CKIα Is Associated with and Phosphorylates Star-PAP and Is Also Required for Expression of Select Star-PAP Target Messenger RNAs*

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Received for publication, January 25, 2008, and in revised form, February 20, 2008 Published, JBC Papers in Press, February 27, 2008, DOI 10.1074/jbc.M800656200

We have recently identified Star-PAP, a nuclear poly(A) polymerase that associates with phosphatidylinositol-4-phosphate 5-kinase Iα (PIPKIα) and is required for the expression of a specific subset of mRNAs. Star-PAP activity is directly modulated by the PIPKIα product phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂), linking nuclear phosphoinositide signaling to gene expression. Here, we show that PI-4,5-P₂-dependent protein kinase activity is also a part of the Star-PAP protein complex. We identify the PI-4,5-P₂-sensitive casein kinase Iα (CKIα) as a protein kinase responsible for this activity and further show that CKIα is capable of directly phosphorylating Star-PAP. Both CKIα and PIPKIα are required for the synthesis of some but not all Star-PAP target mRNA, and like Star-PAP, CKIα is associated with these messages in vivo. Taken together, these data indicate that CKIα, PIPKIα, and Star-PAP function together to modulate the production of specific Star-PAP messages. The Star-PAP complex therefore represents a location where multiple signaling pathways converge to regulate the expression of specific mRNAs.

Polyadenylation of most mRNAs is required for their efficient transcription and export as well as regulating their stability and translational efficiency (1). Polyadenylation of mRNA is achieved through the activity of poly(A) polymerases (PAPs),² There are multiple PAPs in mammalian cells, including PAPα, which is thought to be primarily responsible for the polyadenylation of newly transcribed mRNAs in the nucleus (2, 3). Additionally, there are “non-canonical” PAPs, including Gld2 and Trf4, which regulate stability and degradation of specific RNAs through polyadenylation (4, 5).

Star-PAP is a non-canonical poly(A) polymerase that is distinct from all other currently characterized PAP enzymes (6). Like the canonical PAPα, Star-PAP has an RNA recognition motif and is a nuclear enzyme. However, similar to non-canonical PAPs, the Star-PAP protein complex and architecture differ significantly from PAPα, and consequently, Star-PAP specifically targets a select subset of mRNAs. This suggests that Star-PAP is a hybrid PAP that is required for the 3'-end formation of newly transcribed pre-mRNAs but functions in a regulatory role to control mRNA expression levels. Star-PAP has a unique domain structure relative to all other known PAPs (6). One unique feature is a 205-amino acid proline-rich region (PRR) inserted into the catalytic PAP core. The PRR splits the catalytic PAP domain, and this region represents a potential site for regulation of Star-PAP function.

Poly(A) polymerases operate as large multiprotein complexes responsible for the 3'-processing of RNAs (7, 8). The Star-PAP polyadenylation complex is similar to that of poly(A) polymerases that utilizes mRNA as a substrate and includes cleavage and polyadenylation specificity factor (CPSF) subunits, cleavage stimulatory factor (CstF) subunits, symplekin, and RNA polymerase II (6, 9). However, unlike other poly(A) polymerase complexes, the Star-PAP complex also contains phosphatidylinositol-4-phosphate 5-kinase Iα (PIPKIα). Moreover, Star-PAP poly(A) polymerase activity is stimulated by the PIPKIα product phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂). Functionally, PIPKIα is required for the expression of select Star-PAP target mRNAs (6).

Phosphoinositides, including PI-4,5-P₂, are lipid signaling molecules that act as important regulators of numerous cellular functions (10). Phosphoinositides are generated ubiquitously in cells by multiple families of phosphoinositide kinases, which include PIPKIα (11). Phosphoinositide signaling specificity is based on the spatial restriction of these kinases to specific subcellular locations (12). This is often achieved through specific interactions between the phosphoinositide-generating enzymes and targeting factors, which are themselves often PI-4,5-P₂-regulated proteins (13, 14). This allows for the generation of inositol lipid second messengers at specific sites within the cell that, in turn, allows for precise targeting of individual phosphoinositide-sensitive pathways (12).

In the nucleus, the PI-4,5-P₂ and PIPKIα, as well as Star-PAP, are found in structures called nuclear speckles (6, 15), which are nuclear bodies enriched in factors required for the processing of pre-mRNA (16). The direct interaction between Star-PAP and PIPKIα and the proximity of Star-PAP to PI-4,5-P₂ generation in nuclear speckles suggest that nuclear phosphoinositides are spatially positioned to modulate Star-PAP activity in vivo. The presence of PIPKIα in the Star-PAP complex implies

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2 The abbreviations used are: PAP, poly(A) polymerase; PRR, proline-rich region; CPSF, cleavage and polyadenylation specificity factor; CstF, cleavage stimulatory factor; siRNA, small interfering RNA; MBP, myelin basic protein; tBHQ, tert-butylhydroquinone; CKI, casein kinase I; PI-4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PIPKIα, phosphatidylinositol-4-phosphate 5-kinase Iα.
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that phosphoinositide-based signal transduction pathways may be able to modulate Star-PAP function.

Another PI-4,5-P₂-sensitive protein found at nuclear speckles is the protein kinase CKIα (formerly known as casein kinase Iα) (17). The protein kinase activity of CKIα is specifically inhibited by PI-4,5-P₂ (18). CKIα is a member of the ubiquitously expressed CKI family of constitutively active Ser/Thr-specific protein kinases (19). CKI activity is modulated through the unique recognition motif (S/T)(P)XX(S/T), where S/T represents a phosphoserine or phosphothreonine in the –3 position (20). The activity of CKIs toward their substrates is regulated through this “priming” phosphorylation that precedes phosphorylation by CKIs. However, this is not the only mechanism of regulation for CKI family members.

Because the composition of a polyadenylation complex is important for the function of the associated poly(A) polymerase (5, 9), we looked for additional unique components of the Star-PAP-associated complex relative to PAPα. Identification of other unique Star-PAP-associated factors will lead to a more complete understanding of Star-PAP function and of its differences from other known PAPs. Here, we show that the Star-PAP complex contains unique protein kinase activities compared with PAPα. One of these kinases is the PI-4,5-P₂-sensitive kinase CKIα, which is capable of directly phosphorallyating Star-PAP. Moreover, CKIα and PIPKIα are required for the expression of a subset of Star-PAP target mRNAs. These data further reinforce the existence of a nuclear phosphoinositide-based signaling complex that regulates the Star-PAP polyadenylation complex to modulate expression of specific mRNAs.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Treatments—HEK 293 and normal rat kidney cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum at 37 °C in 5% CO₂. For DNA transfection, cells were transfected using the calcium phosphate method with the indicated amounts of DNA. The growth medium was exchanged after 4 h, and the cells were harvested at the indicated time. siRNA oligonucleotides were transfected using calcium phosphate at a final concentration of 120 nM oligonucleotide/ml of growth medium. Growth medium was replaced 6 h after transfection, and the transfection was repeated 24 h later. Cells were harvested for analysis 72 h after the first transfection. For PIPKIα, the oligonucleotides used were PIPKIα-1 (GGUGCCAUCAGUUAAGGCA) and PIPKIα-3 (GAAGUUGGAGCACUCUUGG). For CKIα, an siGENOME SMARTpool (Dharmacon) was directed against CSNK1A1. To induce a transcriptional antioxidant response, Hu-M. Synthetic PI-4,5-P₂ (Echelon Biosciences Inc.) was resuspended in 10 mM Tris-HCl (pH 7.5) and used at final concentrations of 0.1–100 μM. Synthetic PI-4,5-P₂ (Echelon Biosciences Inc.) was resuspended in 50 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂ and 0.5 mM EGTA for use in kinase assays.

Protein Kinase Assays—Protein kinase assays were performed in 1× kinase buffer. Assays were initiated by the addition of 10 μM ATP and 5 μCi of [γ-³²P]ATP to the reaction mixture. The endogenous kinase activity in the Star-PAP complex was destroyed by heating for 15 min at 65 °C. For inhibitor studies, all reaction components except ATP were incubated with inhibitors for 45 min on ice prior to starting the assay. CKI inhibitors IC261 (IC₅₀ = 11 μM) and CKI-7 (IC₅₀ ~ 6.0 μM) were resuspended in Me₂SO and used at final concentrations of 0.1–100 μM. Synthetic PI-4,5-P₂ (Echelon Biosciences Inc.) was resuspended in 50 mM Tris-HCl (pH 7.9) at 2.5 mM, subjected to bath sonication to form micelles, and used at final concentrations of 12.5–100 μM.

Immunoprecipitation—This was performed as described previously (6).

Quantitative Real-time PCR—This was performed as described previously (6). All mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

RNA Immunoprecipitation—This was performed as described previously (6).

RESULTS

The Star-PAP Complex Contains Protein Kinase Activity—Purification of FLAG-Star-PAP or FLAG-PAPα from HEK 293 cells resulted in the copurification of a large protein complex (6). This complex contained factors essential for the 3’-end formation of mRNAs, including CPSF₇₃, CPSF₁₀₀, Cst₁₆₄, symplekin, and RNA polymerase II (6). In contrast to PAPα, the Star-PAP complex also contained PIPKIα and, accordingly, PI-4-P kinase activity. To enhance the characterization of the Star-PAP complex, we subjected both the purified Star-PAP and
The PI-4,5-P_2-sensitive kinase CKIα Is Associated with the Star-PAP Complex—There are very few protein kinases known to be inhibited by PI-4,5-P_2; one of these is CKIα (17). Accordingly, immunoprecipitation of purified FLAG complexes with a CKIα-specific antibody showed that CKIα copurified specifically with Star-PAP and not with PAPα (Fig. 2A). In addition, immunoprecipitation of endogenous Star-PAP from HEK 293 cells resulted in coprecipitation of endogenous CKIα (Fig. 2B), demonstrating that these two proteins reside in the same complex in vivo. Both Star-PAP (6) and CKIα (17) have been reported to localize at nuclear speckles. Consequently, there was a strong colocalization between FLAG-Star-PAP and endogenous CKIα in the nuclei of normal rat kidney cells at nuclear speckles (Fig. 2C), demonstrating that in vivo Star-PAP and CKIα are present at the same sites within the nucleus and are therefore spatially positioned to interact with each other.

To confirm that the associated CKIα is involved in the phosphorylation of Star-PAP, the ability of CKI-specific inhibitors to block the phosphorylation of FLAG-purified Star-PAP by the associated kinase activity was examined. The CKI inhibitors IC261 (Fig. 2D) and CKI-7 (Fig. 2E) were both able to effectively block the phosphorylation of Star-PAP by the associated kinase activity in a dose-dependent fashion, signifying that CKIα is responsible for at least some of the kinase activity contained in the Star-PAP complex. The presence of CKIα specifically in the Star-PAP complex serves to further demonstrate that although Star-PAP and PAPα both contain the same basic 3′-end formation components, the Star-PAP complex contains additional signal transduction components that serve to distinguish it from the PAPα complex.

CKIα Can Directly Phosphorylate Star-PAP in Vitro on the Proline-rich Insert Region—The ability of IC261 and CKI-7 to block Star-PAP phosphorylation in the context of the purified complex suggests a role for CKIα in Star-PAP phosphorylation, but it does not prove that CKIα itself is directly phosphorylating Star-PAP. To demonstrate direct phosphorylation, purified CKIα was used to phosphorylate FLAG-purified Star-PAP. Prior to the assay, endogenous kinase activity in the FLAG complex was destroyed by heat inactivation. After heat inactivation, there was no detectable phosphorylation of Star-PAP (Fig. 3A). Purified FLAG-CKIα was able to directly phosphate heat-inactivated Star-PAP, whereas the catalytically inactive CKIα mutant K46R (21) was not (Fig. 3A). Similarly, phosphorylation by CKIα was blocked by 50 μM IC261 or 50 μM PI-4,5-P_2 (Fig. 3B). Together, these data indicate that the phosphorylation of Star-PAP is occurring directly by CKIα.

To determine the CKIα phosphorylation site(s) on Star-PAP, a series of FLAG-Star-PAP truncation and deletion mutants (Fig. 3C) was expressed and purified from HEK 293 cells and
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CKIα is associated with Star-PAP and is responsible for Star-PAP phosphorylation. A, FLAG-purified Star-PAP and PAPα complexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FLAG and anti-CKIα antibodies. B, endogenous Star-PAP was immunoprecipitated (IP) from HEK 293 cells. The resulting precipitates were immunoblotted with Star-PAP- and CKIα-specific antibodies. A nonspecific IgG immunoprecipitation was used as a control. C, normal rat kidney cells were transfected with FLAG-Star-PAP, allowing to express for 24 h, and fixed for immunofluorescence. Cells were stained with anti-FLAG (red) and anti-CKIα (green) to determine subcellular localization. Colocalization is shown as yellow in the Merge panel. Nuclei are indicated by staining with 4',6-diamidino-2-phenylindole (DAPI). D, the purified FLAG-Star-PAP complex was incubated with 0, 0.1, 1.0, 10, or 100 μM IC261 (IC_50 = 11 μM) (D) or CKI-7 (IC_50 ~ 6.0 μM) (E) prior to initiation of the kinase reaction by ATP. The arrow indicates Star-PAP protein.

**FIGURE 2.** CKIα is associated with Star-PAP and is responsible for Star-PAP phosphorylation. A, FLAG-purified Star-PAP and PAPα complexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FLAG and anti-CKIα antibodies. B, endogenous Star-PAP was immunoprecipitated (IP) from HEK 293 cells. The resulting precipitates were immunoblotted with Star-PAP- and CKIα-specific antibodies. A nonspecific IgG immunoprecipitation was used as a control. C, normal rat kidney cells were transfected with FLAG-Star-PAP, allowing to express for 24 h, and fixed for immunofluorescence. Cells were stained with anti-FLAG (red) and anti-CKIα (green) to determine subcellular localization. Colocalization is shown as yellow in the Merge panel. Nuclei are indicated by staining with 4',6-diamidino-2-phenylindole (DAPI). D, the purified FLAG-Star-PAP complex was incubated with 0, 0.1, 1.0, 10, or 100 μM IC261 (IC_50 = 11 μM) (D) or CKI-7 (IC_50 ~ 6.0 μM) (E) prior to initiation of the kinase reaction by ATP. The arrow indicates Star-PAP protein.

subjected to the same in vitro kinase assays described above. CKIα was able to phosphorylate all truncation mutants except those that lacked the first half of the proline-rich region (ΔPRR 1/2, amino acids 223–274) that splits the catalytic domain of Star-PAP (Fig. 3D), demonstrating that this region contains the CKIα phosphorylation site(s) in Star-PAP. This region contains nine serine and threonine residues conserved among mammalian species, including two consensus CKIα sites and a number of acidic residues that could contribute to additional CKIα phosphorylation sites (Fig. 3E).

The Star-PAP PRR Is Not Required for CKIα Association with the Star-PAP Complex—The above results leave open the possibility that CKIα does not phosphorylate Star-PAP in the PRR but, rather, that CKIα requires the PRR to associate with the Star-PAP complex and subsequently phosphorylates other sites within Star-PAP. To examine this, the requirement for the Star-PAP PRR for association of CKIα and for protein kinase activity with the Star-PAP complex was assessed. Both full-length Star-PAP and Star-PAPΔPRR expressed and purified from HEK 293 cells associated with endogenous CKIα (Fig. 4A). Furthermore, although FLAG-purified Star-PAPΔPRR could not be phosphorylated by the associated kinases (Fig. 4B), the complex still contained activity toward both casein and MBP similar to that of full-length Star-PAP (Fig. 4C), demonstrating that deletion of the PRR does not disrupt the association of protein kinase activity with the Star-PAP complex. This indicates that the inability of CKIα to phosphorylate Star-PAPΔPRR mutants is most likely due to a deletion of the phosphorylation site(s) and not to a disruption of the Star-PAP/CKIα interaction.

CKIα and PIPKια Are Required for the Expression of Specific Star-PAP Target mRNAs—Star-PAP and PIPKια are required for expression of detoxifying and oxidative stress response mRNAs; however, microarray analysis indicated that Star-PAP is required for the expression of a diverse assortment of mRNAs (6). Therefore, the requirements for CKIα and PIPKια for the expression of a diverse group of Star-PAP target mRNAs were analyzed. A group of mRNAs in which protein products function in a wide array of cellular processes was selected for further analysis, including heme oxygenase 1 (HO-1), NADPH:quinone oxidoreductase 1 (NQO1), cation transporter regulator-like 1 (CHAC1), asparagine synthetase (ASNS), p8 protein homolog (COM1), and secretogranin II (SCG2). The non-target mRNA glutamate-cysteine ligase, catalytic subunit (GCLC) was used as a negative control.

To confirm the requirement for Star-PAP in the expression of these mRNAs, HEK 293 cells were treated with siRNA oligonucleotides specific for Star-PAP. Knockdown of Star-PAP expression resulted in a 5–15-fold decrease in the mRNA species examined (Fig. 5, A and D), indicating that Star-PAP is indeed required for their expression. Treatment of HEK 293 cells with CKIα-specific siRNA resulted in a dramatic decrease in HO-1 and NQO1 mRNAs, whereas other Star-PAP target mRNAs were unaffected (Fig. 5, B and E). Likewise, we found that treatment of cells with PIPKια-specific siRNA resulted in comparable decreases in the same Star-PAP target mRNAs as CKIα siRNA, viz. HO-1 and NQO1 (Fig. 5, C and F). Together, these data raise the possibility that PIPKια and CKIα may be working together to regulate specific Star-PAP target mRNAs.

HO-1 and NQO1 mRNAs encode important detoxifying enzymes involved in protection from reactive oxygen species and cellular injury (22). Both HO-1 and NQO1 are up-regulated in response to oxidative stress through increased transcription (23). We have shown previously that Star-PAP and

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PIPKIα/H9251 are required for the up-regulation of the HO-1 transcript in response to a tBHQ-induced antioxidant response by playing a direct role in the 3′-end formation of this mRNA (6). Pretreatment of cells with the CKI-specific inhibitors CKI-7 and IC261 not only reduced the basal levels of HO-1 mRNA but also effectively blocked HO-1 induction after exposure to 100 μM tBHQ (Fig. 5E). Treatment of HEK 293 cells with CKIα-specific siRNA oligonucleotides also caused a reduction in basal HO-1 levels; however, it did not block HO-1 induction by tBHQ (Fig. 5F).

The requirement for CKIα and PIPKIα specifically for Star-PAP target mRNAs involved in cellular protection suggests that phosphoinositide-based signaling is involved in the Star-PAP regulation of stress response mRNA maturation. However, although CKI inhibitors can effectively block HO-1 induction, CKIα-specific siRNA does not. This suggests that other CKI isoforms, or other protein kinases sensitive to CKI inhibitors, are also involved in the induction of HO-1 mRNA.

**DISCUSSION**

Star-PAP is a novel poly(A) polymerase involved in the regulation of specific mRNA expression levels. Star-PAP differs from both the canonical and non-canonical poly(A) polymerases in several ways, including the presence of the PRR insert in its catalytic domain, a unique association with the PI-4,5-P₂-generating enzyme PIPKIα, and direct regulation by PI-4,5-P₂. Here, we show that Star-PAP also differs markedly from canonical
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FIGURE 4. Kinase activities and CKIα remain associated with Star-PAP when the proline-rich region is deleted. A, full-length FLAG-Star-PAP (FL) and FLAG-Star-PAPΔPRR complexes were expressed and purified from HEK 293 cells. The cell lysate (Lys) and the eluted FLAG affinity-purified complex are shown. Purified complexes were separated by SDS-PAGE and immunoblotted with anti-FLAG and anti-CKIα antibodies. Purified full-length FLAG-Star-PAP and FLAG-Star-PAPΔPRR complexes were tested for associated kinase activity toward themselves (B), 100 μg/ml casein (C), or 100 μg/ml MBP (D) using in vitro protein kinase assays.

PAPα because of the association of protein kinase activity with the purified enzyme complex and that the PI-4,5-P2-sensitive protein kinase CKIα is one of the kinases contributing to this activity. We also found that both PIPKIIα and CKIα are required for the synthesis of the Star-PAP target mRNAs HO-1 and NQO1, which are vital protective enzymes that respond to reactive oxygen species and cellular oxidative stress (22). Furthermore, like Star-PAP, CKIα and PIPKIIα, are required for the expression of some but not all Star-PAP target mRNAs. This suggests that phosphoinositide-based signal transduction does not regulate all aspects of Star-PAP function and, additionally, that maturation of all Star-PAP target mRNAs is not regulated through the same signaling pathway. Furthermore, the fact that the related Star-PAP target mRNAs HO-1 and NQO1 require CKIα and PIPKIIα, whereas unrelated Star-PAP target mRNAs such as CHAC1 do not, reveals that maturation of Star-PAP target mRNAs may be divided into groups based on their function and regulation. Star-PAP target mRNAs may exist as distinct groups, or modules, each of which is regulated by a different signal transduction pathway. For example, the HO-1/NQO1 oxidative stress response module is regulated by a CKIα- and PIPKIIα-based phosphoinositide pathway, whereas other modules may be controlled by different pathways. This arrangement would allow Star-PAP to specifically affect the expression of individual mRNA groups.

It is also notable that CKIα is associated only with Star-PAP target mRNAs, the levels of which it affects. This indicates that the composition of the Star-PAP 3′-processing complex is dynamic and can change to regulate different groups of mRNAs. It is therefore likely that other, as yet unidentified signal transduction components can also be associated in a Star-PAP complex. This suggests a model in which different signal transduction components assemble into the Star-PAP complex to control the expression of specific Star-PAP target mRNA modules. Each of these different Star-PAP complexes would be targeted to a specific Star-PAP module. The activation of specific signal transduction pathways could then direct Star-PAP to participate in the 3′-end formation of select mRNAs to which that specific complex was targeted while not affecting the expression of the others. In this manner, Star-PAP would be positioned to interpret an array of stimuli and coordinate the cellular response by regulating mRNA expression.

It is still unclear how the Star-PAP complex identifies different mRNA modules. It could be that incorporation of certain factors directs the complex to different mRNA targets. Alternatively, association of the Star-PAP complex with members of each mRNA group could recruit different sets of signal transduction components. Identification of more Star-PAP target mRNAs and their inherent modules will be necessary before the mechanism of Star-PAP specificity can be explored.

Although basal expression of HO-1 requires both PIPKIIα and CKIα, CKIα is not required for HO-1 induction, suggesting of which are present in the PAPα complex (Fig. 1A). As with Star-PAP, phosphorylation of these proteins is also inhibited by PI-4,5-P2. This suggests that additional components of the Star-PAP complex may also be regulated by PI-4,5-P2-sensitive protein phosphorylation.

Combined, these data point to the Star-PAP complex as a site where multiple PI-4,5-P2-sensitive components are present to take advantage of locally generated PI-4,5-P2 to regulate Star-PAP function. The presence of phosphoinositides in the nucleus has been well established; however, until recently, the mechanisms by which they affected nuclear events were unknown (25). The data presented here help to establish the mechanism by which nuclear phosphoinositides generated at nuclear speckles regulate mRNA processing and expression.

CKIα and PIPKIIα are required for the expression of some but not all Star-PAP target mRNAs. This suggests that phosphoinositide-based signal transduction does not regulate all aspects of Star-PAP function and, additionally, that maturation of all Star-PAP target mRNAs is not regulated through the same signaling pathway. Furthermore, the fact that the related Star-PAP target mRNAs HO-1 and NQO1 require CKIα and PIPKIIα, whereas unrelated Star-PAP target mRNAs such as CHAC1 do not, reveals that maturation of Star-PAP target mRNAs may be divided into groups based on their function and regulation. Star-PAP target mRNAs may exist as distinct groups, or modules, each of which is regulated by a different signal transduction pathway. For example, the HO-1/NQO1 oxidative stress response module is regulated by a CKIα- and PIPKIIα-based phosphoinositide pathway, whereas other modules may be controlled by different pathways. This arrangement would allow Star-PAP to specifically affect the expression of individual mRNA groups.
overlapping but unique roles for each protein in Star-PAP regulation. However, CKI inhibitors are effective in blocking tBHQ-induced HO-1 mRNA expression. This raises the possibility that other CKI isoforms can compensate for the loss of CKI and can allow Star-PAP-dependent induction of HO-1 mRNA expression. Alternatively, it may be that other CKI isoforms, or even other CKI inhibitor-sensitive protein kinases, are a functional component of the Star-PAP complex and play a role in Star-PAP-dependent HO-1 mRNA induction.

The location of the CKI phosphorylation site in the PRR of Star-PAP that interrupts the catalytic domain means that phosphorylation of Star-PAP could affect its catalytic activity. This could be through either a direct effect of the phosphorylation on activity or the modulation of an interaction with yet unknown regulatory proteins. Alternatively, it could be that this site is somehow important for substrate recognition or even possibly involved in PI-4,5-P_2 binding and modulation of Star-PAP activity. Deletion of either the entire PRR or the portion that contains the CKI phosphorylation site(s) results in a complete loss of Star-PAP poly(A) polymerase activity (data not shown). This may be due to a loss of regulatory elements, but just as likely, it may be due to a general disruption of the structure of the catalytic core of the Star-PAP enzyme.

Phosphorylation of PAPα has been shown to modulate its activity (26). However, on PAPα, this phosphorylation occurs on a serine/threonine-rich region in its carboxyl terminus. A comparable region does not exist in the same location on Star-PAP, so it is likely that the mechanism by which phosphorylation affects Star-PAP function will be different from that by which phosphorylation affects the activity of PAPα. We have

FIGURE 5. CKIα and PIPKα are required for the maintenance of specific Star-PAP mRNAs. A–C, quantitative real-time PCR analysis of mRNA expression levels after treatment with siRNA oligonucleotides specific for Star-PAP (A), CKIα (B), or PIPKα (C) relative to treatment with a control (Ctl) siRNA oligonucleotide. D–F, immunoblotting of representative protein levels from cells used in A–C with anti-Star-PAP (D), anti-CKIα (E), or anti-PIPKα (F) antibodies to demonstrate the extent of siRNA knockdown. G, quantitative real-time PCR analysis of HO-1 message levels from cells treated with 100 μM tBHQ after a 2.5-h pretreatment with the CKI inhibitor IC261 (50 μM) or CKI-7 (250 μM). H, quantitative real-time PCR analysis of HO-1 message levels from CKIα or PIPKα knockdown cells treated with 100 μM tBHQ or Me2SO (control) for 4 h. Quantitative real-time PCR results are the average of three independent experiments. Error bars represent 1 S.D. RNAi, RNA interference. ASNS, asparagine synthetase; GCLC, glutamate-cysteine ligase catalytic subunit.
Although CKIα is one protein kinase component of the Star-PAP complex, it is likely not the only one. The Star-PAP complex contains activity toward both MBP and casein. However, CKIα is unable to directly phosphorylate MBP, suggesting that there is at least one other protein kinase associated with the Star-PAP complex. Additionally, CKI inhibitors are capable of inhibiting casein phosphorylation by the associated kinase activity but are ineffective in blocking MBP phosphorylation (data not shown). Finally, most CKI substrates must first be phosphorylated by another kinase to generate a CKIα phosphorylation site (20). Notably, CKIα is unable to phosphorylate Escherichia coli-expressed, purified Star-PAP (data not shown), which would not be expected to contain the priming phosphorylation.

Together, these data strongly suggest that another kinase is also associated with the purified Star-PAP complex. However, the contribution of this kinase to Star-PAP phosphorylation is still unclear. CKI inhibitors are able to block a majority of Star-PAP phosphorylation in vitro; however, the ability of this kinase to phosphorylate Star-PAP in vivo and/or afterward in response to specific stimuli cannot be discounted. It may be that this unidentified kinase activity represents the CKIα priming kinase. Alternatively, this kinase may be involved in the regulation of other Star-PAP mRNAs, consistent with our model in which distinct Star-PAP complexes are each responsible for controlling the expression of specific mRNA modules.

The data presented here demonstrate an unexpected new nuclear substrate and function for the PI-4,5-P₂-sensitive protein kinase CKIα. CKIα can directly phosphorylate Star-PAP and is required for the expression of the Star-PAP target mRNAs HO-1 and NQO1, which encode critical detoxifying enzymes. The involvement of Star-PAP, PIPKIγ, and CKIα in the expression of these mRNAs suggests that a nuclear phosphoinositide signaling pathway regulates the level of specific mRNAs in response to oxidative stresses. However, the finding that phosphoinositide-based regulation of Star-PAP affects only a subset of Star-PAP target mRNAs demonstrates that Star-PAP is a very sophisticated regulatory enzyme. The association of the basic Star-PAP 3′-processing complex with distinct signal transduction components allows Star-PAP to specifically regulate the expression of discrete groups of mRNAs. In this manner, as depicted in Fig. 7, Star-PAP is capable of interpreting signals from distinct pathways and translating them into unique mRNA expression profiles by modulating the 3′-end formation of specific groups of Star-PAP target mRNAs.

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