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Long-chain acyl-CoA synthetase 4 is regulated by phosphorylation

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ABSTRACT

Long chain acyl CoA synthetase 4 (Acsl4) is a key enzyme in steroidogenesis. It participates in steroid synthesis through of arachidonic acid release and Steroidogenic Acute Regulatory protein (StAR) induction.

Acsl4 prefers arachidonic acid as substrate and acts probably as a homodimer. In steroidogenic cells, it has been demonstrated that Acsl4 is a high turnover protein located mainly in mitochondrial-associated membrane fraction (MAM) bound to other proteins and that it is newly synthesized by hormone stimulation. The synthesis of Acsl4 constitutes an early step in steroidogenesis.

In the steroid synthesis process, activation of kinases plays a very important role. For this reason, the aim of this work was to study Acsl4 as a possible phosphoprotein and try to elucidate the role of its phosphorylation.

We have determined for the first time that Acsl4 is a phosphoprotein whose phosphorylation is hormone-dependent. We also demonstrated that Acsl4 acts effectively as a dimer and that phosphorylation occurs after dimer formation.

Studies *in vitro* demonstrated that Acsl4 is a substrate of both PKA and PKC and its phosphorylation by these kinases regulates its activity.

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1. Introduction

Free fatty acids must be activated to their CoA thioesters before participating in most catabolic and anabolic reactions. Several processes such as incorporation of fatty acids into phospholipids or triacylglycerols, fatty acid elongation, unsaturation and degradation and fatty acylation of proteins require activated fatty acid substrates [1]. In turn, acyl-CoAs up-regulate uncoupling protein in brown adipose tissue and key enzymes of glycolysis, gluconeogenesis, and β-oxidation, are essential for vesicle trafficking, and play a critical role in the transport of fatty acids into cells by making transport unidirectional.

Free fatty acid activation is catalyzed by acyl-CoA synthetases, also known as acid:CoA ligases (AMP-forming) (EC 6.3.1.3). Acyl-CoA synthetases can be divided into five sub-families (1, 3, 4, 5 and 6) on the basis of the chain length of their preferred acyl groups. Long-chain acyl-CoA synthetases (Acsl) is the subfamily preferring fatty acids C12 to C20 as a substrate [2], that might act as homodimer [3].

In particular, Acsl4 shares 68% of its amino acid sequence with Acsl3 while it is poorly related to the other family members [4]. Purified Acsl4 prefers arachidonate as a substrate among other C8–C22 saturated and C4–C22 unsaturated fatty acids [2].

Tissue distribution is different for each Acsl. A striking feature of Acsl4 is its abundance in steroidogenic tissues, especially adrenal gland and ovary. Acsl4 immunoreactivity was been detected in the zona fasciculata and reticularis of the adrenal cortex, in the corpus luteum and stromal luteinized cells of the ovary and in Leydig cells of the testis [4]. Furthermore, Acsl4 is a peripheral membrane protein, located mainly on the mitochondrial-associated membrane fraction (MAM), on peroxisomal membrane and microsomes [5].

It has been demonstrated that Acsl4 expression in the Y1 murine adrenocortical tumor cell line is induced by ACTH and suppressed by glucocorticoids [6]. In Y1 and Leydig cells, its expression is also regulated by EGF, another factor that increases steroid production [7]. Furthermore, hormone stimulation of steroid production through the cAMP-dependent phosphorylation involves new synthesis of Acsl4 as an early step [8].

In hormone-induced steroidogenic cells Acsl4 activity appears to be essential for arachidonic acid release, StAR induction and steroid synthesis [8,9]. In Y1 and MA-10 cells, Acsl4 has been also proven as a high turnover protein [8].

Even if Acsl4 participation in steroidogenesis has been very well determined, its postransductional modifications have not been studied yet.

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Therefore, considering that Acsl4 is a high turnover protein which might act as a dimer, that it is induced by hormonal treatment and is located in MAM bound to other protein, events which could be regulated by phosphorylation, the aim of this work was to study whether Acsl4 is indeed a phosphoprotein and whether at least some of those events are actually regulated by protein phosphorylation.

The studies presented herein show that Acsl4 is a phosphoprotein whose phosphorylation is hormone-dependent and that this modification might regulate its activity.

2. Materials and methods

8Br-cAMP, 22(R)-OH-cholesterol, cycloheximide (CHX), PKC, arachidonic acid and Co-enzyme A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-39 kDa subunit of the NADH-cytochorme c reductase antibody was obtained from Molecular Probes, Inc. (Eugene, OR, USA). PKA catalytic subunit was from New England Biolabs (Beverly, MA, USA). All other reagents were of the highest grade available.

2.1. Cell culture

Murine Y1 adrenocortical tumor cells (ATCC CCL 79) were handled as described in [8].

MA-10 Leydig cell line was generously provided by Dr. Mario Ascoli, University of Iowa, College of Medicine (Iowa City, IA, USA) and was handled as originally described [10].

2.2. Preparation of mitochondrial fraction

Mitochondria and post-mitochondrial fractions (PMTC) were obtained as previously described [11].

2.3. Incorporation of [^{32}P]phosphate and Acsl4 immunoprecipitation

Y1 cultured cells were washed 3 times in phosphate-free Eagle media modified by Dulbecco (Gibco) and incubated for 5 h at 37 °C under 5% $C0_2$/95% air in the same medium containing [^{32}P]phosphate (200 μCi/ml). Then, EGF (10 ng/mL), ACTH (5 mU/mL) or 8Br-cAMP (1 mM) was added and incubated for another 10 min. The cells were washed three times with the media and lysed. Four hundred micrograms of cellular extract was used for Acsl4 immunoprecipitation as described in [8].

2.4. Assay of Acsl4 phosphorylation in vitro by PKA and PKC

Phosphorylation assay by PKA was performed in a final volume of 30 μL containing 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM EGTA, 1 mM, β-mercaptoethanol, 0.1 mM [γ-^{32}P] ATP 10 μCi, 10 UI of PKA catalytic subunit and 3 μg of purified Acsl4 at 30 °C.

Phosphorylation assay by PKC was performed in a final volume of 30 μL containing 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 5 μg phosphatidylserine, 0,4 μg diacylglycerol, 10 μM [γ-^{32}P]ATP 10 μCi, 10 UI PKC catalytic subunit and 3 μg of purified Acsl4 at 30 °C. In the indicated cases kinase reaction was started without radioactive ATP, which was later incorporated. After the phosphorylation assays, proteins were separated by SDS–PAGE, transferred to PVDF membrane and exposed to X-ray film to detect phosphorylated proteins. Then, a Western Blot for Acsl4 detection was made.

For quantification of phosphate incorporated per mole of protein, gels were dried, phosphorylated bands were cut and radioactivity was measured by liquid scintillation counting.

When sequential phosphorylations were done, the reaction conditions were those used for PKC reaction but using 0.1 mM ATP instead.

2.5. Acsl4 activity reaction

Acsl4 was previously phosphorylated by PKA or PKC for 30 min as stated above. Then Acsl4 activity reaction was carried out in a final volume of 90 μL for 30 min at 37 °C in the following conditions: 100 mM Tris–HCl (pH 7.4), 5 mM ATP, 250 μM Coenzyme A, 50 μM arachidonic acid, 0.03% Tritón X-100, 1 μM EDTA, 8 mM $MgCl_2$, 5 mM DTT, 0.4 μCi [$^{1-14}$C] arachidonic acid. The reaction was stopped by the addition of 1% ClH (0.8 mL) and then 4 extractions with cold n-hexane were made. The aqueous phase was measured by liquid scintillation counting.

2.6. Western Blot analysis

Proteins were separated by SDS/PAGE and transferred to poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) as described previously [9]. For separation in non-reducing condition, β-mercaptoethanol free sample buffer was used.

In the case of bidimentional electrophoresis, first dimension isoelectric focusing was done using Immobiline DryStrip precast gels (Amersham) (pH 6–11, 7 cm) according to manufacturer's recommendations. The second dimension was done as indicated above.

Acsl4 protein was detected using anti-Acsl4 antibodies [8] and enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, UK). For quantitative analysis, band intensities were analyzed using ImageQuant 5.2 software.

2.7. Plasmid transfection

MA-10 cells were transiently transfected with pcDNA3 plasmid containing Acsl4 cDNA or empty plasmid as indicated in [7].

2.8. Protein determination and statistical analysis

Protein was quantified by Bradford's method [12] using bovine serum albumin as standard. Statistical analysis was performed by ANOVA followed by the Student–Newman–Kuels test.

3. Results

3.1. Is Acsl4 a phosphoprotein?

Given that steroidogenic hormones trigger kinase activation and that some events as subcellular localization, dimer formation and protein half-life could be regulated by addition of phosphate group, we studied whether Acsl4 can be phosphorylated by hormonal stimuli. For this purpose we immunoprecipited Acsl4 of Y1 cells that were incubated with [^{32}P]phosphate and stimulated by either ACTH, its second messenger 8Br-cAMP or EGF, another well known factor that induces steroidogenesis [7] (Fig 1A).

As it is shown in the autoradiography, we observed a radioactive band of 74 kDa. The identity of this band was confirmed by Western-Blot analysis for Acsl4, which indicated that it is indeed a phosphoprotein (Fig 1B). On the other hand, radioactivity was higher in Acsl4 from stimulated cells compared to the control ones (Fig 1C). This result would indicate that hormonal stimuli induce Acsl4 phosphorylation.

In order to determine Acsl4 phosphorylated forms, proteins from control and stimulated cells were separated by bidimensional

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