Role of Regulatory Subunits and Protein Kinase Inhibitor (PKI) in Determining Nuclear Localization and Activity of the Catalytic Subunit of Protein Kinase A*

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Regulation of protein kinase A by subcellular localization may be critical to target catalytic subunits to specific substrates. We employed epitope-tagged catalytic subunit to correlate subcellular localization and gene-inducing activity in the presence of regulatory subunit or protein kinase inhibitor (PKI). Transiently expressed catalytic subunit distributed throughout the cell and induced gene expression. Co-expression of regulatory subunit or PKI blocked gene induction and prevented nuclear accumulation. A mutant PKI lacking the nuclear export signal blocked gene induction but not nuclear accumulation, demonstrating that nuclear export is not essential to inhibit gene induction. When the catalytic subunit was targeted to the nucleus with a nuclear localization signal, it was not sequestered in the cytoplasm by regulatory subunit, although its activity was completely inhibited. PKI redistributed the nuclear catalytic subunit to the cytoplasm and blocked gene induction, demonstrating that the nuclear export signal of PKI can override a strong nuclear localization signal. With increasing PKI, the export process appeared to saturate, resulting in the return of catalytic subunit to the nucleus. These results demonstrate that both the regulatory subunit and PKI are able to completely inhibit the gene-inducing activity of the catalytic subunit even when the catalytic subunit is forced to concentrate in the nuclear compartment.

The subcellular localization of multifunctional protein kinases is emerging as a critical regulatory mechanism to target activity to sites of second messenger generation or to a specific subset of intracellular substrates (1, 2). One of the important actions of PKA involves the phosphorylation of transcription factors and the induction of specific genes. However, the PKA holoenzyme is normally found to be cytoplasmic, and only the catalytic subunit is thought to be able to enter the nucleus after its release from the holoenzyme. The translocation of the C subunit into the nucleus is the key event associated with the initiation of PKA-mediated induction of transcription. After translocation to the nucleus, the C subunit phosphorylates transcription factors such as CREB, specifically stimulating transcription (3–5).

The C subunit of PKA has been shown to translocate into the nucleus after the microinjection of purified protein into the cytoplasm of cultured cells (6, 7). However, microinjection of preformed holoenzyme injected into the cytoplasm fails to transverse the nuclear membrane and remains cytoplasmically sequestered (8). The nuclear pore complex (NPC) is the primary route for proteins to diffuse back and forth across the nuclear membrane. Studies measuring pore permeability suggest that proteins larger than 23–26 nm in diameter, which corresponds approximately to a 40–60-kDa globular protein, are excluded from passage through the aqueous pore of the NPC (9, 10).

Because the C subunit is approximately 40 kDa, this places it at the upper size limit of proteins that can diffuse passively into the nucleus. Nevertheless, investigation into the mechanism of C subunit translocation into the nucleus has suggested that passive diffusion is sufficient to explain the kinetics and apparent nuclear accumulation of C (11).

The partitioning of C between cytoplasm and nucleus is further complicated by the recent discovery of the nuclear export properties of PKI. Residues 37–46 of PKI contain a leucine-rich region that has been shown to function as a nuclear export signal (NES) (12). PKI appears to transport the C subunit out of the nucleus in a C/PKI complex utilizing an ATP and temperature-dependent mechanism (6, 7). PKI is capable of freely entering the nucleus (6, 8, 13) and actively shuttling the C subunit back into the cytoplasm where the C subunit can recombine with R subunits to form inactive holoenzyme and restore cAMP regulation to the cell.

In this study, we utilize epitope-tagged C subunit to evaluate the ability of either the RII subunit or PKI to prevent nuclear accumulation and inhibit gene induction of a CRE-luciferase reporter. By adding an artificial nuclear localization signal (NLS) to the tagged C subunit we are able to compare the ability of RII and PKI to affect a strong nuclear localization signal and inhibit C subunit activity. Our results are consistent with the current model developed from microinjection experiments and further demonstrate that the entire RII/C holoenzyme complex would be transported to the nucleus if the C subunit contained an exposed NLS. In contrast to RII, PKI is capable of exporting a C subunit with a strong NLS, although export is not a prerequisite for inhibition of gene expression. The export process that is engaged by PKI appears to be saturable, suggesting that free PKI may interact with the export machinery.
Localization and Activity of C Subunit

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—JE G3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) in a 10% CO2 incubator at 37 °C. Before transfection, the cells were passaged onto 24-well plates at 2 × 104 cells/well. The cells were allowed to grow to approximately 50% confluence. The medium was reduced to 250 μl/well 3–6 h before transfection. The cells were transfected by standard calcium phosphate techniques (14), adding 25 μl of the calcium phosphate/DNA precipitate to each well. The α16-raf-luciferase reporter, containing 168 base pairs of the human gondatropin α promoter (3, 15), was used at a concentration of 2.5 ng/well in all transfections as a CAMP responsive reporter. C TAG, NLS-C TAG, and RII TAG were used at a concentration of 2.5 ng/well unless otherwise stated. The RSV-PKI nucleotides of overlap between the 3′ and 5′ flanking a 350-nucleotide fragment and a 150-nucleotide fragment with 25 nucleotides at the initial three codons of the coding sequence that introduced a NcoI site at the initiator methionine (3′-GAATTCGAGGTCGGCGCGATGAGCAGGTATTTGAGTCATTTGAGGAA-GGTGTGAA-3′) with the 5′-NcoI restriction end (5′-CTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAG-3′) which was used to generate the CTAG construct. The PCR product was subcloned into JEG3 cells demonstrated that expression of C was assayed to nuclear translocation, it was critical to determine whether adding the c-Myc epitope to the carboxyl terminus of the C subunit altered activity of the expressed protein. Co-transfection of the CRE-luciferase reporter into JEG-3 cells demonstrated that expression of C was associated with robust stimulation of transcription, suggesting that the recombinant C was enzymatically active (Fig. 1B). However, it was possible that the observed activity was because of C displacing endogenous C subunit from its association with R in hololoenzyme. To address this issue, co-transfections were performed using a tagged mutant C subunit (CQR TAG) that does not associate with R (17). In these experiments, CQR TAG demonstrated comparable levels of transcriptional transactivation of the CRE-luciferase reporter as untagged C subunit and C TAG, suggesting that the addition of the c-Myc tag to the carboxyl-terminal region of the protein did not significantly diminish enzymatic activity (data not shown).

When C TAG and RII were co-transfected to form holoenzyme, the C TAG localized exclusively to the cytoplasm (Fig. 1C, lower panels), and this cytoplasmic sequestration correlated with a complete block in transactivation of the CRE-luciferase reporter construct. This suggests that the expressed C and R subunits appropriately assemble to form the holoenzyme and that this precludes entry into the nucleus, consistent with previous observations that the holoenzyme is too large to diffuse between cytoplasmic and nuclear compartments (7, 8).

In many of the cells transfected with C TAG alone, there appeared to be nuclear accumulation of the protein. This observation was also previously reported using fluorescein-conjugated C subunit introduced by microinjection (7, 8). One possible explanation is that the C subunit contains a weak or perhaps conditional NLS, although fluorescence bleaching and kinetic observations argue against it (11). We examined the ability of a well characterized NLS to modify both the subcellular localization of C and its activity in a gene induction assay. The SV40 T-antigen NLS was spliced onto the amino-terminal region of C as shown to dramatically affect enzymatic activity (18, 19), it was critical to determine whether adding the c-Myc epitope to the carboxyl terminus of C subunit was as was used to generate the C TAG construct. The PCR product was NcoI- and Apo I-digested, and the resulting 1192-base pair fragment was subcloned into ZEM3. The same PCR-based strategy was used to incorporate the SV40 NLS to the amino-terminal end of NLS-CWT, with a 3′ primer complimentary to the carboxyl-terminal coding region of C followed by the c-Myc epitope used in the construction of NLS-C WT and C WT with a 5′ primer complimentary to the RII nucleo-terminal coding region that introduced a NcoI site at the initiator methionine. The C WT construct was generated by PCR mutagenesis using primers mutating the leucines (positions 37, 39, 41, and 44) and the isoleucine (position 46) in the nuclear export signal to alanines. PCR primers were generated internal to the PKI coding sequence, amplifying a 350-nucleotide fragment and a 150-nucleotide fragment with 25 nucleotides of overlap between the 3′ end of the 350-nucleotide fragment and the 5′ end of the 150-nucleotide fragment. The two purified fragments were ligated and then digested with two c-Myc restriction sites to complete the synthesis of the mutated PKIAs before the addition of primers. Primers against the 5′ and 3′ ends of PKI, containing NcoI and ApoI restriction sites, respectively, were added to the reaction, and 13 more cycles were completed. The resulting band purified product was then subcloned into the MT vector already described. The generation of MT-PKI was done using the same 5′ and 3′ primers with NcoI and ApoI sites. The amplified PKI coding region was subsequently subcloned into the MT vector. Multiple clones of both MT-PKI and MT-PKI mut were selected and tested.

Immunocytochemistry—After 12–15 h of zinc treatment, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline. The cells were subsequently reconstituted with the c-Myc antibody (Oncogene Sciences) at a dilution of 1:100 for 1–4 h. The fixed cells were then probed with the biotin-conjugated horse anti-mouse antibody (Vector) at 1:100, followed by incubation with avidin-FITC (Vector) at 1:1000 in 1 × Hepes-buffered saline. After fluorescein isothiocyanate labeling, the cells were incubated in 5 μM propidium iodide to label the nuclei. Slides were prepared as described previously (14). The stained monolayer was examined using a CRM-600 Bio-Rad confocal microscope.

RESULTS

Regulation and Localization of the C Subunit by Association with R—The localization of either free C subunit or assembled R/C holoenzyme was examined by transiently transfecting JEG3 cells with an epitope-tagged C subunit (C TAG) with and without RII. When the C TAG construct was transfected alone, expressed C subunit was localized ubiquitously throughout the cell. Because the carboxyl-terminal region of the C subunit has been shown to dramatically affect enzymatic activity (18, 19), it was critical to determine whether adding the c-Myc epitope to the carboxyl terminus of the C subunit altered activity of the expressed protein. Co-transfection of the CRE-luciferase reporter into JEG3 cells demonstrated that expression of C was associated with robust stimulation of transcription, suggesting that the recombinant C was enzymatically active (Fig. 1B). However, it was possible that the observed activity was because of C displacing endogenous C subunit from its association with R in holoenzyme. To address this issue, co-transfections were performed using a tagged mutant C subunit (CQR TAG) that does not associate with R (17). In these experiments, CQR TAG demonstrated comparable levels of transcriptional transactivation of the CRE-luciferase reporter as untagged C subunit and C TAG, suggesting that the addition of the c-Myc tag to the carboxyl-terminal region of the protein did not significantly diminish enzymatic activity (data not shown).

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Nuclear Translocation and Activation of the NLS-C TAG Holoenzyme—In co-transfection experiments with the RII regulatory subunit, it was observed that NLS-C TAG still localized entirely in the nucleus (Fig. 2C, lower panels). However, although the R subunit did not prevent nuclear translocation, it did prevent transactivation of the CRE-luciferase construct.
That the C subunit was still localized to the nucleus but was not catalytically active suggested that the NLS on the C subunit caused translocation of the entire holoenzyme into the nucleus. To test this hypothesis, an RIITAG expression vector was made. RIITAG expressed alone was only found in the cytoplasmic compartment (Fig. 3 B), and co-transfection with a Ca expression vector had no effect on the localization of the RIITAG protein (Fig. 3 C). However, when RIITAG was co-transfected with NLS-Ca (diagrammed in Fig. 3 A), there was complete nuclear translocation of the RIITAG protein (Fig. 3 D). This confirmed that an active and exposed NLS on the C subunit was sufficient to translocate the entire holoenzyme to the nucleus.

Regulation of C Subunit Subcellular Localization and Trans-activation by PKI—PKI has been shown to actively export the C subunit from the nucleus via the nuclear export signal resident within PKI between residues 37 and 46 (12). To directly correlate PKI-mediated export of the C subunit with the ability of PKI to inhibit C subunit activation of CRE-luciferase expression, co-transfection studies were done with CTAG and PKI. Initial titration studies were performed to determine the level of RSV-PKI necessary to effectively inhibit the majority of C subunit activity (data not shown). Co-transfection with sufficient PKI to inhibit C subunit activity by greater than 70% (Fig. 4 B) resulted in near complete cytoplasmic sequestration of the CTAG protein (Fig. 4 A).
Competition Between Import and Export with NLS-CTAG and PKI—We examined the relative strength of active import and export processes by co-transfecting NLS-CTAG in the presence or absence of PKI. Transfection with NLS-CTAG alone resulted in complete nuclear localization as shown in Fig. 2. Cotransfection of NLS-CTAG with PKI yielded three different staining patterns ranging from completely nuclear to completely cytoplasmic (Fig. 5B). Because endogenous PKI has been shown to be expressed in a cell cycle-dependent fashion, we speculated that there might be cell cycle-dependent expression of export factors leading to the variability in localization observed. However, when cells were synchronized at various points in the cell cycle, the variability in localization persisted, demonstrating it was not a cell cycle effect (data not shown).

One plausible explanation for variable export is that co-transfection of PKI and NLS-CTAG expression vectors results in a variable ratio of PKI to C protein in individual cells. This hypothesis was tested by incrementally increasing PKI and then observing both the CRE-luciferase transactivation and the subcellular localization of NLS-CTAG under each transfection condition. Fig. 6A demonstrates that increasing concentrations of PKI produced a continual decrease in CRE-luciferase transactivation. However, quantitation of staining patterns demonstrated a biphasic localization such that increasing concentrations of PKI shifted NLS-CTAG localization toward the cytoplasm until only about 15% of the cells exhibited nuclear-only staining. Increasing the concentration of PKI past this point resulted in a relative decrease in the cytoplasmic localization and a return to nuclear localization such that 55% of transfected cells were scored as nuclear only. This suggests that there is an optimal ratio of PKI to C for export and that further increases in PKI compete with the C/PKI complex for binding to the export machinery.

The Role of Nuclear Export in PKI Regulation of C Subunit Function—It has been demonstrated that PKI inhibits C subunit activity and actively exports the C subunit out of the nucleus in a C/PKI complex. These activities are encoded in discrete regions of the protein. However, the relative functional impact of these two activities on C subunit-directed gene expression has never been quantitated. The localization of CTAG and its ability to transactivate CRE-luciferase was examined using various concentrations of either wild-type PKI or mutant PKI in which the critical hydrophobic residues within the nuclear export signal had been mutated to alamines (Fig. 7). Titration of the wild-type PKI showed a concentration-dependent effect both with respect to export and inhibition of transactivation of the CRE-luciferase reporter (Fig. 7). Consistent with previous reports (12), the mutant PKI failed to export the C subunit out of the nucleus (Fig. 7C). A comparison of mutant transactivation.
and wild-type PKI demonstrated that mutant PKI was a somewhat less effective inhibitor of CRE-luciferase transactivation at low concentrations (Fig. 7B). However, with higher levels of PKI, both the mutant and wild-type proteins were indistinguishable in their inhibitory activity. This suggests that export of the C subunit may play a particularly important role in the regulation of C subunit activity when PKI is at sub-stoichiometric concentrations relative to C subunit but that export is not essential for complete inhibition of C subunit-induced gene expression.

**DISCUSSION**

The subcellular localization and trafficking of PKA has received a great deal of attention, and several competing mechanisms have been described that may modulate this process. The localization of holoenzyme is most often suggested to be a mechanism to bring PKA closer to selective substrates, increasing substrate specificity as well as the rate of phosphorylation. It is equally plausible that localizing mechanisms could serve to place PKA to sites of cAMP generation and allow regional changes in cAMP to promote PKA activation. Several mechanisms for PKA localization have been elucidated. A collection of anchoring proteins for PKA (AKAPs) have been described that bind with nM affinities to the type II regulatory subunits, and these AKAPs are themselves associated with cytoplasmic membranes, cytoskeletal components, or cytoplasmic organelles (20, 21). Recently several novel AKAPs have been isolated that can also bind to type I regulatory subunits although with much lower affinities (22, 23). The Cα and Cβ1 isoforms of C have also been shown to be amino-terminal myristoylated (24), and this lipid modification might hypothetically play a role in membrane association, although no evidence for this exists to date. The heat-stable inhibitor of PKA, PKI, has also been shown to facilitate nuclear export of C subunits back to the cytoplasm (7, 25), and this may help to terminate the transcriptional response. We have used an epitope-tagging strategy to localize C subunits that were free or bound to either RII or PKI. The nuclear distribution of C subunit was correlated with the transcriptional activation of a cAMP-responsive reporter gene.

![Fig. 5. Localization patterns observed when the NLS-C \textsubscript{TAG} subunit has been cotransfected with PKIα. Panel A, cells transfected with NLS-C\textsubscript{TAG} alone. Panel B, co-transfection of NLS-C\textsubscript{TAG} with PKIα resulted in the emergence of three different localization patterns. In the top row, three examples of predominantly nuclear localization are presented. The second row in panel B (Cyto + Nucleus) depicts examples where strong cytoplasmic and nuclear staining are both evident. The bottom row (Cyto) shows cells with predominantly cytoplasmic sequestration of NLS-C\textsubscript{TAG} with minimal to no protein present in the nucleus.](image)

**Fig. 6. NLS-C\textsubscript{TAG} transcriptional activity and subcellular localization with increasing PKIα concentrations.** JEG-3 cells were transfected with NLS-C\textsubscript{TAG} alone or co-transfected with various concentrations of PKIα. Panel A, the transcriptional activity of NLS-C\textsubscript{TAG} is plotted as -fold induction calculated relative to the normalized levels of CRE-luciferase activity in cells transfected with just the reporter constructs. The amount of NLS-C\textsubscript{TAG} (2.5 ng/well) is constant in all conditions, and the level of RSV-PKIα is increased as shown in the figure. Panel B, the subcellular localization was characterized by visual examination of cells under each transfection condition. The three localization patterns (Nucleus (■), Cyto + Nucleus (□), and Cyto only (□)) observed correspond to those depicted in Fig. 5.
where it can phosphorylate nuclear targets such as CREB and other transcription factors, modulating their activity.

The C subunits do not appear to contain an endogenous NLS, although a consensus sequence (KRVK) resembling the human c-Myc NLS is located at amino acid 189. Mutations of this sequence have been shown to have no effects on nuclear accumulation or transcriptional activity of C. 2 We introduced an exogenous SV40 T-antigen NLS onto the amino terminus of C to test the ability of either RII or PKI to maintain the cytoplasmic localization and compete with a well characterized signal for nuclear import. The presence of an exogenous NLS on the C subunit transported nearly 100% of the protein into the nucleus, and NLS-C was an effective inducer of reporter gene activity. This experiment demonstrates that the C subunit is localized completely to the nucleus when an exposed NLS is fused to the protein and argues that no endogenous NLS is recognized in the free C subunit.

When an RII expression vector was added to the transfections along with the NLS-C TAG, the ability of C to induce gene activity was completely inhibited, but surprisingly, the C subunit remained nuclear. This implies that the NLS-C TAG is forming holoenzyme effectively and that the NLS is capable of targeting this entire holoenzyme to the nucleus where it remains inactive. We tested this idea by constructing a tagged RII expression vector and by following the localization of RII in the presence or absence of NLS-C. As expected, RII remained cytoplasmic when transfected on its own or when transfected with wild-type Co. However, in the presence of NLS-C, the RII was transported into the nucleus, leaving no visible cytoplasmic staining. Despite the known array of cytoplasmic AKAPs, which bind RII with high affinity, a strong NLS was capable of transporting all of the holoenzyme to the nucleus. Although all cells appear to have AKAPs, we have not investigated which AKAPs are present in JEG-3 cells, and it is possible that if higher affinity AKAPs had been present, some of the holoenzyme might have remained in the cytoplasmic compartment.

The egress of C out of the nucleus has been shown to be facilitated by PKI, which not only binds and inhibits C subunit activity but also acts as an adapter to target C to the nuclear export machinery (12). There are three isoforms of PKI (α, β, and γ) (28, 29), and in addition to the C subunit interacting domain, each has a highly conserved leucine-rich NES. These leucine-rich nuclear export sequences have been shown to interact with CRM1 (exportin 1) and together with Ran, form a complex that can be exported through the NPC (30, 31). Once in the cytoplasm, GTPase-activating proteins bind and stimulate the hydrolysis of Ran-bound GTP, causing the complex to dissociate. This process returns the C subunit to the cytoplasm where it is presumed to bind to R subunits when cAMP has returned to basal levels. PKI may therefore function to terminate the nuclear actions of C subunit and rapidly reset the PKA system to respond again.

When C TAG was transfected together with PKI, the C TAG subunit did not appear in the nucleus to a significant extent, and gene expression was severely inhibited. The complex of C and PKI may be too large to diffuse effectively through the NPC, and the C/PKI that does enter the nucleus might be rapidly exported. However, when the NLS-C TAG was transfected with various concentrations of PKI, a more complicated pattern emerged. As the ratio of PKI to NLS-C TAG expression vector increased, the induction of CRE-luciferase progressively decreased to basal levels. The nuclear localization of NLS-C TAG at first diminished with increasing PKI, but then as more PKI expression vector was added to the transfections, the tagged C subunit reappeared in the nucleus and decreased in the cytoplasm. We interpret this to mean that overexpression of PKI interferes with the nuclear export machinery, decreasing the capacity to export the C/PKI complex. This contrasts with previous reports suggesting that the NES on PKI is masked and only becomes available when C binds to PKI (12, 25). One possibility might be that free PKI interacts with components of the export machinery and prevents their normal function without actually being a target for export itself in the absence of C.

Because PKI acts as both an inhibitor and exporter of C subunit, we examined the relative importance of the export process in the control of gene expression. A PKI expression vector was constructed with mutations in the NES (12) that rendered it incapable of interacting with the export machinery but did not affect C subunit binding. As expected, the mutant PKI did not alter the cellular distribution of co-transfected C TAG, which remained distributed in both the cytoplasm and nucleus. However, when the ability of this mutant PKI to inhibit gene induction by C subunit was compared with wild-

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**FIG. 7. Effects of mutagenesis of the NES of PKIα on subcellular localization and transcriptional regulation by C TAG.** Site-directed mutagenesis was performed to mutate the leucines and isoleucines within the hydrophobic NES of PKIα. The wild-type and mutant PKI were then subcloned into the MT/hGH expression vector used to express C TAG. Panel A, a diagram of the PKI constructs with the amino acid sequence of the NES shown. Panel B, the ability of wild-type or mutant PKI to inhibit C subunit-mediated transactivation of the CRE-luciferase construct is shown. Panel C, the subcellular localization patterns observed for C TAG in the presence of various concentrations of wild-type (MT-PKI) or mutant (MT-PKI mut) PKIα is shown.

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[2] J. C. Wiley, L. A. Wailes, R. L. Idzerda, and G. S. McKnight, unpublished observations.
Localization and Activity of C Subunit

Inhibitor and transporter of C subunit in the cell. Our results suggest that the export function of PKI is not essential for terminating gene induction by PKA and that the role of PKI export is more likely related to resetting the system, the C subunit must be rapidly returned to a holoenzyme form. The observation that PKI can act to transport C out of the nucleus suggests that it may play an essential role in the temporal regulation of gene expression.

The ability of the C subunit of PKA to regulate gene expression is well established and thought to be particularly important in the hormonal regulation of genes and the activity-dependent activation of gene expression in neurons. In at least some cases, the C subunit must gain access to the nucleus to phosphorylate nuclear substrates such as CREB and members of the ATF family of transcription factors. However, because C must also be able to phosphorylate cytoplasmic enzymes and membrane-bound receptors and ion channels, it is perhaps not surprising that the endogenous C subunit does not have a strong nuclear localization signal. To terminate gene expression and reset the system, the C subunit must be rapidly returned to a holoenzyme form. The observation that PKI can act to transport C out of the nucleus suggests that it may play an essential role in the temporal regulation of gene expression by cAMP. Further studies to alter the function of PKI in vivo may uncover the physiological significance of PKI as both an inhibitor and transporter of C subunit in the cell.

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