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Mechanism of Repression of the Inhibin α-Subunit Gene by Inducible 3′,5′-Cyclic Adenosine Monophosphate Early Repressor

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The rodent ovary is regulated throughout the reproductive cycle to maintain normal cyclicity. Ovarian follicular development is controlled by changes in gene expression in response to the gonadotropins FSH and LH. The inhibin α-subunit gene belongs to a group of genes that is positively regulated by FSH and negatively regulated by LH. Previous studies established an important role for inducible cAMP early repressor (ICER) in repression of α-inhibin. These current studies investigate the mechanisms of repression by ICER. It is not clear whether all four ICER isoforms expressed in the ovary can act as repressors of the inhibin α-subunit gene. EMSAs demonstrate binding of all isoforms to the inhibin α-subunit CRE (cAMP response element), and transfection studies demonstrate that all isoforms can repress the inhibin α-subunit gene. Repression by ICER is dependent on its binding to DNA as demonstrated by mutations to ICER's DNA-binding domain. These mutational studies also demonstrate that repression by ICER is not dependent on heterodimerization with CREB (CRE-binding protein). Competitive EMSAs show that ICER effectively competes with CREB for binding to the inhibin α CRE *in vitro*. Chromatin immunoprecipitation assays demonstrate a replacement of CREB dimers bound to the inhibin α CRE by ICER dimers in ovarian granulosa cells in response to LH signaling. Thus, there is a temporal association of transcription factors bound to the inhibin α-CRE controlling inhibin α-subunit gene expression. (*Molecular Endocrinology* 20: 584–597, 2006)

THE INTRACELLULAR SIGNALING molecule cAMP functions in all cells to regulate gene expression. Many target genes contain *cis*-acting regulatory sequences called cAMP-response elements, or CREs. The bZIP family of transcription factors contains a number of proteins that are known to bind to CREs. These proteins contain leucine zipper motifs at their carboxy terminus along with a stretch of basic residues, which allows for DNA binding and dimerization (1). Two members of this family are the CRE-binding protein (CREB) and CRE-modulatory protein (CREM).

Both the CREB and CREM family of transcription factors contain multiple exons, and alternative RNA processing events generate protein isoforms that differ in domain structures and function as transcriptional activators or repressors (2, 3). Binding of CREB dimers to a CRE or a CRE-like element in a promoter is important for the transcription of CREB-dependent genes (4, 5). Once phosphorylated on serine 133, CREB can interact with the coactivator protein CBP (CREB-binding protein) (6, 7), and this leads to the recruitment of additional histone acetyltransferases (8, 9) to the promoter. The formation of a multiprotein complex containing CREB, CBP, and the transcriptional machinery initiates gene transcription (10, 11). It is evident that proteins, which prevent the formation of this transcription complex, will attenuate cAMP-responsive gene transcription. There are proteins that inhibit either the recruitment of CBP to the promoter, such as the human T cell leukemia virus type 1 oncoprotein Tax (12), or the interaction of CBP with p300/CBP-associated factor, such as the adenoviral oncoprotein E1A (9, 13). In addition to these proteins, there are bZIP repressors, including a class of truncated isoforms, which can bind to CRE and CRE-like elements (14). One well-characterized repressor isoform of the CREM family is the inducible cAMP early repressor (ICER) (15).

ICER is expressed as a family of four isoforms that are produced from an internal promoter, P2, located in an intron of the CREM gene (15). Alternative splicing of the γ-exon and the ICER DNA-binding domains (DBDs I and II) generates ICER I, Iγ, II, and IIγ. ICER I and Iγ

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Abbreviations: CBP, CREB-binding protein; CG, chorionic gonadotropin; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE-binding protein; CREM, CRE modulatory protein; DBD, DNA-binding domain; DTT, dithiothreitol; GST, glutathione-*S*-transferase; HA, hemagglutinin; Hsp, heat shock protein; ICER, inducible cAMP early repressor; P-CREB, phosphorylated CREB; PMSG, pregnant mare's serum gonadotropin; SDS, sodium dodecyl sulfate.

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mRNA contain sequences encoding both DBD I and II, but the presence of a stop codon at the C terminus of DBD I prevents translation of DBD II. Splicing out DBD I results in the formation of ICERs containing DBD II and ICER II and IIγ. Although the DBDs share significant homology, there are 11 of 57 amino acid differences between DBD I and DBD II. The expression of repressors with alternative DBDs raises the possibility that isoforms containing alternative DBDs may show different sequence specificity or differ in their modes of action as repressors. Additionally, the presence or absence of the γ-exon may have an effect on isoform function.

One feature common to bZIP transcription factors is the high degree of homology in the DBD of these proteins. Different members of this family can form homo- and/or heterodimers and bind to similar DNA-binding sites. When a CRE or CRE-like element is occupied by a dimer of activator isoforms, transcription of a target gene is induced. However, when the same DNA site is occupied by a heterodimer containing a repressor isoform or by repressor homodimers, transcription is repressed, presumably due to impaired interaction with CBP or transcription factor IID or both (16, 17). The occupancy of the CRE by activating or repressing dimers is dependent, presumably, on the relative abundance and affinities of the three classes of dimers for the target CRE.

In the rat ovary, the full-length activator isoform of CREB (18) and four ICER isoforms (19) are expressed. Although the pituitary gonadotropins FSH and LH do not regulate CREB expression, they stimulate CREB phosphorylation (18) leading to its activation. Numerous genes expressed in the ovary are regulated by cAMP-dependent mechanisms downstream of gonadotropin signaling. Previous studies have demonstrated that CREB plays a key role in FSH-stimulated inhibin α-subunit gene expression in ovarian granulosa cells (20), and that ICER plays an important role in the LH-mediated repression of inhibin α-subunit gene promoter activity (19) in rat granulosa cells. However, the mechanism of repression by ICER remains unknown. Also, it is unclear whether all four isoforms of ICER expressed in the ovary are capable of repressing the inhibin α-subunit gene in granulosa cells during the postovulatory period. These studies address these issues. Our experimental findings demonstrate that a predominant mechanism of inhibin α gene repression after the LH surge appears to be the replacement of CREB by ICER on the inhibin promoter. Additionally, all ICER isoforms seem capable of repressing the inhibin α-subunit gene in granulosa cells.

RESULTS

Interaction of ICER Isoforms with CREs

To determine whether deletion of the γ-exon or differences between ICER DBD I and II would have an effect on isoform binding to the noncanonical inhibin α CRE, recombinant HA-ICER protein was made in HeLaT4 cells and used in subsequent EMSAs. A map of the different isoforms is shown in Fig. 1A. Amounts of crude nuclear extracts that contained equivalent levels of ICER protein were ascertained by Western blotting, as shown in Fig. 1B, and identical amounts were then used in EMSAs. As shown in Fig. 1C, all four HA-ICER isoforms bound to the inhibin α CRE probe with similar efficiencies (lanes 3–6). The mobility of these DNA-protein complexes was further reduced when the reactions were incubated with an ICER antibody (lanes 9–12). When two nucleotides were mutated in this binding site (lanes 15–18), the DNA-protein complexes were eliminated, indicating that the mutations abolished binding of ICER isoforms to this site in the promoter.

ICER Isoforms Repress the Inhibin α-Subunit Gene

Because binding studies demonstrated that all four ICER isoforms can bind the inhibin α CRE, transient cotransfection experiments were then performed to compare the efficiencies of ICER isoforms in down-regulating the inhibin α gene promoter. HA-tagged ICER I, Iγ, II, and IIγ expression constructs were cotransfected in a mouse granulosa cell line, GRMO2 (21, 22), with an inhibin α-subunit promoter-luciferase reporter construct, and basal and forskolin-induced luciferase activities were measured. Figure 2A shows the relative luciferase activity normalized for total protein. The inhibin α-subunit promoter demonstrated a basal level of activity that was induced 6-fold by 10 μM forskolin treatment. Cotransfection with each HA-ICER expression construct significantly repressed both basal and forskolin-induced promoter activity. ICER isoforms had larger repressive effects on forskolin-stimulated promoter levels in comparison to basal levels. These results demonstrate that ICER isoforms repress cAMP-responsive transcription with similar efficiencies regardless of the presence of the γ-exon or either DBD I or DBD II. When a control 70-kDa heat shock protein (Hsp70) promoter was cotransfected with each HA-ICER expression construct, as shown in Fig. 2B, promoter activity was not significantly altered, indicating that the repressive effect of each ICER isoform on the inhibin α promoter was specific. Forskolin treatment of the Hsp70 promoter did not alter activity compared with basal (data not shown).

Binding of ICER to the CRE Is Necessary for Down-Regulation of the Inhibin α-Subunit Gene

To determine whether inhibin repression by ICER involves direct DNA binding of ICER to the inhibin α CRE or protein-protein interaction, *e.g*. with activators such as CREB, mutational studies to the ICER DBD were performed. Molecular modeling has demonstrated the

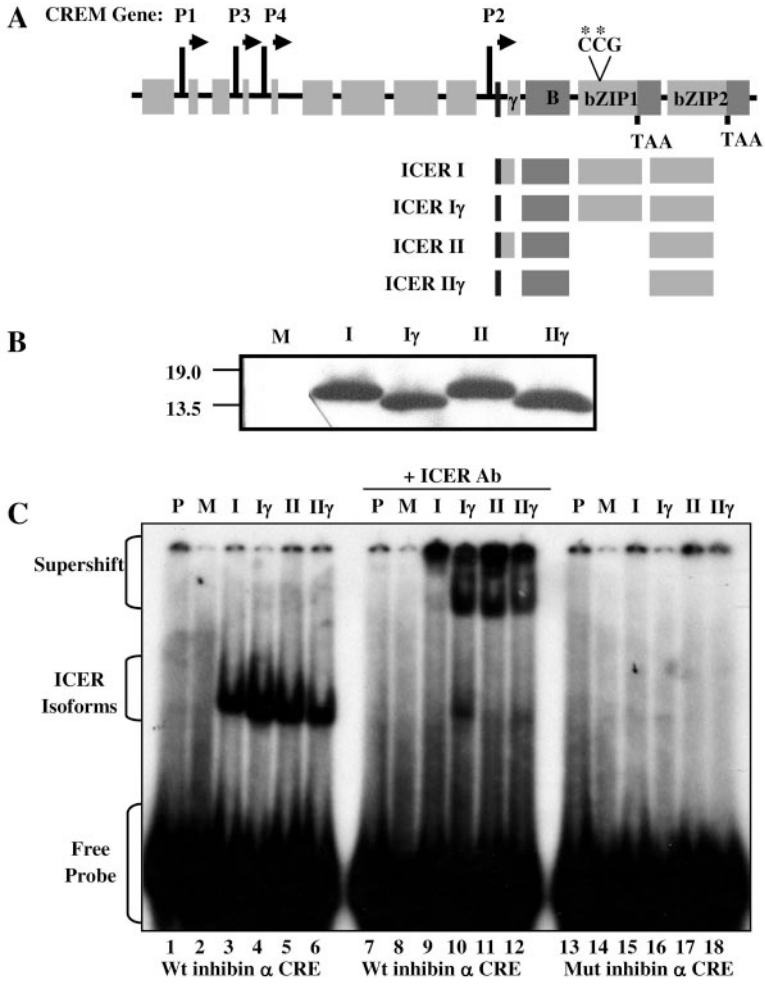

Fig. 1. Binding of Recombinant ICER Isoforms to the Inhibin α-Subunit CRE

A, Schematic depicting the four different ICER isoforms including location of a mutation used in subsequent experiments. B, Western blot showing equivalent amounts of recombinant ICER protein expressed in HeLaT4 cells using the vaccinia-T7 RNA polymerase hybrid expression system. Proteins were electrophoretically transferred to a nitrocellulose membrane and immunostained using an anti-HA antibody followed by a sheep antimouse antibody conjugated to horseradish peroxidase. Enhanced chemiluminescence was used to detect protein complexes. Positions of molecular size standards are indicated. C, Nuclear extracts described in panel B were incubated with ^{32}P-labeled double-stranded oligonucleotide probes spanning the inhibin α-subunit CRE (lanes 1–12) or a mutant CRE (lanes 13–18) that were end labeled using γ [^{32}P]ATP and T4 polynucleotide kinase and gel purified. The binding reaction was incubated with an anti-ICER antibody (lanes 7–12). DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. Probe, without any extract (lanes 1, 7, and 13), and nuclear extracts from mock-transfected cells (lanes 2, 8, and 14) were included in the gel as negative controls. Ab, Antibody; Mut, mutant; Wt, wild type; P, probe alone; M, mock transfected; P1–P4, promoters 1–4; I, ICER I; Iγ, ICER Iγ; II, ICER II; IIγ, ICER IIγ.

importance of key arginines in CREB binding to DNA. One of these arginines corresponds to Arg73 in both DBD I and II in the different ICER isoforms. Therefore, an ICER DBD mutant was generated, ICER I^{R73P}, and recombinant wild-type and mutant proteins were expressed in HeLaT4 cells. Figure 3A is a Western blot demonstrating that recombinant proteins for both ICERs are made. Relative amounts of crude nuclear extracts that contained equivalent ICER protein were also ascertained by Western protein blotting (Fig. 3A). EMSAs were performed using identical amounts of nuclear extracts used in Fig. 3A. Two different double-stranded CRE probes were incubated with these nuclear extracts. As shown in Fig. 3B, ICER I bound to both the inhibin α CRE as well as a consensus CRE, whereas ICER I^{R73P} did not bind to either probe.

To determine whether this ICER DBD mutant was folded correctly and could still dimerize, studies were performed using vaccinia-infected HeLaT4 cells cotransfected with both FLAG-CREB and ICER I^{R73P} expression constructs. Different ratios of the amounts of CREB:ICER I^{R73P} expression constructs were used. Nuclear extracts prepared from these cotransfected cells were then immunoprecipitated using an anti-

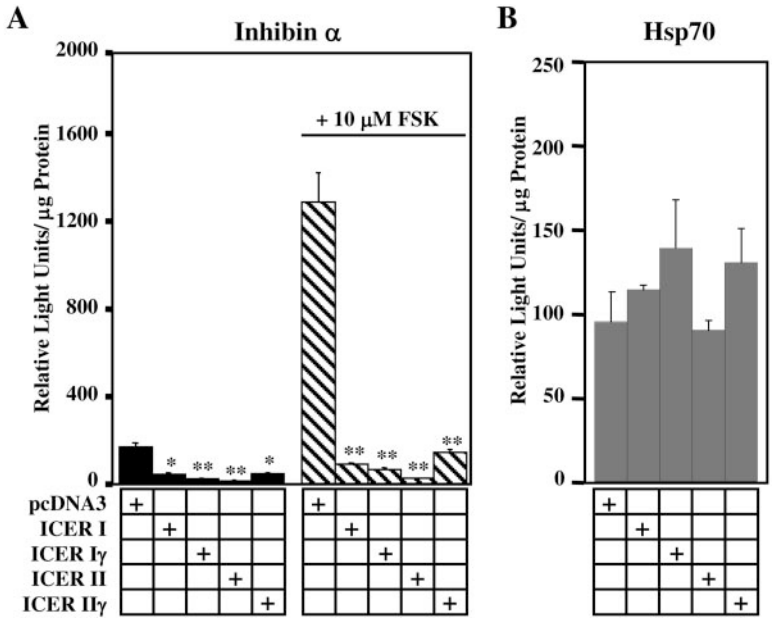

Fig. 2. Repression of Inhibin α-Subunit Gene Promoter Activity by ICER Isoforms in GRMO2 Cells

GRMO2 cells were transfected for 6 h with 500 ng inhibin-luciferase reporter DNA **(A)** or Hsp70-luciferase reporter DNA **(B)** and 100 ng of the indicated DNAs, allowed to recover 16 h, treated with vehicle or 10 μM forskolin for 6 h, and then harvested for luciferase activity. Values are relative light units per μg protein and represent the mean ± SEM (n = 3). These results represent multiple independent experiments (n > 4 independent transfections). Both basal and forskolin activity levels of the inhibin-luciferase reporter cotransfected with each ICER isoform were statistically significant when compared with respective values obtained with cotransfection with pcDNA3: *, $P < 0.05$; **, $P < 0.001$. Activity levels of the Hsp70-Luc construct with each ICER construct were not statistically significant when compared with respective values obtained with cotransfection with pcDNA3. FSK, Forskolin.

ICER antibody. Western protein blot analysis of immunoprecipitates showed that CREB is coimmunoprecipitated with ICER I^{R73P} (Fig. 4, *left, top*). As expected, increasing levels of CREB are immunoprecipitated with increasing amounts of ICER I^{R73P} DNA transfected. Immunoprecipitation using an anti-CREB antibody showed that ICER I^{R73P} is coimmunoprecipitated with CREB (data not shown). These results indicate that ICER I^{R73P} protein is likely to be properly folded and, like wild-type ICER protein (data not shown), has retained the ability to dimerize with other proteins such as CREB.

To determine whether ICER I^{R73P} could functionally repress the inhibin α-subunit gene promoter, GRMO2 cells were transiently cotransfected with an inhibin α-subunit promoter-luciferase reporter and either ICER I or ICER I^{R73P} expression constructs. Figure 5 shows the relative luciferase activity normalized for total protein. The inhibin α-subunit promoter demonstrated a basal level of activity that was induced 4-fold by 10 μM forskolin treatment. Cotransfection with the ICER I expression construct significantly repressed both basal and forskolin-induced promoter activity. However, cotransfection with the ICER I^{R73P} construct did not significantly repress either basal or forskolin-induced activity, indicating that DNA binding is required for repression. Taken together with results from Fig. 4, these data demonstrate that ICER binding to the inhibin α CRE is necessary for repression, and although ICER is capable of heterodimerization with CREB (data not shown), the ability of ICER I^{R73P} to heterodimerize with CREB and not repress inhibin α promoter activity indicates that dimerization is not a major mechanism for repression.

DNA Binding in the Presence of Varied Concentrations of CREB and ICER

To investigate binding of CREB *vs.* ICER to the inhibin α CRE, varying amounts of HA-ICER II expression construct and a constant amount of FLAG-CREB expression construct were transfected into HeLaT4 cells, and nuclear extracts were used in EMSAs. Nuclear extracts were initially assayed for ICER and CREB protein expression by Western protein blotting. As shown in Fig. 6A, uniform CREB protein expression was obtained in all transfected samples. Increasing the amount of ICER DNA transfected resulted in increasing amounts of ICER protein expression obtained. Using the same amount of nuclear extracts processed for Western protein blotting in Fig. 6A, EMSAs were performed. Autoradiograms showing mobility shifts with an inhibin α CRE probe are presented in Fig. 6B. With increasing amounts of ICER DNA transfected, leading to enhanced ICER protein expression (see Fig. 6A), the percentage of ICER

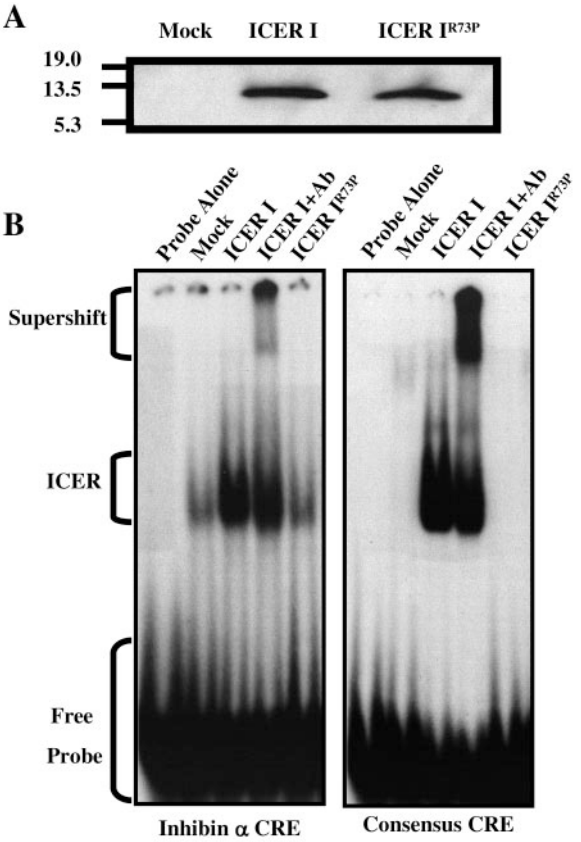

Fig. 3. Failure of ICER I^{R73P} to Bind to the Inhibin α CRE or a Consensus CRE

The vaccinia-T7 RNA polymerase hybrid expression system was used to express recombinant ICER protein in HeLaT4 cells. A, Western blot showing equivalent amounts of ICER I and ICER I^{R73P} protein. Proteins were electrophoretically transferred to a nitrocellulose membrane and immunostained using an ICER antibody that detects both wild-type and mutant ICER, followed by a sheep antimouse antibody conjugated to horseradish peroxidase. Enhanced chemiluminescence was used to detect protein complexes. Positions of molecular size standards are indicated. B, Nuclear extracts described in panel A were incubated with ^{32}P-labeled double-stranded oligonucleotide probes spanning the inhibin α-subunit CRE and a consensus CRE that were end labeled using γ [^{32}P]ATP and T4 polynucleotide kinase and gel purified. DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. Probe, without any extract, and nuclear extract from mock-transfected cells were also included in the gels as negative controls. Ab, Antibody.

dimers bound to the inhibin α CRE increased, whereas the percentage of CREB dimers bound to the inhibin α CRE decreased. The percentage of CREB-ICER dimers bound to the inhibin α CRE increased marginally with increased ICER expression; however, this species represented a minimal amount of the total bound protein. These data are quantified in Fig. 6C. These results demonstrate that although heterodimers between CREB and ICER can bind to the inhibin α CRE, the predominant dimers binding to the inhibin α CRE, when both CREB and ICER are expressed, are ICER dimers. Interestingly, at equivalent levels of FLAG-CREB and ICER DNA transfected, full competition is observed. To compare levels of protein expression for both CREB and ICER when complete competition is observed, equivalent levels of HA-CREB and HA-ICER expression constructs were cotransfected into HeLaT4 cells, and nuclear extracts were processed for Western protein blotting, as shown in Fig. 6D. Although levels of HA-CREB are slightly diminished compared with levels of HA-ICER, the full competition that is observed at these protein levels demonstrates ICER's ability to efficiently compete with CREB for binding to the inhibin α CRE.

Change in Occupancy of the Inhibin α-Subunit Gene Promoter in Response to Gonadotropins

These *in vitro* studies demonstrate a competition between ICER dimer and CREB dimer binding to the inhibin α-subunit CRE. Therefore, we sought to test the hypothesis that ICER replaces CREB on the inhibin α promoter *in vivo* using chromatin immunoprecipitation (ChIP) assays performed with primary rat granulosa cells. Treating immature female rats with pregnant mare's serum gonadotropin (PMSG) or PMSG and subsequently human chorionic gonadotropin (hCG) can recapitulate both the activation and repression of inhibin α mRNA and protein seen in granulosa cells of normal cycling rats. In three independent experiments, immature female rats were injected with PMSG or PMSG/hCG, ovaries were harvested, and primary granulosa cells were extracted for ChIP. A schematic depicting the locations of both distal and proximal primers used in the PCR amplification is shown in Fig. 7A. PCR amplification in the distal region demonstrated lack of binding of all three factors (Fig. 7B). PCR amplification in the proximal region demonstrated that at PMSG 48 h, a time when inhibin levels are maximal and ICER is absent in the ovary, higher levels of CREB are bound to the inhibin α promoter than after the 4-h hCG treatment. Conversely, levels of ICER bound to the proximal inhibin α promoter are higher after the 4-h hCG treatment, a time when inhibin levels have begun to decline and when ICER levels are maximal, than they are at the PMSG 48-h time point. As a control, levels of acetylated histone H4 bound to the inhibin α promoter were examined and, as expected, decreased after the 4-h hCG treatment. Quantification of PCR amplifications from the three independent experiments is depicted in Fig. 7C and demonstrates that these changes in CREB, ICER, and acetylated histone H4 bound to the inhibin α promoter after different hormone treatment are significant. To determine the timeframe of ICER occupancy on the inhibin promoter, ChIPs were performed at multiple time points after hCG stimulation, and Fig. 7D shows that occupancy of the inhibin α promoter by ICER diminishes by 8 h after hCG stimulation. Therefore, immediately after the LH surge, ICER represses inhibin

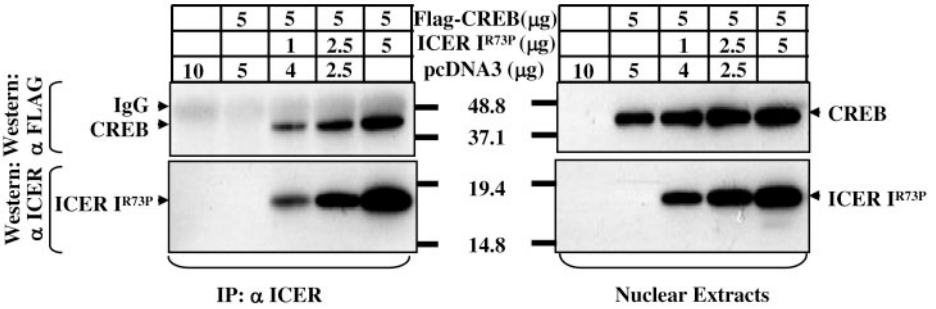

Fig. 4. Interaction between CREB and ICER I^{R73P} Coexpressed in HeLaT4 Cells

Vaccinia-infected HeLaT4 cells were transfected with the indicated amounts of FLAG-CREB and ICER I^{R73P} expression constructs. pcDNA3, the parent vector for expression constructs, was used to balance for total DNA used in each transfection. Nuclear protein extracts (10 μg) were immunoprecipitated using an anti-ICER antibody (*left, top and bottom*). Immunoprecipitates and 10 μg of nuclear protein extracts were size separated on a 13% SDS-PAGE gel and processed for Western protein blotting using either an anti-FLAG antibody (*top, left and right*) or an anti-ICER antibody (*bottom, left and right*). The detection system used was enhanced chemiluminescence. The time for detection was different between blots on the *left vs. right*. The positions of heavy chain immunoglobulins (IgG), FLAG-CREB (CREB), and ICER I^{R73P} (ICER I^{R73P}) are indicated by *arrows*. The positions of molecular size standards are also indicated. IP, Immunoprecipitation.

α-subunit gene expression by transiently replacing CREB on the inhibin promoter.

DISCUSSION

The inhibin α-subunit gene is dynamically regulated by FSH and LH in the rodent ovary. FSH positively regulates the inhibin α-subunit gene whereas immediately after the preovulatory LH surge, inhibin α-subunit gene expression is rapidly down-regulated (23, 24). Down-regulation of inhibin, late in proestrus, facilitates the prolonged release of FSH on the morning of estrus, and this secondary FSH surge is critical to the recruitment of a new cohort of antral follicles into the follicular phase of the estrous cycle (23, 25, 26). Therefore, the down-regulation of the inhibin α-subunit gene, and subsequently dimeric inhibin, is a crucial step for the maintenance of the rodent estrous cycle. Expression of the inhibin α-subunit gene in rat ovarian granulosa cells is dependent, in part, on the interaction of CREB with an atypical CRE at -122 in the promoter of this gene (20). LH-dependent down-regulation of this gene may be orchestrated by molecular events that lead to the occlusion of CREB dimers from the inhibin α-subunit promoter CRE. In an earlier study, we showed that, in response to the preovulatory LH surge, ICER is expressed in ovarian granulosa cells and acts as a repressor of inhibin α-subunit gene transcription. In this study, we addressed whether all ICER isoforms expressed in the ovary are capable of repressing CREB-dependent transcription of the inhibin α-subunit gene. In addition, we also present data that demonstrate that ICER acts as a repressor by competing with CREB for binding to the inhibin α-CRE.

ICER is described as a potent repressor of all cAMP-responsive transcription (15). Numerous reports that demonstrate the transrepression activity of ICER support this hypothesis. ICER binding can potentially down-regulate the promoters of several genes the expression of which is induced by cAMP, *i.e*. as c-*fos*

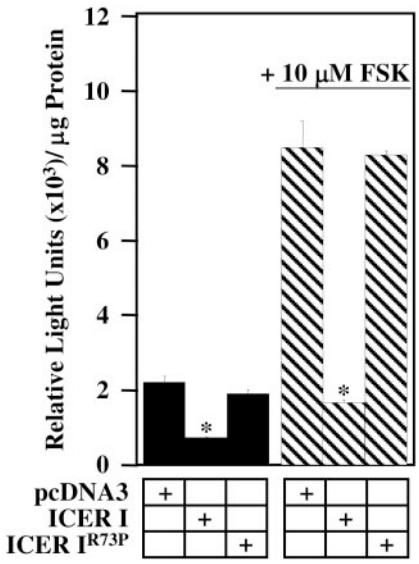

Fig. 5. Failure of ICER I^{R73P} to Fully Repress the Inhibin α-Subunit Gene Promoter in GRMO2 Cells

GRMO2 cells were transfected for 6 h with 500 ng inhibin-luciferase reporter DNA and 100 ng of the indicated DNAs, allowed to recover 16 h, treated with vehicle or 10 μM forskolin for 6 h, and then harvested for luciferase activity. Values are relative light units per μg protein and represent the mean $\pm$ SEM (n = 3). These results represent multiple independent experiments (n $>$ 3 independent transfections). Activity levels of the inhibin-luciferase reporter cotransfected with ICER I (both basal and forskolin stimulated) were statistically significant when compared with respective values obtained with cotransfection with pcDNA3: *, $P < 0.01$. Activity levels of the inhibin-luciferase reporter cotransfected with ICER I^{R73P} were not significant compared with respective values obtained with cotransfection with pcDNA3. FSK, Forskolin.

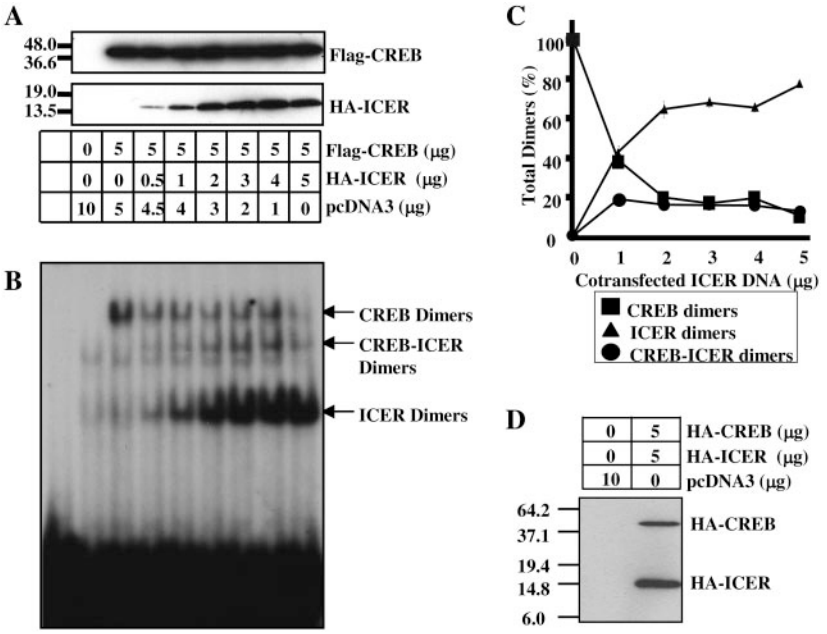

Fig. 6. Competition between CREB and ICER Dimers for Binding to the Inhibin α CRE *in Vitro*

A, Western immunoblots showing the expression of recombinant proteins in nuclear extracts (5 μg) from HeLaT4 cells that were transfected with the indicated amounts of FLAG-CREB and HA-ICER II expression constructs. pcDNA3, the parent vector for expression constructs, was used to balance for total DNA used in each transfection. B, Nuclear extracts (5 μg) described in panel A were incubated with a ^{32}P-labeled oligonucleotide probe that spans the inhibin α-subunit CRE. DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. Protein complexes corresponding to CREB dimers, CREB-ICER dimers, and ICER dimers are indicated by correspondingly labeled *arrows*. Mobility shift reactions containing probe alone or probe and mock-transfected extracts (5 μg) were included as controls. C, Probe bound to the different protein complexes was quantified from four independent experiments and the content of probe, presented as the percentage of total dimers (%) ± SEM (n = 4), was plotted against the amount of ICER DNA cotransfected with CREB DNA. D, Western immunoblot showing the expression of recombinant proteins in nuclear extracts (10 μg) from HeLaT4 cells that were transfected with the indicated amounts of HA-CREB and HA-ICER expression constructs.

(27), somatostatin (27), tyrosine hydroxylase (28, 29), insulin (30), arylalkylamine *N*-acetyltransferase (31–33), creb (34), TSH receptor (35), FSH receptor (36), bcl2 (37), and icer P2 (15). There are four isoforms of ICER produced by alternative splicing of the γ exon and two different DBDs. The functional difference between these ICER isoforms has not been addressed. Differential regulation by the different ICER isoforms would be expected due to differences in exon domains and amino acid sequences in DBDs. Because the inhibin α CRE is a noncanonical CRE, it is reasonable to expect that ICER isoforms with more divergent DBDs, compared with CREB, might have a greater or lesser affinity for a nonconsensus CRE. A comparison between DBDs for CREB and ICER reveals two and 13 amino acid differences between the DBD for CREB and DBD I and II, respectively. Surprisingly, in these studies, we report that all four ICER isoforms bind to the inhibin α-subunit CRE with similar affinities, and we demonstrate that ICERs containing different DBDs are all capable of significantly down-regulating both basal and forskolin-stimulated activity of the inhibin α-subunit promoter.

The ability of all four ICER isoforms to bind to the inhibin α CRE, as well as repress inhibin promoter activity, suggests that binding of ICER isoforms to the CRE, or alternatively, interaction of ICER with CREB and then subsequent binding to the CRE, is necessary for inhibin repression. To test these hypotheses, mutational studies to the ICER DBD were performed. Charged residues, such as arginines, in the basic region of bZIP proteins are typically involved in DNA binding. In the crystal structure of the CREB-DNA complex, Arg294 and Arg301, corresponding to Arg73 and Arg 80 in both ICER I and II, are located at the protein-DNA interface and make extensive contacts with DNA (38). Mutating one or both residues to prolines was expected to abrogate DNA binding activity. A DBD mutant, ICER I^{R73P}, was generated based on molecular modeling, and EMSAs demonstrated that ICER I^{R73P} was unable to bind to either the inhibin α CRE or a consensus CRE. Coimmunoprecipitation studies with CREB and ICER I^{R73P} demonstrate that ICER I^{R73P} can still interact with CREB, which also suggests that ICER I^{R73P} is likely to be properly folded. The importance of ICER binding in repression was then demonstrated in transfection studies using an inhibin promoter-luciferase reporter and ICER I^{R73P}. ICER I^{R73P} was incapable of repressing basal or forskolin-stimulated levels of promoter activity compared

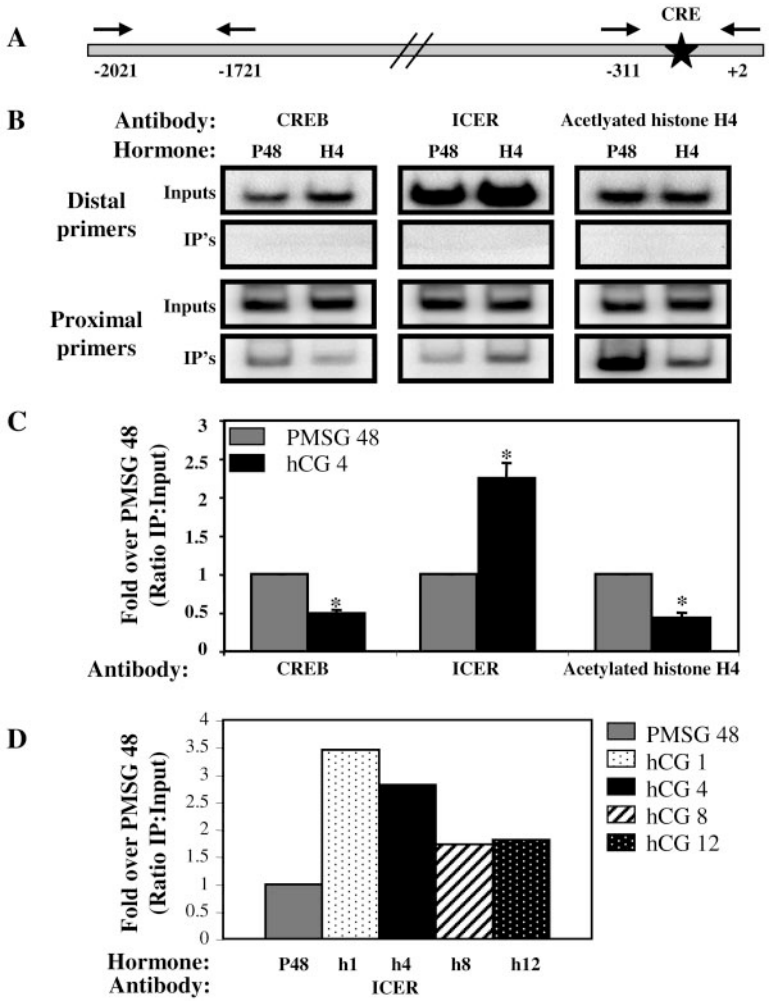

Fig. 7. Temporal Association of CREB and ICER with the Inhibin α-Subunit Promoter in Rat Primary Granulosa Cells

Immature female rats were injected with PMSG for 48 h or PMSG for 48 h and hCG for 4 h. Granulosa cells were isolated at both times and used in ChIP assays. A, Schematic of the inhibin α promoter designating primer location as well as the CRE element. B. IP and input samples were amplified using [^{32}P]dCTP in a PCR reaction, run on a 5% acrylamide gel, and quantified using a phosphor imager. Shown is one representative experiment. C, Quantification of ChIPs representing the ratio of IP sample to input sample using PCR. Results are depicted as fold difference in association of factors after treatment with PMSG for 48 h and hCG for 4 h compared with the levels detected in the cells after treatment with PMSG alone. Means $\pm$ SEM for the proximal inhibin promoter are statistically significant, with *, $P < 0.01$ (n = 3 independent experiments). D, Quantification of ChIPs representing the ratio of IP sample to input sample using PCR. Results are averages from two independent experiments depicted as fold difference in association of factors after treatment with PMSG for 48 h and hCG for the indicated times (hours) compared with the levels detected in the cells after treatment with PMSG alone. IP, Immunoprecipitation.

with wild-type ICER. One possible mechanism of repressing CREB-dependent transcription involves the formation of dimers that are impaired for DNA binding, as observed in the case of the dominant-negative CREB mutant, KCREB (39). However, the inability of ICER I^{R73P} to repress inhibin promoter activity, yet still interact with CREB, indicates that the formation of CREB-ICER heterodimers impaired for DNA binding is not a major mechanism of repression.

In rat ovarian granulosa cells, the levels of CREB protein are constant (18), whereas ICER proteins are strongly induced in response to the LH surge (19). To recapitulate *in vitro* dimer formation on the inhibin α CRE in response to changes in ICER protein, constant levels of CREB DNA and increasing levels of ICER DNA were cotransfected, and protein lysates were used in subsequent EMSAs. Formation of CREB:ICER heterodimers on the DNA is seen, as has been reported earlier (40). However, these EMSAs demonstrate that DNA-bound CREB:ICER heterodimers are formed at low levels compared with CREB and ICER homodimers, suggesting that ICER binds to the inhibin α-CRE primarily as a homodimer. Interestingly, increasing ICER protein expression resulted in increased binding of ICER dimers and decreased binding of CREB dimers. These results indicated that

binding of ICER was directly responsible for the attenuation of CREB-dependent transcription of the inhibin α-subunit gene. At approximately equivalent levels of protein expression, ICER can fully compete with CREB for binding to the inhibin α CRE, suggesting that ICER has a greater affinity for this nonconsensus CRE than CREB.

ICER is not the first truncated CREB/CREM family member to demonstrate repressive activity. For instance, alternative splicing of CREB generates truncated CREB proteins lacking transactivation domains that can act as repressors of CREB-dependent transcription (41). However, these repressors are not expressed in the ovary. In addition, CREB acts as a repressor when Ser133 is mutated (42–44) by competing with wild-type CREB for binding to CREs. One possibility for this competition could be that ICER dimers have a higher affinity than CREB dimers for binding to the inhibin α CRE, which is a nonconsensus CRE. The result from the competitive EMSA using recombinant CREB and ICER proteins supports this hypothesis, because when equal levels of CREB and ICER proteins are expressed, CREB dimers are almost completely competed by ICER dimers. An alternative and not mutually exclusive possibility may be that there are differences in abundance of proteins such that after LH signaling, the number of ICER dimers greatly exceeds the number of CREB dimers present. Thus, there would then be a greater likelihood that an ICER dimer, rather than a CREB dimer, would occupy the CRE. Although CREB mRNA levels are unchanged in the ovary in response to gonadotropin signaling, levels of phosphorylated CREB (P-CREB) do change in response to PMSG and hCG (18). Treatment with PMSG alone results in maximal expression of P-CREB, and subsequent treatment with hCG results in a decrease in P-CREB levels (18) at a time when ICER levels are maximal. It is possible that differences in both affinities and abundance result in the repression of the inhibin α-subunit gene by ICER.

To investigate the association of CREB and ICER dimers with the inhibin α promoter *in vivo*, ChIP assays were performed. At a time when inhibin α levels are elevated, PMSG 48 h, levels of CREB bound to the inhibin α promoter are significantly higher than they are after the 4-h hCG treatment. In direct contrast, at a time when inhibin α levels have significantly declined, hCG 4 h, levels of ICER bound to the inhibin α promoter are significantly higher than they were at the PMSG 48-h time point. The time course of ICER occupancy on the inhibin promoter demonstrates that ICER binding to the promoter peaks 1 h after hCG stimulation, and this is likely sufficient to displace CREB, thus eliminating the effect of CREB phosphorylation present at this early time. ICER is only transiently associated with the inhibin CRE as ICER occupancy diminishes by 8–12 h after hCG stimulation. Yehia and colleagues (45) have investigated the half-life of ICER and determined it to be approximately 5 h. Taken together, these results support the idea that ICER is a transient and immediate repressor of inhibin gene expression. However, down-regulation of the inhibin α-subunit gene is maintained throughout the postovulatory period in the rodent ovary, extending beyond the period of ICER expression. Therefore, the transient nature of ICER expression in the ovary suggests that other transcription factors are necessary to maintain suppression of the inhibin α-subunit gene during ovulation and luteinization. Recently, we have described two distinct mechanisms by which C/EBPβ, another bZIP transcription factor, is capable of repressing the inhibin α-subunit gene in granulosa cells. In addition to binding to an upstream C/EBPβ binding site, dimers of C/EBPβ compete with CREB for binding to the α-CRE (46), similar to the competition seen between CREB and ICER dimers, at a time when ICER expression in the ovary is diminishing. Thus, the temporal participation of multiple factors regulates inhibin α gene expression.

One major physiological question is how granulosa cells and ovarian genes respond differently to FSH and LH. Binding of both FSH and LH to their respective receptors results in increasing intracellular cAMP levels and subsequent signaling through the cAMP-dependent protein kinase A signaling pathway (47–49). The response to cAMP differs at different times during the rat estrous cycle. The simplest explanation of these differential responses is that the level of cAMP produced in response to LH signaling is much higher than the response to FSH signaling (50, 51). This could be due to a difference in number or function of receptors or a difference in the coupling of these receptors to various second messenger systems. Recently the differential effects of FSH and LH on aromatase expression in rat granulosa cells was examined, and these results demonstrate that the combination of receptor density as well as differences in signaling pathways activated contribute to this differential response (52).

Disruption of the CREM gene by homologous recombination generated mice lacking both CREM and ICER expression (53, 54). Although homozygous deficient males show reproductive defects in spermatogenesis, the females are fertile. It is interesting that lack of ICER expression would not have a more profound effect on female reproductive processes; however, detailed reproductive phenotypes of these females are still unknown and would be informative. For example, these animals may be subfertile, a phenotype seen when inhibin α is constitutively expressed in transgenic mice (55). One explanation is that perhaps other factors compensate for this lack of ICER repression. Repressor forms of CREB have been shown to be expressed in the testis (56) but not the ovary (18). Perhaps ICER-deficient animals compensate by generating these CREB repressors in the ovary. Compensation is seen in mice with a disruption of the CREB gene by homologous recombination in mouse embryonic stem cells (57). Although a lower percentage of homozygous null mice survive to adulthood (15%),

these animals are viable with no impairment of growth or development. Levels of CREM isoforms were found to be up-regulated, which likely compensates for the lack of CREB protein, resulting in the absence of any mutant phenotype.

Transcription factors belonging to the bZIP superfamily are important regulators of a number of cellular processes, due, in part, to their ability to function as activators and repressors of gene transcription. The balance of activating proteins to repressing proteins is crucial in regulating gene transcription. Based on these current results, we can propose a mechanism for the attenuation of inhibin α-subunit gene expression in rat ovarian granulosa cells *in vivo*. In response to FSH, P-CREB dimers bind to the α-CRE and initiate gene transcription. Signaling by LH on the afternoon of proestrus causes the induction of another set of bZIP factors, including all four isoforms of ICER. Dimers of each ICER isoform rapidly replace CREB dimers on the α-inhibin CRE causing repression. Expression of ICER isoforms diminishes within hours at the same time expression of another bZIP factor, C/EBPβ, is increased. C/EBPβ represses inhibin α gene expression by binding to an upstream C/EBPβ-binding site and competing with CREB dimers for binding to the α-CRE (46). Certainly, other factors are likely important for regulation at later times. Morales *et al.* (58) have also recently demonstrated that ICER acts as a repressor of the rat ovarian aromatase promoter, another gene similarly regulated to inhibin α by FSH and LH. Regulation of cAMP-responsive genes, such as α-inhibin and aromatase, is a highly coordinated process involving multiple transcription factors, and these studies investigating the relationship between CREB and ICER contribute to understanding these dynamic processes.

MATERIALS AND METHODS

Antiserum against ICER

ICER I was cloned into a glutathione-*S*-transferase (GST) gene fusion vector (Amersham Pharmacia Biotech, Piscataway, NJ). GST-ICER was expressed and purified from *Escherichia coli* cells and sent to Animal Pharm Services, Inc. (San Francisco, CA). A polyclonal antiserum against ICER was produced by immunization of rabbits with GST-ICER. This antiserum recognizes all four ICER isoforms but does not recognize CREB.

Oligonucleotide Primers and Plasmids

Oligonucleotide primers corresponding to nucleotides 1–21 (PRIMER 1) of mouse ICER cDNA (15) and nucleotides 1115 to 1135 (PRIMER 2) of the mouse CREM cDNA (3) were used to amplify ICER cDNAs from proestrus 1800 rat ovarian RNA. The amplified fragments were cloned into the *Eco*RV site of the expression vector pcDNA3 (Invitrogen, San Diego, CA), which includes the human cytomegalovirus promoter and 3′-polyadenylation sequences from the bovine GH gene. A chimeric oligonucleotide primer corresponding to the sequence for hemagglutinin (HA) epitope and nucleotides 1–21 of mouse ICER cDNA (15) and PRIMER 2 were used to generate cDNA encoding HA epitope tagged ICER from specific ICER cDNA clones. The PCR product was cloned into pcDNA3. A FLAG-epitope-tagged CREB expression construct, generously provided by Dr. Jon Kornhauser (Children's Hospital, Boston, MA), was used as a source for subcloning FLAG-CREB into pcDNA3. A chimeric oligonucleotide primer corresponding to the sequence for HA epitope and nucleotides 1–18 of rat CREB cDNA and a primer corresponding to nucleotides 962–979 of rat CREB cDNA was used to generate cDNA encoding HA epitope-tagged CREB from FLAG-CREB expression constructs.

HeLaT4 and GRMO2 Cell Culture

HeLaT4 cells were cultured in DMEM supplemented with 5% fetal bovine serum in a humidified incubator at 37 C and 5% CO_2. GRMO2 cells (provided by N.V. Innogenetics, Ghent, Belgium) were cultured as described (21, 22) in HDTIS (1:1 mixture of DMEM and Ham's F12 medium, 10 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin) supplemented with 2% fetal bovine serum and sodium pyruvate (100 mg/liter) in a humidified incubator at 37 C and 5% CO_2.

Transfection and Luciferase Assays

GRMO2 cells were transiently transfected with DNA (59) using cationic liposomes prepared as described previously (60). Reporter plasmid DNA (500 ng) and 100 ng of ICER expression constructs for each well of a 12-well culture dish were incubated at room temperature with lipofection reagent for 20 min in Optimem. Cells were washed with PBS and incubated with the DNA-lipid mixture for 6 h. After 6 h, cells were aspirated and maintained in fresh HDTIS containing 2% fetal bovine serum for 14–16 h. Fresh medium or medium containing 10 μM forskolin was then added to the cells for 4–6 h. After forskolin treatment, cells were washed twice with PBS and lysates were prepared for luciferase assays.

Transfected cells were lysed on ice in lysis buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100] for 20 min with gentle rocking. Luciferase assays were performed as previously described (61). Cell lysates (100 μl) were added to 400 μl of reaction buffer (25 mM HEPES, pH 7.8; 15 mM $MgSO_4$; 4 mM EGTA; 2.5 mM ATP; 1 mM DTT; 1 μg/ml BSA) and 100 μl of 1 mM luciferin (sodium salt) (Analytical Bioluminescence, San Diego, CA) were added using an automatic injector, and emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence) for 10 sec. Relative light units were normalized for total protein content. Cell lysates (5 μl) were used for a protein assay using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA).

Vaccinia Virus Expression of Recombinant Proteins

The vaccinia T7 RNA polymerase hybrid expression system (62) was used to overexpress proteins used in immunoprecipitation, Western blot analysis, and EMSAs. HeLaT4 cells were infected with vaccinia virus vTF7.3 expressing the bacteriophage T7 RNA polymerase (obtained under license from Dr. Bernard Moss, National Institutes of Health, Bethesda, MD) at a multiplicity of infection of 10 for 35 min in PBS-0.1% BSA. Virus was aspirated, and preincubated DNA-liposome mixture was added to the cells and incubated for 6 h. Cells were washed once with PBS and maintained in culture for an additional 14–16 h, washed with PBS, harvested, and processed for protein isolation.

Preparation of Nuclear Protein Extracts

Small-scale nuclear extracts were prepared as previously described (63). Cultured cells were washed twice with cold

PBS, scraped from the plates in cold PBS with protease inhibitors, and collected in microfuge tubes. Cells were spun at low-speed and PBS was aspirated. The cells were gently resuspended in 5 packed cell volumes of buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 0.5 mM phenylmethylsulfonylfluoride and 1 μg/ml antipain; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 μg/ml pepstatin A) and incubated on ice for 10 min. Lysates were then homogenized on ice using 10 strokes of a glass-glass Dounce homogenizer. The homogenate was centrifuged at 1500 $\times$ *g* for 2 min to collect the nuclear pellet, which was then resuspended in two thirds packed volume of buffer C [20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.2 mM EDTA, 25% (vol/vol) glycerol; 0.5 mM DTT; 0.5 mM phenylmethylsulfonylfluoride; and 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A]. Protein concentrations were estimated using a Bradford colorimetric assay (Bio-Rad).

EMSAs

A 22-bp probe containing a consensus CRE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 36-bp double-stranded oligonucleotide probes that span the inhibin non-consensus CRE with and without a 2-bp mutation were end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega Corp., Madison, WI) and gel purified on a 12% polyacrylamide gel. Nuclear lysates from vaccinia-infected transfected cells were used for each binding reaction. Lysates were incubated in binding buffer [10 mM Tris, pH 7.5; 1 mM $MgCl_2$; 1 mM DTT; 200 ng/μl poly deoxy(inosinic-cytidylic)acid] for 20 min at room temperature with 10,000 cpm of ^{32}P-labeled probe. Where indicated, 1 μl of anti-ICER antibody was added to the reaction before labeled probe, and the reaction was incubated on ice for 60 min. The protein-DNA complexes were resolved on a native 5% polyacrylamide-Tris-borate-EDTA gel. Gels were dried and exposed to x-ray film (Kodak XAR, Eastman Kodak Co., Rochester, NY).

Immunoprecipitation Analysis

HeLaT4 cells were transfected with a total of 5 μg of FLAG-CREB and indicated amounts of ICER I^{R73P} and expressed using the vaccinia T7 hybrid system. Nuclear lysates were prepared as described above. Lysates were incubated with an ICER antibody overnight at 4 C and then with protein A Sepharose beads for 1 h. The beads were washed four times with lysis buffer and once with lysis buffer without detergent. The beads were then boiled with equal volume of 2$\times$ SDS-PAGE sample buffer for 5 min and processed for Western protein blot analysis.

Western Blot Analysis

Protein lysates were boiled for 5 min in sample buffer and size separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to nitrocellulose (BA-85, Schleicher & Schuell, Keene NH). The membrane was washed once with water and blocked with 3% nonfat dry milk in PBS (blocking buffer) for 20 min at room temperature with shaking. The blot was then incubated with primary antibody, HA epitope-specific antibody 12CA5 (provided by Dr. R. A. Lamb, Northwestern University, Evanston, IL) at 1:6000, anti-FLAG at 1:6000 (Sigma Chemical Co., St. Louis, MO), or anti-ICER (1:5000) in blocking buffer for 16–18 h at 4 C with gentle shaking. The blot was washed twice with water followed by 90 min incubation at room temperature with sheep antimouse antibody conjugated to horseradish peroxidase (1:5000) (Promega) or goat antirabbit antibody conjugated to horseradish peroxidase (1:5000) (Promega). The blot was then washed with water twice, PBS/0.05% Tween for 5 min, and water four to five times. The antibody-antigen complexes were visualized using an enhanced chemiluminescence system (ECL-Plus kit, Amersham, Little Chalfont, Buckinghamshire, UK).

Animals and Hormone Treatments

Immature 23- to 25-d-old female Sprague Dawley rats (Charles River Laboratories, Inc., Lexington, MA) were kept on 14-h light, 10-h dark cycles, with lights on at 0500 h. Rats were injected sc with PMSG (10 IU, Sigma) for 48 h and subsequently with 25 IU hCG ip (Sigma) for various times ranging from 1–12 h. Rats were killed at different times after treatment, and ovaries were removed and used immediately to isolate granulosa cells. All animal protocols were approved by the Animal Care and Use Committee of Northwestern University (Evanston, IL), and animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Granulosa Cell Culture

Ovaries from hormonally treated female rats were processed for follicular puncture as described previously (20, 64). Ovaries were collected into serum-free medium (4F) consisting of 15 mM HEPES, pH 7.4, 50% DMEM, and 50% Ham's F12 with transferrin (5 μg/ml), human insulin (2 μg/ml), hydrocortisone (40 ng/ml), and antibiotics. Ovaries were washed once in 4F, incubated at 37 C for 30 min in 4F medium containing 0.5 M sucrose and 10 mM EGTA, and washed once in fresh 4F medium. Using a 25-gauge needle under a dissection microscope, individual follicles were punctured and the granulosa cells were extruded. Cells were then processed for ChIP analysis.

ChIP Assay

The ChIP assay was performed as described previously (65), with minor modifications. Granulosa cells were cross-linked at room temperature for 15 min in 1% formaldehyde. Cells were then sonicated in lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris, pH 8.0; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin; 1 μg/ml antipain; 1 μg/ml leupeptin; 1 μg/ml pepstatin A) 10 times for 20 pulses at 30% output at 4 C. The supernatant was divided into three tubes, and 1% of the sample was stored at $-$20 C for total DNA (inputs) to be processed later with the immunoprecipitates. Samples were precleared in immunoprecipitation buffer (0.01% SDS; 1.1% Triton; 1.2 mM EDTA; 17.6 mM Tris, pH 8.0; 167 mM NaCl; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin; 1 μg/ml antipain; 1 μg/ml leupeptin; 1 μg/ml pepstatin A) and protein A Sepharose beads (Pharmacia Biotech) at 4 C for 45 min with rotating. Beads were spun out, and 5 μl of antiacetylated H4 (Upstate Biotechnology, Lake Placid, NY), anti-CREB (Santa Cruz), or anti-ICER were added and incubated with rotating for 48 h at 4 C. Immunoprecipitates were recovered by Protein A Sepharose beads and washed for 10 min and 3 min with wash buffer 2 (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris, pH 8.0; and 150 mM NaCl), for 1 min with wash buffer 1 (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris, pH 8.0; and 500 mM NaCl), and twice for 3 min with immunoprecipitation buffer. Immune complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$ for a total of 30 min at room temperature with rotating. Immunoprecipitates and inputs were reverse cross-linked with 200 mM NaCl at 65 C for 4 h. Samples were then incubated overnight at 50 C with 10 mM EDTA, 40 mM Tris, pH 6.5, and 40 ng/ml proteinase K. DNA was extracted by phenol/chloroform extraction and ethanol precipitation. DNA was amplified by PCR with incorporation of [^{32}P]dCTP into the PCR product with a temperature cycle of 30 sec at 94 C, 30 sec at 60 C, and 1 min at 72 C for 30 cycles. Primers used for PCR correspond to the inhibin α promoter region ($-$2021 to

−2000) (5′-GAA TTC AAT ATT CCT CAG GAC-3′) and (−1742 to −1721) (5′-AAG TTC CGC TGC TAC GTG ATG-3′) or (−311 to −290) (5′-TGG CCA ACC CTA AGC ACC CTG-3′) and (−21 to +2) (5′-TTC CCT CCC CAT CCC ACT GCT TG-3′).

Statistical Analysis

Student's unpaired two-tailed *t* test was performed using statistical analysis functions in Prism 3.0. Differences between the activities of the indicated constructs and fold differences were considered statistically significant at $P < 0.05$.

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