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cAMP stimulation of STAR expression and cholesterol metabolism is modulated by co-expression of labile suppressors of transcription and mRNA turnover

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Abstract

The steroidogenic acute regulatory protein (StAR) is generated in rodents from 1.6kb and 3.5kb mRNA formed by alternative polyadenylation. The zinc finger protein, TIS11B (also Znf36L1), is elevated by cAMP in adrenal cells in parallel with StAR mRNA. TIS11b selectively destabilizes the 3.5kb mRNA through AU-rich sequences at the end of the 3'UTR. siRNA suppression shows that TIS11b surprisingly increases StAR protein and cholesterol metabolism. StAR transcription is directly activated by PKA phosphorylation. cAMP responsive element binding protein 1 (CREB) phosphorylation is a key step leading to recruitment of the co-activator, CREB binding protein (CBP). A second protein, CREB regulated transcription coactivator (TORC/CRTC), enhances this recruitment, but is inhibited by Salt inducible kinase (SIK). Basal StAR transcription is constrained through this phosphorylation of TORC. PKA provides an alternative stimulation by phosphorylating SIK, which prevents TORC inactivation. PKA stimulation of StAR nuclear transcripts substantially precedes TORC recruitment to the StAR promoter, which may, therefore, mediate a later step in mRNA production. Inhibition of SIK by staurosporine elevates StAR transcription and TORC recruitment to maximum levels, but without CREB phosphorylation. TORC suppression by SIK evidently limits basal StAR transcription. Staurosporine and cAMP stimulate synergistically. SIK targets the phosphatase, PP2a (activation), and Type2 histone deacetylases (inhibition), which may each contribute to suppression. Staurosporine stimulation through SIK inhibition is repeated in cAMP stimulation of many steroidogenic genes regulated by steroidogenic factor 1 (SF-1) and CREB. TIS11b and SIK may combine to attenuate StAR expression when hormonal stimuli decline.

Keywords

StAR; cholesterol metabolism; cAMP; SIK; TIS11b; adrenal

1. Introduction

The steroidogenic acute regulatory protein (StAR) is a central regulator in steroidogenesis. StAR stimulates the initial mitochondrial metabolism of cholesterol to pregnenolone by enhancing cholesterol transfer from the outer membrane to Cytochrome P450 11A1 (P450_{scc}) in the innermembrane (Jefcoate, 2002; Stocco, 2001). Adrenocorticotrophic hormone (ACTH) stimulates the functions of StAR in the adrenal cortex cells through increases in cAMP and cAMP-protein kinase A (PKA) activity. Inhibition of PKA causes a relatively rapid loss of StAR expression (Zhao et al., 2005). StAR expression in mice also exhibits an ACTH-independent circadian rhythm in the adrenal gland, driven by endogenous clock factors. These changes correlate with the fluctuation in serum corticosterone (Son et al., 2008). Each of these stimulations is countered by StAR mRNA removal, with a half time of less than 2hr. In this review, we address mechanisms, which can regulate this relatively rapid turnover of StAR.

PKA not only elevates StAR mRNA, but also acts directly on StAR protein to stimulate activity. Cytoplasmic (Type 2) and nuclear forms (Type 1) of PKA regulatory subunit control these processes in different ways, as shown by the selective stimulatory effects of cAMP analogs that are relatively Type-specific (Dyson et al., 2009). The Type 1 form activates transcription, while the Type 2 form provides the essential phosphorylation of the protein. StAR-mediated cholesterol metabolism depends on the mitochondrial import of newly synthesized and phosphorylated protein, providing a functional link to availability of mRNA (Artemenko et al., 2001). Co-translational phosphorylation of StAR by PKA coupled through the PKA-anchoring adapter protein, peripheral benzodiazepine receptor-associated protein (PAP7), facilitates the cholesterol-transporting activity of StAR in steroidogenic cells (Liu et al., 2006). A kinase anchor protein, 121 (Akap 121), which also complexes with PKA regulatory subunits, facilitates StAR translation, and also targets the transcript to the mitochondria (Dyson et al., 2008).

The control of StAR mRNA by PKA involves two additional proteins, SIK and the zinc finger protein, Znf36L1, which is otherwise known as TIS11b (or BRF1). The transcription of each is stimulated by PKA in parallel with StAR transcription. SIK represses StAR transcription (Takemori et al., 2007), while TIS11b decreases the stability of StAR mRNA (Duan et al., 2009). StAR is transcribed in most species as both a short (1.6kb) and a long (3.0–3.5kb) mRNA. These mRNA species arise from alternative polyadenylation sites located in exon 7. TIS11b exclusively targets the long StAR mRNA, which contains multiple conserved binding elements in the extended sequence. This contribution to hormonal control may partly explain the generation of this extended mRNA, which is also intrinsically more labile in cells than the shorter 1.6kb mRNA (Duan and Jefcoate, 2007; Zhao et al., 2005). In this review, we show that the considerable extra energy expenditure that is required for synthesis of this long mRNA is used to provide a refined control of StAR expression. We suggest that both modulator proteins rapidly enhance the attenuation of StAR following removal of the hormonal stimulus. This hypothesis is also consistent with the requirement of continuous StAR translation and phosphorylation to maintain the mitochondrial cholesterol transport activity.

2. StAR Transcription by CREB and SF-1

PKA activates the transcription of StAR as well as other steroidogenic genes. PKA activation of StAR transcription requires the cooperative actions of multiple transcription factors, including cAMP response element-binding (CREB) (Clem et al., 2005), CCAAT-enhancer-binding protein (C/EBP β), steroidogenic factor 1 (SF-1) (Manna et al., 2003) and GATA4 (Silverman et al., 2006). The phosphorylation of CREB leads to recruitment of the

co-activator CREB binding protein (CBP/p300), while SF-1 activation has been linked to recruitment of another co-activator, steroid receptor coactivator 1 (SRC-1) (Yang et al., 2009). The proximal region of the mouse StAR promoter contains three canonical 5' CRE half-sites (TGAC) that are bound by the activated CREB/ATF-1/CBP-p300 complex (Clem et al., 2005; Manna et al., 2002). The cooperation between SF-1 and CREB depends on additional complex changes on SF-1. Phosphorylation on S203 by a variety of kinases has been implicated in the activity (Yang et al., 2009). Sewer and coworkers have demonstrated physiological effects of ligand binding to SF-1: inhibition by sphingosine and stimulation by phosphatidic acid. PKA can modify the SF-1 activity through effects on the enzymes that control the levels of these lipids, including ceramidase, sphingosine kinase and diglyceride kinase theta (Li et al., 2007). Hammer and collaborators have demonstrated an alternate modulation of SF-1: activation through coordination of phosphorylation by cyclin-dependent kinase 7 (CDK7) at S203 and inhibition by sumoylation at adjacent K194 (Yang et al., 2009).

2.1 Intervention by SIK in StAR transcription

SIK was discovered in the adrenal cortex as a mediator of responses to elevated dietary sodium (Lin et al., 2000). Three forms of SIK, that are broadly distributed in multiple cell types, share extensive homologies with the catalytic subunit of AMP-dependent protein kinase (AMPK), a major regulator of energy homeostasis in cells. SIK prevents recruitment of the histone acetyl transferase, CBP, to CREB, which is critical to transcriptional activity. This association is enhanced by a set of proteins called TORCs (or more recently CRTC), which are inhibited by SIK activity (Takemori et al., 2007; Takemori and Okamoto, 2008). CBP binds the central KID region of CREB following phosphorylation at S133 by several kinases, including PKA. The C-terminal region of CREB interacts with the N-terminal region of TORC, which in turn further enhances CBP binding. TORC2, the predominant form in adrenal cells, is inhibited when phosphorylated on S171 by SIK. This additionally leads to nuclear export and sequestration of TORC2 by 14-3-3 in the cytoplasm (Al-Hakim et al., 2005). TORC also appears to directly interact with CREB. TORC may enhance the cooperation between CBP and CREB without the direct intervention of PKA.

TORC2 is highly expressed in Y-1 (adrenocortical tumor cell line that synthesizes steroids de novo, PN=55, ATCC) and MA10 (a clonal strain of mouse Leydig tumor cells, PN=16) cell lines. Both lines have been extensively used for steroidogenesis studies. When these cells are transfected with a TORC2-GFP expression vector, the fusion protein is initially located in the cytoplasm. When Br-cAMP is added to the cell, the loss of SIK activity causes TORC2-GFP to migrate slowly to the nucleus over the course of 1- to 6hr (Lee, unpublished).

Phosphorylation of SIK at S577 by PKA inhibits SIK activity. Interestingly, this also effects migration to the cytoplasm. Phospho-TORC is dephosphorylated by the calcium-dependent phosphatase, calcineurin (PP2B). A PKA resistant S577A SIK extensively inhibits stimulation of StAR expression by Br-cAMP (Takemori et al., 2007). PKA activation of TORC through this inhibitory phosphorylation of SIK, therefore, provides an alternative activation of CREB. Evidently, the nuclear cytoplasmic shuttling of these proteins is a key part of the activation process.

The natural multi-ring compound, Staurosporine, provides further insight into this process. Staurosporine competes with ATP for the catalytic site of many kinases, including SIK, for which inhibition is particularly potent (EC₅₀ in adrenal cells =5-10nM). Surprisingly, Staurosporine is almost as effective as cAMP in stimulating not only StAR mRNA in mouse adrenal Y-1 cells, but also many other steroidogenic genes. In the human adrenal H295 cell line, forskolin and staurosporine produce similar stimulations of StAR and CYPs (11A1,

11B1, 11B2 and 21A1), which are each regulated by SF1 and CREB. The forskolin effects are each blocked by S577A SIK (Takemori et al., 2007). We have identified 8 additional matches between Br-cAMP and staurosporine in Y-1 cells including, adrenodoxin, P450 oxidoreductase, low-density lipoprotein (LDL) receptor and scavenger receptor class B type 1 (SRB1) (data not shown). Clearly, the relief of SIK inhibition of TORC is limiting for most steroidogenic genes in adrenal cells.

Following staurosporine inhibition of SIK, transcription is effectively stimulated without phosphorylation of CREB. A similar active interaction of TORC with CBP, but without CREB phosphorylation, may also occur with PKA. Chromatin immunoprecipitation (ChIP) analyses show sequential binding of factors to the StAR promoter following cAMP or ACTH activation (Hiroi et al., 2004; Yang et al., 2009). In Y-1 cells, Br-cAMP substantially increases of nuclear transcripts in 15 min and produces a steady state level in 30 min (Fig. 1B). However, the mRNA only starts to increase at 30 min. This indicates a delay of 45-60 min between generation of nuclear transcripts and completion of the processing that creates mature StAR mRNA. Staurosporine generates nuclear transcripts more slowly than Br-cAMP, but this then catches up by eliminating most of this processing delay. ChIP assays show that the initial TORC recruitment to the StAR proximal promoter takes much longer (60 min) than p-CREB (within 15 min), which matches the increase in nuclear transcripts (Fig. 2). This slow participation of TORC is consistent with the redistribution of SIK and TORC between the nucleus and cytoplasm. Transcription may, therefore, be initiated by the rapid recruitment of pCREB and SF-1. For some reason, there is a switch to a CREB/TORC mechanism after nuclear transcripts reach steady state levels. By contrast, staurosporine inhibition of SIK directs TORC to CREB much more rapidly, but appreciably earlier than the activation of transcription. Presumably, an additional step such as recruitment of SF1 is needed for the increased transcription.

The following model (Fig. 3A) summarizes SIK participation in StAR regulation. Under basal conditions, SIK maintains TORC in an inactive state that cannot interact with unmodified CREB and which relocates a large proportion of TORC to 14-3-3 in the cytosol (Screaton et al., 2004). Activation of PKA initiates StAR transcription through phosphorylation of CREB and activation of SF-1. As SIK becomes inhibited by PKA, TORC reverts to a de-phosphorylated state through the activity of calcineurin/PP2B and then shifts to the nucleus. TORC then takes over the function of pCREB on the StAR promoter by combining with unphosphorylated CREB. The activation of calcineurin by cytoplasmic Ca^{2+} leads to synergy between cAMP and Ca^{2+} in the activation of TORC, CREB and CBP. When SIK is inhibited by staurosporine, TORC rapidly moves on to StAR, presumably by binding to unphosphorylated CREB. At low concentrations of cAMP, constitutive SIK attenuates cAMP responsiveness, while, under chronic stimulation, SIK/TORC becomes a predominant influence over StAR expression. When the chronic PKA stimulus is withdrawn, restoration of SIK activity through dephosphorylation can rapidly stop StAR transcription. Later passage Y-1 cells show much slower increases in StAR mRNA. SIK may contribute to this delay and to the slow stimulation of StAR transcription in MA10 cells.

2.2 Other regulatory features of SIK

SIK exhibits multiple additional functions, which may impact on StAR regulation. SIK increases the activity of the key phosphatase, PP2a, via inhibitory phosphorylation of a suppressor protein phosphatase, methylesterase-1 (PME1) (Sjostrom et al., 2007). This PP2a activity results in dephosphorylation of Na/K ATPase at the plasma membrane (activity stimulated) and presumably reversal of multiple other effects of serine kinases. Notable effects of PP2a include dephosphorylation of Raf kinase (activity increased) and extracellular signal-regulated kinase (Erk) (activity attenuated) (Adams et al., 2005). In Y-1

cells, staurosporine increases Erk phosphorylation, potentially through a lowering of PP2a activity. The use of MEK inhibitors shows that increased Erk activity contributes to the staurosporine stimulation of StAR. We see partial inhibition of staurosporine stimulation of StAR expression by MEK inhibitors. SF-1 phosphorylation at S203, mediated by Erk activation, may contribute to the staurosporine stimulation of StAR transcription.

SIK phosphorylation of type 2 histone deacetylases (HDACs) decreases their activity, thus increasing the acetylation of many proteins (Berdeaux et al., 2007). Significantly, we find that HDAC inhibitors extensively attenuate the PKA stimulation of StAR expression. SIK can also affect other PKA-dependent processes that regulate StAR, including the formation of SF1 ligands (ceramidase, sphingosine kinase and diglyceride kinase theta) (Li et al., 2007), either through CREB or through an increase in PP2a. These alternative contributions by SIK will require careful evaluation.

The expression of SIK in Y-1 cells and the stimulation by PKA also involves rapid and complex regulation. SIK mRNA increases over 5-fold to a new steady state within 30 min. This response is typical of mRNA stabilization that will be described in the next section. Staurosporine produces a similar, but slower stimulation, suggesting that SIK transcription is auto-regulated. SIK transcription may, therefore, be regulated by CREB/SF-1 and subject to similar SIK controls as those described for StAR. Interestingly, the rapid increase in SIK mRNA produced by PKA stimulation does not result in increased SIK protein. The process that increases SIK mRNA may, therefore, delay translation of the protein, thus minimizing the impact of SIK during the PKA stimulatory phase.

3. The role of the 3'UTR in StAR regulation

In rodent steroidogenic cells, cAMP stimulates two major StAR mRNA (1.6kb and 3.5kb), as well as a minor 2.8kb mRNA (Clark et al., 1995). These mRNA arise from different polyadenylation sequences in the terminal exon 7 and, therefore, differ only in their 3' untranslated region (3'UTR). When expressed through vector transfection, each forms StAR protein in proportion to the level of mRNA (Duan et al., 2009; Duan and Jefcoate, 2007). The 1.6kb transcript has a 0.7kb 3'UTR, while the 3.5kb transcript has a 2.6kb 3'UTR, including a 300 base region at the 3' end that contains the AU-rich elements (AURE), which can regulate many functions of mRNA (Ariyoshi et al., 1998; Duan and Jefcoate, 2007). Mouse, rat, bovine and human StAR retain similar polyadenylation sites in exon 7 that direct equivalent and extended transcripts (3- to 3.5-kb) (Clark and Combs, 1999; Haidan et al., 2000; Ivell et al., 2000). The appreciable delay in the conversion of StAR nuclear transcripts to mature mRNA suggests that PKA may initiate a relatively complex process at this stage. This may include a coordinated splicing and selection of the 3.5kb polyadenylation site. The recruitment of TORC to the StAR gene at this stage suggests a possible participation in this splicing process.

The stability of many labile mRNA is regulated by signal transduction pathways, most commonly through the interaction between the AURE, defined by the sequence AUUUA, and AURE-binding proteins (AURE-BP) (Guhaniyogi and Brewer, 2001; Ross, 1995). The AURE-BP can additionally determine mRNA location and translation. Stabilization and destabilization of such mRNA provides the means to rapidly change their expression levels. The core AUUUA is often found in repeating sequences, including nonamers of UUAUUUA (U/A)(U/A). They also accompany more U-rich elements (Guhaniyogi and Brewer, 2001). Over 900 genes in the human genome database have been found to contain AURE within their 3'UTRs, underlying the importance of this sequence element (Bakheet et al., 2001). Many of these genes express early response transcription factors (fos, jun, myc), cytokine (tumor necrosis factor α (TNF α)) and inflammatory regulators (cyclooxygenase-2

(COX2), endothelial nitric oxide synthase (eNOS) and cytochrome P450 1B1 (CYP1B1)). These proteins, like StAR, mediate cellular responses to a changing environment. Stabilization generates an elevated steady state with a halftime equal to that of the halftime for mRNA degradation. These fast responding transcripts degrade with halftimes as short as 10-15 min and, therefore, can respond to stabilizing factors equally rapidly.

StAR exhibits regulation representative of a sub-group of these genes. In earlier publications, we showed that the 3.5kb StAR mRNA is preferentially synthesized relative to the 1.6kb mRNA after ACTH stimulation *in vivo* (Artemenko et al., 2001). Br-cAMP stimulation in Y-1 and MA10 cells exhibited a preference for the 3.5kb mRNA (Zhao et al., 2005). We have identified two regions in the extended StAR 3'UTR (a basal instability region and the AURE) that selectively enhance basal transcript destabilization (Duan and Jefcoate, 2007). Interestingly, SIK mRNA (4.4kb) (Stephenson et al., 2004) and TIS11b mRNA each respond rapidly to cAMP and contain AURE at the end of the 3'UTR.

3.1 Proteins that bind AU-rich sequences in the 3'UTR of mRNA

A number of proteins interact with AURE sequences. The TTP family of tandem zinc finger proteins, which includes TTP/ZFP36, TIS11b/ZFP36L1 and TIS11d/ZFP36L2, directly bind an extended AURE and promote degradation of the host transcript (Ogilvie et al., 2005). TIS11b plays a crucial role in development since deletion is lethal in mouse embryos at gestational day (gd) 8. This probably arises through a failure to repress the expression of vascular endothelial growth factor (VEGF) mRNA during a critical phase of vascular development (Stumpo et al., 2004). This regulation of VEGF by TIS11b is also seen in adrenal cells (Ciais et al., 2004).

The central RNA binding domain (RBD) of these proteins interacts with AURE, while the N- and C-terminal domains recruit enzymes involved in the mRNA degradation pathway. A crystal structure shows that the TIS11d tandem zinc finger domains bind as a homodimer to the 8 base sequence, UAUUUAAU (Hudson et al., 2004). Mouse, rat, bovine and human StAR sequences each have 2 or 3 repeats of this octamer in the extended 3'UTR (Zhao et al., 2005).

TIS11b commonly functions in combination with other AU binding proteins (AU-BPs), which are also expressed abundantly in adrenal cells, including human antigen R (HuR) and alternatively spliced isoforms of AU-rich RNA binding factor 1 (AUF1/hnRNPd) (Duan et al., 2009). The four AUF isoforms (38-45 kD) can either stabilize or destabilize transcripts and play an important role in splicing and packaging mRNA as ribonucleoproteins for export to the cytoplasm (Raineri et al., 2004). The destabilizing AURE-BPs, such as TIS11b, recruit proteins, which enhance the de-adenylation and de-capping processes. These steps initiate the complex formation, which effects RNase degradation of the mRNA. The stabilizing AURE-BPs, such as HuR, protect the message from access to this degradation machinery. Inhibition of translation also commonly inhibits this process, including for StAR mRNA (Zhao et al., 2005). Protein complexes that form at sequences in the translated region compete with the transiting ribosome to regulate mRNA degradation (Lemm and Ross, 2002). Stress particles, that include TIS11b, sequester translationally arrested mRNA through AURE interactions (Kedersha et al., 2005). These particles provide a back-up system for important cell transcripts when cells become stressed.

HuR, which typically causes mRNA stabilization, can compete with destabilizing AURE-BP, such as TIS11b (Chen et al., 2002; Myer et al., 1997; Raineri et al., 2004). HuR and TIS11b, respectively, stabilize and destabilize the VEGF mRNA in adrenal cells through interaction with two AURE in the 3'UTR (Ciais et al., 2004). HuR recognizes a different sequence (nnUUnUUU) (Meisner et al., 2004). Direct competition between TIS11b and

stabilizing effect of HuR has been observed for VEGF mRNA, through a HuR binding sequence that overlaps with a TIS11b sequence (Cherradi et al., 2006). The sequence overlap between the HuR and TIS11b binding sequences is not seen in the StAR 3'UTR, and suppression of HuR with a siRNA had no effect on StAR expression (Duan et al., 2009).

3.2 TIS 11b modulation of StAR stimulation by cAMP

TIS11b mRNA (Fig. 4A) and multi-phosphorylated TIS11b protein (Fig. 5A) are rapidly stimulated by Br-cAMP in Y-1 or MA10 cells and by ACTH in primary bovine bovine adrenocortical cells (BAC), each in parallel with increased StAR expression (Chinn et al., 2002; Duan et al., 2009). Suppression of TIS11b specifically increases expression of 3.5kb StAR mRNA (Fig. 4B). TIS11b suppression by a specific siRNA in either MA-10 or Y-1 cells doubles the 3.5kb StAR mRNA, while leaving the 1.5kb form unaffected. The proportion of 3.5kb mRNA rises along with the total mRNA. Surprisingly, StAR protein levels are halved by suppression of TIS11b (compare Figures 4B and 5A), suggesting that TIS11b additionally stimulates StAR translation. This stimulation can clearly arise either through the interaction by TIS11b with the AURE sites on the 3.5kb mRNA or indirectly by targeting another translation factor.

The decrease in cholesterol metabolism associated with the removal of TIS11b (Fig. 5B) is consistent with the dependence of intra-mitochondrial cholesterol transport on continuous translation of StAR protein (Artemenko et al., 2001). This further suggests that TIS11b has a critical impact on StAR activity. Possibilities for this coupling between mRNA turnover, translation and activity are discussed further in a later section.

3.3 Establishing TIS11b regulatory sites in the StAR 3'UTR

We have used specific mutations in the 3'UTR of StAR linked to luciferase reporters to identify functional sites. We show that TIS11b-mediated mRNA turnover depends on the AU-rich octomer repeats that bind TIS11b dimers (Fig. 6). Direct TIS11b binding to these sites in the extended StAR mRNA 3'UTR was demonstrated in cultured BAC (Duan et al., 2009).

cAMP stimulation of TIS11b doubles 3.5 kb StAR mRNA degradation without affecting the 1.6 kb mRNA, as evidenced by the selective increase when TIS11b is removed (Fig. 4B). At the same time, SIK suppresses transcription through a process that is blocked by cAMP. We rationalize these conflicting effects by proposing that they combine as twin suppressors of StAR mRNA when cAMP declines (Fig. 3B). SIK becomes reactivated, thus actively suppressing transcription, while the previously stimulated levels of TIS11b enhance removal of the predominant 3.5kb mRNA.

The StAR 3'UTR sequences exhibit appreciable species diversity, while retaining the multiple polyadenylation and AURE sequences at similar locations (Fig. 7). The 3.5kb rodent StAR mRNA exhibits less basal stability than the alternative 1.6kb mRNA, mostly due to sequences immediately upstream of the AURE that we indicate here for the mouse (Zhao et al., 2005). While this sequence is very different in bovine and human, StAR 3'UTR secondary structure may be conserved. The UAUUUAUU octomers occur as three repeats in a conserved 90 base sequence near the 3'ends of the mouse and rat StAR 3.5kb mRNA. Two repeats of this octomer are found in the bovine sequence at a similar location, but separated by 40 bases. Three octomer repeats are found in the equivalent extended human StAR mRNA, although again with very different spacing and surrounding sequences (Fig. 7). ACTH selectively stimulates the long human form of StAR mRNA in H295R cells (Clark and Combs, 1999).

3.4 TIS11b as a regulator of StAR translation and activity

The inverse relationship between StAR protein and mRNA in response to TIS11b suppression can arise from direct interactions only on the 3.5kb mRNA. Indirect effects of TIS11b on genes involved in protein translation are also feasible. Although expression vectors which specifically deliver either StAR 1.6 or 3.5kb mRNA are similarly translated (Duan et al., 2009), additional post-transcriptional steps on the natural gene may be more selective. Importantly, ribonucleoprotein (mRNP) complexes are assembled in the nucleus during splicing of the primary transcript, but prior to transport of mature mRNAs to the cytoplasm (Matsumoto et al., 1998). This nuclear packaging of mRNAs can determine post-transcriptional regulation. We have noted the delay associated with the conversion of nuclear StAR transcripts to mature mRNA (Fig. 1). TIS11b, HuR and AUF1 each undergo constant CRM1-dependent nuclear-cytoplasmic shuttling, directed by localization signals (Fan and Steitz, 1998; Phillips et al., 2002; Sarkar et al., 2003). Binding of these proteins to AURE may play important roles in controlling the processing, splicing and polyadenylation of the StAR nuclear transcript, together with the nuclear export and eventual translation. AUF1/AURE complexes have been implicated in such processing, which may include selection of the StAR 3'UTR polyadenylation site (Raineri et al., 2004).

Contrary to our findings for StAR, TIS11b has previously been shown to inhibit translation (Kedersha et al., 2005). However, inhibition of StAR translation by cycloheximide substantially suppresses StAR mRNA turnover, suggesting that TIS11b could participate in such an integration of translation and mRNA turnover (Zhao et al., 2005). For several key mRNA, including c-myc and c-fos, specific proteins bind to elements in the translated sequence to stall the ribosome and, thereby, to decrease endonuclease degradation of the mRNA (Lemm and Ross 2002, Noubissi et al., 2006). For c-fos, this translation-linked mRNA stabilization involves an AUF1-AURE complex (Grosset et al 2000). Displacement of this complex, by passage of the ribosome, opens up the mRNA to endonuclease degradation. The StAR translated sequence includes a region with homology to this c-fos regulatory sequence (Zhao et al., 2005). TIS11b could enhance translation and degradation of the StAR 3.5kb mRNA by displacing AUF1 from the AURE and restoration of ribosome passage along the mRNA. c-Myc also uses rare codons (arginine/CGA and threonine/ACA) that requires a low abundance aminoacyl transfer RNA to stall ribosome transit (Lemm and Ross, 2002). These codons are also present in the StAR sequence.

Interestingly, these rare codons are present in the StAR sequence at the end of the N-terminal mitochondrial import sequence. A slowing of StAR translation, as the mitochondrial import sequence is completed, may be important for StAR activity. The unusual plasticity of the StAR molten globule structure (Bose et al., 2008) and the importance of coupling import to cholesterol transport activity have been frequently discussed (Artemenko et al., 2001). StAR translation at the surface of the mitochondrion may be additionally controlled by AKAP 121, a protein that binds regulatory subunits of PKA (Dyson et al., 2008). AKAP 121 has been linked to targeting of manganese superoxide dismutase (MnSOD) mRNA to mitochondria. AKAP121 binds to a KH stem-loop secondary structure formed in the 3'UTR (Ginsberg et al., 2003). The presence of such secondary structures in the StAR 3'UTR remains to be identified. Functional interactions between TIS11b and AKAP121 on 3.5kb StAR mRNA need to be considered.

3.5 TIS11b phosphorylation and further regulation

The phosphorylation state of TIS11b critically determines the location and turnover of TIS11b and the participation in post-transcriptional regulation of StAR. TIS11b is phosphorylated at multiple sites. At least 4 products of different mobility are apparent in Y-1 and MA10 cells (Fig. 5). These products correspond to phosphorylated forms, as evidenced

by their conversion to a single, more mobile product after phosphatase treatment of the cell extracts (Duan et al 2009). Serine phosphorylations at S92 and S203 have been established through removal of mobility changes by S/A substitutions (Schmidlin et al., 2004). Phosphorylation attenuates TIS11b activity in part through directing sequestration by cytoplasmic 14-3-3 anchor proteins. Akt and p38 are important kinase contributors (Schmidlin et al., 2004). The extensive phosphorylation of TIS11b in these cells suggests that the effectiveness of the TIS11b is partially compromised. The role of SIK, both in direct phosphorylation and as a stimulant of PP2a activity, suggests that these proteins may crosstalk.

SIK may also participate in the rapid PKA-mediated transcription of TIS11b (Fig. 3B). Importantly, TIS11b RNA also contains the sequence elements at the end of the 3'UTR, which bind TIS11b homodimers. Thus, like SIK, TIS11b has the features needed for self-regulation.

4. General Conclusions

SIK and TIS11b are each co-expressed with StAR during stimulation of adrenal and testis cells by cAMP. Each represses StAR expression; SIK through an effect on CREB-mediated transcription and TIS11b by binding to specific 8 base elements. These are conserved, exclusively, in the extended sequence of 3.5kb mRNA, which arise from alternative polyadenylation. This link between TIS11b and the StAR 3.5kb mRNA provides an explanation for the extra energy expended on the synthesis of this longer and more labile transcript. By utilizing mRNA stabilization as a means of up-regulation, StAR joins many genes that function through acute regulation. These include early response transcription factors, cytokines and chemokines. The observed crosstalk between Znf36 family members and micro RNAs (MiRs) (Asirvatham et al., 2009) provides a new direction for this research.

SIK and TIS11b are genes that express mRNA, which contain repeat AURE. Each exhibits rapid changes in mRNA expression. Each undergoes nuclear-cytoplasmic shuttling and binds, when phosphorylated, to 14-3-3, like an inactive phosphoprotein. This CRM-mediated process is shared with TORC/CRTC, the key mediator of SIK effects on StAR.

SIK may also suppress StAR by other processes. These include direct targeting of Type 2 HDACs and PP2a and secondary effects mediated by other CREB/TORC-regulated genes. The manner in which staurosporine matches cAMP stimulation and suppressions of the latter by constitutively active S577ASIK for over 10 genes emphasize the dominant role of SIK/TORC regulation in the control of steroidogenic genes (Takemori et al., 2007). The stimulatory effects of staurosporine also establish that SIK functions to suppress basal steroidogenic activity at multiple genes. TIS11b, on the other hand, only targets genes with the appropriate AURE in the mRNA, which is much rarer among these genes.

The parallel stimulations by cAMP of these relative labile mRNA encoding SIK, TIS11b and StAR represent a major commitment of extra energy to the rapid removal of StAR. We have proposed that this attenuation process provides a novel mechanism to inactivate StAR when hormonal stimulation ceases. It is also likely that this combination contributes to the unique circadian regulation of StAR in the adrenal, which is controlled by Bmal and Clock (Son et al., 2008). This exclusive regulation of StAR among steroidogenic genes mediates the cycling of glucocorticoids, which plays a crucial role in mammalian energy homeostasis.

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Abbreviation

ACTH	Adrenocorticotrophic hormone
AKAP 121	A-kinase anchor protein 121
AUF1	AU-rich RNA binding factor 1
AURE-BPs	AURE-binding proteins
C/EBP β	CCAAT-enhancer-binding protein
CBP	CREB binding protein
CDK7	cyclin-dependent kinase 7
ChIP	chromatin immunoprecipitation
CREB	cAMP responsive element binding protein 1
HDACs	histone deacetylases
HuR	human antigen R
P450_{scc}	cytochrome P450 11A1
PME-1	protein phosphatase methylesterase-1
SF-1	steroidogenic factor 1
SIK1	Salt inducible kinase 1
StAR	steroidogenic acute regulatory protein
TORC/CRTC	CREB regulated transcription coactivator

Figure 1. Staurosporine and Br-cAMP are comparably effective in the stimulation of steroidogenic genes in Y-1 cells

A. Design of primers to measure, respectively, StAR primary nuclear transcripts and mRNA.

B. Stimulation of nuclear transcripts in Y-1 cells by 400uM Br-cAMP and by 30nM staurosporine. Response to Br-cAMP is significant within 15 min ($n=3$, $P<0.05$) and at steady state within 30 min. Staurosporine produces a much slower response, reaching steady state at 60 min.

C. Stimulation of mRNA in the same samples. There were no increases in mRNA at 15 min. There is a delay of 45-60 min between the increase in nuclear RNA and mRNA following Br-cAMP stimulation. This difference was much smaller for staurosporine.

Figure 2. The participation of TORC2/CRTC2 in the stimulation of StAR transcription by Br-cAMP is a relatively late event

Chromatin immunoprecipitation (ChIP) assays were carried out on Y-1 cell samples analyzed in Figure 1. ChIP analyses, with antibodies recognizing the indicated nuclear factors, were carried out on pooled cultures at the indicated time points after Br-cAMP stimulation of Y-1 cells (mean of 2 experiments). Immunoprecipitated, cross-linked DNA quantified by qPCR was compared to similar determinations on DNA obtained prior to immunoprecipitation (Input). Results for ChIP are expressed as percent of input. A) pCREB antibody; B) TORC antibody.

Figure 3. Schematic presentation of StAR suppression by SIK and TIS11b

A. SIK-mediated suppression of the activation of CREB by CRTC/TORC.

SIK is activated by a combination of two kinases, LKB1 and GSK3 β (in combination with autophosphorylation). This involves phosphorylation at, respectively, T182 and S186. SIK activity is suppressed by PKA phosphorylation at S577 and by staurosporine inhibition. SIK inhibits TORC by phosphorylation at S171, which effects translocation to 14-3-3 in the cytoplasm. SIK may also function through the activation of PP2a, which may, additionally, enhance other PKA-dependent processes, including enzymes that affect SF1 ligands. SIK also inhibits Type 2 HDACs (see text for references).

B. Post-transcriptional regulation of the 3.5kb StAR mRNA by TIS11b.

cAMP/PKA stimulation of StAR transcription produces 3.5kb (major product), 2.8kb (minor) and 1.6kb mRNA through alternative polyadenylation of nuclear transcripts. PKA also stimulates expression of TIS11b, a protein which specifically enhances degradation of the 3.5kb mRNA. TIS11b binds as a homodimer to three UAUUUAU AURE elements in the extended 3'UTR of the 3.5kb mRNA. The increase in TIS11b expression may share some regulatory features with the parallel increase in StAR mRNA, including mediation by CREB and SF-1 and suppression by SIK. The 3'UTR of TIS11b mRNA has elements that recognize TIS11b, suggesting post-transcriptional auto-regulation.

Figure 4. Effects of suppressing TIS11b on stimulation of StAR mRNA expression

A. Time course for stimulation of TIS11b mRNA by Br-cAMP (1mM) in MA10 and Y-1 cells.

B. MA10 cells were pre-treated with siRNA for TIS11b or scrambled control siRNA for 48h and subsequently stimulated by 1mM Br-cAMP for the indicated times. Northern blots were carried out using a 32-P labeled probe specific for TIS11b. siRNA-TIS11b doubled the StAR 3.5kb mRNA in 3-24 h determinations (average increase), while producing no effect on the 1.6kb mRNA.

Cultures were carried out on three separate dishes for each data point. Stimulations of 3.5kb mRNA by siRNA-TIS11b was significant compared to scrambled siRNA treatments ($p < 0.05$) at each time point (3-24hr). Data, presented with permission, are previously published in *Mol Endocrinol* 23, 497-509 (2009).

Figure 5. Effects of suppressing TIS11b on stimulation of StAR protein expression and activity MA10 cells were treated with a siRNA mixture that targeted TIS11b (or scrambled control siRNA) for 48h. The cells were subsequently stimulated with Br-cAMP (1mM) for the indicated times.

A. Immunoblots for TIS11b (4 phosphorylated forms—removed by phosphatase treatment), StAR, cyclooxygenase 2 and actin standard. When TIS11b is removed by siRNA, StAR protein declines by an average of two-fold, while 3.5kb mRNA increases (Figure 4B).

B. Cholesterol metabolism was measured, as previously described (Artimenko et al 2001). TIS11b siRNA decreased cholesterol conversion to pregnenolone (3 β -hydroxysterol dehydrogenase inhibited during 5 min assay) in proportion to loss of StAR protein. Cultures were carried out on three separate dishes for each data point. The effects of siRNA-TIS11b were significant compared to scrambled siRNA treatments ($p < 0.05$) at each time point. Data, presented with permission, are previously published in *Mol Endocrinol* 23, 497-509 (2009).

Figure 6. Effects on mutating TIS11b recognition sequence on degradation of StAR 3'UTR
 MA-10 cells were transfected with the indicated amounts of TIS11b expression vector and the indicated luciferase construct in which the 3'UTR contains a 300bp sequence from StAR 3'UTR that contains three AURE (ARE). In ARE12m, the upstream pair of AURE are mutated, as indicated, to prevent TIS11b binding. The pGL3P control contains only the SV40 polyadenylation sequence used in the ARE and ARE12m constructs. Luciferase activity was measured 24h after transfection. The greater loss of ARE activity indicates a significant effect of TIS11b acting through the AURE sequence (triplicate transfections, $p < 0.05$). Data, presented with permission, are previously published in *Mol Endocrinol* 23, 497-509 (2009).

Figure 7. Interspecies differences in the organization of StAR 3'UTR sequences

Comparison of the distribution of polyadenylation (AAUAAA) and TIS11b binding AURE sites (UAUUUUUU) in the StAR 3'UTR from mouse and humans. There is minimal conservation of sequence at other sites. The basal instability sequence upstream of AURE in the mouse 3'UTR causes substantially increased turnover when included in 3'UTR reporters, similar to those described in Figure 6 (see Duan et al 2007).