Phosphorylation of Raf-1 by p21-activated Kinase 1 and Src Regulates Raf-1 Autoinhibition* 

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Exposure of cells to mitogens or growth factors stimulates Raf-1 activity through a complex mechanism that involves binding to active Ras, phosphorylation on multiple residues, and protein-protein interactions. Recently it was shown that the amino terminus of Raf-1 contains an autoregulatory domain that can inhibit its activity in Xenopus oocytes. In the present work we show that expression of the Raf-1 autoinhibitory domain blocks extracellular signal-regulated kinase 2 activation by the Raf-1 catalytic domain in mammalian cells. We also show that phosphorylation of Raf-1 on serine 338 by PAK1 and tyrosines 340 and 341 by Src relieves autoinhibition and that this occurs through a specific decrease in the binding of the Raf-1 regulatory domain to its catalytic domain. In addition, we demonstrate that phosphorylation of Raf-1 on serine 338 and tyrosines 340 and 341 relieves autoinhibition, is unlikely to regulate autoinhibition. These results demonstrate that the autoinhibitory domain of Raf-1 is functional in mammalian cells and that its interaction with the Raf-1 catalytic domain is regulated by phosphorylation of serine 338 and tyrosines 340 and 341.

Activation of the extracellular signal-regulated kinases (ERK) 1 and 2 is required for cellular proliferation in many cell types and is also a requisite event in neoplastic transformation (1–4). The activities of ERKs 1 and 2 are regulated through the activation of a sequentially acting protein kinase cascade, known generically as a mitogen-activated protein kinase module. At the top of this cascade is the serine/threonine protein kinase Raf-1. Once activated, Raf-1 phosphorylates and activates the mitogen and extracellular signal-regulated kinase 491 and serine 494, two phosphorylation sites in the catalytic domain that are required for Raf-1 activation, is unlikely to regulate autoinhibition. These results demonstrate that the autoinhibitory domain of Raf-1 is functional in mammalian cells and that its interaction with the Raf-1 catalytic domain is regulated by phosphorylation of serine 338 and tyrosines 340 and 341.

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‡The abbreviations used are: ERK, extracellular signal-regulated kinase; RBD, Ras binding domain; CRD, cysteine-rich domain; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PAK, p21-activated kinase; HA, hemagglutinin; MBP, myelin basic protein; RafCAT, Raf-1 catalytic domain.

Actions, and perhaps direct interaction with lipids (5–7). Binding to active Ras relocalizes Raf-1 from the cytosol to the plasma membrane. This interaction is initially mediated by a conserved domain in the Raf-1 amino terminus known as the Ras binding domain (RBD). Binding of Ras to the RBD then promotes contact with an adjacent domain known as the cysteine-rich domain (CRD), and binding of Ras to both of these domains is required for full activation of Raf-1 (5). Interaction between Ras and Raf-1 also stimulates the release of 14–3-3 from its amino-terminal binding site, which is centered on serine 259, and the subsequent dephosphorylation of this site (8–10). This is thought to contribute to the separation of the catalytic domain from the amino-terminal regulatory domain.

Activation of Raf-1 by Ras is also accompanied by phosphorylation on multiple residues, including serines 338 and 339 and tyrosines 340 and 341. Phosphorylation of these sites is essential for Raf-1 activation by extracellular ligands such as epidermal growth factor, phorbol esters, and integrin binding (11–13). Kinases that catalyze the phosphorylation of these sites in the cell are the p21-activated kinases (PAKs) 1–3, which phosphorylate serines 338 and 339 (13, 14), and the Src family of tyrosine kinases, which phosphorylate tyrosines 340 and 341 (15, 16). In addition, Raf-1 activation may require phosphorylation on two conserved sites within the activation loop of its kinase domain (threonine 491 and serine 494) (17). Kinases that phosphorylate these residues have not been identified. Despite the clear importance of phosphorylation of Raf-1 on each of these sites, it is not yet understood how these events contribute to Raf-1 activation.

Previously it was shown (18) that the amino terminus of Raf-1 contains an autoinhibitory domain that can block the function of the Raf-1 catalytic domain in Xenopus oocytes. This supports a model in which the catalytic activity of inactive Raf-1 is inhibited by interaction with the autoinhibitory domain. The mechanism whereby the catalytic domain is released from the autoinhibitory domain has not been determined. In this report we show that the autoinhibitory domain of Raf-1 can block the ability of a separately expressed Raf-1 catalytic domain to stimulate ERK2 activity in mammalian cells. We also show that this domain minimally consists of the first 147 amino acids of Raf-1 and encompasses portions of both the RBD and the CRD. We also demonstrate that phosphorylation of Raf-1 on serine 338 and tyrosines 340 and 341 relieves this autoinhibitory effect and that this occurs through a reduction in the affinity of the amino terminus for the phosphorylated catalytic domain. Furthermore, we demonstrate that putative phosphorylation sites within the kinase loop (threonine 491 and serine 494) are not likely to regulate autoinhibition. These data demonstrate that phosphorylation of Raf-1 on serines 338 and 339 and tyrosines 340 and 341 contributes to Raf-1 activation by blocking the ability of the autoinhibitory domain to regulate Raf-1 catalytic activity.

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Regulation of Raf-1 Autoinhibition

Characterization of the Raf-1 Autoinhibitory Domain in Mammalian Cells—Cutler et al. (18) reported that expression of the Raf-1 amino terminus (amino acids 1–330) in Xenopus oocytes blocked both germinal vesicle breakdown and mitogen-activated protein kinase activation stimulated by coexpression of the Raf-1 catalytic domain. To test whether the Raf-1 amino terminus could function as an autoinhibitory domain in mammalian cells, we tested its ability to block ERK2 activation stimulated by expression of the Raf-1 catalytic domain. HEK 293 cells were cotransfected with hemagglutinin epitope-tagged ERK2 (HA-ERK2) and a constitutively active Raf-1 catalytic domain construct (Raf BXB, amino acids 1–25 and 304–648) (19), either with or without increasing amounts of Raf-1 1–330 (1, 3, or 10 μg). Two days later the HA-ERK2 was immunoprecipitated and assayed for kinase activity (IP kinase assay) using myelin basic protein (MBP) as a substrate. The data shown are representative of four independent experiments.

RESULTS

Plasmids and Recombinant Proteins—Hemagglutinin epitope-tagged ERK2, Raf BXB (amino acids 1–25 and 304–648), and constitutively active PAK1 (PAK1 L107F) were as previously described (19, 20). Constitutively active chicken Src (21) was subcloned into pCMV5. Point mutations in pCMV5/Raf BXB plasmids were produced by PCR and sequenced to confirm correct amplification. Raf-1 1–330, 1–330 C165/166SS, 1–256, 1–186, 1–147, 51–131, and 139–186 were produced by PCR and cloned into pCMV Flag BD (Sigma). Recombinant baculovirus expressing hexahistidine-tagged RafCAT (amino acids 204–648) was produced using the plasmid pFastBac HTB, essentially as described by the manufacturer (Invitrogen). GST-Raf-1 1–330 protein was produced in BL21DE3 Escherichia coli. Specifically, cells were grown to A600 = 0.8 at 37 °C and induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C. Cells were frozen and subsequently lysed by sonication in 10 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfon fluoride). Insoluble protein was pelleted by centrifugation at 35,000 × g for 30 min at 4 °C. GST-Raf-1 1–330 was purified from the soluble lysate by affinity chromatography using glutathione-agarose. Specifically, 2 ml of a 50% slurry of pre-swelled glutathione-agarose was added to the soluble lysate and incubated at 4 °C for 1 h. Bound GST-Raf-1 1–330 protein was precipitated by centrifugation, and the slurry was washed three times with lysis buffer. The beads were then resuspended in an equal volume of phosphate-buffered saline and stored at 4 °C. Hexahistidine-tagged RafCAT protein was produced in SF9 cells grown in Sf-900 II SFM (Invitrogen) by infection with a recombinant baculovirus for 48 h. Cells were pelleted at 500 × g and stored at –80 °C. Cell pellets were lysed by homogenization in hypotonic lysis buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 80 mM glycerin, 10 μg/ml pepstatin A, 10 mM sodium orthovanadate, 1 mM sodium orthovanadate, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfon fluoride). Unbroken cells and nuclei were pelleted by centrifugation. Triton X-100 was added to the lysate to a final concentration of 0.5%, and insoluble material was pelleted by centrifugation at 35,000 × g for 30 min at 4 °C. RafCAT protein was purified by NTA–NTA affinity chromatography using a 10-250 mM imidazole gradient, essentially as described by the manufacturer (Qiagen).

Transfection, Immunoprecipitation, and in Vitro Kinase Assays—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 10 μg/ml penicillin/streptomycin (all cell culture reagents from Invitrogen). Cells in 60-mm dishes were transfected. The amounts transfected for other constructs were as noted in the corresponding figure legends. Twenty hours after transfection, the culture medium was replaced with Dulbecco’s modified Eagle’s medium without serum, and the cells were incubated for a further 24 h. Cells were lysed in 0.5 ml of Triton lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 0.5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM phenylmethylsulfon fluoride). Insoluble proteins were pelleted by centrifugation at 16,000 × g for 10 min at 4 °C, and the soluble supernatant was stored at –80 °C. Immunoprecipitation–kinase assays measuring hemagglutinin epitope-tagged ERK2 activity were performed as previously described (19). Briefly, HA-ERK2 was immunoprecipitated from soluble cell lysates using 2 μg of mouse anti-HA antibody (Santa Cruz Biotechnology) and 40 μl of a 50% slurry of protein A-Sepharose (Amersham Biosciences). Immunoprecipitates were washed three times with 20 ml Tris-HCl, pH 8.0, 500 mM NaCl, and once with 20 ml Tris-HCl, pH 8.0. The kinase activity of immunoprecipitated HA-ERK2 was assayed using 10 μg of myelin basic protein (MBP) (Sigma) as a substrate. Incubations were for 30 min at 30 °C in kinase buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, 100 μM ATP, 2 μCi of γ-32P-ATP (ICN Biomedicals)). Phosphorylated MBP was resolved by 15% SDS-PAGE. After drying, the gel was exposed to autoradiography film, and the phosphorylation of MBP was quantitated by scintillation counting of the excised MBP.

RafCAT Binding Assays—RafCAT protein (1 μg) was phosphorylated or not by incubation in kinase buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 100 μM ATP) for 30 min at 30 °C with or without recombinant GST-PAK1 (22) or recombinant Src (Upstate Biotechnology Inc.). It was previously determined that after 30 min the phosphorylation of RafCAT by either kinase had reached a maximum. After phosphorylation, one-tenth of the phosphorylated RafCAT (100 ng) was mixed with 10 μg of GST-Raf-1 1–330 bound to glutathione-agarose, and phosphate-buffered saline was added to a final volume of 300 μl. Proteins were mixed for 1 h at 4 °C. Glutathione-agarose–protein complexes were then pelleted by centrifugation, and the supernatant was saved to test for the presence of unbound RafCAT protein. The pellets were washed three times with 1 ml of ice-cold phosphate-buffered saline. Pelleted complexes were solubilized with an equal volume of 2× Laemmli sample buffer. Bound and unbound proteins were loaded on 12% SDS-PAGE and transferred to nitrocellulose. Western blots were then performed for RafCAT (rabbit anti-Raf-1, C-12, Santa Cruz Biotechnology), phospho-serine 338 (rat anti-Raf-1 phospho-serine 338, Upstate Biotechnology Inc.), and phospho-thyro-tyrosine 4G10, Upstate Biotechnology Inc.). Western blots were quantitated by densitometry.
For each Raf-1 amino-terminal construct transfected, domain. Numbers refer to the amino acid residues. The three regions conserved among Raf proteins (CR1, CR2, and CR3) are shown. RBD, the Ras binding domain. CRD, the cysteine-rich domain. Arrows denote the activating phosphorylation sites in the CR3 (catalytic) domain. B, deletion analysis of the Raf-1 autoinhibitory domain. HEK 293 cells were transiently transfected with HA-ERK2, Raf BXB, and the Raf-1 amino-terminal constructs shown. HA-ERK2 activity was monitored by IP kinase assay using MBP as a substrate. MBP phosphorylation was quantified by scintillation counting. Values are listed as the percent of activation achieved by expression of Raf BXB alone. The Raf-1 amino-terminal constructs expressed are shown on the bottom of the graph. 1–330 CC/SS, Raf-1 1–330 containing the double C165/168S substitution, which inactivates the zinc finger. For each Raf-1 amino-terminal construct transfected, dark bars refer to 1× plasmid transfected, light bars refer to 3× plasmid transfected. Results are the average of at least three independent experiments. Errors are the S. E. of the mean.

Fig. 2. Characterization of the Raf-1 autoinhibitory domain. A, schematic description of Raf-1 domains and phosphorylation sites. Numbers refer to the amino acid residues. The three regions conserved among Raf proteins (CR1, CR2, and CR3) are shown. RBD, the Ras binding domain. CRD, the cysteine-rich domain. Arrows denote the activating phosphorylation sites in the CR3 (catalytic) domain. B, deletion analysis of the Raf-1 autoinhibitory domain. HEK 293 cells were transiently transfected with HA-ERK2, Raf BXB, and the Raf-1 amino-terminal constructs shown. HA-ERK2 activity was monitored by IP kinase assay using MBP as a substrate. MBP phosphorylation was quantified by scintillation counting. Values are listed as the percent of activation achieved by expression of Raf BXB alone. The Raf-1 amino-terminal constructs expressed are shown on the bottom of the graph. 1–330 CC/SS, Raf-1 1–330 containing the double C165/168S substitution, which inactivates the zinc finger. For each Raf-1 amino-terminal construct transfected, dark bars refer to 1× plasmid transfected, light bars refer to 3× plasmid transfected. Results are the average of at least three independent experiments. Errors are the S. E. of the mean.
these sites are involved in the Raf-1 autoinhibition mechanism. We first substituted these sites with alanine (TS/AA), which precludes their phosphorylation in the cell. As shown in Fig. 5A, this largely blocked the ability of Raf BXB to stimulate ERK2 activity (lane 7). This was most likely due to a reduction in Raf BXB kinase activity, because these sites were shown to be required for the kinase activity of full-length Raf-1 (17). However, some ERK2 activation was still apparent, and this was effectively blocked by coexpression of Raf-1 1–330 (lane 8). In addition, autoinhibition of this Raf BXB mutant was counteracted by coexpression of active PAK1 or active Src (lanes 9 and 10). Thus, mutation of threonine 491 and serine 494 to nonphosphorylatable residues does not affect the autoinhibition mechanism.

To mimic the phosphorylation of these sites, we replaced them with acidic residues (TS/ED) (17). Expression of this Raf BXB mutant stimulated ERK2 activity to a greater degree than the TS/AA mutant (compare lanes 7 and 11). However, this Raf BXB mutant was still subject to autoinhibition by the Raf-1 amino terminus (lane 12). This suggests that phosphorylation of these sites is not required for relief of autoinhibition. Furthermore, autoinhibition of Raf BXB TS/ED was effectively blocked by coexpression of active PAK1 or active Src (lanes 13 and 14). Fig. 5B represents the mean of seven independent experiments. Thus, these results demonstrate that phosphorylation of threonine 491 and serine 494 is not required to block autoinhibition and indicate that phosphorylation of these sites is required for other steps within the Raf-1 activation mechanism.

Phosphorylation of Raf-1 by PAK1 or Src Decreases the Affinity of the Autoinhibitory Domain for the Catalytic Domain—Using purified proteins, we next examined whether phosphorylation of the Raf-1 catalytic domain by PAK1 or Src affected the ability of the autoinhibitory domain to bind to the catalytic domain. For these experiments, Raf-1 1–330 was expressed in bacteria as a GST fusion protein, and the Raf-1 catalytic domain (RafCAT, residues 304–648) was expressed as a hexa-histidine-tagged fusion protein in insect cells (it is insoluble in bacteria). To measure the binding of these proteins, an excess of GST/Raf-1 1–330 was incubated with RafCAT, and the binding was allowed to proceed to equilibrium. GST/Raf-1 1–330 was then precipitated using glutathione-agarose, and the amount of RafCAT protein still present in the supernatant was also measured. Under these conditions, ~10% of the RafCAT protein did not bind to Raf-1 1–330 (Fig. 6). Furthermore, coprecipitation of RafCAT with the Raf-1 amino terminus required the presence of Raf-1 1–330 because RafCAT did not coprecipitate with glutathione beads bound to glutathione S-transferase alone (data not shown). However, when RafCAT was phosphorylated with Src prior to incubation with Raf-1 1–330, the
amount of free RafCAT increased to ~30%. The reduction in binding was even more apparent if one measured only the tyrosine-phosphorylated RafCAT population (40% unbound).

Similar results were observed when RafCAT was phosphorylated by recombinant PAK1 prior to incubation with the Raf-1 amino terminus. Thus, these data indicate that phosphorylation of Raf-1 on serine 338 or tyrosines 340 and 341 reduces the affinity of the autoinhibitory domain for the catalytic domain.

DISCUSSION

Raf-1 activation by growth factors is a complex process that entails recruitment to the plasma membrane by active Ras and the subsequent phosphorylation of Raf-1 on multiple sites. The role of phosphorylation within this mechanism has remained unclear. Previous work has shown that Raf-1 contains an autoinhibitory domain within the first 330 amino acids of its amino terminus (18). This was demonstrated by measuring the ability of the Raf-1 amino terminus to inhibit meiotic maturation in *Xenopus* oocytes stimulated by expression of the Raf-1 catalytic domain. It was also shown that regulation of autoinhibition did not correlate with changes in 14–3–3 binding to the Raf-1 amino terminus and that it depended on the integrity of the CRD. In the present study we have shown that the Raf-1 autoinhibitory domain can block the ability of the Raf-1 catalytic domain to stimulate ERK2 activity in mammalian cells. Furthermore, this domain minimally consists of the first 147 amino acids of Raf-1, although sequences within the CRD carboxyl-terminal to residue 147 may contribute to autoregulation. We also show that phosphorylation of Raf-1 on serine 338 and tyrosines 340 and 341 blocks autoinhibition and that this is due to a reduction in the affinity of the autoinhibitory domain for the phosphorylated, catalytic domain. Thus, these results indicate that phosphorylation of Raf-1 on these sites is required for activation because this modification blocks autoinhibition.

Through extensive deletion analysis we have more precisely defined the region encompassing the autoinhibitory domain. This domain includes sequences within the RBD (amino acids 51–131) and the CRD (amino acids 139–186) but does not include the CR2 domain of Raf-1 (amino acids 255–268). A coincidence of autoinhibitory and small G protein binding domains exists in other kinases. For example, PAK1 contains an autoinhibitory domain that partially coincides with its Rac/Cdc42 binding domain (20, 29). In addition, the interaction of the PAK1 autoinhibitory domain with the catalytic domain is regulated by phosphorylation (30, 31).

Interestingly, in contrast to the results of Cutler *et al.* (18), we found that an intact zinc finger within the CRD domain was not required for autoinhibition. This is based on our definition of the minimal autoinhibitory domain (residues 1–147), which does not include the zinc finger region, and the finding that disruption of the zinc finger by mutation of two key cysteine residues to serine (C165/186S) only slightly affected autoregulation. This was proposed because expression of RafCAT containing a phosphorylation mimic at Y340 (Y340D) precluded inhibition of meiotic maturation by the Raf-1 regulatory domain (18). In addition, it was recently published that phosphorylation of residues between serine 338 and tyrosine 341 was necessary for high affinity interaction between Raf-1 and MEK1 (32). Our results are

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**Fig. 5.** Threonine 491 and serine 494 are not required for autoinhibition by the Raf-1 amino terminus. A, HEK 293 cells were transiently transfected with HA-ERK2 and either Raf BXB, Raf BXB T491A/S494A (TS/AA), or Raf BXB T491E/S494D (TS/ED). Where indicated, the cells were also transfected with Raf-1 1–330, constitutively active PAK1, or constitutively active Src. HA-ERK2 activity was measured by IP kinase assay. The panel is an autoradiograph of phosphorylated MBP. Fold refers to the fold increase in ERK2 activity relative to cells transfected with ERK2 alone. B, quantification of the effects of mutating kinase loop phosphorylation sites on Raf-1 autoinhibition. Results are the mean of seven independent experiments. Errors are S. E. of the mean.

**Fig. 6.** Phosphorylation of the Raf-1 catalytic domain on serine 338 or tyrosine 341 reduces the binding of the Raf-1 autoinhibitory domain to the catalytic domain. Hexahistidine-tagged Raf-1 catalytic domain (RafCAT) was incubated at 4 °C with GST-Raf-1 1–330 prebound to glutathione-agarose beads. Prior to incubation with the Raf-1 amino terminus, RafCAT was phosphorylated or not by incubation in kinase buffer with or without recombinant PAK1 or recombinant Src. After binding had reached equilibrium, GST-Raf-1 1–330 was precipitated by centrifugation. RafCAT associated with the GST-Raf-1 1–330, as well as that still in solution (unbound), was measured by Western blot with an antibody specific for the Raf-1 catalytic domain. Western blots were quantified by densitometry. Shown is the average of three independent experiments. Errors are the S.E. of the mean.
consistent with the idea that phosphorylation of serine 338 and/or tyrosine 340 is required to block autoinhibition. We cannot, however, preclude a role for the phosphorylation of these sites in regulating the affinity of Raf-1 for MEK1. In fact, it is possible that phosphorylation of serine 338 and tyrosine 340 serves a dual role in stimulating the activity of Raf-1 toward MEK1, namely to block autoinhibition and to increase interaction between Raf-1 and MEK1.

We have also found that phosphorylation of threonine 491 and serine 494 is not likely to be involved in the regulation of autoinhibition, because substitution of these sites with acidic residues, which mimics their phosphorylation, does not affect autoinhibition. These sites are located within the activation loop of the Raf-1 kinase domain, and in many kinases phosphorylation of sites within the activation loop directly affects catalytic activity (33, 34). Thus, given our data, we would predict that phosphorylation of threonine 491 and serine 494 is necessary for an increase in Raf-1 catalytic activity rather than a relief of autoinhibition.

In conclusion, our data detail a role for phosphorylation of Raf-1 on serine 338 and tyrosines 340 and 341 in the Raf-1 activation mechanism. Specifically, phosphorylation of these sites prevents interaction between the autoinhibitory and catalytic domains. Given this data, we can refine a common model for growth factor-mediated Raf-1 activation (5, 17). In this model, inactive Raf-1 is recruited to the plasma membrane by GTP-bound Ras. Ras first binds to the RBD of Raf-1 and then interacts with the CRD. Binding of Ras to these domains causes 14–33 to release from its amino-terminal binding site (serine 259), thereby allowing Raf-1 to further unfold. Dephosphorylation of serine 259 may also occur at this time. Raf-1 is then phosphorylated on serine 338 and tyrosines 340 and 341 by kinases whose activities are also regulated by Ras. Phosphorylation of these sites locks Raf-1 in an open conformation by preventing interaction between the catalytic and autoinhibitory domains. However, full activation of Raf-1 would not occur until it is phosphorylated on threonine 491 and serine 494 by one or more as yet unidentified kinases. Once Raf-1 is phosphorylated on all four sites it is fully active and able to phosphorylate its downstream target MEK. Refinement of this model remains an area for further study.

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