitation to neutralize the anti-PKC δ antibody. 3Y1-vSrc + PNP, 30 mM phenylphosphate was included to neutralize the anti-phosphotyrosine antibody. 3Y1 Lysate (containing 20 μg total protein) was loaded prior to immunoprecipitation. B is identical to A except that the lysates were denatured (D) by treatment with 1% SDS and boiled for 10 min prior to immunoprecipitation.

**Y327FS12A** has an additional change at Ser-12 to Ala (19); the LN mutation has 4 additional amino acids at the amino terminus (MAAA) (20) as was included in the c-Src Y327F context as described for the S12A mutation (19); the SH2 deletion of c-Src Y327F-ASH2 has a disruption of the SH2 domain in which amino acids 148–187 have been deleted (21), and this mutation was placed in the c-Src 527 context as with the LN and S12A mutations (19). All Src constructs were in the pEVX expression vector (22, 23).

**Antibodies—**Anti-phosphotyrosine monoclonal antibody (4G10) (Upstate Biotechnology) was used for Western blots, and monoclonal anti-phosphotyrosine (PY20) (Transduction Laboratories) was used for immunoprecipitations. For Src, a monoclonal antibody from Oncogene Sciences was used for Western blots, and a monoclonal antibody from Upstate Biotechnology was used for immunoprecipitations. For PKC δ, a polyclonal antibody obtained from Life Technologies, Inc. was used for Western blots and a polyclonal antibody obtained from Calbiochem was used for immunoprecipitations. Protein-tyrosine phosphatase 1B was obtained from Upstate Biotechnology.

**Cell Lysate Preparation and Subcellular Fractionation—**Cells grew to approximately 85% confluence in 150-mm culture dishes and then were shifted to Dulbecco’s modified Eagle’s medium containing 0.5% serum for 24 h. Cells were washed three times with ice-cold isosotic buffer (phosphate-buffered saline (PBS), 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH2PO4, 4.2 mM Na2HPO4, pH 7.2). For subcellular fractionation, cells from 150-mm dishes were washed and then scraped into 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.5, 5 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 2 mM dithiothreitol, 200 μM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cells were then disrupted with 20 strokes in a Dounce homogenizer (type B pestle), and the lysate was centrifuged at 100,000 × g for 1 h. The supernatant was collected as the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton X-100. After incubation for 30 min at 4 °C, the suspension was centrifuged at 100,000 × g for 1 h. The supernatant was collected as the membrane fraction. For whole cell lysates, cells were treated with 1 ml of homogenization buffer containing 1% Triton X-100 followed by centrifugation at 100,000 × g for 1 h. The supernatant was collected and used as the whole cell lysate.

**Immunoprecipitation—**Cell lysates or cell fractions prepared as described above were incubated with appropriate antibodies at 4 °C overnight. Antigen-antibody complexes were recovered using protein A-agarose beads (Santa Cruz Biotechnology). For immunoprecipitations with mouse monoclonal antibodies, rabbit anti-mouse IgG was added to the lysates for an additional hour of incubation prior to recovery with protein A. The immunoprecipitates were washed three times with immunoprecipitation wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5% Nonidet P-40, 1% Triton X-100).

**Western Blot Analysis—**Samples were normalized to contain equal amounts of protein in the total cell lysate or from cytosolic and membrane fractions prior to immunoprecipitation. The immunoprecipitated samples were subjected to SDS-polyacrylamide gel electrophoresis using an 8% acrylamide separating gel followed by transfer to nitrocellulose as described previously (4, 24). After blocking at 4 °C overnight with 5% nonfat dry milk in PBS buffer, nitrocellulose filters were incubated with appropriate primary antibodies. Depending on the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham Corp.) or the super signal system (Pierce).

**Assay of In Vitro PKC Activity—**PKC activity of tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ was determined according to protocols described by Denning et al. (13). Cell lysates from v-Src-transformed cells were immunoprecipitated with anti-phosphotyrosine antibody, and phosphorytose-containing proteins were recovered with protein A-agarose beads. The supernatant was used as the source of non-tyrosine-phosphorylated PKC δ. The anti-phosphotyrosine immunoprecipitate pellet was resuspended in homogenization buffer containing 30 mM phenylphosphate to release the tyrosine-phosphorylated proteins. The antibodies were recovered by centrifugation, and the supernatant was used as the source of tyrosine-phosphorylated PKC δ. Both the tyrosine-phosphorylated and non-tyrosine-phosphorylated preparations were then immunoprecipitated with anti-PKC δ antibody. The immunoprecipitates were washed three times with immunoprecipitation buffer and twice with 20 mM HEPES, pH 7.5, and 10 mM MgCl2 followed by resuspension in 100 μl of kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM histone type III, 60 μM/ml phosphatidylserine, and TPA at 1 μM if included). [γ-32P]ATP (10 μCi, 3000 Ci/mmol) was present at 100 μM. PKC activity was then determined as described previously (24). The PKC δ levels in the assays was determined by Western blot analysis, and activity was normalized to these levels.

**RESULTS**

**PKC δ Is Tyrosine-phosphorylated in v-Src-transformed 3Y1 Cells—**In v-Src-transformed 3Y1 cells, the δ isoform of PKC is preferentially associated with the membrane relative to the parental 3Y1 cells (4). It was recently reported that PKC δ can be phosphorylated on tyrosine (13–16) and that Src family kinases can phosphorylate PKC δ on tyrosine in vitro (14, 17). We therefore investigated tyrosine phosphorylation of PKC δ in v-Src-transformed 3Y1 rat fibroblasts, where the expression of v-Src results in increased membrane association of PKC δ. 3Y1 cells and v-Src-transformed 3Y1 cells were lysed and subjected to immunoprecipitation with antibodies against either phosphotyrosine (Tyr(P)) or PKC δ. The immunoprecipitates were then subjected to Western blot analysis using either anti-Tyr(P) or anti-PKC δ antibody. As shown in Fig. 1A, anti-Tyr(P) antibody precipitated a protein from v-Src-transformed 3Y1 cells that could be recognized by the anti-PKC δ antibody and reciprocally, the 80-kDa protein precipitated by the anti-PKC δ antibody from the v-Src-transformed cells was recognized by the anti-Tyr(P) antibody. These results were observed only in the v-Src-transformed cells. As expected, PKC δ depletion by prolonged treatment with phorbol ester ablated precipitation of PKC δ by the anti-Tyr(P) antibody, and treatment with phenyl phosphate (a phosphotyrosine analog) ablated precipitation of PKC δ by anti-Tyr(P) antibody. As expected, the...
SDS and heated at 100 °C for 10 min prior to immunoprecipitation of PKC δ cells has reduced enzymatic activity—Tyrosine phosphorylation is specific for the isoforms v-Src-transformed 3Y1 cells. Tyrosine phosphorylation of PKC δ has been reported to both enhance (14) and reduce (13, 15) the kinase activity of PKC δ. We therefore compared the kinase activity of tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ. Sequenatal immunoprecipitation with anti-Tyr(P) and anti-PKC δ antibodies was used to separate tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ isolated from v-Src-transformed cells as described under “Experimental Procedures.” We then examined the in vitro kinase activity as described previously (24). As shown in Fig. 3, the kinase activity of the tyrosine-phosphorylated PKC δ was reduced by about 33% of the non-tyrosine-phosphorylated PKC δ for both basal and TPA-induced enzymatic activity. Consistent with tyrosine phosphorylation having an inhibitory effect on the kinase activity of PKC δ, treatment of the tyrosine-phosphorylated PKC δ with protein-tyrosine phosphatase 1B (PTP1B) restored the kinase activity to about 70% of that observed in the non-tyrosine-phosphorylated PKC δ (Fig. 3). These data suggest that tyrosine phosphorylation of PKC δ reduces the enzymatic activity of PKC δ in v-Src-transformed cells.

PKC δ Associates with v-Src—Since v-Src was shown previously to be able to phosphorylate PKC δ directly in vitro (17), we further explored the possibility that PKC δ may be a substrate of v-Src in vivo by examining it for an association between PKC δ and v-Src. The results of co-immunoprecipitation experiments are shown in Fig. 4. When cell lysates were im-

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**Fig. 2.** Tyrosine-phosphorylated PKC δ is primarily associated with the membrane fraction. Lysates from membrane (M) and cytosolic (C) cell fractions were prepared as described under “Experimental Procedures” and immunoprecipitated with anti-PKC δ antibody. The immunoprecipitates were then subjected to Western blot analysis using anti-PKC δ or anti-Tyr(P) (anti-P-Tyr) antibodies as indicated. Cells were either treated (TPA) or untreated (Con.) with 100 nM TPA for 30 min prior to preparation of subcellular fractions. The relative amounts of PKC δ in the different fractions was determined by densitometer scanning of autoradiographs.

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**Fig. 3.** Tyrosine-phosphorylated PKC δ from v-Src-transformed cells has reduced enzymatic activity. Cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins were recovered with protein A-agrose and then eluted with phenyl phosphate as described under “Experimental Procedures.” PKC δ was then precipitated with anti-PKC δ antibody from both the eluted tyrosine-phosphorylated proteins and the supernatant of the anti-phosphotyrosine immunoprecipitation. PKC δ activity was determined in the presence (+) and absence (−) of TPA (1 μM). Treatment with protein-tyrosine phosphatase 1B (PTP1B) was performed after recovery with anti-PKC δ antibody and was for 30 min according to the vendor’s instructions. PKC δ was then recovered from the PTP1B assay mixture by centrifugation of the antibody-bound PKC δ. PKC δ kinase activity was determined by phosphorylation of histone type IIIS as described under “Experimental Procedures.” Kinase activity values were normalized to PKC δ levels in the immunoprecipitates as determined by Western blot analysis. Relative PKC δ activity represents the PKC δ activity normalized to the activity present in the non-phosphorylated PKC δ in the absence of TPA which was given a value of 100%. Error bars represent the range of values for at least two experiments where values varied by less than 10%.

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**Fig. 4.** PKC δ associates with v-Src. Lysates from 3Y1 or v-Src-transformed 3Y1 cells (3Y1-vSrc) were immunoprecipitated (IP) with either anti-v-Src, anti-PKC δ, or anti-PKC ε antibody and then subjected to Western blot analysis using the anti-PKC δ and anti-v-Src antibodies as shown. The 3Y1 and 3Y1-vSrc lysates were subjected to Western blot analysis without prior immunoprecipitation and represented 2% of the lysate used for the immunoprecipitations.
munoprecipitated with v-Src antibody and then Western-blot-
ted with anti-PKC\(\delta\) antibody, PKC\(\delta\) was detected in v-Src
immunoprecipitates from v-Src-transformed 3Y1 cells, but not
the parental 3Y1 cells. In the reciprocal experiment, where
anti-PKC\(\delta\) immunoprecipitates were Western-blotted with an-
ti-v-Src antibody, the PKC\(\delta\) antibody co-precipitated v-Src
protein. v-Src was not detected in anti-PKC\(\delta\) immuno-
precipitates (Fig. 4). The amount of v-Src in the anti-PKC\(\delta\)
immunoprecipitates is estimated to be about 1–2% of the total v-Src,
and the amount of PKC\(\delta\) in the anti-v-Src precipitates is also
estimated to be about 1–2% of the total PKC\(\delta\).

**Interaction between PKC\(\delta\) and Src Mutants**—To further
investigate the interaction between PKC\(\delta\) and Src, we charac-
terized the interaction between PKC\(\delta\) and Src in cells overex-
pressing c-Src and several c-Src mutants (Fig. 5A). Cell lines
that overexpress the c-Src genes were established and expres-
sion levels of the c-Src proteins were determined by Western
blot analysis (Fig. 5B). We first examined the interaction be-
tween PKC\(\delta\) and c-Src and an activated mutant of c-Src that
has the Tyr at 527 converted to Phe (c-Src Y527F) (18, 20). As
shown in Fig. 5C, very little Src protein was present in anti-
PKC\(\delta\) immunoprecipitates from cells overexpressing c-Src.

Consistent with this observation, little or no tyrosine phospho-
rylation of PKC\(\delta\) was detected in the c-Src-overexpressing cells
(Fig. 5C). In contrast, activated c-Src Y527F was associated
with PKC\(\delta\), and PKC\(\delta\) was tyrosine-phosphorylated, although
not quite to the level observed in cells expressing v-Src. How-
ever, c-Src Y527F was as active as v-Src in inducing tyrosine
phosphorylation of PKC\(\delta\) if TPA was added to stimulate mem-
brane association of PKC\(\delta\).
phosphorylation of PKC δ. A mutation to the SH2 domain of c-Src 527 had little or no effect upon either tyrosine phosphorylation of PKCδ or the association between Src and PKC δ (Fig. 5C). Lastly we examined the effect of an amino-terminal modification of c-Src 527 that prevents membrane association but not kinase activity. This mutant protein (c-Src 527-LN) failed to associate with PKC δ and did not stimulate tyrosine phosphorylation of PKC δ. These data indicate that the interaction between Src and PKC δ requires both Src tyrosine kinase activity and membrane localization. Phosphorylation of Src at Ser-12 may lead to the dissociation of a Src-PKC δ complex, since a mutation at this site increased the Src-PKC δ interaction.

DISCUSSION

We have demonstrated that in cells transformed by v-Src, PKC δ is phosphorylated on tyrosine and is associated with v-Src. This interaction requires active, membrane-localized Src kinase. The association between Src and PKCδ was not significantly affected by SH2 deletion but was greatly enhanced by a mutation to the PKC phosphorylation site on Src at Ser-12. The tyrosine-phosphorylated PKC δ had reduced kinase activity relative to the non-tyrosine-phosphorylated PKC δ. We previously reported that PKC δ becomes preferentially associated with the membrane in response to the kinase activity of v-Src (4). The increase in membrane association of PKC isoforms has been widely used to demonstrate PKC isoform activation. The finding here that tyrosine phosphorylation of PKC δ inhibits its kinase activity suggests that regulation of novel PKC isoforms involves more than DG-mediated recruitment to the membrane.

It was previously reported that PKC δ could be phosphorylated on tyrosine in response to phorbol esters that activate PKC (14). However, in 3Y1 cells and in 3Y1 cells overexpressing wild type c-Src or activated c-Src that was not membrane-localized (c-Src Y527F-LN), we did not see an increase in PKC δ tyrosine phosphorylation in response to TPA. On the other hand, in cells expressing activated membrane-bound c-Src Y527F, we did detect a TPA-induced increase in PKC δ tyrosine phosphorylation. These data suggest that tyrosine phosphorylation of PKC δ in response to TPA is dependent upon an active membrane-bound tyrosine kinase and is consistent with the hypothesis that TPA-induced tyrosine phosphorylation of PKC δ is a secondary effect of TPA-induced membrane localization.

Overexpression of PKC δ has previously been reported to inhibit cell growth (8). Our previous observation that PKC δ became membrane-associated in response to the mitogenic stimuli of v-Src (4) was surprising since membrane association of PKC isoforms has been widely used to imply activation. The finding here that PKC δ becomes phosphorylated and has a reduced kinase activity in v-Src-transformed cells is perhaps consistent with the previous reports that PKC δ is an inhibitor of cell growth. The increased DG levels observed in response to v-Src (26) may reflect a requirement for activation of the α PKC isoform, which also becomes membrane-bound in response to v-Src (4). PKC α has been reported to phosphorylate Raf, which contributes to the activation of Raf (27). Since Raf is required for transformation by v-Src (28), it is possible that activation of PKC α and phosphorylation of Raf is required for the mitogenic signals activated by the tyrosine kinase activity of v-Src. The increased DG needed for PKC α activation may be causing the PKC δ recruitment to the membrane. However, since PKC δ is inhibitory for mitogenic signals, there may be a mechanism whereby tyrosine phosphorylation, which correlates well with mitogenic signals, results in down-regulation of the enzymatic activity of PKC δ.

Although PKC δ becomes membrane-associated in v-Src-transformed cells, there is no change in the subcellular distribution of the ε PKC isoform, which is also a DG-dependent Ca2+-independent PKC isoform (4). The preferential increase in membrane association of PKC δ over PKC ε observed in v-Src-transformed cells suggests that there may be some functional significance for the observed membrane association of PKC δ in response to v-Src. Several recent reports have suggested kinase-independent roles for PKC isoforms (10–12). It is possible that increased membrane association of PKC δ and down-regulation of its enzymatic activity indicate a kinase-independent function for PKC δ. Alternatively, Src could be a critical substrate for PKC δ and that upon phosphorylation of Ser-12 there is a reciprocal tyrosine phosphorylation that serves as a negative feedback control mechanism for PKC δ. A mutation to c-Src at Ser-12 was previously shown to be required for the enhanced responsiveness to β-adrenergic agonists in cells overexpressing c-Src (29). Thus, the interaction between Src and PKC δ may also be important for regulating other indirect effects of Src.

The effect of the Ser-12 mutant on both association and tyrosine phosphorylation further supports the hypothesis that PKC δ is a direct substrate of Src. The dependence of the association on an active kinase suggests that interaction occurs only when Src has been activated. It is still not clear as to what role(s) c-Src plays in cell physiology, and while the data presented here with cells overexpressing activated forms of Src do not prove that PKC δ is a normal cellular target of c-Src, the data do show that PKC δ could be regulated by Src or perhaps a related Src family kinase. Perhaps more importantly, the data presented here in cells transformed by v-Src demonstrate that v-Src can associate with and down-regulate a protein kinase that has been strongly associated with inhibiting cell growth. The ability to down-regulate this inhibitory PKC isoform may be important for the transforming ability of v-Src.

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