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A homogeneous scintillation proximity assay for acetyl coenzyme A carboxylase coupled to fatty acid synthase

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Abstract

We have devised a rapid and sensitive homogeneous assay for acetyl CoA carboxylase (ACC) in a scintillation proximity assay format suitable for high-throughput screening. In this assay, ACC is coupled to fatty acid synthase (FAS). Malonyl CoA, the product of the ACC reaction, and acetyl CoA serve as substrates for FAS to synthesize palmitic acid. When [^{3}H]acetyl CoA is used in the ACC/FAS coupled system, [^{3}H]palmitic acid, the final product, is readily detected by scintillation proximity in a FlashPlate or Image FlashPlate coated with phospholipid. The [^{3}H]palmitic acid binds to the coated phospholipid through hydrophobic interaction which brings it into close proximity of the scintillant on the FlashPlate or the Image FlashPlate, yielding photons that are read in a TopCount or LeadSeeker, respectively. The current assay consists of simple reagent addition, incubation, and detection of signal. The signal is ~30-fold over the background and the Z' value is ~0.80, suggesting that this assay is robust and highly reproducible. To our knowledge this ACC/FAS coupled scintillation proximity assay is the only assay format that is compatible with high-throughput screening for systematic search of inhibitors against mammalian ACC.

Keywords: Acetyl CoA carboxylase; Fatty acid synthase; Scintillation proximity assay; High-throughput screening

Acetyl CoA carboxylase (ACC)[1] (EC 6.4.1.2) catalyzes the rate-limiting reaction in the fatty acid biosynthesis pathway. Malonyl CoA, the product of the ACC enzymatic reaction, is an important intermediate product of fatty acid metabolism that is involved in various physiological processes. In lipogenic tissues, such as liver and adipose tissue, malonyl CoA is the two-carbon donor of de novo synthesis of long-chain fatty acids [1]. In the fatty-acid-consuming tissues, malonyl CoA is important in gauging the rate of fatty acid β-oxidation [2]. The rising level of tissue malonyl CoA in animals in an anabolic state allosterically inhibits carnitine palmitoyl transferase 1 (CPT1), a critical enzyme that transfers the long chain fatty acid into the mitochondria for β-oxidation [3]. Recently, the level of hypothalamic malonyl CoA has also been proposed to serve as an indicator of energy status and to mediate feeding behavior [4]. Also, in the pancreatic β-islet, malonyl CoA may also play an important regulatory role in insulin secretion [5,6].

In mammals, including humans, there are two ACC isoforms, ACC1 and ACC2. ACC1 (MW ~ 265 kDa) is enriched in lipogenic tissues such as liver and adipose tissue and primarily involved in fatty acid synthesis [7,8]. ACC1 activity is regulated in response to dietary and hormonal states. Under catabolic conditions or in diabetes, ACC1 activity is reduced through decreased transcription [9] and by phosphorylation of the ACC1 protein [10,11]. When the animal is starved and refed with high-carbohydrate/low-fat diet, the liver ACC1 content and activity are induced significantly [9,12]. In contrast, ACC2 (MW ~ 280 kDa) is expressed primarily in fatty-acid-consuming tissues, such as heart, skeletal muscle, and liver [7,8]. The distribution

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[1] *Abbreviations used:* ACC, acetyl coenzyme A carboxylase; FAS, fatty acid synthase; SPA, scintillation proximity assay; HTS, high-throughput screening; CPT1, carnitine palmitoyl transferase 1; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; RT, room temperature; S/B, signal/background.

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of the two ACC isoforms is also distinct at the cellular level. ACC1 is located in the cytosol where fatty acid synthesis takes place, whereas ACC2 is attached to the outer leaf of mitochondria through a stretch of hydrophobic sequence that is not found in ACC1. The close proximity of ACC2 and CPT1 suggests that its primary role is to regulate β-oxidation of fatty acids [13].

Emerging evidence suggests that ACC2 or both ACCs are good pharmacological targets for the treatment of metabolic disorders including obesity, insulin resistance, and dyslipidemia. Abu-Elheiga and co-workers [14] demonstrated that ACC2 knockout mice have exhibited healthy metabolic phenotypes including less weight gain and lower fat content. This healthy phenotype appears to result from a continued high rate of fatty acid β-oxidation in skeletal muscle where the malonyl CoA concentration is decreased as a consequence of genetic deletion of ACC2. In a high-fat/high-carbohydrate-diet-induced obesity/diabetes mouse model, ACC2 deletion also prevents the development of obesity and insulin resistance [15]. More recently, Harwood et al. [16] demonstrated that a class of isozyme-nonselective inhibitors of mammalian ACCs (pan-ACC inhibitors) reduced the level of fatty acid and triglyceride syntheses and increased the rate of fatty acid β-oxidation in rodents, indicating that a pan-ACC inhibitor could be used in the treatment of metabolic disorders.

Despite the importance of ACC isozymes as potential drug targets, progress toward finding highly potent inhibitors has been relatively slow. One of the key obstacles appears to be the lack of a convenient assay that can be transformed into a high-throughput-screening (HTS)-compatible format. The most commonly used ACC assay is the CO_2 fixation assay [17]. In this assay, ACC, substrates, and cofactors are incubated with $H[^{14}C]O_3^-$ and quenched with acid followed by heating to remove unreacted $H[^{14}C]O_3^-$ as $[^{14}C]O_2$. The remaining acid-stable [^{14}C]malonyl CoA retained is counted in a scintillation counter [17]. Although the CO_2 fixation assay has been adapted into 96-well microtiter plate format [16], this is a multistep, time-consuming, and labor-intensive assay.

The ACC enzyme reaction can also be monitored by the rate of ATP consumption. In a traditional protocol, ACC is coupled to an ATP-regeneration system where the regeneration of ATP is coupled to the consumption of NADH using pyruvate kinase and lactate dehydrogenase and can be monitored continuously in a spectrophotometer [17]. Alternatively, inorganic phosphate produced in the reaction can be measured using a malachite green/ammonium molybdate/HCl reagent [18]. More recently, a scintillation proximity assay (SPA) for measuring the inorganic phosphate produced from [γ-^{33}P]ATP was reported using streptavidin-coated SPA beads or wheat-germ agglutinin imaging beads. This SPA format was proposed for HTS against bacterial ACC enzyme [18]. Despite this progress, the shortcomings of these methods of assaying ACC that measure ATP consumption are their sensitivity to ATPases and the requirement for highly pure ACC protein.

In this paper, we describe an ACC assay protocol that overcomes all of these drawbacks. In this new assay ACC is coupled with FAS (see Eqs. (1)–(3)). [^{3}H]Palmitic acid, the ultimate product of the ACC/FAS coupled reaction, is measured in a phospholipid-coated FlashPlate or Image FlashPlate by the principle of SPA that was used for FAS assay [19].

ACC reaction:

$$7[^3\text{H}]\text{Acetyl CoA} + 7\text{NaHCO}_3 + 7\text{ATP} \rightarrow 7[^3\text{H}]\text{Malonyl CoA} + 7\text{ADP} + 7\text{P}_\text{i}. \quad (1)$$

FAS reaction:

$$[^3\text{H}]\text{Acetyl CoA} + 7[^3\text{H}]\text{Malonyl CoA} + 14\text{NADPH} \rightarrow [^3\text{H}]\text{Palmitate} + 14\text{NADP} + 8\text{CoA}. \quad (2)$$

ACC/FAS coupled reaction:

$$8[^3\text{H}]\text{Acetyl CoA} + 7\text{NaHCO}_3 + 7\text{ATP} + 14\text{NADPH} \rightarrow [^3\text{H}]\text{Palmitate} + 7\text{ADP} + 14\text{NADP} + 8\text{CoA} + 7\text{P}_\text{i}. \quad (3)$$

This novel ACC/FAS coupled SPA is a convenient, sensitive, highly reproducible assay and requires less pure enzyme preparation. To our knowledge, this is the first ACC assay format that is truly compatible with HTS for a systematic search for mammalian ACC inhibitors.

Materials and methods

Reagents

Custom-made 384-well phospholipid FlashPlates (SMP-501) (FlashPlate is a white polystyrene microplate, coated with a thin layer of polystyrene-based scintillant that is used for homogeneous radiometric assays, based upon the principle of scintillation proximity) and Image FlashPlates (52113) (Image FlashPlate is a 384-shallow-well microplate coated with a scintillant that emits in the red region which combines ultraminiaturization with superior sensitivity) were obtained from PerkinElmer Life and Analytical Sciences; [^{3}H]acetyl CoA was obtained from GE Healthcare (5.2 Ci/mmol) or PerkinElmer Life and Analytical Sciences (3.7 Ci/mmol); acetyl CoA was from Sigma; complete protease inhibitor cocktail was from Roche Diagnostics; and monomeric avidin Sepharose was from Pierce. All other reagents were of analytical grade and obtained from Sigma.

ACC and FAS enzyme

Rat ACC and FAS enzymes were purified by a rapid purification procedure from frozen livers from rats that were treated by a fasting/refeeding cycle. The protocol was adopted from [20] with some modifications. All operations were carried out at 4 °C or on ice. In a typical preparation, 100