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Stimulation of StAR expression by cAMP is controlled by inhibition of highly inducible SIK1 via CRTC2, a co-activator of CREB

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Abstract

In mouse steroidogenic cells the activation of cholesterol metabolism is mediated by steroidogenic acute regulatory protein (StAR). Here, we visualized a coordinated regulation of StAR transcription, splicing and post-transcriptional processing, which are synchronized by salt inducible kinase (SIK1) and CREB-regulated transcription coactivator (CRTC2). To detect primary RNA (pRNA), spliced primary RNA (Sp-RNA) and mRNA in single cells, we generated probe sets by using fluorescence in situ hybridization (FISH). These methods allowed us to address the nature of StAR gene expression and to visualize protein–nucleic acid interactions through direct detection. We show that SIK1 represses StAR expression in Y1 adrenal and MA10 testis cells through inhibition of processing mediated by CRTC2. Digital image analysis matches qPCR analyses of the total cell culture. Evidence is presented for spatially separate accumulation of StAR pRNA and Sp-RNA at the gene loci in the nucleus. These findings establish that cAMP, SIK and CRTC mediate StAR expression through activation of individual StAR gene loci.

Keywords

StAR; SIK; CRTC; Splicing; Fluorescence in situ hybridization

1. Introduction

The steroidogenic acute regulatory protein (StAR) plays a central role in steroidogenesis by facilitating the transfer of cholesterol to cytochrome P450 11A1 (P450_{scc}) in the inner mitochondrial membrane (Jefcoate, 2002; Stocco, 2001). StAR activity is, therefore, a major determinant of the levels of circulating glucocorticoids, which respond to both diet and stress (Kanter et al., 2001; Saltzman et al., 2006; Son et al., 2008). Removal of the

adrenocorticotrophic hormone (ACTH) stimulus or inhibition of protein synthesis stops cholesterol transfer activity within minutes, consistent with the ultradian rhythm (Ariyoshi et al., 1998; Saltzman et al., 2000, 2004; Ulrich-Lai et al., 2006).

Okamoto and Takemori showed that the kinase, salt inducible kinase 1 (SIK1), is a critical mediator of PKA activity (Berdeaux et al., 2007; Katoh et al., 2004; Okamoto et al., 2004; Takemori et al., 2009). This kinase phosphorylates and inactivates cAMP response element-binding protein (CREB) regulated transcription coactivator 2 (CRTC2) proteins, which facilitate the recruitment of CREB and the histone acetyl transferase, CREB-binding protein (CBP), to StAR (Altarejos and Montminy, 2011; Conkright et al., 2003; Dentin et al., 2008; Ferreri et al., 1994; Radhakrishnan et al., 1997, 1999; Uebi et al., 2010; Yamamoto et al., 1988). SIK1 is inhibited by PKA and this is essential for sustained cAMP-induced transcription of StAR (Okamoto et al., 2004). Inhibition of SIK1 with very low concentrations of staurosporine stimulates StAR transcription almost as effectively as Br-cAMP (Jefcoate et al., 2011). Phosphorylated forms of SIK bind to the scaffold protein, 14-3-3, in the cytoplasm (Al-Hakim et al., 2005; Sreaton et al., 2004). We have developed our hypothesis to envisage a coordinated regulation of StAR transcription, splicing and post-transcriptional processing, which are coordinated by SIK and the zinc finger protein ZFP36L1/TIS11b, and respond to PKA in parallel with StAR stimulation (Ciais et al., 2004; Duan et al., 2009; Jefcoate et al., 2011).

We present data sets obtained on single adrenal and testis cells, which used two novel techniques: the real time measurement of intra-cellular transfer of SIK1 and CRTC2 using GFP chimeric proteins, and the localization of StAR initial nuclear RNA transcripts (nuc-RNA) and mRNA using tandem DNA oligonucleotides labeled with either Cy3 or Cy5 in RNA fluorescence in situ hybridization (FISH) (Vargas et al., 2011). The appearance of nuc-RNA in nuclear speckles and subsequent mRNA in the cytoplasm was detected in single cells and shown to be compatible with the time courses of nuc-RNA and mRNA determined by qPCR on whole cell populations. This method also provides a general approach for quantitative dissection of expression steps at designated gene loci in cultured cells or in tissue sections, providing a new level of precision to histology.

In this study, we examine the early steps in the stimulation of StAR expression in mouse adrenal Y-1 and testis MA10 cells. These lines exhibit similar maximum expression levels, with much higher basal expression in the adrenal cells. This basal StAR mRNA supports the rapid stimulation of cholesterol metabolism in these cells, which peaks within 15 min, prior to any response in the testis cells. We use qPCR primer pairs that differentially quantify transcription initiation, elongation and splicing. We uncover a novel control point for StAR expression as transcription reaches early exon 7 in both cell types, which remarkably precedes both further elongation to the alternative polyadenylation sites and splicing of all 6 introns. The relationship of the cAMP-dependent steps to parallel processes involving the induction of SIK1 and the involvement of CRTC forms is examined. The potential for SIK1 and CRTC2 as mediators of ultradian signaling is further considered.

2. Materials and methods

2.1. Cell culture

Adrenal cell experiments were conducted with a Y-1 subclone provided by Dr. Bernard Schimmer (University of Toronto). MA-10 mouse Leydig tumor cells were a generous gift from Dr. Mario Ascoli (University of Iowa College of Medicine). Cell culture was performed as described previously (Duan et al., 2009).

2.2. Quantitative real time RT-PCR

Total RNA (1–5 µg) isolated with Trizol reagent (Invitrogen) was treated with DNase I (Promega) followed by cDNA synthesis using the GoScript™ Reverse Transcription System (Promega). Quantitative RT-PCRs were carried out as described previously (Duan et al., 2009). The analysis of relative RNA levels was performed using a delta- C_T ($-C_T$) relative quantification model with actin and RN18s as reference genes. Each value is expressed as fold change relative to the RNA level of the control value.

2.3. Western blot

Cells were harvested and lysed with ProteoJET™ membrane protein extraction buffer (Fermentas, USA) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF), Phosphatase inhibitor (20×: 125 mM NaF, 250 mM B-glycerophosphate, 250 mM para-nitrophenyl phosphate, 25 mM NaVO₃) and protease inhibitor (Sigma, P8340) were added. Extracts were quantified by BCA protein assay kit (Pierce Bio-technology, Inc., Rockford, IL, USA). Proteins were incubated with an antibody raised against recombinant mouse StAR protein (Dr. Dale Buck Hales, University of Illinois at Chicago, USA), CRTC2 (Dr. H. Takemori, National Institute of Biomedical Innovation, Osaka, Japan) for 1 h. Protein bands were visualized by ECL reagent (Amersham Biosciences) and exposing the membrane against Hyperfilm (Amersham Biosciences).

2.4. Immunocytochemistry

Glass coverslips with plated cells were washed with sterile PBS, placed in 6 well plate. To determine the subcellular localization of SIK1 and CRTC2, cells were fixed, permeabilized with 0.2% Triton X-100 (Calbiochem) on ice. Cells were blocked with 5% BSA in PBS-Tween 20 (PBST) including 0.3M of glycine. Cells were incubated with the primary antibody in PBST (1:200 dilution). Cells were imaged using Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) antibody (Molecular Probes/Invitrogen). Images were captured by Olympus IX81 motorized inverted microscope, from 0.1 to 25 µm Z-sections and deconvoluted with Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.). The image stacks were projected onto a single plane or 3D image. The Nearest Neighbors deconvolution is a rapid way to deblur fluorescence data. The algorithm uses the plane above and below the plane of interest to compute and subtract the fraction of the data that are out-of-focus information. 3D image was represented with the imajaJ software, which is a public domain, Java-based image processing program developed at the National Institutes of Health.

2.5. Fluorescence in situ hybridization (FISH)

The Stellaris FISH method has been demonstrated to be effective at visualizing well-expressed mRNA transcripts in most cells. Glass coverslips with plated cells were washed with sterile PBS, placed in 6 well plate. Cells were plated on glass coverslips, fixed with 1% paraformaldehyde and permeabilized in ice cold 0.2% TritonX100. Cells were stored at 4 °C in 70% ethanol overnight and hybridization was performed the next day. In order to detect individual molecules of mRNA, Stellaris FISH probe sets consist of multiple singly labeled oligonucleotides (generally 30–48) designed to hybridize along targeted RNA transcripts. Hybridization was performed in dark, humidified condition. Samples were re-suspended in 2× SSC and added GLOX buffer without enzymes for equilibration, incubated then re-suspended in GLOX buffer with enzymes (glucose oxidase and catalase). Again, antifade reagent was used prior to Z-stack imaging.

2.6. Combined immunofluorescence and FISH

Additional cells were treated with the addition of RNase inhibitor (Promega) during block and antibody exposures of the immunohistochemistry procedure then subsequently treated for FISH. The combined fluorescence was imaged using the aforementioned Z-stack analysis.

2.7. Transfection

Transfection was performed using DreamFect™ Gold solution (OZ Biosciences) and combiMag solution. The final lipoplex mixtures were added to the cells growing in serum-containing culture medium and incubated for 4 hrs on the magnetic plate at 37 °C in a CO₂ incubator. SIK1-GFP, pTarget-SIK1, SIK1-S577A-GFP, pTarget-S577A-SIK1, DR-defective SIK1-GFP, and CRTC2-GFP plasmids were gifts from Dr. H. Takemori. Cells were cotransfected with pTAL-Cre firefly luciferase plasmid (Clontech). Targeted fragment of the 5'-StAR gene (m254-StAR) was placed upstream of the luciferase reporter gene into the pGL2 basic vector (a generous gift from Dr. Barbara Clark). Luciferase assays were performed using a Luciferase Assay kit (Promega) according to manufacturer's instructions.

2.8. Data analysis

Results were expressed as means ± SEM of triplicates. One-way ANOVA with Tukey's post-hoc test was used to compare different samples, or two-way ANOVA with Bonferroni's post-hoc test was used for comparison of different groups. Differences among means were considered as significantly different at $P < 0.05$. Data were analyzed by using the GraphPad PRISM software (San Diego, CA).

3. Results

3.1. Rapid generation of initial StAR pre-RNA

StAR is expressed in Y-1 and MA10 cells as both 3.5 and 1.6 kb mRNA forms (Ariyoshi et al., 1998; Duan and Jefcoate, 2007). At shorter stimulation times, the long transcript predominates, including in rat adrenals (Ariyoshi et al., 1998). Although the two cell lines exhibit similar maximum expression of StAR after 3 h of stimulation they exhibit very

different basal expression and steroidogenesis response kinetics. Y-1 cells, like primary adrenal cells, exhibit basal activity, which is about 10% of the 3 h stimulated level. The basal mRNA is sufficient to mediate maximum stimulation of steroidogenesis within 15 min. This process depends on translation of new StAR protein from StAR mRNA located at the mitochondria and direct phosphorylation by Type2 PKA (Artemenko et al., 2001; Dyson et al., 2009) (Fig. 1A). For MA10 cells, basal StAR expression is essentially undetectable and peak steroidogenesis comparable to Y-1 cells is only realized after near maximum StAR expression. StAR activity is therefore determined by transcription, which is mediated by SIK/CRTC in addition to the StAR phosphorylation.

We have assessed the transcriptional response of Y-1 adrenal cells at an optimum stimulatory concentration of Br-cAMP (1 mM). StAR pre-mRNA reaches steady state at about 30 min. mRNA increases during this steady state period in an approximately linear manner (Fig. 1B). There is no significant difference between transcription in exon 1 or intron 6 (Fig. 1C). By contrast, there was a delay of 15 min before transcription of exon 7 at the end of the translated sequence, as indicated by the TAA termination site. Primers that measure the removal of, respectively, introns 1 and 5 or the extension of transcription to the end of the 3'UTR show the same delay (Fig. 1D). This suggests that transcription is stalled at some point in late intron 6 or early exon 7, and that further transcription beyond this point is necessary for removal of the introns. This stimulation of Y-1 cells is superimposed on an appreciable constitutive expression that is at least 10-fold higher than in MA10 testis cells (Fig. 1E).

To characterize the “pause site” further, we measured the stimulation of transcripts in Y-1 cells extending from the end of exon 6 to the beginning of exon 7 (Fig. 1F). Thus, not only is transcription continuing into exon 7, but splicing also remains minimal. More precisely, there is a similar fivefold stimulation by Br-cAMP up to sequences close to the translation termination TAA sequence. The 20 base sequence that overlaps the TAA and later sequences in the 3'UTR failed to respond, indicating the pause is proximal to the TAA sequence.

3.2. Direct localization of StAR transcripts in the nuclei of single MA10 cells by high sensitivity FISH

In order to learn more about the transcription and splicing of StAR RNA and the subsequent transfer to mitochondria, we applied a method of enhanced FISH to StAR transcripts. Short oligonucleotides targeting adjacent RNA sequences bind cooperatively. We designed sets of 30 or more 20mers to distinguish StAR primary and spliced transcripts, each set with different Alexa fluorescent labeling (Fig. 2A). The StAR primary transcripts are recognized by 20mers targeting intron 1, while spliced transcripts are selectively targeted by 20mers that are distributed across the exons, such that contiguous cooperative binding is only achieved after removal of the introns. These probe sets are sufficiently sensitive to detect single StAR primary (StAR pRNA) and spliced transcripts (StAR Sp-RNA).

In MA10 cells, these probe sets fail to detect any StAR RNA in unstimulated cells (Fig. 2B). However, 15 min after stimulation by 1 mM Br-cAMP, StAR pRNA appears at single nuclear sites in about 30% of the cells. The number of cells with such sites increases

progressively with time, with a second site appearing from 30 min onwards. After 60 min, 85% of the cells express StAR pRNA, mostly at two distinct sites. We have termed these sites StAR pRNA loci. Preliminary studies with pRNA probes demonstrate co-localization of the previously detected RNA with the StAR alleles, although presently with modest efficiency. The average intensity of the loci also increases with time of stimulation, albeit with a wide range that presumably reflects the accumulation of transcripts.

3.3. Splicing of StAR transcripts is delayed at nuclear loci in single cells

StAR Sp-RNA probes fail to detect loci in MA10 cells at 15 min, despite the presence of p-RNA loci, indicating that Sp-RNA oligomers do not appreciably recognize the primary transcripts. This selectivity is critical since there is the potential for crossover recognition of exons in the primary transcripts. The distinct responses of the two probe sets validate our prediction that the excision of the introns is necessary to establish binding synergy for StAR Sp-RNA. The Sp-RNA probes follow a similar, but delayed trend in eventually detecting two loci in most cells after 60 min. When we added both probe sets together, the pRNA loci clearly overlap (Fig. 2C). Close inspection shows that the overlap is not exact and that the proportions of pRNA and Sp-RNA vary. The time difference between pRNA appearance and Sp-RNA appearance is likely to correspond to the delay in splicing detected by q-PCR (Fig. 1).

The proportion of cells that express StAR loci increases in parallel with the number of detectable loci per cell (Fig. 2D). There is a marked decline in pRNA for many cells between 60 min and 180 min, which indicates a cessation of transcription initiation at these StAR loci (Fig. 2B). The mean levels of expression for each pRNA locus follow this same temporal profile. The linear increases in StAR pre-mRNA, quantified by qPCR, in the macro population thus represent an asynchronous entry of different cells and their StAR alleles into the transcription process. The steady state for StAR pre-mRNA in the macro-culture corresponds to near complete activation of StAR alleles. At later times (60–180 min) third loci appear, probably reflecting the tetraploid state for this MA10 tumor cell line.

StAR mRNA was not detectable in the MA10 cytoplasm at 60 min, but was highly visible after 180 min. StAR protein, as expected, overlapped almost completely with mitochondria (Fig. 2E). Newly stimulated StAR mRNA overlapped extensively with some, but not all StAR protein (Fig. 2F).

3.4. Y-1 cells exhibit basal active loci and cytoplasmic mRNA

Unlike MA10 cells, Y-1 cells exhibit both pRNA and Sp-RNA at nuclear loci and mRNA in the cytoplasm (Fig. 3A). Y-1 cells deliver a maximum steroidogenic response within 15 min, prior to any increase in cytoplasmic mRNA (Artemenko et al., 2001). Interestingly, the amounts of basal StAR pRNA, Sp-RNA and cytoplasmic mRNA in each cell are unevenly matched, suggesting a degree of independent control at each step involving these StAR RNA species. The stimulation by Br-cAMP of each at the loci is substantial compared to this basal expression (Fig. 3B) and involves asynchronous further activation of two loci, similar to that seen in MA10 cells.

The overlap of pRNA (green) and Sp-RNA (red), which appears as yellow fluorescence, is variable in the XY plane (Fig. 3B, 60 min loci). Cytoplasmic mRNA appears at 60 min (earlier than in MA10 cells), but again increases substantially at 180 min. Interestingly, the increases in StAR mRNA, which is visualized in both cell types, lag appreciably behind increases seen on Northern blots and in the translated StAR protein, which are each substantial within 60 min (Duan and Jefcoate, 2004, 2007). Possibly, the most readily translated StAR mRNA is protected from the probes, either as ribonucleoprotein complexes or as polyribosomes.

We further examined the overlap of pRNA and Sp-RNA through the process of Z-slicing. This procedure involves taking images at different depths in the cell by shifting the focal plane in incremental steps. This is diagrammed in Fig. 3C. The pRNA always appears at the top, the Sp-RNA at the bottom. This 1- μ m spread greatly exceeds the dimensions of the associated StAR RNA, due to the dispersion of the fluorescence. The 60 min loci are shown in a series of XY planes at different depths and also projected from the side (XZ) (Fig. 3D). Similar images are seen in MA10 cells.

The fluorescence intensity for pRNA and Sp-RNA has been determined from the respective pixel densities, and is shown in combination with the XY images (Fig. 3E). The center of the pRNA image is consistently displaced from the center of the lower Sp-RNA image by 0.3–0.4 μ m. This distance should represent the physical separation of the StAR Sp-RNA from the site of primary transcription.

3.5. Expression and activity of SIK1 in adrenal cells

SIK1 mRNA is very rapidly and extensively induced in Y-1 cells by ACTH (Takemori et al., 2003) and in rat adrenals in vivo (Liu et al., 2012). Essentially the same stimulation is produced in these Y-1 cells by Br-cAMP (Fig. 4A). There is a delay of 15 min before a 20-fold rise between 15 and 60 min. SIK2 is expressed under basal conditions at levels comparable to the peak level of SIK1. The increase in SIK1 protein is appreciably delayed with increases only initiated as mRNA levels peaked (Liu et al., 2012). This delay suggests a further control over translation.

SIK forms control the PKA activation of many genes by restraining the essential CRTC co-activators in the cytoplasm. The regulation of StAR in adrenal cells most likely involves basal restraint by the constitutive SIK2, but more refined control by the highly inducible SIK1. To test the role of SIK1 further, we investigated the effects of the S577A mutation of SIK1, which is resistant to inhibition by cAMP and PKA. Y-1 cells were co-transfected with SIK1-S577A and a tandem-CRE luciferase reporter, which is activated by CREB binding to the CRE repeats (Fig. 4B). Basal activity was unaffected, but the stimulatory effect of Br-cAMP was suppressed by 75% (Fig. 4C). In this case, CRTC enhances the effectiveness of p-CREB to bind the key histone acetyl transferase CBP. The proximal StAR promoter (–250 bp) luciferase reporter responds to CREB through a half site shared with SF1, and through a combination of interactions with other nuclear factors (Clark and Combs, 1999). A similar transfection experiment mediated near complete inhibition of both basal and stimulated activity (Fig. 4C). The inhibitory effect is much more severe for the more complex interactions of CREB in the proximal StAR promoter. We also measured the effect of this

SIK-S577A transfection on the total StAR expression (Fig. 4D). A short expression period of 3 h was ineffective, but extensive suppression was seen after 24 h. SIK1 transfection did not enhance the effect of native SIK.

3.6. Translocation of SIK1 in Y-1 cells

This mechanism is substantially dependent on the movement of SIK 1 and CRTC forms. To follow the rapid movement of SIK1 and CRTC forms in these cells, we used fluorescent GFP fusion proteins that retain normal activity. Following transfection, SIK1-GFP primarily localized to the nucleus (approximately 70%) in Y-1 cells. Following stimulation by Br-cAMP, most of SIK1 exited to the cytoplasm within 5 min, and transfer was complete in 15 min (Fig. 4E). By contrast, SIK1-GFP-S577A was located exclusively in nuclear speckles before and after Br-cAMP stimulation (not shown). Expression of this fluorescent derivative allowed us to track the extent of StAR expression in relation to the presence of the PKA-resistant SIK1 form in single cells. Transfection of SIK1-GFP-S577A generated strong GFP fluorescence in nuclei of about 15% of the Y-1 cells, whereas other cells had undetectable levels. The transfected cells exhibited complete suppression of StAR RNA in both the nuclear loci and the cytoplasm, whereas cells that did not visibly express the vector had normal expression (Fig. 4F). Similar levels of expression extended to cells comparably transfected with a GFP control or SIK1-GFP (not shown). The low acute effects of SIK1-GFP-S577A compared to this acute suppression of StAR transcription in individual cells can be substantially attributed to the low transfection efficiency. Low efficiency transfection that is undetectable by GFP-fluorescence may cause the slower loss of total StAR shown in Fig. 4D.

3.7. CRTC2 transfer to the nucleus is a key step in the activation of StAR expression by Br-cAMP

We tested GFP chimeras of each CRTC form for responses to Br-cAMP in Y-1 cells. CRTC1-GFP and CRTC3-GFP were each mostly cytoplasmic and unresponsive to Br-cAMP stimulation (Fig. 5A). CRTC2-GFP is initially present in the cytoplasm, but moves over several hours to speckle sites in the nucleus. This process is completely prevented after 3 h by co-transfection with SIK1-S577A. Time lapse imaging of the transfer stimulated by Br-cAMP shows that the transfer to the nucleus is almost complete in 5 min, but is followed by intra-nuclear accumulation in speckle-like structures (Fig. 5B). This transfer of CRTC2-GFP was slower in MA10 cells compared to Y-1 cells (Fig. 5C). The transfer rate declined when the concentration was decreased from 1 mM to 0.1 mM. The transfer rates were very similar in all transfected cells, again in contrast to the heterogeneity of initiation of StAR transcription. This asynchrony does not arise from cell differences in these transfer processes.

CRTC2 is restrained in the cytoplasm through phosphorylation of S307 by SIK2 and subsequent binding to the protein, 14-3-3 (Uebi et al., 2010). Following inhibition of SIK2 by PKA, dephosphorylation at S307 is mediated by calcineurin/PP2B, a Ca-dependent phosphatase. This dephosphorylation then leads to rapid nuclear transfer. A second phosphorylation by SIK2 targets S171 (Uebi et al., 2010). The dephosphorylation at S171, which is necessary for CREB activity, occurs in the nucleus and is mediated by PP1 or PP4.

The use of selective inhibitors, however, shows some limitations to this model. Cyclosporine A (CsA), which inhibits calcineurin, slows the Br-cAMP stimulated transfer of CRTC2 (Fig. 5D). However, this produces only minimal decreases in the StAR expression stimulated by Br-cAMP, the cAMP stimulant forskolin (Fig. 5D). Stimulation by ACTH, which may in part function through elevation of Ca, is inhibited, possibly due to a greater contribution from calcineurin.

Okadaic acid (OA) potently inhibits PP2A (at 1 nM), whereas inhibition of PP1 and PP4 phosphatases requires much higher concentrations (100 nM). Such high levels of OA did not prevent Br-cAMP from rapidly equalizing the nuclear and cytoplasmic levels of CRTC2-GFP (Fig. 5E). However, there is a progressive loss of CRTC2-GFP, which is also seen for native CRTC2. The StAR expression after 180 min decreases with the loss of CRTC2. OA may promote enhanced phosphorylation of CRTC2 at sites that trigger proteolytic degradation. However, preliminary studies of primary transcription, using either p-RNA probes in single cells or by qPCR on the macro cultures, show that OA does not affect transcription after 60 min stimulation. CRTC2 may participate differently at early and later stages in StAR expression.

The two forms of SIK that are expressed in Y-1 and MA10 cells each have homologous sequences surrounding the respective PKA inhibition sites (SIK1-S577; SIK2 –S587) (Fig. 6A). There is a nuclear import/export site adjacent to this PKA site exclusively in SIK1. Y-1 cells express all three forms of CRTC. Fig. 6B diagrams their structures and identifies the phosphorylation sites targeted by SIK forms in the central domain. There are additional differences in potential phosphorylation sites in the N and C-terminal domains, which lead to their different functions (Takemori et al., 2003).

Figure 6C shows the effects of SIK1 inhibition in relation to the two step activation of CRTC2. Following inhibition of SIK2 by PKA S307 is dephosphorylated by PP1B/calcineurin in the cytoplasm (inhibited by cyclosporine/CsA) leading to nuclear transfer. This is insufficient for activation of CREB and StAR transcription. A second dephosphorylation by nuclear serine phosphatases (PP1 or PP4) at S171 is necessary, which is inhibited by relatively high levels of okadaic acid (OA) while also promoting degradation. S577-dephospho-SIK1 co-localizes with CRTC2 immediately after nuclear transfer of the latter thus efficiently restoring the inactive dually phosphorylated CRTC2. SIK1 appears in this state after the peak of cAMP produced by a pulse of ACTH declines.

4. Discussion

Y-1 adrenal cells and MA10 testis cells exhibit similar peak levels of hormonally stimulated steroidogenesis, but differ in the kinetics of their respective responses (Artemenko et al., 2001; Stocco and Kilgore, 1988). A basic mechanism for StAR activity on mitochondria has been developed for MA10 cells. The distinctive acute stimulation of adrenal cells in culture and of rat adrenals in vivo (Lightman, 2008; Liu et al., 2012; Sarabdjitsingh et al., 2010) suggests that the adrenal mechanism has additional features (Jefcoate, 2002). Here, high sensitivity FISH microscopy reveals new features of the transcriptional control of StAR in response to Br-cAMP, at the level of single cells that, nevertheless, match the responses

determined in macro-cultures by q-PCR. The two cell lines share many features, but several features are unique to adrenal cells. We further establish the essential role of the SIK/CRTC mechanism developed by Okamoto and Takemori (Okamoto et al., 2004; Takemori et al., 2002) as a mediator of the PKA control in each cell type.

These new features of StAR transcription also provide a novel perspective on the relationship between transcription and splicing (Parton et al., 2014). First, the PKA stimulation of transcriptional elongation stalls near the translation termination site in exon 7, without initiation of splicing or reaching the alternative polyadenylation sites (Duan and Jefcoate, 2007; Duan et al., 2009). FISH analysis of this stimulation of Y-1 and MA10 cells separates transcription and splicing directly at the StAR gene. The 15–30 min delay in splicing is recognized by the early absence of spliced transcripts at the gene locus (Sp-RNA). These StAR loci are activated through asynchronous processes in the individual cells, which also appear independently controlled. This sequence occurs over the 60 min period that corresponds to generation of the pre-mRNA steady state in the Y-1 and MA10 macro-cultures. The delay in splicing is not apparent for the loci initiated after 30 min, suggesting that a second PKA-dependent process occurs similarly in all cells, thereby overcoming the delay. Elongation of the 3'UTR to the polyadenylation sites and the splicing of all 6 introns subsequently occur inseparably.

The FISH imaging identifies two major differences between the adrenal and testis cells that are also seen in the qPCR: a decline in transcription exclusively in MA10 cells between 60 and 180 min, and basal expression of StAR RNA specifically in Y-1 cells. The appreciable basal transcription, splicing and mRNA export in Y-1 cells mediate the acute steroidogenic response. The intensity of pRNA in these constitutive loci in Y-1 cells corresponds to no more than 10% of the steady levels observed after 60 min of stimulation, in either cell type. These constitutive loci in Y-1 cells evidently retain some splicing activity.

In both cell types, the StAR loci have similar spatial features: spliced transcripts, which are always separated below and laterally from the primary transcripts. These active StAR loci are fixed close to the midline of the nucleus. This Sp-RNA satellite locus may provide a site for processing of the StAR mRNA as ribonucleoprotein complexes prior to export to the cytoplasm (Kohler and Hurt, 2007). The mRNA detected by Sp-RNA 20mers not only appears in the cytoplasm approximately 60 min after the splicing, but also lags behind both the StAR detected by qPCR and protein translation. Much of the FISH detection is spatially separated from StAR protein and the mitochondria. The earliest transfer to, of, StAR mRNA to mitochondria may be protected by the associated ribonucleoprotein.

The asynchrony of StAR locus transcription does not derive from the CRTC2 activation, since CRTC2-GFP transfers similarly in all the transfected Y-1 cells. This redistribution also matches the nuclear transfer of CRTC2 in the rat adrenal following ACTH stimulation (Liu et al., 2013). The rates are fast enough to match the activation of the StAR transcription at the loci. The remarkable induction of SIK1 between 15 and 30 min and the high basal level of SIK2 are conserved in MA10 cells (not shown) and in rat adrenals in vivo (Liu et al., 2013). The basal restraint of CRTC2 in the cytoplasm should, therefore, be determined by the phosphorylation delivered by SIK2, which is then reversed by cAMP and PKA. The

cytoplasmic location of SIK2 also fits this role. The induction of SIK, however, lags behind the early primary StAR transcripts, while the effectiveness is further lessened by delayed translation (Liu et al., 2012). SIK1 only becomes effective and nuclear when dephosphorylated by PP2A as cAMP levels decline. The relative effects of cyclosporin A and okadaic acid on StAR transcription suggest that the nuclear dephosphorylation at CRTC2-S171 has more impact than a slowing of nuclear transfer. The slow association of CRTC2 with nuclear speckle structures may have functional significance, including reversal of the stalled elongation.

The comprehensive inhibitory effects of the PKA-resistant SIK1-GFP-S577A on the earliest stages of StAR transcription in single cells demonstrate the potency of the nuclear-unphosphorylated SIK1 as an inhibitor of CRTC2. The preference of CRTC1 and CRTC3 GFP fusion proteins for the cytoplasm suggests that they have different roles from CRTC2.

The added commitment of cell energy needed for this indirect cAMP activation mechanism suggests that SIK1 has a key role in shutting down StAR in the decline phase of pulsatile endocrine signaling. The preferred induction of the labile 3.5 kb transcript and early increases in the mRNA degradation mediator, ZFP36L1/TIS11b (Duan et al., 2009), and the Creb antagonist, Crem (Manna et al., 2002), support this perspective.

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Abbreviation

ACTH	adrenocorticotrophic hormone
CBP	CREB binding protein
CREB	cAMP responsive element binding protein 1
CRTC	CREB regulated transcription coactivator
CsA	cyclosporine A
FISH	fluorescence in situ hybridization
OA	okadaic acid, P450 _{scc} , cytochrome P450 11A1
SF-1	steroidogenic factor 1
pRNA	primary RNA
SIK1	salt inducible kinase 1
Sp-RNA	spliced primary RNA
StAR	steroidogenic acute regulatory protein

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Fig. 1.

Characterization of delayed splicing of StAR transcription in Y1 cells. (A) Distinction between Y-1 cells and MA10 cells for stimulation of cholesterol metabolism in relation to StAR expression. (B) Time course for stimulation of StAR expression in Y1 cells by Br-cAMP (1 mM). (C) Stimulation of transcription shown at three positions of elongation. (D) Stimulation of spliced transcripts created at two positions by splicing and at the end of the 3'UTR. (E) Basal and 15 min levels of StAR pRNA and mRNA in Y1 and MA10 cells. (F) Stimulation of StAR transcripts extended to different positions in intron 6 and early exon 7. One-way ANOVA with Tukey's post test (F) or two-way ANOVA with Bonferroni's post test (B–E) was used to compare different samples. Error bars show means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 2. Stimulation of StAR pRNA and mRNA in the nucleus and cytoplasm of MA10 cells. (A) 20 mers used for FISH of pRNA and Sp-RNA. (B) Detection of pRNA (green) and Sp-RNA (red) in typical MA10 cells at different times of stimulation. (C) Sixty minute stimulation using dual addition of pRNA and Sp-RNA 20mers. (D) (Left) Percentage of cells showing StAR loci compared to average number of loci/cell. (Right) Percentage of cells expressing loci compared to StAR pre-mRNA levels determined by qPCR. (E) Distribution of mitochondria detected by mitotracker compared to immunofluorescence of StAR protein after 180 min stimulation. (F) Overlap of StAR mRNA (Sp-RNA 20mers) and StAR protein after 180 min stimulation.

Fig. 3.

Dissection of Y1 StAR loci. (A) Basal expression of STAR pRNA and Sp-RNA at high sensitivity in the same Y1 cells. (B) Dual labeling of representative Y1 cells at low sensitivity after 60 and 180 min of stimulation by Br-cAMP (1 mM). (C) Diagram of z-stacking of a typical StAR locus after 60 min of stimulation. Three XY planes are shown at different depths along the z- axis. pRNA is shown as green, Sp-RNA as red. (D) Dual labeling of two 60 min loci viewed in a XY plane at the nuclear midline and from a lateral position (XZ plane). (E) Quantification of pRNA and Sp-RNA for different slices of a 60 min locus in the z-stack (4 shown).

Fig. 4. Expression, movement and activity of SIK1 in Y1 cells. (A) Induction of *Sik1* and *Sik2* mRNA in Y1 cells by Br-cAMP (1 mM). (B) Design for transfections of Y1 cells. (C) Effects of co-transfected SIK1-577A (and control vector) with 250bp-StAR luc and multi CRE luc. (D) Effects of transfected SIK1-577A and *Sik1* (and control vector) on a 24 h stimulation of StAR expression by Br-cAMP (1 mM). (E) Stimulation of nuclear exit of SIK1-GFP. (F) Impact of SIK1-GFP-SIK1-577A on the stimulation of sp-RNA/mRNA in Y1 cells after 180 min. One-way ANOVA with Tukey's post test (A) or two-way ANOVA with Bonferroni's post test (C) was used to compare different samples. Error bars show means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 5.

Stimulation of transfer of CRTC2-GFP in relation to StAR expression. (A) Location of CRTC-GFP forms in Y1 cells. Effects of Br-cAMP and co-transfected SIK1-577A. (B) Re-distribution of CRTC2-GFP in Y1 cells stimulated by Br-cAMP (1 mM). (C) Re-distribution of CRTC2-GFP in MA10 cells stimulated by Br-cAMP (1 mM/0.1 mM). (D) Effect of CsA (20uM) on the transfer of CRTC2-GFP in MA10 cells (1 mM Br-cAMP) in relation to the stimulation of StAR mRNA (180 min) by Br-cAMP and ACTH. (E) Effect of OA (100 nM) on the transfer of CRTC2-GFP promoted by Br-cAMP (1 mM). This is compared to effect on CRTC2 protein and StAR protein (180 min; 62.5/125 nM OA). Two-way ANOVA with Bonferroni's post test (D) was used to compare different samples. Error bars show means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 6.
Scheme for effects of cAMP and PKA on SIK forms and on the ensuing re-distribution
CRTC2 in relation to activation of StAR loci. (A) SIK1/SIK2 domains. (B) CRTC1/CRTC2/
CRTC3 domains. (C) Inhibition effects of CsA and OA are included.