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An adrenocorticotropin-regulated phosphoprotein intermediary in steroid synthesis is similar to an acyl-CoA thioesterase enzyme

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We have previously reported the purification of a phosphoprotein (p43) intermediary in steroid synthesis from adrenal zona fasciculata [Paz C., Dada, L. A., Cornejo Maciel, M. F., Mele, P. G., Cymeryng, C. B., Neuman, I., Mendez, C. F., Finkielstein, C. V., Solano, A. R., Park, M., Fischer, W. H., Towbin, H., Scartazzini, R. & Podestá, E. J. (1994) *Eur. J. Biochem. 224*, 709−716]. Here, we describe the cloning and sequencing of a cDNA encoding p43 as well as the hormonal regulation of the p43 transcript. The protein resulted homologous to a very recently described mitochondrial peroxisome-proliferator-induced very-long-chain acyl-CoA thioesterase (MTE-I). The deduced amino acid sequence of the protein shows consensus sites for phosphorylation by different protein kinases, and a lipase serine motif. Antibodies raised against a synthetic peptide that includes the lipase serine motif and against the N-terminal region of p43 block the action of the protein. The transcript of p43 was detected in ovary of pseudopregnant rats, rat adrenal zona fasciculata and glomerulosa, mouse Leydig tumor cell line (MA-10), rat brain and human placenta. Inhibition of adrenocorticotropin hormone (ACTH) release and steroid synthesis by dexamethasone produced a dose-dependent decrease in the abundance of the adrenal transcript. The transcript was induced by *in vivo* stimulation of the adrenals with ACTH. The effect had a rapid onset (5 min), reached maximal stimulation (62%) at 15 min, and returned to basal levels at 30 min. The effect of ACTH on the p43 transcript was inhibited by actinomycin D and enhanced by cycloheximide. Our results provide the first evidence linking acyl-CoA thioesterases with very-long-chain specificities, and a protein intermediary in steroid synthesis, thereby supporting a regulatory role for acyl-CoA thioesterases in steroidogenic tissues.

Keywords: acyl-CoA thioesterase; arachidonic acid; steroidogenesis; phosphorylation; hormone.

It is well established that protein phosphorylation plays an obligatory role in the mechanism of action of luteinizing and adrenocorticotropin hormones (LH and ACTH) in Leydig and adrenal cells, respectively [1−7]. One of the processes along the steroidogenic pathway postulated to be regulated by protein phosphorylation is the release of arachidonic acid (AA) [8−11]. It is accepted that AA and the lipoxygenase products also play an obligatory role as intermediaries in the mechanism of action of LH and ACTH [9, 11−16].

Searching for the phosphoprotein involved in steroid synthesis through the release of AA, we identified a cytosolic phosphoprotein. The steroidogenic activity of the soluble partially purified protein was blocked by the use of inhibitors of AA release. This inhibition could be overcome by addition of exogenous AA [17], thereby concluding that this protein regulates steroid synthesis through the activation of AA release [9, 17]. The activity of the protein was dependent on cAMP and cAMP-dependent protein kinase [17]. The cytosolic fraction of a series of mutants of an adrenal tumor cell line (Y-1) defective in adenylyl cyclase or protein kinase A failed to induce steroidogenesis [17].

The protein was isolated and purified to homogeneity, resulting in a phosphoprotein of 43 kDa (p43) [18]. ACTH treatment of rat adrenal glands resulted in the appearance of multiple phosphorylated forms of the protein which were sensitive to acid phosphatase treatment. Therefore, It was concluded that the activity of p43 and the state of phosphorylation were dependent on ACTH [19].

In this paper, we further study the molecular characterization of this phosphoprotein. We have now cloned and sequenced a cDNA encoding p43 from which its primary structure is deduced. The protein is homologous to a very recently described mitochondrial-peroxisome-proliferator-induced acyl-CoA thioesterase (MTE-I) [20].

Although the physiological role of acyl-CoA thioesterases is not completely understood, it has been suggested that the enzyme may play a role in the release of a number of important signal-transduction molecules covalently bound to lipids [21]. This paper reports that a phosphoprotein intermediary in the steroidogenic pathway is structurally related to acyl-CoA thioesterases. It also demonstrates a rapid and transient effect of ACTH on the expression of the gene. Given the obligatory role of the protein in the activation of steroidogenesis through AA release, we propose the name arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) for p43.

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Abbreviations. ARTISt, arachidonic-acid-related thioesterase involved in steroidogenesis; ACTH, adrenocorticotropin hormone; dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; AA, arachidonic acid; RT-PCR, reverse-transcription PCR; CTE-I, cytosolic acyl-CoA thioesterase I; MTE-I, mitochondrial acyl-CoA thioesterase.

MATERIALS AND METHODS

Animals. Adult Wistar rats (90 days old) were used throughout. Ovaries from pseudopregnant rats were obtained as previously described [22]. For reverse-transcriptase PCR (RT-PCR) and 5′ RACE experiments, rats were injected with 2.5 mg/kg dexamethasone 5 h before sacrifice and ACTH (200 μg/kg) 15 min before sacrifice. In all cases, animals were sacrificed by decapitation and the tissues rapidly excised and kept on ice.

General methods. Standard molecular cloning techniques were employed [23]. DNA clones were sequenced by the dideoxy chain termination method [24] using a Sequenase 2.0 sequencing kit, or sequenced directly using the Sequenase PCR Product Sequencing Kit (United States Biochemical Corp.). Both strands were completely sequenced. Oligonucleotides were purchased from CyberSyn.

Screening of rat adrenal λZapII and kidney λgt11 cDNA libraries. Appropriate *Escherichia coli* cells and λZapII or λgt11 phage were mixed and plated on agar plates and incubated overnight at 37 °C. The plaques were lifted onto nylon using standard protocols [23]. The filters were then hybridized and washed as described elsewhere [23]. The probe used was a labeled 795-bp PCR product obtained after PCR amplification with 5′TGCTGGCTGGGAAGGGGCTTT3′ (oligo60) and 5′TCATCTGAAATGATATTCTTA3′ using the 1.4-kb fragment (L11811) as a template.

***In vitro* transcription and translation of the 1.4-kb fragment.** *In vitro* transcription and translation was performed using a TNT *in vitro* translation system (Promega) according to the instructions of the manufacturer. [^{35}S]Methionine (800 Ci/mmol) was used to radiolabel the translated protein. The product was resolved by SDS/PAGE, electrophoretically transferred to nitrocellulose membranes and subjected to Western blot and autoradiography.

Reverse-transcription PCR (RT-PCR). Total RNA (1 μg) isolated from rat adrenal zona fasciculata was used to synthesize cDNA. The reaction mixture contained 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiotreitol, 400 μM each dNTP, oligo d(T) (50 pmol) as primer and 200 U SuperScript reverse transcriptase II. The mixture was successively incubated at 42 °C for 50 min and at 70 °C for 15 min. The incubation proceeded at 37 °C for a further 30 min after addition of 1 μl RNase mix. The cDNA was purified by the GlassMax DNA isolation Spin Cartridge (Gibco BRL). PCR was carried out with 2 μl reverse-transcription products in 50 μl reactions, using TaKaRa *Taq* polymerase (Takara Shuzo Co.) in PCR buffer containing 1.5 mM $MgCl_2$ and 10 mM dNTP mixture. Primers, sense oligo60 and antisense 5′CTGTTTGTGGAAGAAAGTCTG3′ (oligo5RC) were used to amplify the 702-bp specific fragment. To obtain the 5′ region, the PCR reaction was performed using the sense primer 5′AATGCTACGCTGAGCCTGGAGCCCGGGAG3′ and antisense primer 5′GGGTCCAGGTTCTGGGGGCA3′. Cycling conditions were 1 min at 94 °C, 30 s at 55 °C and 2 min at 72 °C, 35 cycles using a thermal cycling programmable heating block (Perkin-Elmer-Cetus Instruments model 9600). PCR products were analyzed by Southern blotting [25]. The amplification product corresponding to the positive signal was excised from the agarose gel and subcloned into pGEM-T easy vector (Promega) for sequence analysis after purification with GeneCleanKit (BIO 101).

RNA isolation for Northern-blot analysis. Total RNA from different tissues was extracted by the method of Chomczynski [26]. Northern-blot analysis was performed as described [23]. Autoradiograms were quantified using a Shimadzu laser densitometer.

5′RACE. Rapid amplification of 5′ ends from p43 cDNA was carried out using poly(A) RNA (1 μg) from adrenal zona fasciculata as described [27] using the 5′ RACE system for rapid amplification of cDNA ends (Gibco BRL). Gene-specific primers were oligo5RC and 5′GGTTTCCATGGTCTTGGG3′ for first and second rounds of PCR, respectively.

Sequence homology and alignment search. Sequence homology was analyzed and alignments confirmed using the BLAST search server at the National Center for Biotechnology Information.

Table 1. Amino acid sequences of different peptides of p43. Peptides were obtained after trypsin or N-Asp endoproteinase (*FR64, *FR54 and *FR28) digestion of the protein and HPLC separation of the fragments as described elsewhere [18].

Peptide nomenclature	Sequence
FG7	NATLSLEPGS (amino terminal)[a]
A10A	ADAGGELDLA[a]
V15Q	VVELEVLDGHEPDGGQR[a]
FR56	IEYFEEAVNYL
G11K	GPGIGLLGISK[a]
F7R	FMAPGVR[a]
FR38	LAPEQPVTLRFR20TMETMR
FR11	GH(W)EVK
FR33	GALFR
FR40	EPERPLWRLVK
FR64	DAWQQLQTFFHKQLSGK[b]
FR54	DEPLSITVRGLAPEQPVTLRA(A)L[b]
FR28	DEKGALFRAHARYRA[b]

[a] Peptides described in [18].
[b] Less than 50% confidence.

RESULTS

In a previous report, we described the amino acid sequence of five peptides obtained by trypsin digestion of p43 [18]. The authenticity of these peptides was validated by the detection of the protein both in native [19] and denatured forms (one-dimensional and two-dimensional gel electrophoresis) with anti-peptide Ig [18, 19]. Further digestions of purified p43 with trypsin and N-Asp endoproteinase produced nine new peptides which were microsequenced (Table 1).

Screening of a protein data bank with the sequences shown in Table 1 revealed no significant similarities with other proteins [18]. However, searching the GenBank database, we found a 463-bp and a 341-bp sequence (L11810 and L11811, respectively) corresponding to both ends of a 1.4-kb cDNA fragment isolated by a cyclic screening procedure that was used to eliminate previously identified cDNA clones from a mouse hepatic cDNA library [28]. The 341-bp sequence had an open reading frame of 113 amino acids, including the sequences matching two of the peptides obtained by trypsin digestion of the protein, V15Q and F7R (100% and 85.7% similarity, respectively) [29]. Therefore, in order to investigate whether this cDNA corresponds to the sequence coding for p43, the sequence of the 1.4-kb cDNA clone was completed. This sequence has a 1008-bp open reading frame encoding 335 amino acids and a 309-bp 3′ untranslated region. A putative polyadenylation signal, AATAAA, was located at positions 1230 and 1278, 68 bp and 20 bp upstream from the poly(A) tail, respectively. The deduced amino acid sequence included five other peptides (Table 1), FR20, FR56, FR11, G11K and FR64 (66.7, 91, 66.7, 100 and 82.4% similarity, respectively), suggesting that this cDNA was associated with p43 and suitable for use as a probe for library screening.

The 1.4-kb fragment was cloned in an expression vector as described in Materials and Methods. The product of the *in vitro*

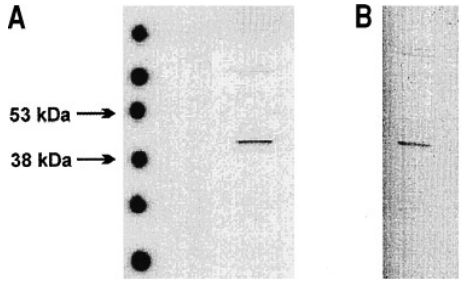

Fig. 1. *In vitro* transcription and translation. 1 μg circular plasmid (pET-1.4 kb) was incubated in a TNT reticulocite-transcription-coupled translation system following the instructions of the manufacturer. Synthesized proteins were then subjected to SDS/PAGE, Western-blot analysis (B) and autoradiography of ^{35}S-labeled proteins (A). Primary antibody used was F7R (1:500 dilution). Arrows on the left indicate the mass (in kDa) of molecular markers.

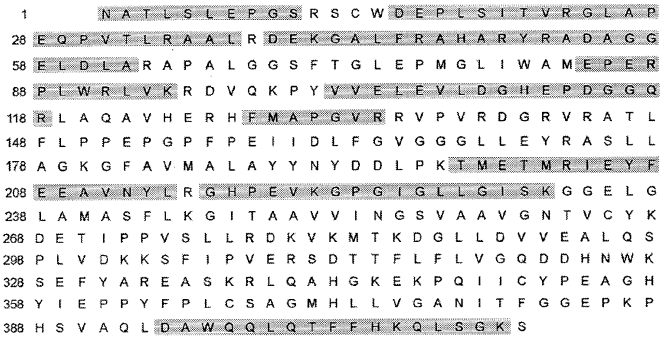

Fig. 2. Deduced amino acid sequence of rat ARTISt cDNA. Schematic presentation of the complete deduced amino acid sequence of rat ARTISt cDNA. Boxes indicate the sequence of peptides obtained after endoproteinase digestion of the purified protein.

transcription and translation assay was subjected to SDS/PAGE, immunoblot and autoradiography. The translated protein exhibited a molecular mass of 40 480 Da and was recognized by anti-peptide Ig raised against p43 in a Western-blot analysis (Fig. 1).

RT-PCR of total rat adrenal zona fasciculata RNA using internal specific oligonucleotides from the coding region of the 1.4-kb cDNA produced an amplification product of 702 bp. The size of the product was as expected and its sequence was similar (91 %) to the mouse liver cDNA. Taking together, the previous data indicate that the cDNA comprises the partial sequence of p43.

In order to obtain the full-length cDNA coding for rat p43, a rat adrenal λZapII cDNA library was screened using the 795-bp probe. Screening of independent recombinants (3×10^5) resulted in the isolation of two clones related to p43. The largest clone isolated was 1130 bp with an open reading frame encoding a 311-amino-acid polypeptide followed by a termination codon (TAG) and 197 bp of 3′ untranslated region not including the poly(A) tail. The second clone was identical, although its sequence spanned 1149 bp. The authenticity of the coding region of this clone was confirmed by the detection of seven of p43 peptide sequences (V15Q, F7R, FR20, FR56, FR11, G11K and FR64) within its sequence. The homology score was 100% for all peptides, confirming the data obtained after amino acid sequence. The order of the peptides within the sequence of the 1.4-kb cDNA fragment was as already described [19]. Both isolated clones finished almost at the same 5′ region and did not contain the N-terminal peptide nor an in-frame initiation codon (ATG). As for the liver cDNA fragment [29], we were unable to detect the 5′ region using an adrenal cDNA library.

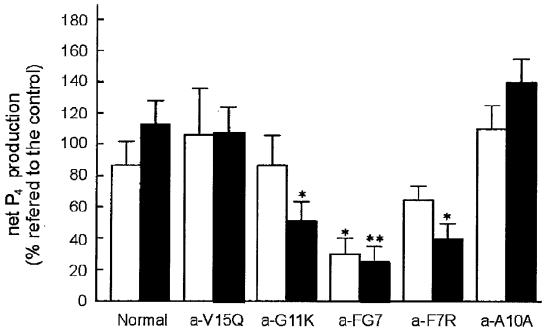

Fig. 3. Effect of anti-peptide Ig on ARTISt bioactivity. Partially purified ARTISt was incubated in the absence (control) or in the presence of normal rabbit serum or anti-peptide Ig (anti-V15Q, anti-G11K, anti-FG7, anti-F7R and anti-A10A) at two different dilutions 1:500 (open bars) or 1:100 (closed bars). The activity was measured by an *in vitro* recombination assay as described elsewhere [38]. Values represent net progesterone production (% of control) and are shown as the mean ± SD, $n = 4$. *, $P < 0.01$; **, $P < 0.001$.

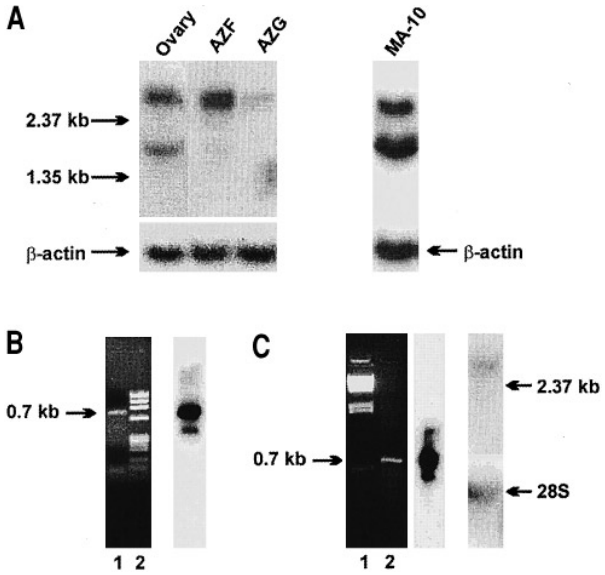

Fig. 4. ARTISt mRNA in various steroidogenic tissues. (A) Total RNA (24 μg) isolated from indicated tissues was subjected to Northern-blot analysis using a 702-bp PCR product and β-actin cDNA as probes. (B) RT-PCR of human placenta. Total RNA (1 μg) was subjected to RT-PCR as described in Materials and Methods. Left panel, amplification product was resolved on a 1.5% agarose gel (lane 1). Molecular markers (ϕX174 RF DNA/HaeIII) are shown in lane 2. Right panel, Southern-blot analysis. (C) RT-PCR of rat brain. Total RNA (1 μg) was subjected to RT-PCR as described in Materials and Methods. Left panel, amplification product was resolved on a 1.5% agarose gel (lane 2). Molecular markers (λ DNA/*Hin*dIII) are shown in lane 1. Central panel, Southern-blot analysis. Right panel, Northern-blot analysis of total RNA (24 μg) hybridized sequentially with the 702-bp fragment and 28 S ribosomal RNA as probes.

Therefore, a kidney cDNA library was screened using the data reported by Warden et al. [29], indicating the major sites of expression for the 1.4-kb cDNA so that the possibilities of isolating the complete cDNA and/or obtaining the lacking 5′ region were increased. The screening resulted in the detection and isolation of 30 positive clones. Positive clones were amplified by PCR and 300−400 bp from both ends directly sequenced. Four clones were shown to be related to p43 and were named E312, F411, B13 and C71.

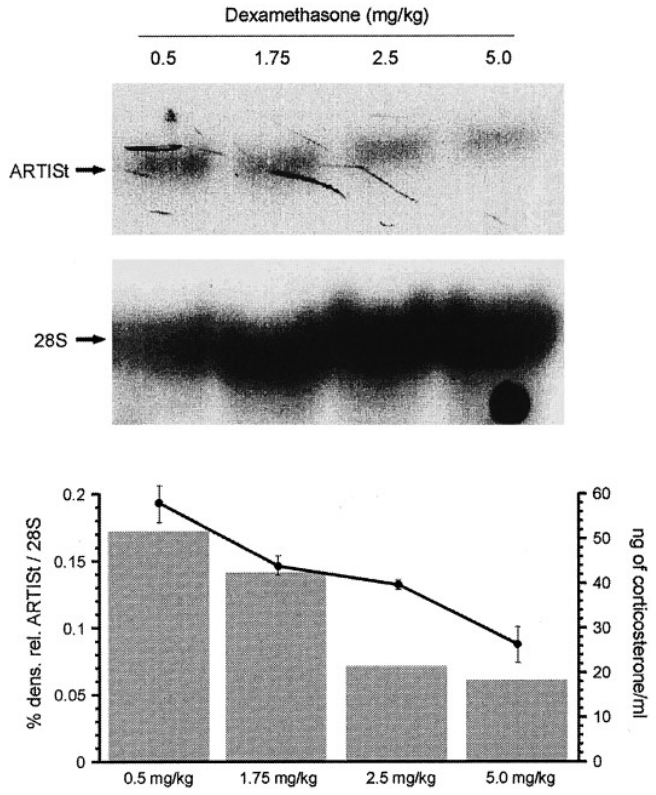

Fig. 5. Effect of dexamethasone on the abundance of ARTISt mRNA on rat adrenal zona fasciculata. Rats were injected subcutaneously with various doses of dexamethasone as indicated. Autoradiography of Northern blots hybridized with a 702-bp fragment (upper panel) or 28 S ribosomal RNA (middle panel) as probes. Lower panel, relative abundance of ARTISt mRNA (bars) and serum corticosterone levels (●). mRNA levels were quantified by densitometric scanning of the autoradiograms and related to those of 28 S ribosomal RNA. Corticosterone was measured by radioimmunoassay [7]. Values are expressed as mean ± SD, $n = 3$, except for RNA in which three separated RNA samples were pooled before analysis.

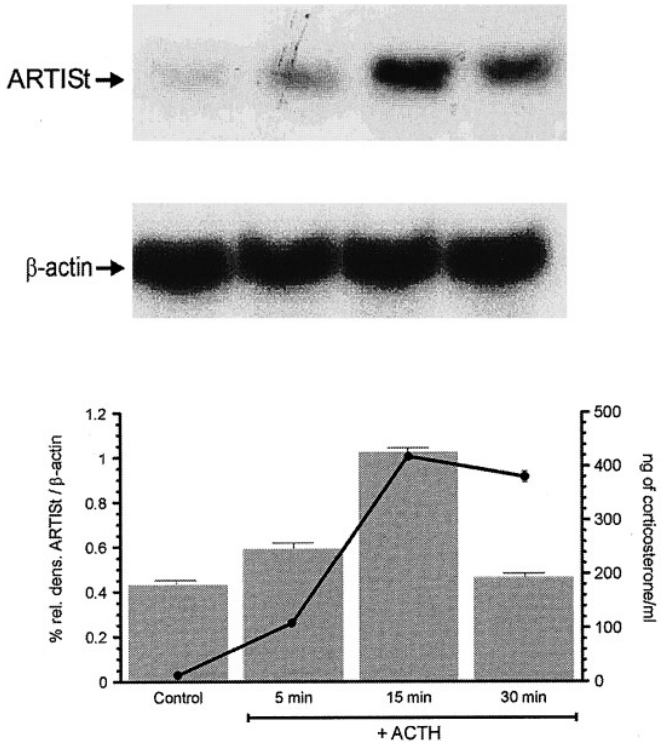

Fig. 6. Time-course effect of ACTH on ARTISt mRNA abundance in rat adrenal zona fasciculata. Rats were injected subcutaneously with 2.5 mg/kg dexamethasone 5 h before sacrifice (control). A group of animals was also injected with ACTH (200 μg/kg) 30, 15 and 5 min before sacrifice. Total RNA (24 μg) was analyzed by Northern blot with a 702-bp fragment (upper panel) or *β*-actin (middle panel) as probes. Relative abundance of ARTISt mRNA and serum corticosterone levels (lower panel) were determined as explained in Fig. 5 and expressed as the mean ± SD, $n = 3$.

Clone E312 (1275 bp) included 40 bp located upstream of the outmost 5′ region of the described cDNA. Clones E312 and F411 were identical, except that they were inverted in relation to the 5′ screening primer amplimer and exhibited a new 140-bp sequence included in position 416 of the clones. Clones B13 and C71 contained 1070-bp and 1711-bp inserts, respectively. None of them included the 140-bp region and both expanded to the 3′ untranslated region and did not include the poly(A) tail. As reported for adrenal cDNA, isolated clones from kidney did not contain an in-frame initiation codon nor the N-terminal region, suggesting that those cDNAs lacked part of the 5′-end region.

To obtain the sequence of the 5′ region, the 5′ RACE technique using total RNA and/or purified mRNA isolated from adrenal zona fasciculata and kidney was used. The amplification products were subjected to Southern-blot analysis and sequencing. The nucleotide sequence showed an open reading frame of 434 bp encoding 144 amino acids, expanding the sequence 131 bp towards the 5′ end. The sequence of this fragment included the peptides V15Q, F7R, a new described peptide named FR40 (100% similarity) and ended with a sequence coding for seven out of 10 amino acids of another peptide of p43 (A10A) located towards the 5′ end. Similar results were obtained by changing the experimental approach using the Marathon cDNA amplification kit (Clontech; data not shown).

The combined sequence spanned 1261 bp and contained an open reading frame encoding 354 amino acids. This sequence was used to screen the GenBank database and the partial deduced amino acid sequence of rat adrenal p43 showed 90% similarity with a newly entered sequence corresponding to mouse acyl-CoA thioesterase (accession number Y14004). Interestingly, within the 5′ coding region of the new sequence, some of the peptides described in Table 1 were identified, such as FG7, FR54, FR38, FR28 and FR33, matching with 60, 87, 100, 100 and 100% similarity, respectively, and that could not be detected in our sequence.

Very recently [20], the sequence of a mitochondrial very-long-chain acyl-CoA thioesterase (MTE-I) has been reported. The deduced amino acid sequence of p43 resulted identical (100% similarity) to MTE-I. The sequence spanned 1711 bp, including 100 bp in the 5′ non-coding region and a coding region of 1359 bp. The sequence also includes 150 bp coding towards the 5′ end containing five of the peptides of p43, including its amino terminal region (Table 1). The deduced amino acid sequence of p43 is shown (Fig. 2).

The sequence of p43 shows four consensus sites for protein kinase A, two for Ca/calmodulin-dependent protein kinase, five for protein kinase C and two for casein kinase II [30]. Contained in the sequence, there is also a lipase serine motif within the G11K peptide (prosite sequence motif database) as well as a Gly-Xaa-His motif close to the C-terminal region which has been shown to be necessary for the hydrolytic activity of other thioesterases [31]. An extensive search in DNA sequence databases showed a high degree of overall sequence similarity only to human, rat and mouse bile acid CoA: amino acid N-acyltransferase (BAT L34081, rat Kan-1 protein D43964 and mouse Kan-1 U95215).

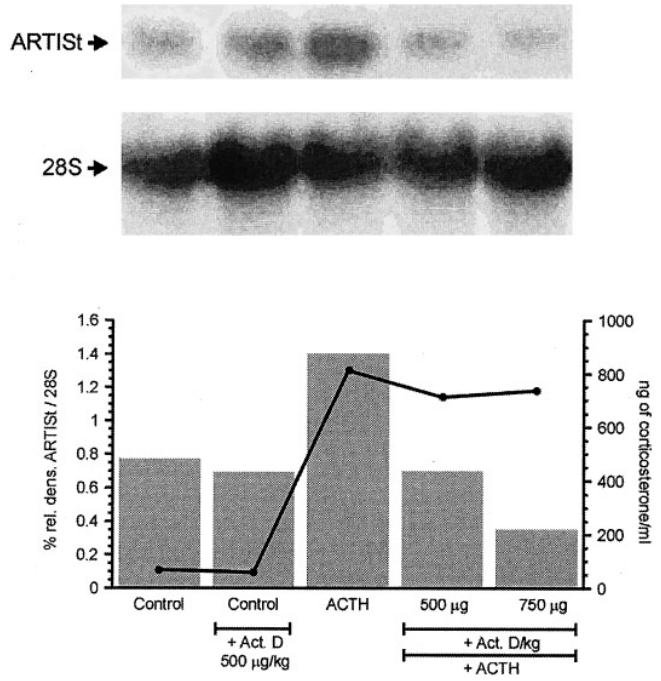

Fig. 7. Effect of actinomycin D on ARTISt mRNA abundance in rat adrenal zona fasciculata. Rats were injected subcutaneously with 2.5 mg/kg dexamethasone 5 h before sacrifice. When administered, actinomycin D (Act. D) was injected at the indicated doses 45 min and ACTH (200 µg/kg) 15 min before sacrifice. Results were obtained and represented as explained in Fig. 5. Error bars in corticosterone determinations are less than 5% and are therefore not visible.

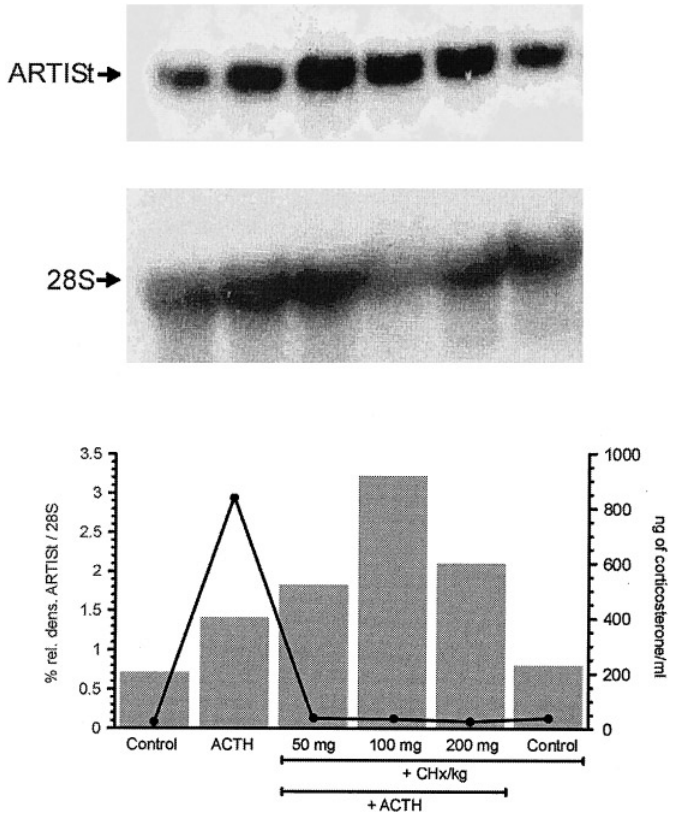

Fig. 8. Effect of cycloheximide (CHx) on ARTISt mRNA abundance in rat adrenal zona fasciculata. Rats were injected subcutaneously with 2.5 mg/kg dexamethasone 5 h before sacrifice. When administered, CHx was injected at the indicated doses 45 min and ACTH (200 µg/kg) 15 min before sacrifice. Results were obtained and represented as explained in Fig. 5. Error bars in corticosterone determinations are less than 5% and are therefore not visible.

Antibodies raised against a synthetic peptide that includes the lipase serine motif (anti-G11K) and against the N-terminal region of p43 (anti-FG7) blocked the action of p43 (Fig. 3). A third antiserum (anti-F7R), although raised against a synthetic peptide with no identifiable active motif, also blocked the steroidogenic activity of p43. Two other antibodies (anti-V15Q and anti-A10A) had no effect on progesterone synthesis.

We next studied the presence of the p43 transcript in steroidogenic tissues. mRNA encoding p43 was detected by Northern blotting (Fig. 4A) as two transcripts in ovary of pseudopregnant rats and the Leydig tumor cell line (MA-10) and as a single transcript in rat adrenal zona fasciculata and glomerulosa and rat brain. The transcript was also detected by RT-PCR (Fig. 4B and C) in human placenta and rat brain. The RT-PCR product from human placenta was sequenced and found to be identical to a hypothetical protein ZAP128 from human brain (L40401) described in GenBank, lacking both the N-terminal region and homology with p43 in its 5′ region.

Dexamethasone produced a dose-dependent decrease in the abundance of the adrenal transcript (Fig. 5). In order to corroborate that the effect of dexamethasone was due to a reduction in circulating levels of ACTH, a second group of animals was injected with ACTH following dexamethasone administration. The transcript was induced by *in vivo* stimulation of the adrenals with ACTH. The effect had a rapid onset (5 min), reached maximal stimulation (62%) at 15 min, and returned to basal levels at 30 min (Fig. 6). The effect of ACTH on the p43 transcript was inhibited by actinomycin D and enhanced by cycloheximide (CHx) (Figs 7 and 8). The hormonal effect on the abundance of the p43 transcript was also observed when MA-10 cells were stimulated with chorionic gonadotropin (data not shown).

DISCUSSION

We have previously purified a phosphoprotein with a mass of 43 kDa (p43) that plays an obligatory role in the regulation of steroid synthesis. Here, we describe the sequence of 14 peptides, the cloning of the cDNA corresponding to p43, as well as the regulation of its transcript by ACTH. During the course of preparation of this report the molecular cloning and sequencing of a protein, mitochondrial-peroxisome-proliferator-induced very-long-chain acyl-CoA thioesterase (MTE-I), was reported [20] that is homologous to p43. MTE-I was purified to apparent homogeneity and found to be most active with substrates with chain lengths of C14−C20 [32]. According to its mitochondrial localization, the sequence of MTE-I includes a mitochondrial leader peptide. The N-terminal region of the mature form of MTE-I was suggested to be located at position 43 [20]. Interestingly, the N-terminal of p43, as obtained by Edman degradation of the purified protein, matches MTE-I from position 43. The deduced amino acid sequence contains all internal peptides of p43 that were microsequenced, accounting for 34.8% of the total protein with 100% similarity. Given the obligatory role of the protein in the activation of steroidogenesis through the release of arachidonic acid, we propose the name arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) for p43.

ARTISt plays an obligatory role in steroid synthesis, possibly regulating AA release. So far, no evidence has been presented showing the participation of acyl-CoA thioesterases in the mechanism of action of hormones that regulate steroidogenesis. However, a regulatory role for acyl-CoA thioesterases has

been suggested on the basis of the expression of the protein in steroidogenic tissues [33]. Our results provide the first evidence to our knowledge linking acyl-CoA thioesterases, with very-long-chain specificities, and a protein intermediary in steroid synthesis, thereby supporting a regulatory role for acyl-CoA thioesterases in steroidogenic tissues. The question as to how the acyl-CoA thioesterase activity could regulate either directly or indirectly AA release is still unresolved. It is, however, of interest that the enzyme shows highest substrate specificity when assayed with arachidoyl-CoA or palmitoyl-CoA [32].

Very-long-chain acyl-CoA hydrolases are widely distributed among organisms and cell types. The thioesterase homologous to ARTISt includes a lipase serine motif that is not inhibited by serine esterase inhibitors [32]. The serine esterase motif, the resistance to serine esterase inhibitors and the sensitivity to thiol-reactive agents suggests that the enzyme represent a novel kind of mechanism for thioester hydrolysis [33].

Analysis of the deduced amino acid sequence of ARTISt isolated from different species shows both structural and functional conservation of the lipase serine motif of the enzyme. Conservation of this active site during the course of evolution will then indicate the importance of the structure for enzyme functionality.

In accordance with the postulated obligatory role for ARTISt in steroidogenesis, we detected the presence of mRNA for ARTISt in all steroidogenic tissues. Although several proteins are described as intermediates in the regulation of steroid synthesis by trophic hormones, evidence showing that the hormone can regulate the transcript of these or other proteins during the early phase of the response is lacking. Here, we present evidence for very early regulation of ARTISt transcript. This regulation is rapid and transient, since ACTH affects the amount of transcript as early as 5 min, returning to basal levels in 30 min. Our data indicate that ACTH could exert its action by both regulating mRNA stabilization and *de novo* transcription. The increase in the abundance of ARTISt mRNA after cycloheximide treatment suggests that the messenger encoding ARTISt is intrinsically unstable. It is known that drugs which inhibit protein synthesis can increase messenger stability both at the transcriptional and post-transcriptional levels [34]. It seems then possible that mRNA stabilization could support the initial phase of steroid synthesis. This is in accordance with the observation that actinomycin D is not able to inhibit steroid synthesis in the early phase of steroidogenesis but it does in the sustained phase [35].

We have isolated ARTISt from the cytosol of adrenal zona fasciculata cells. Although the homology of our sequence with that of a mitochondrial protein seems unexpected, it agrees with our previous detection by Western-blot analysis of a 43-kDa protein in cytosol and mitochondrial matrix and a 55-kDa protein in rat adrenal mitochondrial membrane [36]. The actual mechanism of action of the protein in the different compartments remains unclear.

The finding that the deduced amino acid sequence of the cDNA clone shows consensus sites for different protein kinases is in accordance with the phosphoprotein nature of ARTISt. This coincides also with the observation that the activity of ARTISt is hormone-dependent and operates under several different signal-transduction mechanisms [9, 19]. So far, there is no description to our knowledge of a possible regulation of acyl-CoA thioesterase activity by protein phosphorylation. Our data suggest that protein phosphorylation could play a role in the regulation of this type of enzymes.

The amino acid sequence also shows a lipase serine motif, as well as sites for N-glycosylation and myristiolation. It is noteworthy that the lipase serine motif is contained within the sequence of the G11K peptide, coinciding with the inhibition of the biological activity of ARTISt produced by an antibody raised against a synthetic peptide matching that sequence. Antibodies against the N-terminus and the F7R peptides of ARTISt also inhibit its steroidogenic activity. Although these regions do not match any known active motif, they may play an important role in the mechanism of action of this protein.

ARTISt cDNA is also similar (92% similarity) to a cDNA newly entered in the GenBank database and encoding a peroxisome-proliferator-induced acyl-CoA thioesterase which was partially purified from rat liver cytosol and named CTE-I [37].

Svensson et al. [20] showed that the enzyme is constitutively expressed in heart. It is of interest that we also found ARTISt and its transcript in non-steroidogenic tissues (kidney, liver and heart) and the protein isolated from heart stimulated with isoproterenol was able to induce steroid synthesis in isolated adrenal mitochondria using a recombinant heterologous *in vitro* assay (Neuman, I. et al., unpublished results).

Although the acyl-CoA thioesterases are found in all organisms and are present in several cellular compartments, the physiological significance is not well established, particularly for the novel thioesterases present in cytosol and mitochondria. Given the fact that ARTISt is an obligatory component of the steroidogenic pathway and that it is homologous to this novel enzyme, a physiological role for this kind of acyl-CoA thioesterases can be established as an intermediary in the regulation of steroid synthesis. This information opens the possibility to investigate how an acyl-CoA thioesterase can regulate arachidonic acid release and steroidogenesis.

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