Study on Expression of Human Acetyl-CoA Carboxylase 2 and Malonyl-CoA Decarboxylase Using Silkworm-based BmNPV Bacmid Expression System and their Functional Analysis

THESIS

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カイコ BmNPV バクミド発現系を用いたヒトアセチル-CoA カルボキシラーゼ 2 およびマロニル-CoA デカルボキシラーゼの発現とその機能解析に関する研究

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2014 年 12 月

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CERTIFICATE OF APPROVAL

Ph.D. Thesis

This is to certify that the Ph.D thesis entitled:
Study on Expression of Human Acetyl-CoA Carboxylase 2 and Malonyl-CoA Decarboxylase Using Silkworm-based BmNPV Bacmid Expression System and their Functional Analysis

by

In-Wook Hwang

has been approved by the Thesis Committee on January 29, 2015 as satisfactory for the thesis requirement for the degree of Doctor of Philosophy

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List of Abbreviations

aa	Amino acid
ACADL	Long chain acyl-CoA dehydrogenase
ACADM	Medium chain acyl-CoA dehydrogenase
ACC	Acetyl-CoA carboxylase
AcNPV	*Autographa california* nucleopolyhedrovirus
ACP	Acyl-carrier protein
ACS	Acyl-CoA synthase
AFM	Atomic force microscopy
AMPK	Adenosine monophosphate-activated protein kinase
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
BES	Baculovirus expression system
BEVS	Baculovirus expression vector system
BmNPV	*Bombyx mori* nucleopolyhedrovirus
BV	Budded virus
CAT	Carnitine translocase
CBB	Coomassie Brilliant Blue
cDNA	DNA molecule produced from RNA template during reverse transcription
CPT1	Carnitine palmitoyltransferase 1
CT	carboxyltransferase
C-terminus	Carboxyl terminal end
DDT	Dithiothreitol
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FACS	Fatty acyl-CoA synthase
FAS	Fatty acid synthase
kDa	Kilodalton
GFP	Green fluorescent protein
GV	Granulovirus

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IPTG	Isopropyl beta-D-1-thiogalactopyranoside
*lac*Z	Gene encoding-galactosidase from *E. coli*
LCFA	long chain fatty acid
MCD	Malonyl-CoA decarboxylase
MW	Molecular weight
N-terminus	Amino terminal end
PCR	Polymerase chain reaction
PDV	polyhedral derived virus
PKC	Protein kinase C
PMP	Protein metallophosphatases
PPARγ	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
TCA	Tricarboxylic acid
VLDL	Very-low-density lipoprotein
X-gal	5-bromo-4-chloro-3-indolyl-4-galactoside

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Chapter 3

Thesis Outline

The general objectives of this thesis were to study the production of human acetyl-coA carboxyalase 2 (ACC2) and malonyl-coA decarboxylase (MCD) and to evaluate the functions of purified proteins. For these objectives, silkworm-based BmNPV bacmid expression system was applied to express the ACC2 and MCD. As a result, these proteins were successfully expressed by silkworm and purified by affinity column chromatography. Biological functions of purified proteins were evaluated by each activity assay. To investigate the effects of translational modifications such as phosphorylation, expressed proteins were dephosphorylated by phosphatase or site-directed mutation, and its activity was analyzed. The thesis is organized as follows:

Chapter 1 provides a general review of lipid metabolism, ACCs, MCD, baculovirus expression system and the objectives of the thesis. The biological interactions of ACCs and MCD were introduced. This chapter concludes by raising the current issues of the biological functions of ACCs and MCD, and the silkworm-based baculovirus expression system.

Chapter 2 describes in detail the processes and methods involved in constructing BmNPV/ Δ148aa-hACC2 bacmid and expressing hACC2 in silkworm-based expression system. The biotinylation, phosphorylation and polymerization of hACC2 expressed in silkworm pupae and their carboxylase activities by alteration of these post-translational modifications were investigated.

Chapter 3 describes in detail the processes and methods involved in constructing BmNPV/

Δ39aa-hMCD bacmid and expressing hMCD in silkworm-based expression system. The phosphorylation and degree of polymerization of hMCD expressed in silkworm larvae and pupae, and their decarboxylase activities by alteration of these post-translational modifications were investigated.

Chapter 4 concludes the work. The main findings of this study from the former chapters were summarized. Several recommendations were also presented for further work.

Chapter 1

Introduction

1.1 Lipid metabolism associated with acetyl-CoA carboxylase and malonyl-CoA decarboxylase

1.1.1 Fatty acid biosynthesis

Lipid metabolism refers to the processes that involve the intercourse and degradation of lipids. The types of lipids involved include bile salts, cholesterols, eicosanoids, glycolipids, ketone bodies, fatty acids, steroid, triacylglycerols. Among them, the fatty acid is a carboxylic acid with a long aliphatic chain, which is either saturated or unsaturated (Nutting et al., 1999). Fatty acids can be derived from triglycerides or phospholipids. When metabolized, they yield large amounts of ATP. Therefore, it is important sources of fuel. Fatty acid biosynthesis is the creation of fatty acids from acetyl-CoA and malonyl-CoA precursors by fatty acid synthases. It is an important part of the lipogenesis process, which together with glycolysis stands behind creating fats from blood sugar in living organisms (Ganguly, 1960). Fatty acid biosynthesis occurs similarly to β-oxidation. Acetyl groups are added to a growing chain, but the mechanism of the pathway is distinctly different from the reverse of β-oxidation. Fatty acid biosynthesis occurs in the cytosol. It uses a moiety called acyl-carrier protein (ACP) instead of CoA and the reducing agent NADPH. There are structural similarities between the phosphopantetheine moiety of ACP and the Coenzyme A portion of CoA (Synak and Budohoski, 2000). Fatty acid biosynthesis can be broken in to three separate pathways shown below synthesis of palmitate from acetyl-CoA, elongation of palmitate and desaturation. Fatty acid biosynthesis and breakdown occur by different pathways, are catalyzed by different sets of enzymes, and take place in different parts of the cell. Moreover, malonyl-CoA participates in the biosynthesis of fatty acids but not in their breakdown (Wakil et al., 1983). Acetyl-CoA is generated in two ways in the mitochondria by fatty acid β-oxidation,

pyruvate dehydrogenase and dihydrolipoyl transacetylase, and transported out of the mitochondria to the cytosol. This is accomplished using the tricarboxylate transport system in the inner mitochondrial membrane (Ohlrogge and Jaworski, 1997). Acetyl-CoA is used in synthesis of citrate when combined with oxaloacetate. Citrate transferred into the cytosol is broken back to oxaloacetate and acetyl-CoA by ATP-citrate lyase. Oxaloacetate can be reduced to malate by malate dehydrogenase and NADH. Malate can be converted to pyruvate by malic enzyme and $NADP^+$. The resulting pyruvate is permeable to the inner mitochondrial membrane. Inside the mitochondria, pyruvate can be converted to oxaloacetate by pyruvate carboxylase, completing the cycle (Jungas, 1968). An alternative path is to transport malate across the inner membrane and convert it to oxaloacetate (Fig. 1.1).

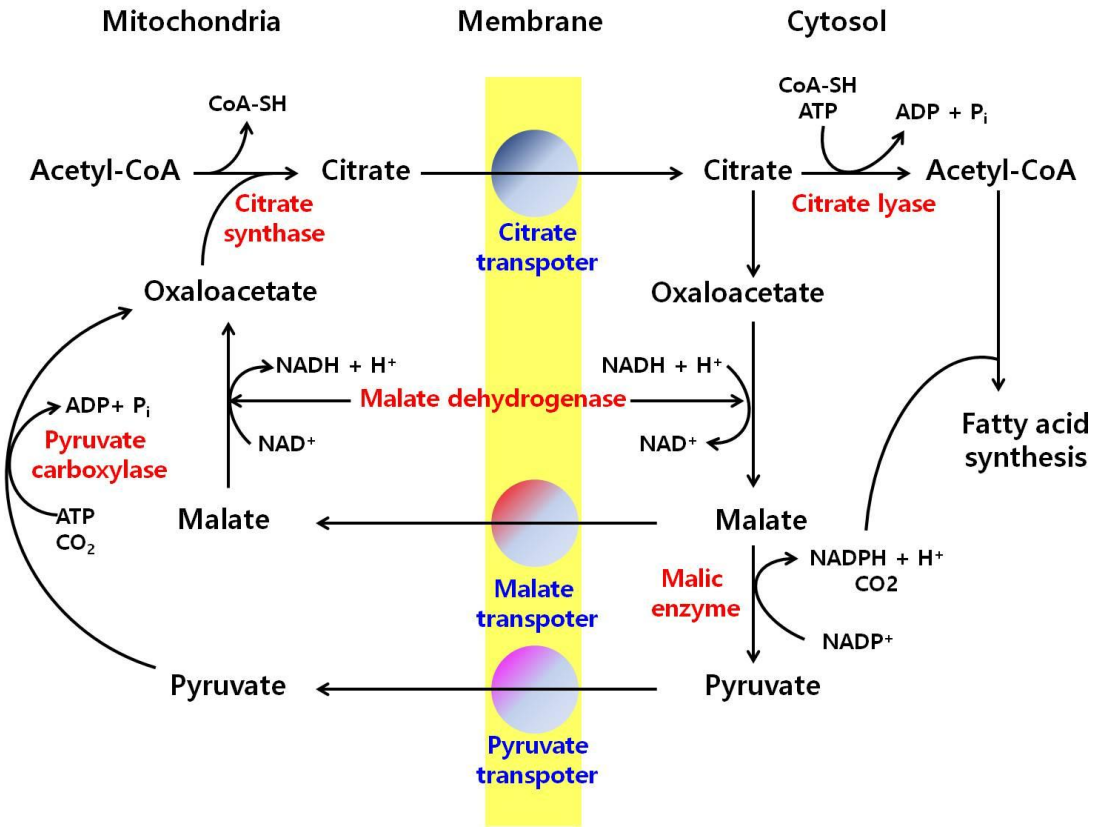

Fig. 1.1 The first phase of fatty acid synthesis starting with acetyl-CoA and oxaloacetate, and ending with pyruvate.

The first step of fatty acid biosynthesis is catalyzed by acetyl-CoA carboxylase (ACC). The ACC contains biotin, and adds a CO_2 to the methyl end of acetyl-CoA. This reaction process requires an energy. The ACC is an interesting enzyme. Studies of the ACC from birds and mammals indicate that it forms long linear polymers. The polymer appears to be the active form of the ACC, whereas monomeric forms are inactive (Kim et al., 2010). Citrate shifts the polymer - monomer equilibrium towards polymer formation. Palmitoyl-CoA shifts the equilibrium towards monomer formation. Another regulation of ACC is by hormones. Glucagon, epinephrine and norepinephrine trigger a cAMP dependent phosphorylation of the enzyme that shifts the equilibrium towards monomer formation. In contrast, insulin stimulates desphosphorylation, favoring polymerization. The enzymes responsible for phosphorylating ACC are cAMP-dependent protein kinase and AMP-dependent protein kinase (Wakil and Abu-Elheiga, 2009). The ACC of *E. coli* is regulated by guanine nucleotides, which are a function of those cells growth requirements. This multifunctional enzyme catalyzes the seven different reactions whereby two carbon units from malonyl-CoA are linked together, to form palmitoyl-CoA. The enzyme complex can exist as both a monomer and dimer. The dimeric form is the fully functional form of the enzyme. The overall synthesis of palmitate from acetyl-CoA requires 14 NADPHs, and 7ATPs (Jungas, 1968).

Steps of fatty acid synthesis starting with acetyl-CoA and malonyl-CoA are shown in figure 1.2. The reactions are as follows transfer of the malonyl group of malonyl-CoA to ACP. Addition of an acetyl group from malonyl-ACP between the thioester bond of the acetyl-ACP molecule. Reduction of the β-keto group to a β-hydroxyl group with NADPH. Dehydration

between the α and β carbons. Reduction of the trans double bond by NADPH. The acetyl group of reaction is replaced by the growing acyl-ACP molecule. The product of this series of reactions, palmitoyl-ACP can be cleaved to palmitate and ACP by the enzyme palmitoyl thioesterase. The multiple enzymatic activities integrated into fatty acid synthase complex are related to the growing fatty acid being "swung" into the appropriate catalytic region of the synthase (Hillgartner et al., 1995). The product of fatty acid synthesis, palmitate, is one of many fatty acids synthesized by cells. Elongase is enzyme that acts to lengthen palmitate to produce many of the other fatty acids. Elongase is present in mitochondria and the endoplasmic reticulum. Elongation using elongase in the mitochondrion involves a mechanism that is essentially the reverse of β-oxidation except substitution of NADPH for $FADH_2$ in the last reaction (Ohlrogge and Jaworski, 1997).

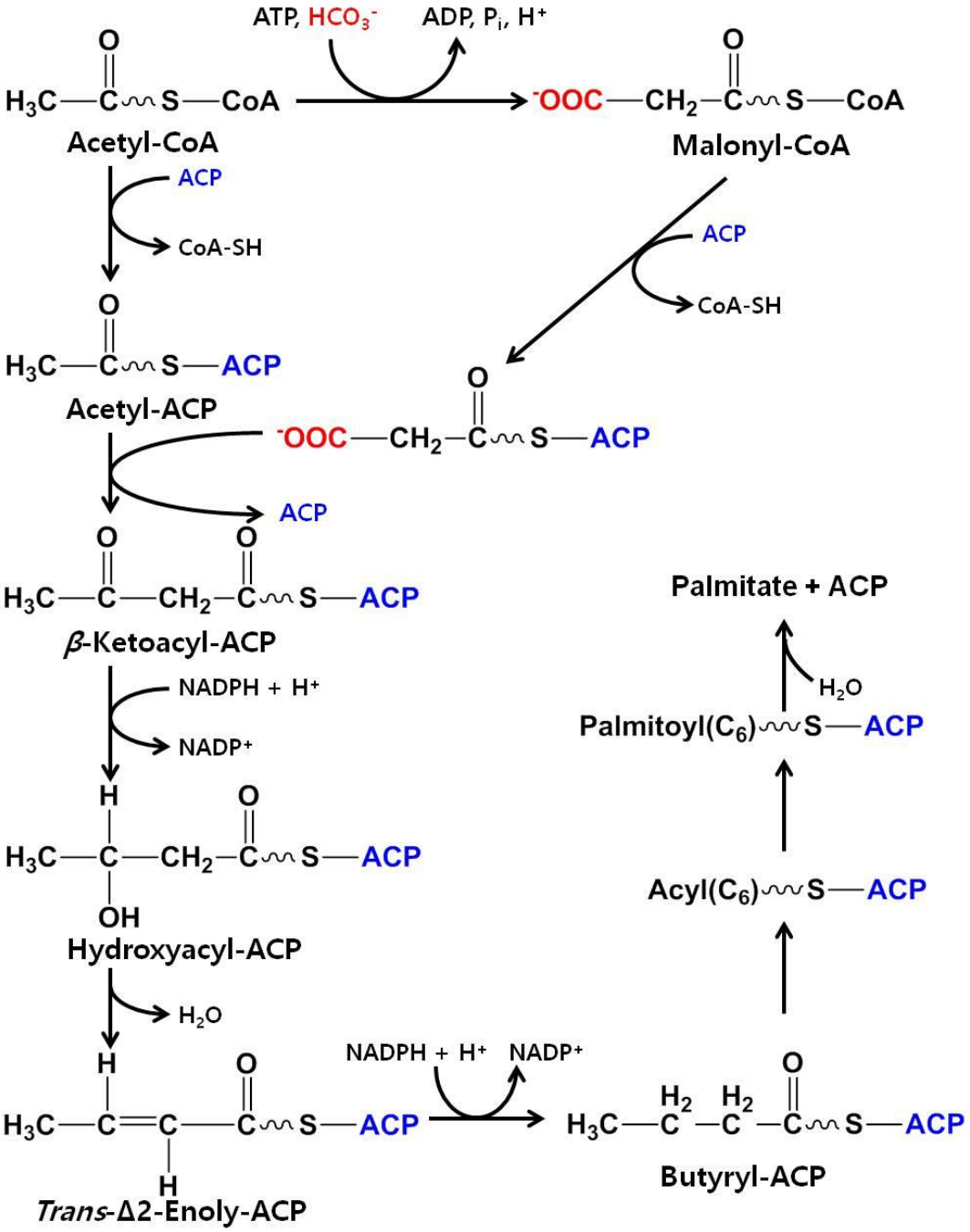

Fig. 1.2 Fatty acid synthesis pathway. ACP; acyl carrier protein.

1.1.2 Fatty acid oxidation

Fatty acids provide highly efficient energy storage, storing much more energy for their weight than carbohydrates. Fatty acids are stored as triglycerides in adipose tissue, in which each triglyceride molecule contains three fatty acids and one glycerol. Triglycerides form fatty droplets that exclude water and take up minimal space. Fatty acids are also more highly reduced than carbohydrates, so they provide more energy during oxidation (Lopaschuk et al., 2010). Fatty acid β-oxidation is a multi process by which fatty acids are broken down by various tissues to produce energy. Fatty acids enter the cell by fatty acid transporters on the cell surface. After enter the cell, fatty acyl-CoA synthase (FACS), forming long chain acyl-CoA, adds a CoA group to the fatty acid. Carnitine palmitoyltransferase 1 (CPT1) converts the long chain acyl-CoA to long chain acylcarnitine. This reaction allows that the fatty acids are transported across the mitochondrial membrane by carnitine translocase (CAT). In inside of mitochondria, the long chain acylcarnitine is converted to long chain acyl-CoA by CPT2. The long chain acyl-CoA can enter the fatty acid β-oxidation pathway, resulting in the production of one acetyl-CoA from each cycle of β-oxidation. This acetyl-CoA enters the mitochondrial tricarboxylic acid (TCA) cycle (Synak and Budohoski, 2000). Four main enzymes involved in fatty acid ß-oxidation include: At the end of each β-oxidation cycle, two new molecules are formed, an acetyl-CoA and an acyl-CoA that is two carbons shorter. During β-oxidation, NADH and $FADH_2$ are formed. One $FADH_2$ is produced during the reaction catalyzed by acyl-CoA dehydrogenase. A NADH is produced during the reaction catalyzed by hydroxyacyl-CoA dehydrogenase. The $FADH_2$ and NADH produced during the process of fatty acid β-oxidation are used by the electron transport chain to produce ATP (Oresic, 2009). An overview of fatty acid oxidation is shown in Figure 1.3.

The regulation of fatty acids can occur at the level of fatty acid entry in to the cell. AMP-activated protein kinase (AMPK), protein kinase C (PKC), and peroxisome proliferator-activated receptor gamma (PPARγ) up-regulate the CD36/FATP activity. Regulation also occurs by the regulation of the acetyl-CoA and malonyl-CoA levels. AMPK inhibits acetyl-CoA carboxylase (ACC), resulting in increased acetyl-CoA levels/decreased malonyl-CoA levels and increased fatty acid oxidation. Malonyl-CoA inhibits fatty acid oxidation by inhibiting CPT1 (Nutting et al., 1999). Transcriptional regulation is also involved in regulating fatty acid β-oxidation. Some transcription factors regulate the expression of proteins involved in fatty acid β-oxidation. The PPARs and a transcription factor co-activator PGC-1α are the most well known transcriptional regulators of fatty acid β-oxidation. PPARs and Retinoid X receptor heterodimerize and bind to gene promoters containing the PPAR response element. Examples of proteins involved in fatty acid β-oxidation that are transcriptionally regulated by the PPARs include FATP, acyl-CoA synthetase (ACS), CD36/FAT, Malonyl CoA decarboxylase (MCD), CPT1, long chain acyl-CoA dehydrogenase (ACADL), and medium chain acyl-CoA dehydrogenase (ACADM). Ligands that bind to and modulate the activity of PPARα, δ, and γ include fatty acids. The transcriptional co-activator PGC-1α binds to and increases the activity of PPARs and ERRα to regulate fatty acid β-oxidation. PGC-1α modulates the activity of a number of transcription factors that can increase the expression of proteins involved in fatty acid β-oxidation, the TCA cycle, and the electron transport chain (Ros, 2000).

Defects in fat metabolism associated with clinical disorders include carnitine or carnitine transferase deficiency, which can lead to weakness and muscle pain during exercise. Defects in acyl-CoA dehydrogenase appear to be involved in some cases of sudden infant death syndrome. Metabolic syndromes such as obesity/diabetes can lead to enlarged adipocytes,

resulting in chronically high circulating free fatty acid supplied as triacylglycerol or VLDL (Bleicher and Lacko, 1992). Chronic free fatty acid may upregulate PPAR alpha and PGC1 alpha pathways directly protoming β-oxidation and decreasing glucose transport (GLUT4) and oxidative glycolysis (PDK4). Hormonal changes in adipose tissue regulate AMPK and ACC (Nakamura and Tando, 2000).

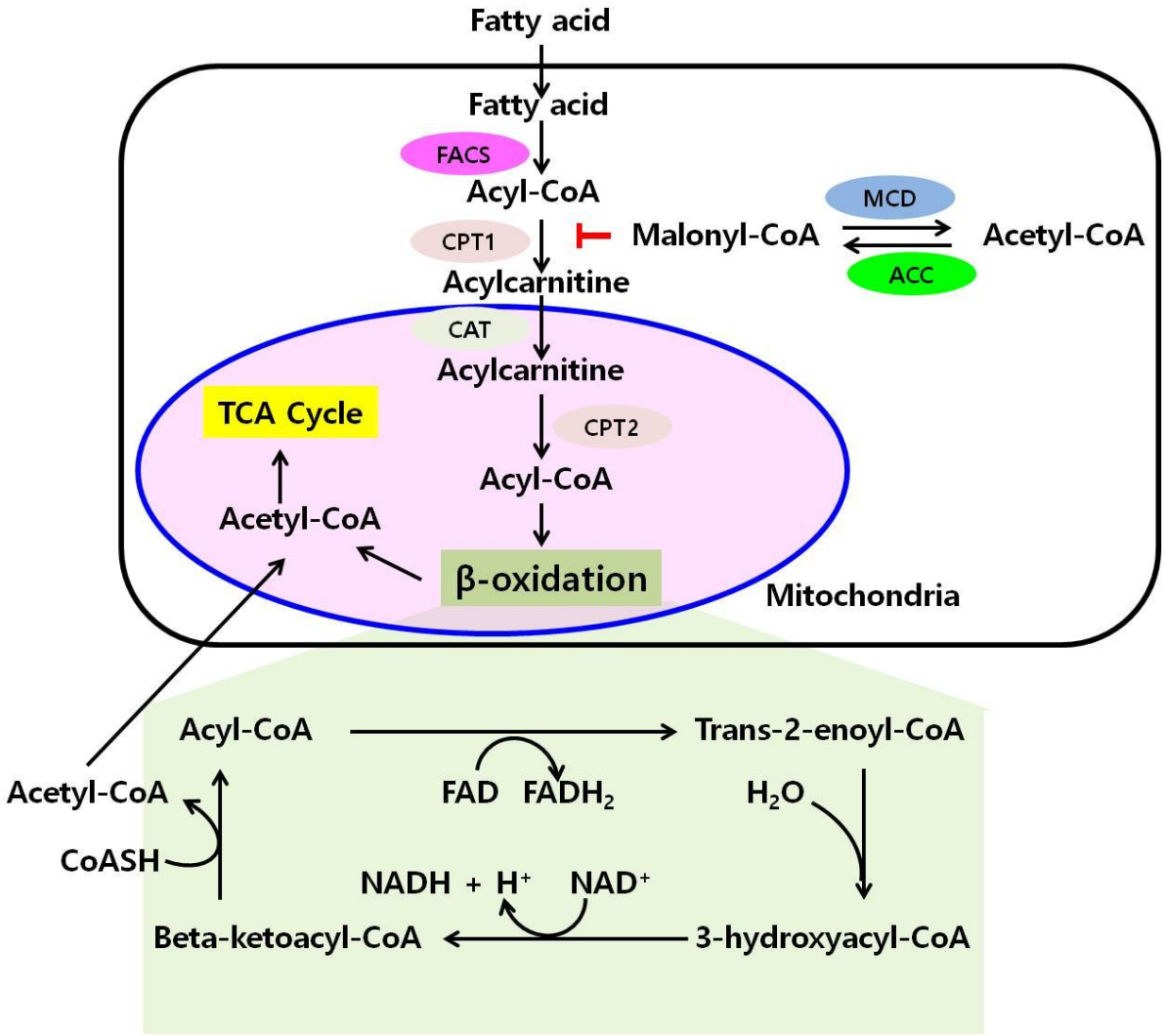

Fig. 1.3 Overview of fatty acid oxidation. ACC; acetyl-CoA carboxylase, CAT; carnitine translocase, CPT; Carnitine palmitoyltransferase, FACS; fatty acyl-CoA synthase, MCD; malonyl-CoA decarboxylase, TCA; tricarboxylic acid.

1.1.3 Acetyl-CoA carboxylase (ACC)

Acetyl-CoA carboxylase (ACC) plays an important role in fatty acid biosynthesis in most of living organisms including animals. The metabolism of carbohydrates to fatty acids is biochemically important for several reasons. First, fatty acids are fuel molecules which provide the major source of energy during rest or exercise. The oxidation of triacylglycerols, which are the storage form of fatty acids, is an important step in meeting certain energy requirements of the cell (Weatherly et al., 2004). Another biochemical reason for the need in fatty acids is that they are building blocks of phospholipids which are important components of biological membranes. Still more basis for the importance of fatty acids include their attachment to proteins to direct them to the right locations within the cell as well as their ability to serve as hormones and other intracellular messengers. It is easy to see that the needs for these long hydrocarbon chains with their terminal carboxylate group, coined fatty acids, are very important for the cells of an organism, and therefore, the need for ACC (Tong and Harwood, 2006).

ACC catalyzes conversion of acetyl-CoA to malonyl-CoA by ATP-dependent carboxylation, which is the first committed step in fatty acid biosynthesis. ACC produces malonyl-CoA by using bicarbonate and ATP. The two step reaction is first carried about by the BC domain in which the prosthetic biotin is carboxylated at the expense of a molecule of ATP. Biotin is covalently bound to the ACC at the terminal amino group of a lysine residue to

the terminal carboxyl group of the biotin side chain. A carboxybiotin intermediate is formed in which the CO_2 is carried on active nitrogen. The second half of the reaction is carried out by the CT domain of ACC in which an acetyl-CoA carbanion performs nucleophilic attack on the carboxybiotinl-enzyme. Once the CO_2 has been placed onto acetyl-CoA, the biotin prosthetic group on the ACC is regenerated and malonyl-CoA is formed (Zagnitko et al., 2001). In this conversion, ACC is a multifunctional enzyme: the ATP-dependent carboxylation of enzyme-bound biotin followed by the transfer of the carboxy group to acetyl-CoA. The biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyltransferase (CT) functions are contained on a single, large (~250 kDa) multi-domain polypeptide in the ACCs found in mammals, yeast, fungi, and plant cytosols. These three enzyme domains are fused in eukaryotic ACCs to form one polypeptide and are separated in *E. coli*, and other bacteria. In contrast, there are four polypeptide chains comprising the multi-subunit enzyme of prokaryotic ACCs with BC, BCCP, and two subunits making up the CT (Mao et al., 2006). ACC is a key regulatory enzyme for fatty acid metabolism. It reacts to changes in its environment such as phosphorylation and dephosphorylation as well as allosteric regulation by nearby metabolites (Zhang et al., 2003). This puts it as a pharmaceutical target for the treatment of obesity, Type II diabetes, cancer and microbial infections. In addition, the product of the carboxylase reaction plays a role in the regulation of fatty acid degradation. Malonyl-CoA is a key metabolic signal for the control of fatty acid production and utilization in response to dietary changes and altered nutritional requirements in animals (Abu-Elheiga et al., 2003; Oh et al., 2005).

As mentioned above, ACC is regulated by its immediate environment. It is inhibited by phosphorylation of a serine residue just before the BC domain as well as at the site located between the BC and CT domains. AMP-dependent protein kinase inactivates ACC thus

diminishing the production of fatty acids (Gao et al., 2007). Contrary to the inactivation of ACC, dephosphorylation by a protein phosphatase activates ACC. In addition, ACC is also allosterically stimulated by citrate, a nearby metabolite. Citrate acts by polymerizing the inactive form of ACC thereby decreasing the inhibition opposed on the enzyme (Zhang et al., 2004). Counteracting citrate is palmitoyl CoA, which is abundant in excess of fatty acids. Palmitoyl CoA acts to disassemble the active form of ACC, thus diminishing the production of fatty acids. Hormones such as epinephrine, insulin and glucagon also regulate ACC. Glucagon and epinephrine switch off fatty acid synthesis by keeping ACC in its inactive state during periods of low energy. Fatty acids can not be synthesized when energy is low because it takes high energy concentrations to make them. On the other hand, the insulin is present at high energy status and stimulates ACC by activating the phosphatase required to remove the phosphate off of inactive enzyme, thus increasing malonyl-CoA synthesis (Zagnitko et al., 2001).

In fatty acid metabolism, there are two distinct ACC forms: ACC1 and ACC2. They are generally expressed in all tissues. However, the ACC1 is more expressed in lipogenic tissues (adipose and liver), whereas the ACC2 is mainly expressed in heart and muscle (Abu-Elheiga et al., 1997). They are encoded by different genes localized at chromosomes 17q12 and 12q23, respectively (Abu-Elheiga et al., 1995). Differences of their cellular localization and molecular weight is due to the first 218 amino acids and hydrophobic N-terminal 20 amino acids of ACC2 (Abu-Elheiga et al., 2000). The ACC1 generally plays for the fatty acid synthesis in the cytosol. In contrast, the ACC2 acts as inhibitor of CPT1 activity and the transfer of the fatty acyl group for β-oxidation (Wakil and Abu-Elheiga, 2009). In this study, we investigated the ACC2 production in silkworms and their characteristics. Therefore, we more describe roles of ACC2 in tissues. In liver and adipose tissues, the malonyl-CoA levels

of ACC2 knockout and wild type mice were similar. In heart and soleus muscle, the malonyl-CoA levels of ACC2 knockout mice were lower than that of wild type mice. The rates of fatty acid oxidation in the ACC2 knockout mice were remarkably higher than that of wild type mice. Thus, this result suggests that the malonyl-CoA produced by ACC2 mainly regulates fatty acid oxidation (Abu-Elheiga et al., 2001a). In addition, under the high fat diet, peripheral and hepatic insulin sensitivities of ACC2 knockout mice were increased. These improvements in insulin-stimulated glucose metabolism were associated with increased insulin-stimulated glucose uptake, decreased lipid accumulation and increased insulin-stimulated AKT activity (Choi et al., 2007). Recent researches in fatty acid metabolism focus on the ACC inhibition for the gene therapy. Selective inhibitors of ACCs and specific ACC2 inhibitors have been investigated (Tong and Harwood, 2006). However, some inhibitors may not be specific against ACCs and they may target other pathways. Their efficiency as potential drugs for the metabolic disorder remains to be determined. Nevertheless, according to previous findings, the ACC2 inactivation can be a promising and valuable attempt for the future treatment of obesity and type II diabetes.

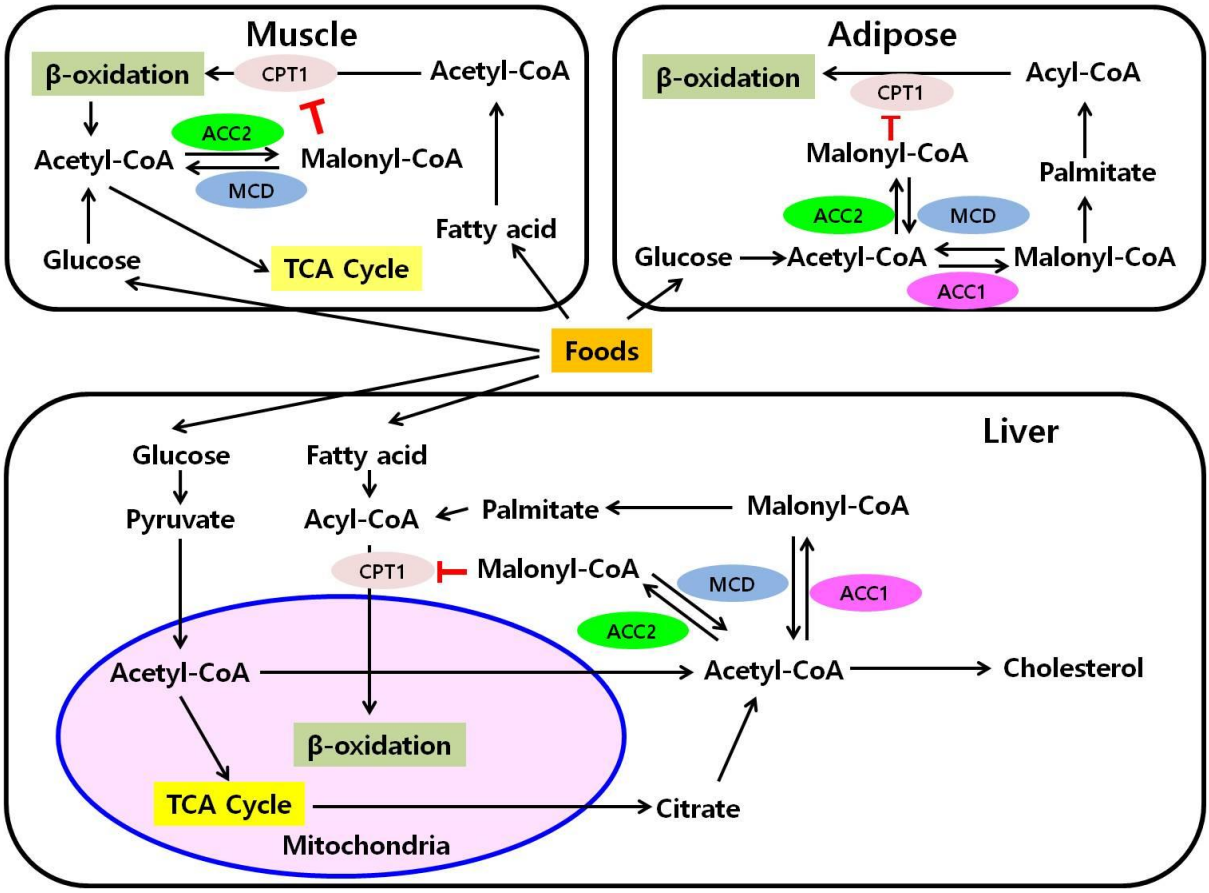

Fig. 1.4 Different roles ACC1, ACC2 and MCD in muscle, adipose and liver tissues.

1.1.4 Malonyl-CoA decarboxylase (MCD)

Malonyl-CoA decarboxylase (MCD, also known as MLYCD) catalyzes the conversion of malonyl-CoA into acetyl-CoA and carbon dioxide. It is involved in fatty acid biosynthesis and associated with MCD deficiency and encoded by the MLYCD gene in humans (FitzPatrick et al., 1999). Various tissues use fatty acids as a major source of energy. These fatty acids are formed and degraded by MCD (Gao et al., 1999). The body also uses fatty acids to form cell membranes, produce hormones, and conducts many other critical processes. As mentioned above, MCD is responsible for the chemical reaction that converts malonyl-CoA into acetyl-CoA. This reaction is an important step in the breakdown of fatty acids (Saggerson, 2008). This enzyme functions in several components of the cell such as

mitochondria and peroxisomes. It also functions in the fluid that surrounds these cell structures (Surendran et al., 2001). MCD is found in several tissues including liver, brain, heart and skeletal muscle tissues. It is localized in the mitochondria, peroxisome and cytosol. MCD deficiency study has been demonstrated the metabolic role of MCD by a rare inborn error of metabolism known as malonic aciduria (Bennett et al., 2001). Depending on the tissue and the metabolic status, MCD can regulate either fatty acid synthesis or oxidation. Malonyl-CoA regulated by MCD serves as the predominant substrate for the synthesis of fatty acids by the cytosol fatty acid synthase complex (FAS) in lipogenic tissues. In case of non-lipogenic tissues, malonyl-CoA functions as an important signaling molecule by its allosteric inhibition of carnitine palmitoyltransferase-1 (CPT-1), the gatekeeper of mitochondrial fatty acid uptake that transports long-chain acyl-CoAs into the mitochondria and is the rate limiting enzymatic step of mitochondrial fatty acid β-oxidation (McGarry and Brown, 1997).

Activity of malonyl-CoA decarboxylase does not require any cofactors or metal ions. Formation of disulfide bonds leads to positive cooperativity between active sites and increases the affinity for malonyl-CoA and the catalytic efficiency (Zhou et al., 2004). When an isolated soleus is deprived of glucose, malonyl-CoA levels diminished by 50%, and it suggests that malonyl-CoA utilization as well as synthesis may be regulated (Alam and Saggerson, 1998; Saha et al., 1995). Evidence has been reported that MCD exists in both cardiac and skeletal muscle. In skeletal muscle, MCD activity is similar to that of ACC and is 10-40 folds higher than that of fatty acid synthase (Goodwin and Taegtmeyer, 1999). In heart, a 10-fold increase in fatty acid oxidation observed between days one and seven after birth is accompanied by 80-95% decreases in ACC activity and malonyl CoA levels and a significant, although more modest (40%), increase in MCD activity (Sakamoto et al., 2000). Increases in

MCD activity have been observed in rat liver during starvation and in skeletal muscle in response to electrically induced contractions (Dyck et al., 2000; Saha et al., 2000). The increase in activity was attributable to activation of AMP-activated protein kinase (AMPK) an enzyme that also phosphorylates and inhibits ACC. The role of MCD in regulating the concentration of malonyl-CoA remains open to question as are the mechanisms by which its activity is regulated (Jang et al., 1989). The increase in MCD activity is accompanied by decrease in ACC activity and malonyl-CoA concentration. Such a coordinate regulation of ACC and MCD attributable to AMPK has also been observed in rat muscle made to contract by electrical stimulation of the sciatic nerve *in vivo* and following incubation of the rat extension digitorum longus muscle with the AMPK activator AICAR. MCD and ACC2 might be jointly regulated by AMPK. The importance of this dual control of MCD and ACC2 activities to the regulation of the cytosolic concentration of malonyl-CoA and secondarily to fatty acid oxidation remains to be determined (Park et al., 2002).

In the fatty acid biosynthesis MCD selectively removes malonyl-CoA and thus assures that methyl-malonyl-CoA is the only chain elongating substrate for fatty acid synthase and that fatty acids with multiple methyl side chains are produced. In peroxisomes it may be involved in degrading intraperoxisomal malonyl-CoA, which is generated by the peroxisomal beta-oxidation of odd chain-length dicarboxylic fatty acids. Plays a role in the metabolic balance between glucose and lipid oxidation in muscle independent of alterations in insulin signaling. May play a role in controlling the extent of ischemic injury by promoting glucose oxidation (Aparicio et al., 2013).

1.1.5 Necessity of acetyl-CoA carboxylase 2 and malonyl-CoA decarboxylase studies

The ACC2 and MCD have a strong impact on the lipid and overall energy metabolism.

They have important role because catalyses the malonyl-CoA and acetyl-CoA formation and an essential enzymes for fatty acid synthesis and oxidation. The overall significance of these enzymes has been reported with ACC and MCD deficient studies (Abu-Elheiga et al., 2001a; Brown et al., 1984; Choi et al., 2007; Haan et al., 1986; Kolwicz et al., 2012; Krawinkel et al., 1994; MacPhee et al., 1993; Matalon et al., 1993; Yano et al., 1997). Todays, the regulation of these enzymes activities has been recognized as an interesting therapeutic target for lipid metabolism disorder such as cancer, cardiovascular disease, obesity and type II diabetes (Tong and Harwood, 2006). In this respect, to investigate the detailed reaction mechanism and functional analysis of these enzymes, they have to be produced sufficient amounts and purified into high purity. Moreover, post-translational modifications of expressed enzymes are also important for the identification of enzyme characteristics. The expression studies of these enzymes have been generally used *E. coli* or insect cell lines (Cheng et al., 2007; Kim et al., 2007; Kwon et al., 2013; Lee et al., 2002; Zhou et al., 2004). Although *E. coli* expression system is wildly used for the protein production, the proteins expressed by *E. coli* often show the poor post-translational modifications. In case of insect cell lines, it often provides insufficient amounts of recombinant protein. Therefore, more versatile, fast, easy, inexpensive and advanced expression system using eukaryote is required. In addition, ACC2 and MCD expressed by advanced expression system have to be evaluated their post-translational modifications and investigated alteration in functions by removing the post-translational modifications.

1.2 Baculovirus expression system

Baculovirus expression system (BES) is one of the most powerful eukaryotic expression systems, and it can be variously applied on proteomics (O'Reilly DR, Miller LK, 1992). The

BES is a helper-independent virus system in contrast with adenovirus or retrovirus expression system. Therefore, this system has been world-widely used for various gene expression including animal, plant, fungus, bacteria and virus due to the simple expression method (Kidd and Emery, 1993). Since first developed in 1980's, various baculovirus expression vectors have been developed and it has been used by quite a number of researchers. As a result, currently, they have a place representing the expression systems (Fraser, 1992). In recent years, application fields of BES have been more expanded such as medicine industry for gene therapy. Consequently, the research and development of medicine using BES has flourished (Hüser and Hofmann, 2003). In this chapter, we describe the characteristics of baculovirus, construction of expression systems and their prospect.

1.2.1 Characteristics of baculovirus

The baculoviruses known as vector system for gene expression is non pathogenic virus to human or vertebrate animals that has pathogenicity to only insect species. These baculoviruses are rod-shaped viruses that can be divided to two groups of genus: nucleopolyhedroviruses (NPV) and granuloviruses (GV). Up-to-date, they have been isolated from about 600 of insect hosts (Rohrmann, 1992). Among them, the immature (larval) forms of moth species are the most common hosts. In early studies, baculoviruses were used and studied widely as biopesticides in crop fields. Since the 1990s they have been utilized for producing complex eukaryotic proteins in insect cell cultures. These recombinant proteins have been used in research and as vaccines in both human and veterinary medical treatments For example, the most widely used vaccine for prevention of H5N1 avian influenza in chickens was produced in a baculovirus expression vector. More recently it has been found that baculoviruses can transduce mammalian cells with a suitable promoter. These medical

and potential medical uses have accelerated the number of publications on baculoviruses since 1995 (Lackner et al., 2008).

In baculovirus expression system, the NPV is commonly used for expression of proteins. The structure of NPV consists of rod-shaped nucleocapsid with about 88-153 kb of double-stranded circular supercoiled DNA (Burgess, 1977). This nuleocapsid forms about 20-50 nm in diameter, 200-400 nm in length of virus particle with membrane. This virus particle is inserted in about 28-33 kDa of polyhedrin. The size of this pholyhedrin virus is about 0.5-15 μm (Fig.1.5). This polyhedrin protects and maintains the activity of virus particle in the natural environment (Blissard and Rohrmann, 1990; Rohrmann, 1986). The proliferation process of NPV can be divided into two types by the source of infection: PDV (polyhedral derived virus) as a first infectious pathogen and BV (budded virus) as a secondary infectious pathogen. Typically, the initial infection occurs when a susceptible host insect feeds on plants that are contaminated with the PDV. The protein matrix dissolves in the alkaline environment (pH 10) of the host stomach. The releasing PDV fuse to the columnar epithelial cell membrane of the host intestine and are taken into the cell in endosomes. Nucleocapsids escape from the endosomes and are transported to nucleus. This step is possibly mediated by actin filaments. Viral transcription and replication occur in the cell nucleus and new BV particles are budded out from the basolateral side to spread the infection systemically. During budding, BV acquires a loosely fitting host cell membrane with expressed and displayed viral glycoproteins (Summers and Smith, 1978). Baculovirus infection can be divided to three distinct phases, early (0–6 h post-infection), late (6–24 h p.i.) and very late phase (18–24 to 72 h p.i.) (O'Reilly DR, Miller LK, 1992). While BV is produced in the late phase, the PDV form is produced in the very late phase acquiring the envelope from host cell nucleus and embedded in the matrix of occlusion body protein. These occlusion bodies are released when

cells lyse to further spread baculovirus infection to next host. The extensive lysis of cells frequently causes the host insect to literally disintegrate, thus the reason for the historic name "wilting disease" (Ayres et al., 1994). The complete PDV-polyhedrin particles are resistant to heat and light inactivation, whereas the naked BV virion is more sensitive to environment (Kool and Vlak, 1993).

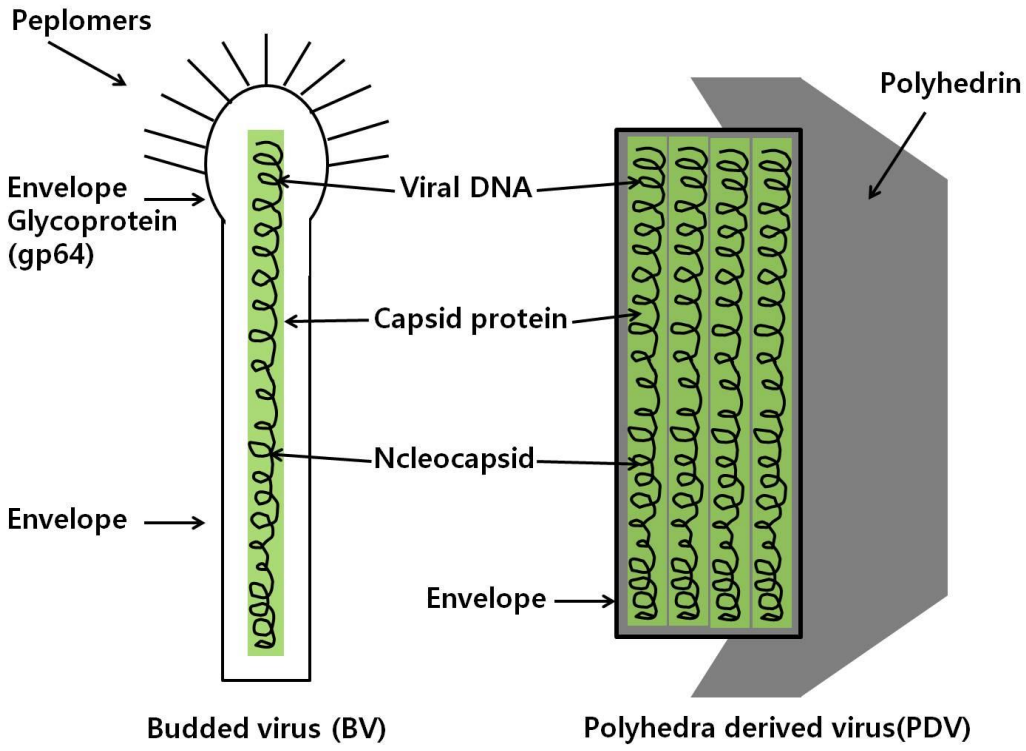

Fig. 1. 5 Comparison of two types of baculoviruses. PDV and BV act as primary and secondary infectious pathogens, respectively.

1.2.2 Baculovirus expression vector systems

1.2.2.1 Development of baulovirus expression vector systems

The polyhedrin of baculovirus is synthesized until 30-50% of total protein in an infected cell. This phenomenon was identified that occurred by strong promoter. Consequently, baculovirus expression vector system was developed and that can express the foreign gene using promoter (Smith et al., 1983a). On the study of baculovirus expression vector system, Smith et al (1983b) first reported the human β-interferon expression using expression vector system combined spodoptera frugiperda (Sf) culture cell line with *Autographa california* NPV (AcNPV). Since that time, Maeda et al (1985) successfully expressed the human α-interferon using expression vector system combined *Bombyx mori* NPV (BmNPV) with silkworm larva. Thus, these two kinds of vector systems are widely used for protein expression studies.

1.2.2.2 Characteristics of baculovirus expression vector systems

The reason of increased interest on development of baculovirus expression vector systems is that BEVS can remedy the disadvantage and remain the advantage of existing expression systems. In BEVS, the expressed proteins have similar characteristics with original protein by using the eukaryotic cell line. Moreover, the productivity and production speed are superior in comparison with other expression vector system using animal cell lines (Emery, 1992). Often, recombinant proteins expressed in bacterial systems are insoluble, aggregated and incorrectly folded. In contrast, proteins expressed in BEVS are, in most cases, soluble and functionally active (Marston, 1986). Thus, there has been a growing interest in BEVS studies.

The expressed recombinant proteins derived from baculovirus are usually localized in the

same subcellular compartment as the genuine protein. Nuclear proteins are transported to the insect nucleus, membrane proteins are anchored into the cell membrane, and secreted proteins are secreted by infected insect cells. In the baculovirus expression systems, several post-translational modifications have been reported to occur including glycosylation, phosphorylation, acylation, amidation, carboxymethylation, isoprenylation, signal peptide cleavage and proteolytic cleavage. The sites of these modifications are often identical to those of the authentic protein in its native cellular environment (Höss et al., 1990). However, the BEVS can express the gene of interest at a high expression rate which may overwhelm the ability of the cell to modify the protein product (Kloc et al., 1991). This often results in lower levels of glycosylation or phosphorylation of the target protein than in the native cell line. Also, tissue- or species-specific post-translational modifications will not be performed in the BV, unless the modifying enzyme is being co-expressed (Kuroda et al., 1991). Compared to other eukaryotic expression systems using animal cell lines, the most outstanding characteristic of the BEVS is its potential to achieve high levels of expression of a cloned gene (Baixeras et al., 1990). The BEVS has proven particularly useful in the production of large quantities of proteins for structural and activity analysis. The highest expression level reported is 50% of the total cellular protein of an infected insect cell corresponding to approximately 1g of recombinant protein per 1×10^9 cells (Brandt-Carlson and Butel, 1991). However, many recombinant proteins are not produced at such high amounts and it is usually difficult to predict the amount of protein expression. Therefore, many researchers are continuing to study for the optimized protein production (Caroni et al., 1991). Insect cells have the capability to perform intron/exon splicing. However, certain virus-, tissue- or species-specific splicing patterns will not be obtained if they require the presence of particular splicing factors which are not available in the infected insect cell environment

(Christensen et al., 1993). In general, for high protein expression levels, a cDNA insert rather than a genomic DNA fragment is recommended. The expandability of the capsid structure of Baculoviruses allows the packaging and expression of very large genes. There is no known upper size limit for the insertion of foreign sequences into the BV genome (Mattion et al., 1991). BEVS has the capability to express two or more genes simultaneously within single infected insect cells. Protein complexes that depend on dimer or multidimer formation for activity can be assembled. A well known example is the formation of complete virus capsids from a variety of viruses which have been assembled in vitro, using BEVS, by co-expressing the capsid subunits simultaneously (Hsu et al., 1991). The BEVS produces over-expressed recombinant proteins containing proper folding, disulfide bond formation and oligomerization. Moreover, this system is capable of performing several post-translational modifications. This leads to a protein that is similar to its native counterpart, both structurally and functionally (Kidd and Emery, 1993).

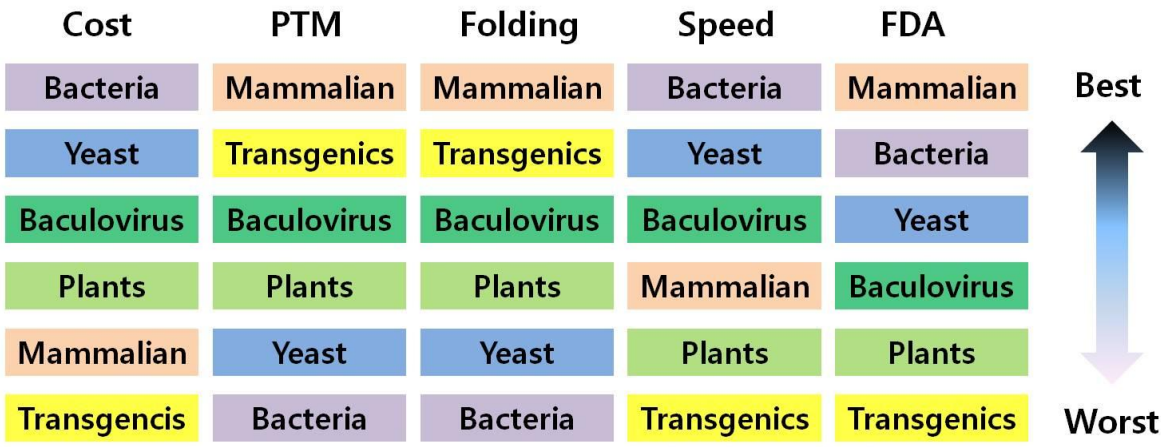

Fig. 1.6 Comparison of strengths and weaknesses in various expression systems. PTM; post-translational modification, FDA; Food and Drug Administration.

1.2.3 Protein production using silkworm larvae and pupae

The best advantage of BmNPV expression vector system is that can produce using silkworms. The use of silkworms as a living bio-factory for protein production has been explored as an alternative to cell-culture technology (Medin et al., 1990). A wide variety of recombinant proteins have been efficiently expressed in insect larvae using recombinant baculoviruses as vectors, including enzymes, antibodies, hormones, vaccines, cytokines and diagnostic proteins (Mathavan et al., 1995). Most of these antigens were processed correctly after synthesis, and their antigenic activities remained intact in soluble larval protein extracts (Kuroda et al., 1989). The advantages of using silkwroms as a recombinant protein production system include the dramatic reduction in production costs with respect to insect cell cultures, increased recombinant protein yields, the absence of high-tech fermentation procedures, reduced development times and easy production scale-up. These advantages make the use of insect larvae as a bio-factory a legitimate alternative to standard cell-culture fermentation systems (Millán et al., 2010).

1.2.4 *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid expression system

In BES, there are two kinds of baculoviruses: *Autographacalifornia* nucleopolyhedrovirus (AcNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV). One big difference of these baculoviruses is their host specificities. The BmNPV can infect silkworm and its cell lines, however, the AcNPV can infect only *Spodoptera frugiperda* and *Tricoplusia ni* (Luckow et al., 1993). In addition, the difference between silkworm and its cell lines is that the protein production using silkworm is maximum 100-fold higher than that of using *B. mori* cell lines.

Thus, the BmNPV baculovirus expression system using silkworm is one of the most effective systems for large amount of recombinant protein production and will be very attractive protein production system (Zhu et al., 1998). However, these expression systems require long time (at least 40 days) for the preparation of recombinant baculovirus. Therefore, firstly the AcNPV bacmid (a baculovirus shuttle vector) system had been developed for effective gene expression. This AcNPV bacmid can be replicated as a large plasmid and generate the recombinant virus DNA by the site specific transposition in *E. coli*, and remains infectious with insect cells (Kato et al., 2004). Afterward, the BmNPV bacmid system also had been developed for BmNPV directly infectious to silkworm. Motohashi et al (2005) reported the establishment of the first practical BmNPV bacmid system for expression of green fluorescent protein (GFP) gene in *B. mori* silkworm larvae, pupae and its cell line. They demonstrated the high levels of protein expression and recombinant bacmid could directly inject into silkworms. Since the development of BmNPV bacmid system by Motohashi et al (2005), it has been widely used due to the low cost, convenience, and high safety for biohazard. In this study, we also used this BmNPV bacmid system for expression of recombinant proteins.

1.2.5 Bac-to-Bac system for *Bombyx mori* nucleopolyhedrovirus bacmid

Todays, approximately thousand genes have been studied and successfully expressed using recombinant AcNPV and BmNPV expression system. As mentioned above, the conventional preparation of recombinant virus to express foreign genes is very time consuming due to the requirement of multiple rounds for amplification and purification of recombinant virus (Acharya et al., 2002) (Fig. 1.7). Recently, therefore, the newly developed

Bac-to-Bac system for BmNPV has overcome this drawback (Luckow et al., 1993). The procedure of Bac-to-Bac system is illustrated in Figure 1.8. This Bac-to-Bac system is a rapid and efficient method to generate recombinant baculoviruses. pFastbac1 and pFastbac dual plasmids are generally used to generate viruses which will express single and fused recombinant proteins, respectively. Target genes are inserted into the multi cloning site of pFastbac plasmid. Then, the recombinant pFastbac-target gene can be transformed into a baculovirus shuttle vector (bacmid) in *E. coli* by site-specific transposition (Luckow et al., 1993). The bacmid contains mini-F replicon, segment of DNA encoding the *lac*Zα and Kanamycin resistance marker. The bacmid can complement a *lac*Z deletion present on the chromosome to form colonies that are blue color in the presence of X-gal or Bluo-gal and Isopropyl β-D-1-thiogalactopyranoside (IPTG). These recombinant bacmids are produced by transposing a mini-Tn7 element from a donor plasmid to the mini-*att*Tn7 attachment site on the bacmid. The Tn7 transposition is provided by a helper plasmid. The helper plasmid provides resistance to tetracycline and encodes the transposase (Hitchman et al., 2009). This bacmid using bac-to-bac system to insert target genes has various advantages in the production of recombinant baculocviruses. The recombinant virus DNA can be isolated from selected colonies by blue white screening. This system also provides significantly reduced time to identify and purify the recombinant virus due to the elimination of multiple rounds of plaque purification. Traditional methods take times about 4 to 6 weeks, whereas this bac-to-bac system takes only 7 to 10 days. Thus, superior advantage of this system is that it allows the rapid and simultaneous isolation of recombinant viruses, and it is reasonable method for the protein expression for functional and structural studies (Belzhelarskaia, 2011).

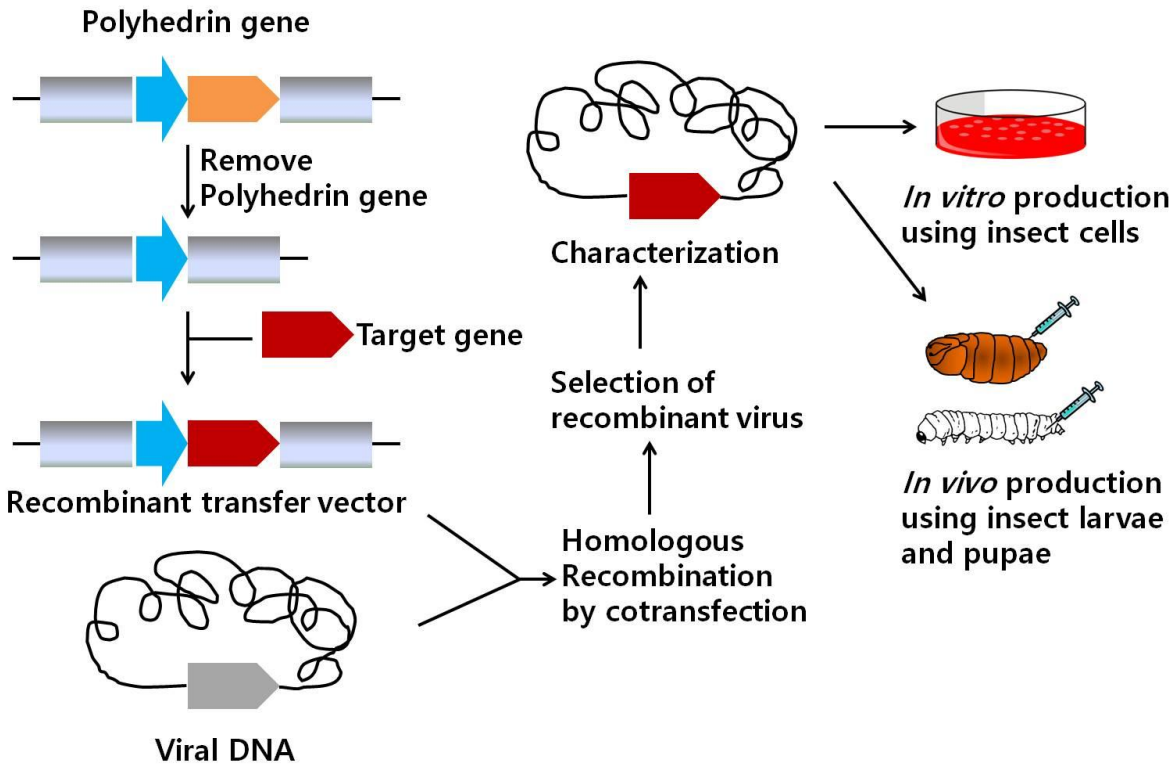

Fig. 1.7 Construction and production of conventional baculovirus expression vector system.

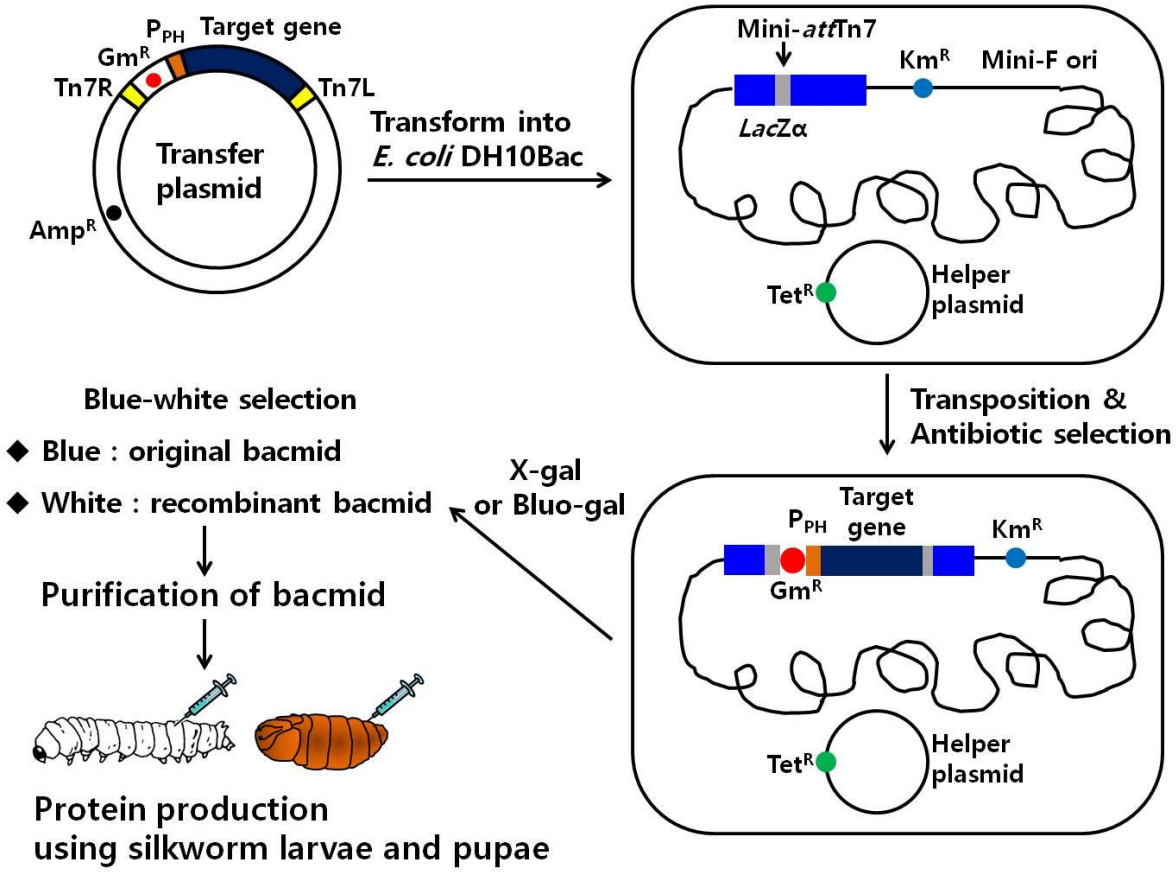

Fig. 1.8 Construction of recombinant bacmid by Bac to Bac system. Amp^R; Ampiciliin resistance, Gm^R; Gentamycin resistance, Km^R; kanamycin resistance, Tet^R; tetracycline resistance, P_{PH}; polyhedrin promoter.

1.2.6 Cysteine protease and chitinase deficient BmNPV bacmid

As described above, the bacmid system is very useful tool for the production of recombinant eukaryotic proteins due to the remove baculovirus amplification step. However, after infection by recombinant bacmid, the silkworm larvae were liquefied at around 5 days. In recombinant proteins production using silkworm larvae, host liquefaction often leads to the loss of proteins. This host liquefaction is caused by a papain type cysteine protease with

cathepsin L-like characteristics, and a chitinase (Hawtin et al., 1997; Ohkawa et al., 1994; Slack et al., 1995). To overcome this drawback, BmNPV lacking the cysteine-protease gene was constructed, and firefly luciferase and human growth factor were very efficiently produced in the silkworm (Suzuki et al., 1997). This was due to the markedly reduced degradation that is usually caused by the cysteine protease. In addition, recently, a BmNPV-*CP*$^{-}$-*Chi*$^{-}$ (cysteine protease and chitinase deleted BmNPV) bacmid was developed using lambda recombination system (Park et al., 2008a). This bacmid system leads to improved human β3GnT2 fused to GFP_{UV} (green fluorescent protein excited with UV light) production in silkworm larvae. Thus, BmNPV-*CP*$^{-}$-*Chi*$^{-}$ bacmid can be utilized as a very effective expression vector for the stable production of target foreign proteins, particularly unstable proteins susceptible to degradation by cysteine proteases. In this respect, the BmNPV-*CP*$^{-}$-*Chi*$^{-}$ bacmid is remarkable expression tool as an expression vector for the production of recombinant protein in silkworms.

1.2.7 Prospect of baculovirus expression system

Recently, the baculovirus expression system has been more studied about application on medical science such as a gene therapy than that of uncomplicated gene over-expression (Pieroni and La Monica, 2001). Interestingly, baculovirus can infiltrate into all most of animal cell lines and it is very safe virus for human. Therefore, some researchers trying to use for human as a gene transporter and they are also doing the clinical test research (Hüser and Hofmann, 2003). Moreover, the development of medicine such as a vaccine using BES is already in progress (Kost and Condreay, 2002). Thus, the utilization and demand of BES will be widened to improve the human health in the future.

1.3 Objectives of this study

The primary objectives of this work were production of human acetyl-CoA carboxylase 2 and malonyl-CoA decarboxylase and investigation of effects of post-translational modifications on enzyme functions. To achieve these goals, we used silkworm-based baculovirus expression system for expression the proteins and evaluated the enzyme activity by alteration of post-translational modifications. Detailed experiments were described below:

1) Expression and purification of ACC2 and MCD by baculovirus expression system using silkworm larvae and pupae.
2) Confirmation of post-translational modifications such as a biotionylation and phosphorylation of purified ACC2 and MCD by western blot analysis using ACC2 and MCD specific antibodies.
3) Dephosphorylation of ACC2 and MCD by treatment of phosphatase and site-directed mutagenesis, respectively.
4) Evaluation of citrate-induced polymerization of ACC2 by citrate concentration.
5) Investigation of effects of dephosphorylation and polymerization on enzyme activity.

Chapter 2

Human acetyl-CoA carboxylase 2 expressed in silkworm *Bombyx mori* possesses post-translational biotinylation and phosphorylation

2.1 Introduction

Acetyl-CoA carboxylases (ACCs) are biotin-dependent enzymes catalyzing the production of malonyl-CoA from acetyl-CoA, a critical metabolic intermediate in lipid metabolism (Brownsey et al., 2006; Kim, 1997; Saggerson, 2008; Tong, 2013; Wakil and Abu-Elheiga, 2009). Two different isoforms of ACC, ACC1 and ACC2, partake in lipid metabolism in humans and mammals (Abu-Elheiga et al., 1995, 1997; Ha et al., 1996). ACC1, encoded by *ACACA*, predominantly exists in cytosol of lipogenic tissues such as adipose and liver where malonyl-CoA functions as a substrate for long chain fatty acids synthesis. In contrast, *ACACB*-encoded ACC2 is associated with the outer membrane of mitochondria in the oxidative tissues such as heart, liver and skeletal muscle where malonyl-CoA is utilized as a negative regulator of fatty acid oxidation. Due to the bifunctional roles in catabolic and anabolic metabolism, ACC functions as a bioenergetics controller to promote stem cell function and tissue regeneration to regulate lipid homeostasis (Folmes et al., 2013; Fullerton et al., 2013; Knobloch et al., 2013; Park et al., 2013). Moreover, ACC2 knockout demonstrates anti-obesity effects and prevention of cardiac remodelling (Abu-Elheiga et al., 2001b, 2003; Kolwicz et al., 2012). Therefore, ACC activity regulation has been recognized as an attractive therapeutic target for dysregulated lipid metabolism such as obesity, type 2 diabetes, cancer, and cardiovascular disease (Tong and Harwood, 2006).

Eukaryotic ACCs, unlike prokaryotic ACCs composed of three separate functional proteins, comprises three distinctive functional domains, including a biotin carboxylase (BC) domain, a biotin carboxyl carrier protein domain (BCCP), and a carboxyltransferase (CT) domain, to carry out multiple functions. Integral in catalysis is the biotin, a prosthetic group attached to lysine residue within the BCCP domain (Bianchi et al., 1990; Cronan and Waldrop, 2002; Tanabe et al., 1975; Tong, 2013). The BC domain catalyzes the Mg-ATP

dependent carboxylation of biotin to form carboxybiotin using bicarbonate as the CO_2 donor (reaction 1). Then, the carboxybiotin is transferred to the CT domain mediating the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA (reaction 2). Besides these core catalytic reactions, ACC activities are allosterically regulated by multiple factors including phosphorylation/dephosphorylation and citrate (Beaty and Lane, 1983a; Brownsey et al., 2006; Ha et al., 1994; Meredith and Lane, 1978; Munday et al., 1988; Wojtaszewski et al., 2003). Thus, ACC with post-translational biotinylation and properly regulated by allosteric modulators is essential in evaluating the full functionality of multi-step reactions to unfold its functional mechanisms and systematic inhibitor discovery efforts.

$$ATP\text{-}Mg_2^{+} + HCO_3^{-} + \text{ACC-biotin} \leftrightarrow \text{ACC-biotin-}CO_2^{-} + ADP + P_i \quad \text{(reaction 1)}$$

$$\text{ACC-biotin-}CO_2^{-} + \text{acetyl-CoA} \leftrightarrow \text{malonyl-CoA} + \text{ACC-biotin} \quad \text{(reaction 2)}$$

The baculovirus expression system has been considered as the most efficient eukaryotic heterologous protein expression system as the host insect cells can implement foolproof post-translational modifications similar to higher eukaryotes (Kost et al., 2005; Possee, 1997). Developed in the 1980s, two types of baculovirus expression systems, i.e., Autographacalifornia multiple nucleopolyhedrovirus (AcMNPV) and Bombyx mori nucleopolyhedrovirus (BmNPV) systems, have been widely used (Kost et al., 2005; Maeda, 1989). We developed a BmNPV bacmid, an Escherichia coli and B. mori hybrid shuttle vector, to expedite the heterologous protein production platform without construction and amplification of viruses in Bombyx mori culture cells as a recombinant BmNPV DNA can be directly injected into silkworm pupae or larvae (Hiyoshi et al., 2007; Motohashi et al., 2005; Park et al., 2008b). Using the BmNPV bacmid system, intracellular, extracelluar and membrane proteins have been successfully generated with proper folding and post-translational modifications (Kato et al., 2010, 2012; Otsuki et al., 2013).

Here, we examined whether the recombinant human ACC2, produced using silkworm BmNPV bacmid-based approach, secures proper post-translational modifications to fulfil the essential catalysis and allosteric modulation. We report that ACC2, demonstrating consistent catalytic activities with proper post-translational biotinylation and phosphorylation, is regulated by allosteric modulator. Thus, silkworm-based BmNPV system provides a reliable large-scale protein production platform for structural and function studies as well as drug discovery applications implementing essential post-translational modifications.

2.2 Materials and Methods

2.2.1 Construction of recombinant hACC2 BmNPV bacmid

The human ACC2 (2548 amino acids) is associated with the mitochondria membrane through N-terminal 148 hydrophobic amino acids classified as mitochondrial attachment and target sequences (Tong, 2013). To increase the solubility of recombinant hACC2, these hydrophobic residues were excluded using polymerase chain reaction (PCR) with the following set of primers, 5´-GCCGTCGACATGTCCAAAGAAGACAAGAAGCAG-3´ (forward), 5´-GCTCTAGATTACTTGTCATCGTCATCCTTGTAGTCGGTGGAGGCCGG GCTGTCCATG-3´ (reverse), and designated as Δ148aa-hACC2. The PCR cycle was performed following 30 cycles of denaturation at 94℃ for 30s, annealing at 55℃ for 30s, and extension at 72℃ for 7 min, followed by a final extension at 72 ℃ for 10 min. The complementary DNA of human ACC2 from Mammalian Gene Collection (Thermo Scientific, Pittsburgh, PA, USA) was used as a template. The resultant PCR product was digested with

SalI and XbaI followed by purification with a GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, UK). The purified DNA fragment was ligated into pFastbac 1 vector, which was transformed into E. coli competent DH5α cells (Invitrogen, Carlsbad, CA, USA) and cultured on a solid LB medium containing 100 μg/mL of ampicillin at 37℃ for 18 h to generate recombinant plasmid. The plasmid containing human Δ148aa-hACC2 gene was isolated and identified by DNA sequencing. Finally, E. coli BmDH10bac-CP–-Chi– competent cells containing the cysteine proteinase- and chitinase-deficient BmNPV bacmid (Park et al., 2008b) were transformed with the pFastbac1-Δ148aa-hACC2 and cultured on a solid LB medium containing 50 μg/mL of kanamycin, 7 μg/mL of gentamycin, 10 μg/mL of tetracycline, 40 μg/mL of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μg/mL of 5-bromo-4-chloro-3-indolyl-4-galactoside (X-Gal) (Takara Bio Inc., Otsu Shiga, Japan) at 37℃ for 18 h. The bacmid containing BmNPV-Δ148aa-hACC2 was isolated from white positive colonies.

2.2.2 Expression and purification of recombinant hACC2 in silkworm

Silkworm pupae were used for the expression of recombinant Δ148aa-hACC2 as a bioreactor. To produce recombinant protein in pupae, 10 μg of BmNPV-Δ148aa-hACC2 bacmid DNA was directly injected with DMRIE-C reagent (Invitrogen) into the dorsal of pupae. The injected pupae were reared at 27℃ for 6–7 days, and stored at –80℃ until further analysis. Protein purification was carried out at 4℃ to minimize aggregation and protease activity. Five pupae were homogenized in 10 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.1% TritonX-100) containing an EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany) using a homogenizer (GLH-115, Yamato, Tokyo, Japan). Cell

debris was removed by pelleting through centrifugation at 12,000 *g* for 30 min. The supernatant was filtered using a 0.45 μm syringe filter and loaded onto a 500 μL of Anti-FLAG M2 antibody Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with equilibration buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.02% TritonX-100). The column was washed with 2.5 mL of equilibration buffer and eluted with elution buffer (100 μg/mL FLAG peptide in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4). The eluted Δ148aa-hACC2 was collected and concentrated using a 100 K Amicon Ultra centrifugal filter (Millipore, Billerica, MA, USA).

2.2.3 Confirmation of biotinylation and phosphorylation by Western blotting

The post-translational biotinylation and phosphorylation of purified Δ148aa-hACC2 were measured by Western blotting analysis. Prior to electrophoresis, purified sample was boiled for 5 min at 95℃ with protein denaturing buffer (Nacalai Tesque, Kyoto, Japan). Samples were electrophoresed in a 5% SDS-PAGE gel with the Mini-protean system (Bio-Rad, Hercules, CA, USA) at 150 V for 45–60 min in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3 and 0.1% SDS). The separated proteins on a SDS-PAGE gel were transferred to PVDF membranes (GE Healthcare) by electroblotting on a wet blotter (Bio-Rad) at 15 V for 1 h. To detect the purified Δ148aa-hACC2 and their biotinylation and phosphorylation, several specific antibodies were used. A mouse anti-FLAG antibody (Wako Pure Chem. Ind. Ltd., Osaka, Japan) was used to detect purified Δ148aa-hACC2 as a primary antibody. A monoclonal anti-phosphoserine antibody (Sigma-Aldrich) was used for phosphorylation dete ction as a primary antibody. An anti-mouse IgG-HRP (GE Healthcare) was used for above both cases as a secondary antibody. A goat anti-biotin antibody (Abcam, Cambridge, MA,

USA) and streptavidin HRP conjugate (Thermo Scientific, Rockford, IL, USA) were used for biotinylation detection as a primary antibody. A rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-mouse IgG-HRP were used as a secondary antibody.

2.2.4 Dephosphorylation of Δ148aa-hACC2

Dephosphorylation was carried out using Lambda protein phosphatase (Lambda PP; New England Biolabs, lpswich, MA, USA). The purified Δ148aa-hACC2 was incubated with 0.5 μL of Lambda PP, 1X NEBuffer for protein metallophosphatases (PMP) and 1 mM $MnCl_2$ at 30℃ for 0, 1, 3, 6 h. Sterilized water instead of Lambda PP was used as negative control. D ephosphorylation was confirmed by Western blot using a monoclonal anti-phosphoserine antibody (Sigma-Aldrich) produced in mouse and an anti-mouse IgG-HRP (GE Healthcare). The activity of dephosphorylated Δ148aa-hACC2 was determined using ACC assay.

2.2.5 Acetyl-CoA carboxylase assay

To measure ACC activity, 4 μL of purified Δ148aa-hACC2 was incubated with 36 μL of reaction buffer (50 mM of HEPES, pH 7.4, 5 mM of $NaHCO_3$, 10 mM of $MgCl_2$, 10 mM of sodium citrate, 0.5% of DMSO, 4 mM of ATP and 0.4 mM of acetyl-CoA) at 37℃. The reaction was terminated by addition of 4 μL of 100% trichloroacetic acid. The produced phosphate during reaction was determined using SensoLyte® MG Phosphate Assay Kit, Colorimetric (AnaSpec, Fremont, CA, USA) by measuring the absorbance at 655 nm. Protein concentration was determined using BCA protein assay kit (Thermo Scientific).

2.2.6 Citrate-induced polymerization of Δ148aa-hACC2

In order to confirm the allosteric regulation of purified Δ148aa-hACC2 from silkworm, citrate-induced polymerization was evaluated. The purified Δ148aa-hACC2 was incubated with 50 mM of HEPES (pH 7.4), 1 mM of dithiothreitol (DTT) and different concentration of citrate at 37℃ for 30 min. The polymerization was evaluated by Native-PAGE and western boltting. Purified proteins were prepared in a non-denaturing sample buffer (Native sample buffer, Bio-Rad). Samples were electrophoresed in a 5% Native-PAGE (without SDS) with the Mini-protean system (Bio-Rad) at 150 V for 45–60 min in Tris-glycine buffer (25 mM Tris and 250 mM glycine, pH 8.3). Next, western blotting protocol was used as described above. The antibodies for detecting polymerized Δ148aa-hACC2 were same as used for detecting purified Δ148aa-hACC2. The activity of Δ148aa-hACC2 by citrate concentration dependent polymerization was determined by ACC assay.

2.2.7 Atomic force microscopy

Nanoscale AFM imaging was employed to investigate the dynamic forms polymers due to allosteric regulation of hACC2 by citrate. The Δ148aa-hACC2 was incubated with or without 15 mM citrate for 20 min at 37℃. The resultant mixtures were placed on the fleshly cleaved mica surface and incubated for several hours in a moisture chamber. After washing with water and drying under nitrogen, the samples were subjected to tapping mode AFM imaging on the Nanoscope IV PicoForce Multimode AFM, equipped with an E-scanner and a rectangular-shaped silicon cantilever (Bruker, Madison, WI, USA) with a 42 N/m spring

constant and a resonant frequency of ~300 kHz at ambient environment (Park and Terzic, 2010; Park et al., 2008c). Images (512×512 pixels/image) were collected from each sample with maximum image size of 5 × 5 μm, and analyzed using the Nanoscope Version 6.13 software (Bruker).

2.3 Results

2.3.1 Expression and purification of recombinant Δ148aa-hACC2

Human ACC2 is a large polypeptide comprised of a mitochondrial attachment domain, a mitochondrial target sequence domain, a biotin carboxylase domain, a biotin carboxyl carrier protein domain, and a carboxytransferase domain (Fig. 2.1A) (Bianchi et al., 1990; Tanabe et al., 1975; Tong, 2013). Biotin is covalently attached to lysine within BCCP domain through post-translational modification and several serine residues are phosphorylated by protein kinases (Beaty and Lane, 1983a; Brownsey et al., 2006; Ha et al., 1994; Meredith and Lane, 1978). We deleted the N-terminal 148 hydrophobic amino acids to enhance the solubility of heterologous protein, which retains core functional modules (Fig. 2.1B). In particular, to prevent protease activity and liquefaction of heterologous proteins in silkworm-based expression system, *E. coli* BmDH10bac-*CP*$^{-}$-*Chi*$^{-}$ competent cells were employed.

Recombinant Δ148aa-hACC2 with a C-terminal FLAG tag was purified using an anti-FLAG M2 affinity gel column. Eluted with FLAG peptides, the enriched protein migrated to ~260 kDa, a predicted molecular weight, on SDS-PAGE based on comparison with molecular weight markers (Fig. 2.2A). Western blot analysis using a FLAG-specific antibody confirmed

the expression of hACC2 (Fig. 2.2B). In addition, the yield of final purified Δ148aa-hACC2 was 495 μg/pupa. This pupae-based recombinant protein expression provided a high yield of purified Δ148aa-hACC2 compared to expression in silkworm larvae (150 μg/larva) (Park et al., 2013). The purified hACC2 displayed significant homogeneity on SDS-PAGE and Western blot analysis, thereby further functional analysis was carried out using this enriched Δ148aa-hACC2.

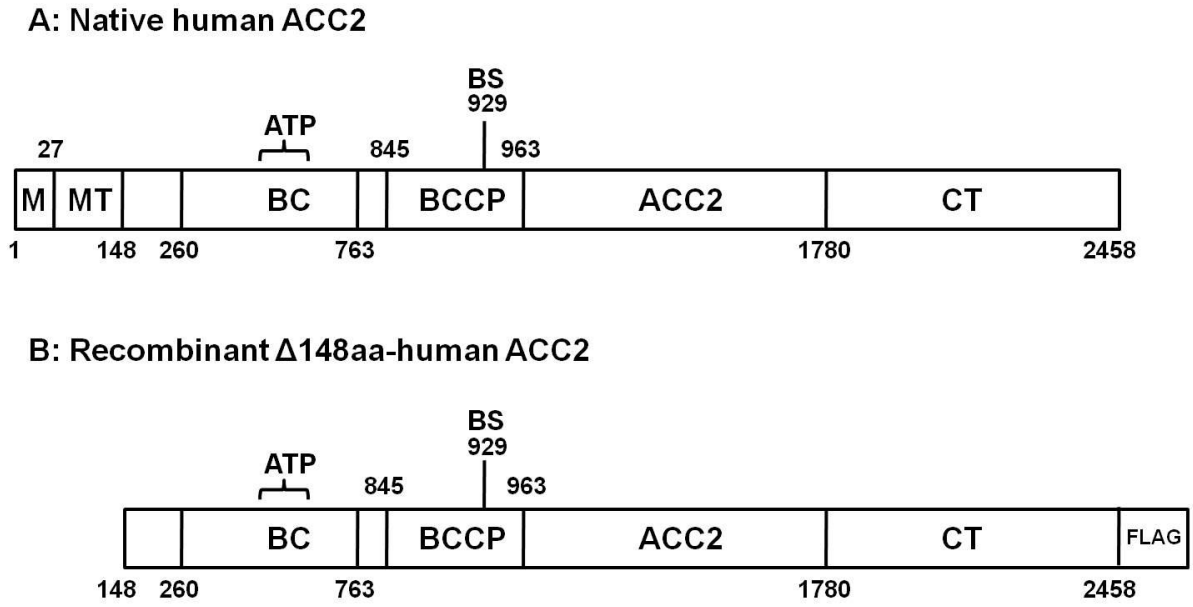

Fig. 2.1 A schematic presentation of human ACC2 domains. (A) Native hACC2. ACC2, human acetyl-CoA carboxylase 2; ATP, ATP-grasp domain; BC, biotin carboxylase domain; BCCP, biotin carboxyl carrier protein domain; BS, biotinylation site; CT, carboxyltransferase domain; M, membrane attachment domain; MT, mitochondria targeting sequence. (B) Recombinant Δ148aa-hACC2. N-terminal 148 amino acids were deleted for increasing the solubility. FLAG was tagged at its C-terminus for affinity purification.

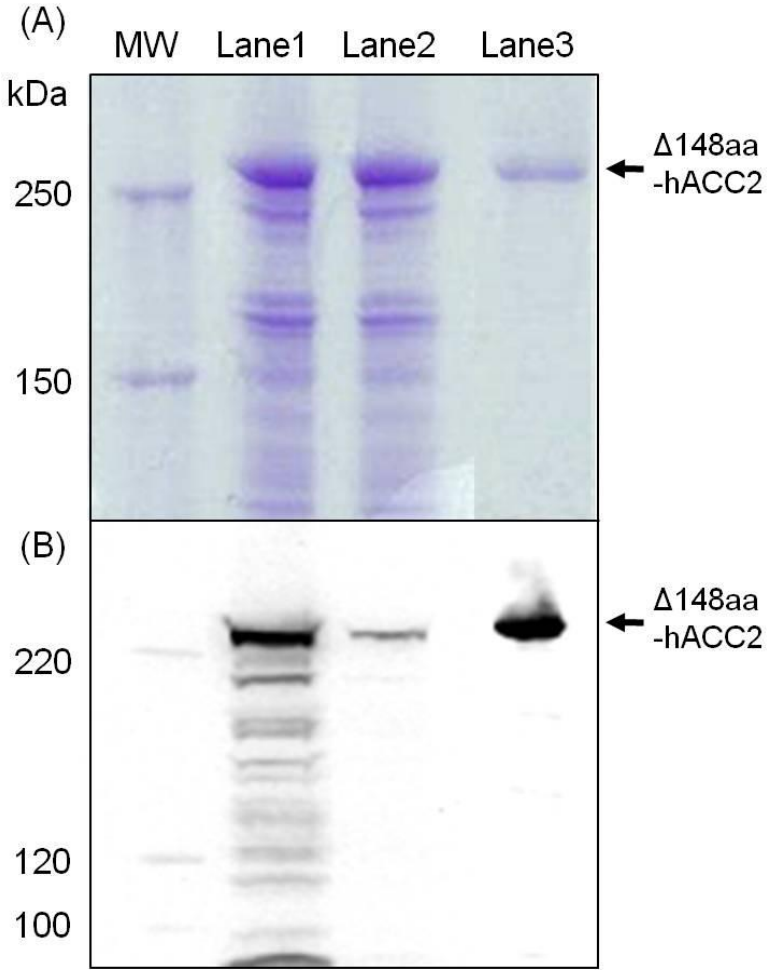

Fig. 2.2 The expression of recombinant Δ148aa-hACC2 was confirmed by analysis of SDS-PAGE (A) and Western blot (B). MW, molecular weight markers; Lane 1, protein extracts after infection; Lane 2, flow through during FLAG-tag purification; Lane 3, purified and concentrated Δ148aa-hACC2. An anti-FLAG M2 antibody and an anti mouse IgG-HRP were used to detect purified Δ148aa-hACC2.

2.3.2 Biotinylation of Δ148aa-hACC2

The post-translational modification with biotin in ACC2 is essential to implement

catalytic function. The biotin binding residue in human ACC2 has not been clearly identified, yet structural studies using nuclear magnetic resonance suggests that biotin is attached to lysine 929 within a BCCP domain (Lee et al., 2008). The biotinylation of Δ148aa-hACC2 from the silkworm was analyzed by Western blotting using an anti-biotin antibody. To further validate biotinylation of the Δ148aa-hACC2, streptavidin HRP conjugate was employed to detect the biotin group as streptavidin is known to interact with biotin with very high affinity. Although the hACC2-anti-biotin band was detected more intensely than the streptavidin bound band (Fig. 2.3), biotin specific detection using two different methods confirmed hACC2 biotinylation. Collectively, without additional supplement of biotin to generate biotinylated ACC observed in *Trichoplusiani* cells (Kim et al., 2007), silkworm enables to produce biotinylated heterologous proteins.

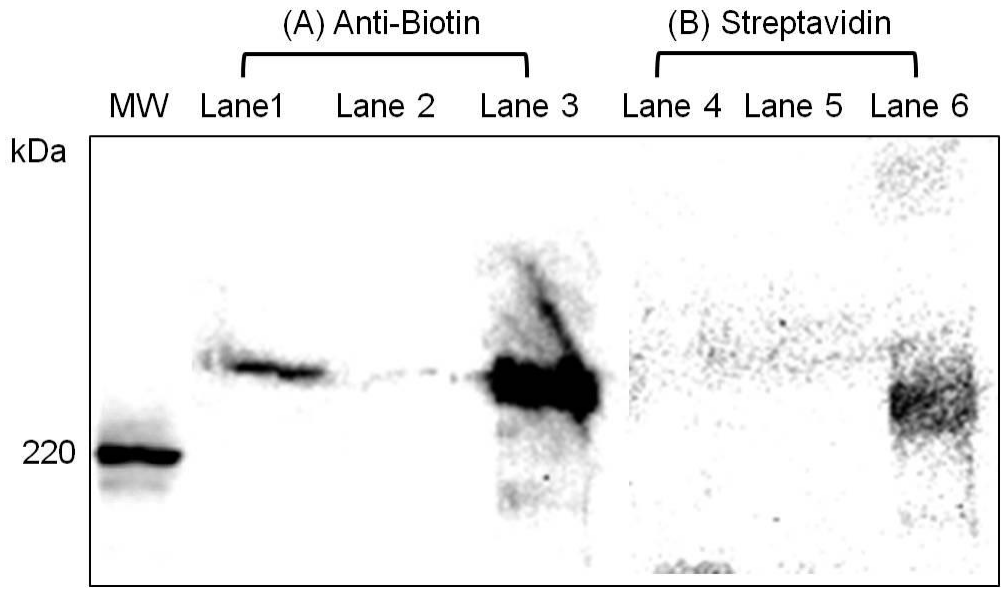

Fig. 2.3 The purified Δ148aa-hACC2 possesses post-translational biotinylation confirmed by Western blot analysis using an anti-biotin antibody (A) and streptavidin HRP conjugate (B). MW, molecular weight markers; Lane 1 and 4, protein extracts after infection; Lane 2 and 5, flow through during FLAG-tag purification; Lane 3 and 6, purified and concentrated Δ148aa-hACC2. An anti-biotin antibody and a streptavidin HRP conjugate were used as primary antibodies. A rabbit anti-goat IgG-HRP and an anti-mouse IgG-HRP were used as secondary antibodies.

2.3.3 Phosphorylation and dephosphorylation of Δ148aa-hACC2

Adenosine monophosphate-activated protein kinase (AMPK) mediated phosphorylation is other layer of post-translational modification to allosterically regulate ACC catalytic function. Phosphorylation inactivates ACC catalytic activity whereas dephosphorylation activates the

enzymatic function. Notably, phosphorylation of Ser222 in hACC2 (Ser212 in mouse ACC2) has been recognized as a vital process for homeostatic lipid metabolism (Fullerton et al., 2013; Wakil and Abu-Elheiga, 2009). Consistent with these findings, the crystal structure of biotin carboxylase domain of hACC2 has revealed that the phosphorylation of Ser222 disrupts the polymerization of ACC2, a widely recognized mechanism in modulating catalytic function (Cho et al., 2010; Lee et al., 2008).

We evaluated post-translational phosphorylation of recombinant Δ148aa-hACC2, and then whether the phosphorylated protein could be effectively dephosphorylated accompanying the changes of catalytic function. Western blotting analysis using a monoclonal anti-phosphoserine antibody demonstrated the phosphorylation of Δ148aa-hACC2 purified from silkworm pupae (Fig. 2.4A). The addition of Lambda protein phosphatase, a Mn2+-dependent dephosphorylation enzyme, gradually decreased the phosphorylation compared with control (Fig. 2.4A), yet dephosphorylation was not completely achieved in Δ148aa-hACC2 up to 6 h incubation. This finding suggests that some of phorsphorylation sites in full length hACC2 could be readily inaccessible by Lambda protein phosphatase unlike isolated functional domains such as a biotin carboxylase domain (Kwon et al., 2013).

The effect of dephosphorylation was assessed by measuring the catalytic function of Δ148aa-hACC2 (Fig. 2.4B). Δ148aa-hACC2 was treated with and without Lambda protein phosphatase for 2 h and then catalytic activity was measured as a function of incubation time. Purified Δ148aa-hACC2 with indigenous post-translational phosphorylation provided a specific activity of 0.786 ± 0.229 nmol/mg/min (n=6), whereas phosphatase treated Δ148aa-hACC2 protein yielded a specific activity of 1.336 ± 0.441 nmol/mg/min (n=6), about 2-fold increase. These measurements are consistent with the findings observed in knockin mice

samples where Ser212 (mouse sequence) is replaced with alanine so that the critical serine phosphorylation was ablated (Fullerton et al., 2013).

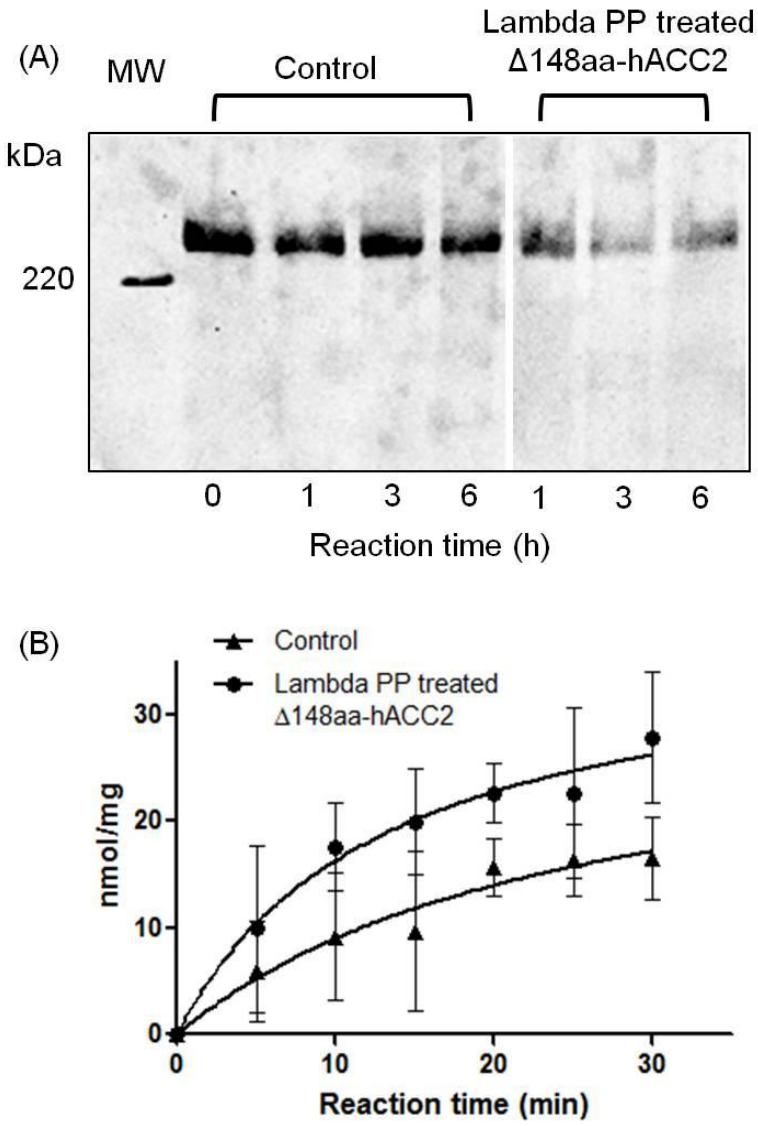

Fig. 2.4 Dephosphorylation of purified Δ148aa-hACC2 influences catalytic function. (A) Dephosphorylation of Δ148aa-hACC2 treated with Lambda PP was assessed by Western blotting using a monoclonal anti-phosphoserine antibody produced in mouse and an anti-mouse IgG-HRP. (B) Lambda PP treated Δ148aa-hACC2 enhanced catalytic activity.

2.3.4 Allosteric activation of Δ148aa-hACC2 by citrate

Citrate-induced polymerization has been extensively employed to understand the regulatory mechanism of ACC, although the concentrations of citrate required for allosteric activation are much higher than that present at physiological locale (Beaty and Lane, 1983a, 1983b; Gregolin et al., 1966; Kim et al., 2010). Upon incubation with citrate, ACC polymerizes into filamentous structures containing 10–20 protomer units with increased functional activity (Kim et al., 2007; Locke et al., 2008). Regardless of biological significance of citrate in ACC regulation the citrate binding sites have not been identified.

The citrate-induced allosteric activation of Δ148aa-hACC2 was investigated by measuring the modulation of structural and functional properties. Incubation of Δ148aa-hACC2 with citrate generated the formation of high molecular weight polymers detected on Native-PAGE, which was increased with rising citrate concentrations (Fig. 2.5A). This polymerization results indicate that Δ148aa-hACC2 derived from pupae consists of dimers and tetramers based on comparison with molecular weight markers, and increased citrate concentrations led to produce tetramers, by decreasing dimers (Fig. 2.5A). Consistent with dose-dependent polymerization, catalytic activity of Δ148aa-hACC2 was also enhanced with increasing citrate concentrations (Fig. 2.5B). Incubation of Δ148aa-hACC2 with 5, 10 and 20 mM citrate produced specific activity of 1.363 ± 0.279, 2.246 ± 0.870 and 4.186 ± 0.200 nmol/mg/min (*n*=4), respectively. These values indicated that when the citrate concentration was increased by 2folds, the activities were also increased by about 2 folds in proportion to citrate concentration (Fig. 2.5B). Furthermore, when the concentration of citrate exceeds 20 mM, the activation curve follows a sigmoidal response, consistent with previous findings

(Cheng et al., 2007).

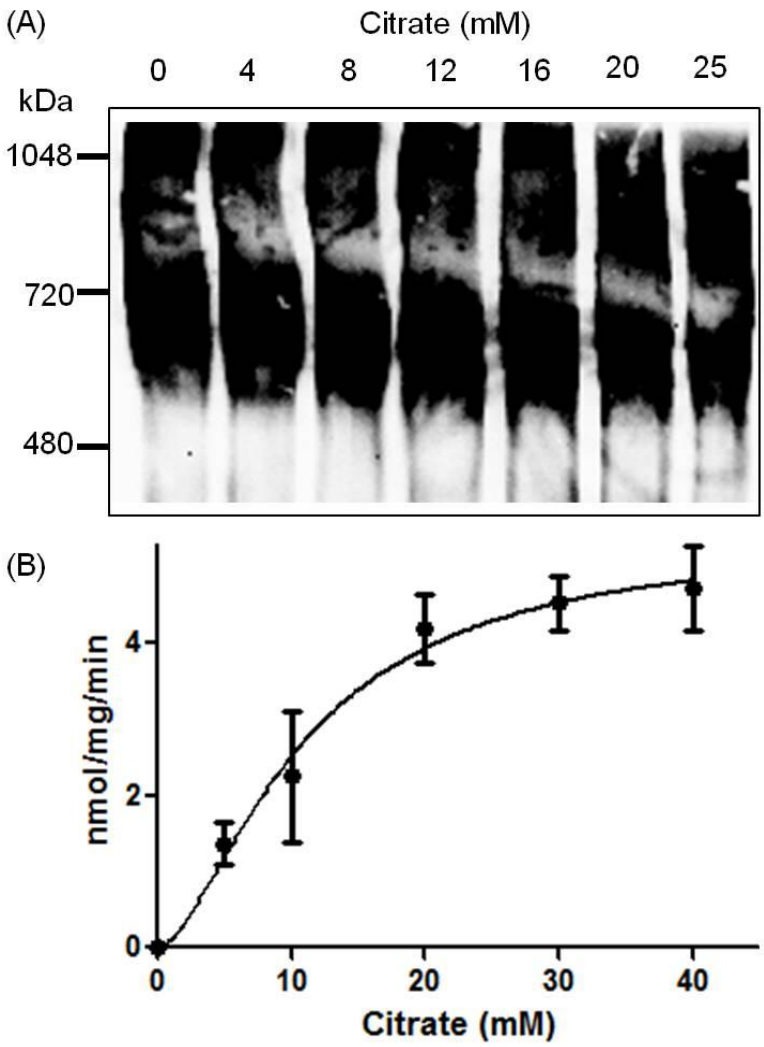

Fig. 2.5 Polymerization and enzyme activities of Δ148aa-hACC2 were modulated by citrate concentration. (A) Degree of polymerization of Δ148aa-hACC2 by different concentration of citrate (0, 4, 8, 12, 16, 20, 25 mM). The polymerization was confirmed using Native-PAGE. (B) Enzymatic activities by citrate concentration. All data are means ± S.D. from 3 separate experiments.

The structural changes of Δ148aa-hACC2 by citrate were also evaluated using high-resolution AFM at a nanoscale resolution (Fig. 2.6). The purified Δ148aa-hACC2 without citrate showed almost homogeneous particle distribution. However, citrate addition to Δ148aa-hACC2 generated filamentous polymeric forms, significantly larger than Δ148aa-hACC2 alone. These findings not only support the formation of high molecular weight polymers observed in Native-PAGE, but validate that Δ148aa-hACC2 produced in silkworm possess full functionality with proper allosteric modulations.

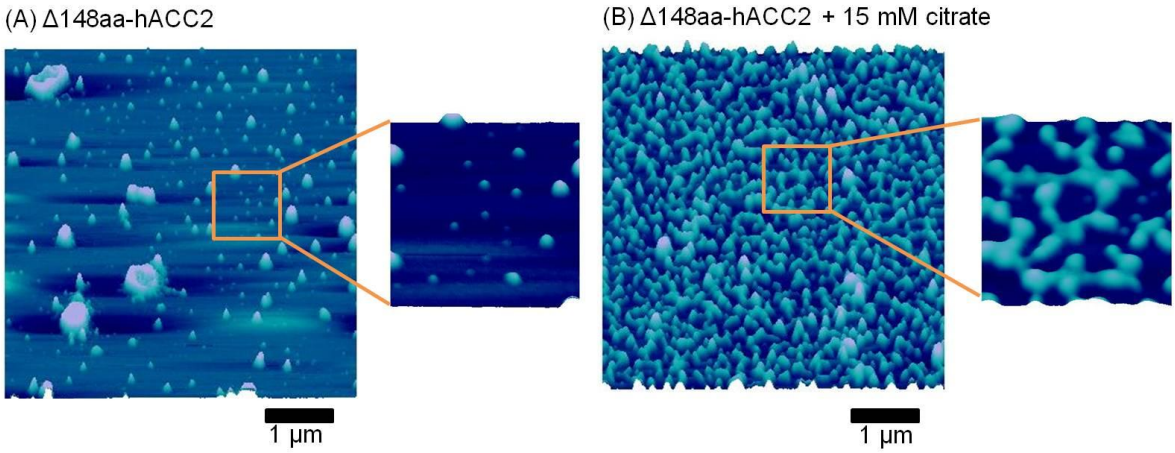

Fig. 2.6 Atomic force microscopy nanoscale images of Δ148aa-hACC2. (A) Δ148aa-hACC2 alone. (B) Δ148aa-hACC2 with 15 mM citrate after 20 min incubation at 37℃. Citrate induced the filaments formation.

2.4 Discussion

Acetyl-CoA carboxylase is a multidomain and multifunctional protein working as a energetic controller in homeostatic lipid metabolism participating in fatty acid synthesis and

fatty acid oxidation (Tong, 2013; Wakil and Abu-Elheiga, 2009). Catalytic function of ACC through a biotin prosthetic group could be allosterically regulated by multiple factors including post-translational phosphorylation and dephosphorylation (Wakil and Abu-Elheiga, 2009). In addition, tertiary level regulation of ACC with small acidic proteins, i.e., Spot14 and Mig12, has been recently identified (Colbert et al., 2010; Kim et al., 2010; Knobloch et al., 2013; Park et al., 2013). Particularly, due to the patho-physiological relevance of ACC2 in lipid metabolic syndrome associated with obesity, type 2 diabetes, cancer, and cardiovascular disease, ACC2 activity regulation has been considered as a candidate target for therapeutic interventions, which essentially requires authentic bioengineered recombinant proteins (Abu-Elheiga et al., 2001b; Tong, 2013; Tong and Harwood, 2006). Here, we successfully produced the high fidelity human ACC2 using silkworm BmNPV system armed with proper post-translational modification machinery. The heterologous ACC2 harbors all the necessary post-translational biotinylation and phosphorylation, vital to maintain the functional integrity. Thus, the silkworm BmNPV bacmid system provides a reliable large-scale production platform for eukaryotic proteins required post-translational modifications.

Biotin is a water-soluble vitamin serving as a vital prosthetic group involved in five carboxylases in human (Zempleni et al. 2009). In hACC2, biotin is covalently linked to lysine 929 within a BCCP domain and forms carboxybiotin using bicarbonate as the CO_2 donor. Following a large conformational change, the carboxyl group from carboxybiotin is transferred to acetyl-CoA to produce malonyl-CoA (Tong, 2013). We revealed post-translational biotinylation of functional recombinant hACC2 from silkworm, without any additional supplement of biotin under silkworm rearing conditions. We believe that this is the first demonstration of post-translational biotinylation in proteins expressed in silkworm *Bombyx mori*.

ACC phosphorylation/dephosphorylation is one of allosteric regulatory mechanisms. Phosphorylation of ACC by AMP-activated protein kinase and cAMP-dependent protein kinase inhibits the enzymatic activity of ACC, whereas the dephosphorylation activates the catalytic function (Munday et al., 1988). Although several phosphorylation sites have been identified, Ser212 (mouse sequence) phosphorylation was recently recognized as an integral process in activity modulation (Fullerton et al., 2013) where knockin mice with substitution of Ser212 with alanine displayed an increased ACC2 activity. Consistent with this, we demonstrated about 2-fold increased specific activity in dephosphorylated hACC2 compared to phosphorylated one, underscoring that the critical serine residue is fully accessible to phosphatase protein. ACC is also allosterically activated by citrate, which is a metabolic intermediate produced in mitochondrial tricarboxylic acid cycle. Although the concentrations of citrate required increasing enzymatic activity of ACC are much higher than physiological concentration of citrate, citrate has been widely used to modulate ACC function (Beaty and Lane, 1983a; Cheng et al., 2007; Thampy and Wakil, 1988). We clearly demonstrated citrated-induced hACC2 catalytic function enhancement and filamentous polymer formation. In summary, a multifunctional human ACC2 was successfully produced using silkworm-based protein expression system. Heterologous ACC2 was correctly folded with post-translational biotinylation and phosphorylation, retaining catalytic activity and citrate-induced allosteric regulation. Moreover, silkworm demonstrated a high yield of recombinant ACC2 production. Thus, silkworm-based BmNPV expression method equipped with proper post-translational modification machinery provides a novel large-scale eukaryotic protein production platform for structural and functional research particularly in the application of therapeutic drug discovery application.

Chapter 3

Phosphorylation of Ser-204 and Tyr-405 in human malonyl-CoA decarboxylase expressed in silkworm *Bombyx mori* regulates catalytic decarboxylase activity

3.1 Introduction

Malonyl-CoA decarboxylase (MCD, E.C.4.1.1.9), encoded by *MLYCD*, is the crucial metabolic enzyme responsible for sustaining the homeostatic lipid metabolism (Dyck et al., 2006; Folmes et al., 2013; Saggerson, 2008). Due to the importance of cellular functions in fatty acid metabolism, MCD is ubiquitously expressed in all living organisms locating in mitochondria, peroxisome and cytoplasm (Buckner et al., 1976; Kim et al., 1979; Scholte, 1969). In humans, the deficiency of MCD (OMIM 248360) precipitates a broad spectrum of disorders including cardiomyopathy, hypoglycaemia, hypotonia, mild mental retardation, metabolic acidosis, malonic aciduria, seizures and vomiting (Brown et al., 1984; Haan et al., 1986; Krawinkel et al., 1994; MacPhee et al., 1993; Matalon et al., 1993; Xue et al., 2012; Yano et al., 1997).

MCD participates in the degradation reaction of malonyl-CoA, an integral metabolic intermediate in anabolic/catabolic lipid metabolism. Malonyl-CoA is a committed substrate for *de novo* fatty acid biosynthesis, yet the abundant malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT-1), a rate-limiting step for long-chain fatty acid transport into mitochondria and subsequent lipid β-oxidation (Kim et al., 1989; Pender et al., 2006). The inhibition of MCD, reduces fatty acid β-oxidation, and accelerates glucose oxidation, producing a metabolic switch in energy substrate preference (Dyck et al., 2006). Consequently, the regulation of MCD activity to modulate the intracellular malonyl-CoA levels has been increasingly accepted as the potential therapeutic applications to mitigate metabolic disorders.

Post-translational modification of MCD modulates enzymatic function (Dyck et al., 2000; Laurent et al., 2013; Park et al., 2002; Saha et al., 2000; Sambandam et al., 2004; Voilley et al., 1999). For example, MCD has several different acetylation sites and MCD deacetylation

inhibits the decarboxylase activity thereby promoting de novo lipogenesis, whereas MCD acetylation enhances fatty acid oxidation (Laurent et al., 2013). In contrast, the catalytic activity associated with MCD phosphorylation/dephosphorylation has not been clearly documented due to the lack of identifying the phosphorylation sites and the production of sufficient authentic MCD with suitable post-translational modification (Dyck et al., 2000; Park et al., 2002; Saha et al., 2000; Sambandam et al., 2004; Voilley et al., 1999).

E. coli expression system has been wildly used for recombinant protein production, yet the proteins expressed in *E. coli* often show poor post-translational modifications (Kamionka, 2011). To secure the eukaryotic protein production with implementing proper post-translational modifications, a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid, a hybrid shuttle vector for an *E. coli* and *B. mori*, has been developed (Kato et al., 2010; Motohashi et al., 2005). With this system, we have successfully produced cellular, mitochondrial, and membrane proteins with proper folding and post-translational modifications (Dojima et al., 2009; Du et al., 2009; Hwang et al., 2014).

Here, we examined whether phosphorylation of human MCD (hMCD) generated using silkworm-based BmNPV bacmid expression system modulates the essential decarboxylase activity. We report that the recombinant hMCD displays the phosphorylation with consistent catalytic activity. Point mutation in the phosphorylation sites in Ser-204 and Tyr-405 dampens enzymatic activity of hMCD, underscoring the regulation of catalytic function by phosphorylation. In addition, silkworm-based BmNPV bacmid expression system provides a reliable recombinant eukaryotic protein production modality with post-translational phosphorylation for functional analysis of human MCD.

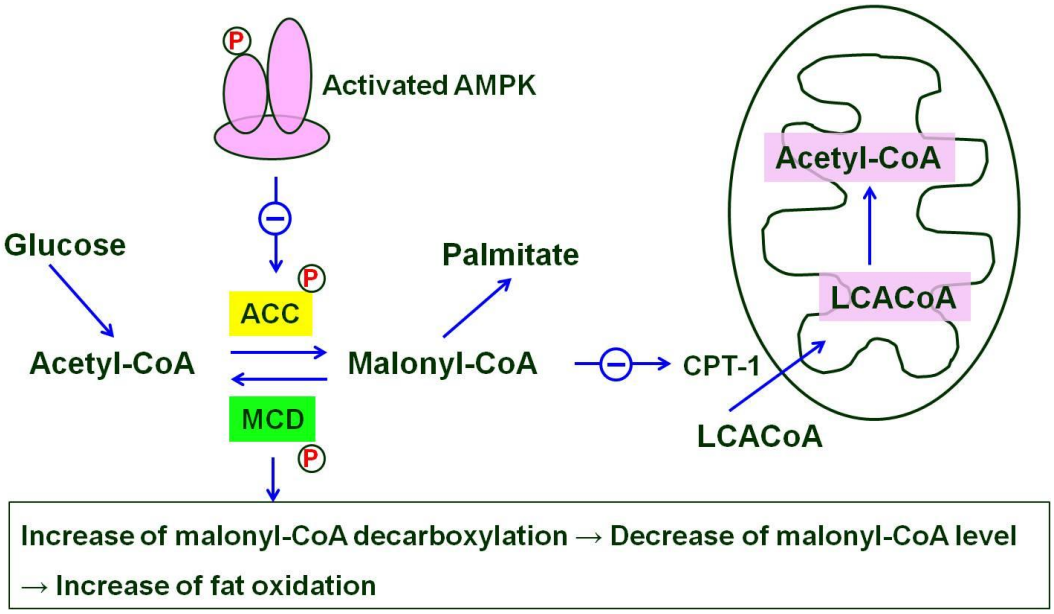

Fig. 3.1 The regulation of lipid homeostasis and function of malonyl-CoA decarboxylase. Malonyl-CoA acts as an inhibitor of CPT-1 and regulates fatty acid oxidation by controlling long chain fatty acyl CoA entry into the mitochondria. MCD catalyzes the conversion of malonyl-CoA into acetyl-CoA. The activated AMPK leads to the phosphorylation of ACC and MCD. This phosphorylation promotes increased malonyl-CoA decarboxylation and decreased malonyl-CoA levels. Consequently, fat oxidation is increased.

3.2 Materials and Methods

3.2.1 Construction of recombinant Δ39aa-hMCD BmNPV bacmid and their mutants

The overall strategy for the construction and expression of Δ39aa-hMCD BmNPV bacmid is shown in Figure 3.2. The complementary DNA of human MCD from Mammalian Gene

Collection (GenBank EAW95513.1, Thermo Scientific, Pittsburgh, PA, USA) was used as a template. N-terminal 39 amino acids of hMCD, which is a putative mitochondria targeting sequence (Fig. 3.2), were deleted using conventional polymerase chain reaction (PCR) with a pair of primers containing the *BamH*I/*Xho*I restriction cloning site: 5´-GCGGATCCCACCATGGACTACAAGGATGACGATGACAAGATGGACGAGCTGCTGC GCCGC-3´ (forward), 5´-GCCTCGAGTCAGAGCTTGCTGTTCTTTTGAAACTG-3´ (reverse). Deletion of this mitochondria targeting sequence leads to high protein expression and does not affect the enzyme activity (Zhou et al., 2004). In addition, kozak consensus sequence and FLAG tag sequence were attached at N-terminus for high expression levels in baculovirus expression system and for purification of expressed protein, repectively. The PCR cycle was conducted following 40 cycles of denaturation at 98℃ for 10s, annealing at 55℃ for 5s, and extension at 72℃ for 10s using PrimeSTAR® Max premix kit (Takara Bio Inc., Otsu, Shiga, Japan). The resultant PCR product (Δ39aa-hMCD gene) was digested with *BamH*I and *Xho*I followed by purification with a GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, UK). The purified DNA fragment was ligated into pFastbac 1 vector, which was transformed into *E. coli* competent DH5α cells (Invitrogen, Carlsbad, CA, USA) and cultured on a solid LB medium containing 100 μg/mL of ampicillin at 37℃ for 18 h. The plasmid containing Δ39aa-hMCD gene was isolated and its sequence was confirmed by DNA sequencing. Finally, *E. coli* BmDH10bac-*CP*⁻-*Chi*⁻ competent cells containing the cysteine proteinase- and chitinase-deficient BmNPV bacmid (Park et al., 2008b) were transformed with the pFastbac1-Δ39aa-hMCD and cultured on a solid LB medium containing 50 μg/mL of kanamycin, 7 μg/mL of gentamycin, 10 μg/mL of tetracycline, 40 μg/mL of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μg/mL of 5-bromo-4-chloro-3-indolyl-4-galactoside (X-Gal) (Takara Bio Inc., Otsu Shiga, Japan) at

37℃ for 18 h. To confirm the BmNPV-Δ39aa-hMCD, bacmid PCR of white colonies was conducted using M13 primers. The PCR cycle was conducted following 30 cycles of denaturation at 98℃ for 10 s, annealing at 55℃ for 10 s, and extension at 72℃ for 40 s using SapphireAmp Fast PCR Master Mix kit (Takara Bio Inc., Otsu, Shiga, Japan). The recombinant BmNPV bacmid (BmNPV-Δ39aa-hMCD) was isolated from positive colonies confirmed by bacmid PCR.

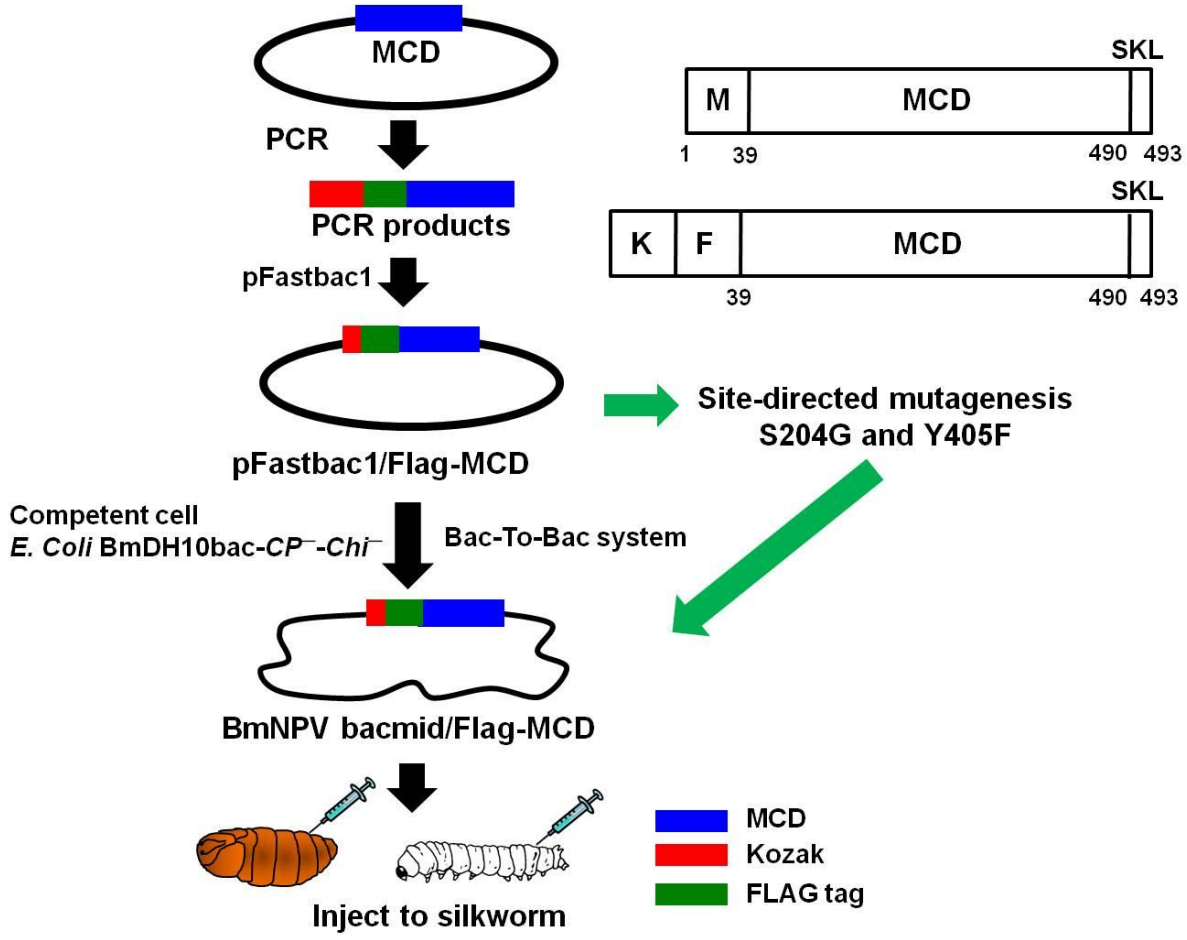

Fig. 3.2 Schematic diagram of recombinant Δ39aa-hMCD bacmid construction and expression in silkworm. The Δ39aa-hMCD gene was amplified by PCR. This PCR fragment was digested by restriction enzymes and ligated into pFastbac1 vector. The pFastbac-Δ39aa-hMCD was transformed into *E. coli* BmDH10Bac harboring cysteine protease- and chitinase-

deficient *Bombyx mori* nucleopolyhedrovirus (BmNPV-*CP*¯-*Chi*¯). The constructed recombinant bacmid was directly injected into the dorsum of the larvae on the first day of their fifth-instar and pupae. M, mitochondrial-targeting sequence; K, Kozak sequence; F, FLAG tag; SKL, peroxisomal-targeting sequence. Black and green arrows denote bacmid constructions for Δ39aa-hMCD and for Δ39aa-hMCD mutants, respectively.

MCD has several putative phosphorylation sites (Fig. 3.3A) and might be regulated by phosphorylation reaction. Thus, mutation study of these phosphorylation sites is required for functional study of MCD. Although phosphorylation studies on rat MCD have been much reported, phosphorylation effects on human MCD are still a lack of scientific evidence to prove them. Therefore, we conducted two kinds of mutation (serine204→glycine204, named S204G and tyrosine405→phenylalanine405, named Y405F) for functional study according to the previous reports (Voilley et al., 1999) and LC/MS/MS result. For the construction of mutated hMCD BmNPV bacmid, the QuikChangeII XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocols. The pFastbac-Δ39aa-hMCDs containing the desired mutation were produced using already constructed pFastbac-Δ39aa-hMCD by PCR reaction with primer sets containing changed base designed for mutation: 5´-GGGTTACCTGGCATGGACCGTGTGAAGTGCTTC-3´(forward for S204G), 5´- GAAGCACTTCACACGGTCCATGCCAGGTAACCC-3´ (reverse for S204G), 5´-AGGCTGTGCGCCTGGTTCCTGTATGGAGAGAAG-3´ (forward for Y405F), 5´-CTTCTCTCCATACAGGAACCAGGCGCACAGCCT-3´(reverse for Y405F). The PCR cy cle was conducted following 18 cycles of denaturation at 95℃ for 15s, annealing at 60℃ for 30s, and extension at 68℃ for 6.5 min using QuikChangeII XL Site-Directed

Mutagenesis Kit (Stratagene). After *Dpn* I treatment at 37℃ for 3 hr, *Dpn* I-treated plasmids were transformed into XL10-Gold ultracompetent cell (stratagene). The plasmid containing pFastbac-Δ39aa-hMCD mutant genes were isolated and its sequence was confirmed by DNA sequencing. The following procedure is the same as that of the wild type of human MCD.

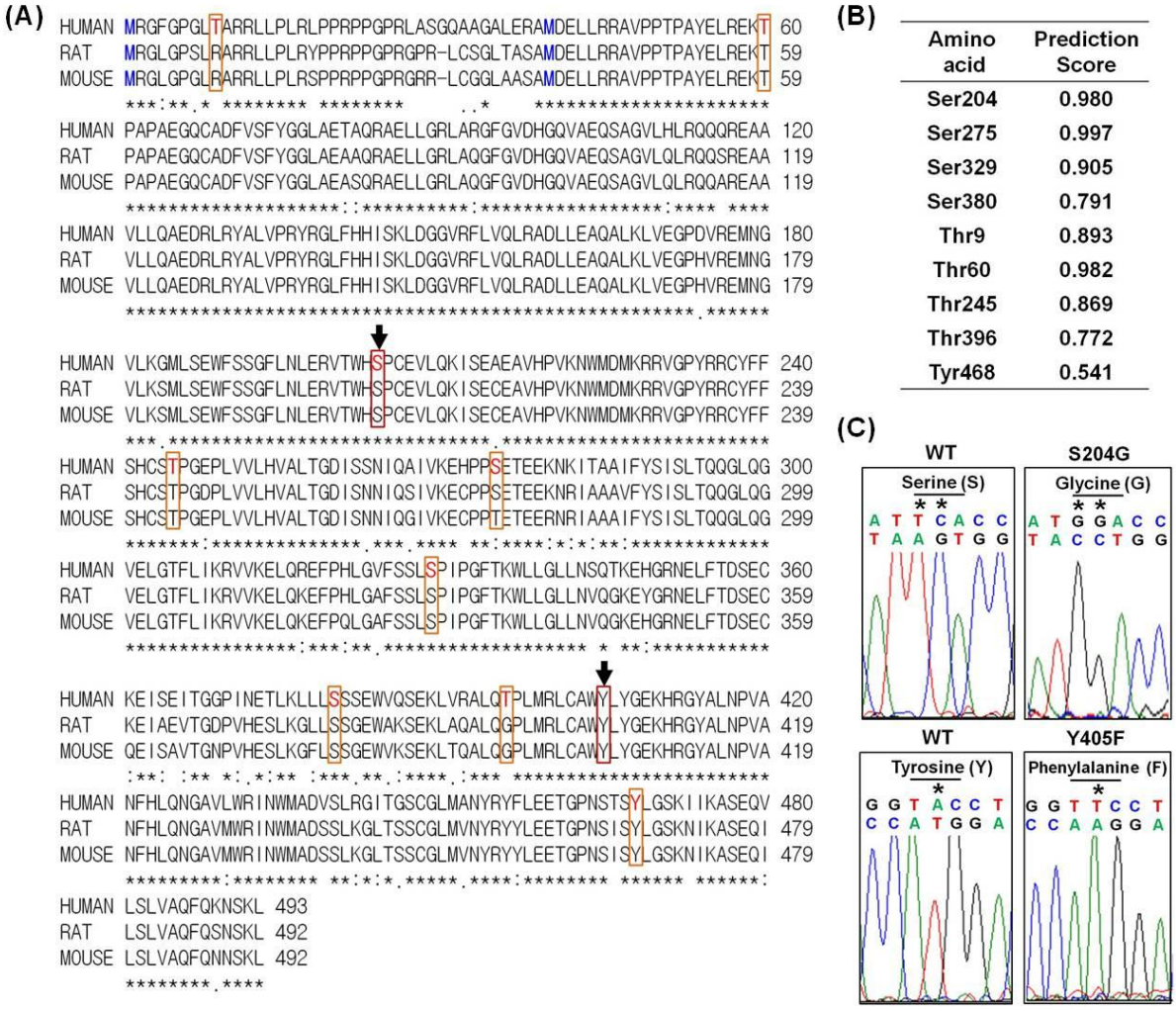

Fig. 3.3 Amino acid sequence alignments of human (*Homo sapiens*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*) MCD (A). Blue, two initiating methionines; Red, putative phosphorylation site residues of hMCD by Netphos 2.0 prediction server; Arrow,

point mutation sites for dephosphorylation of hMCD in this study. (B) Predicted phosphorylation sites of hMCD. Prediction score is the probability of phosphorylation (*max* = 1). (C) A point mutation in hMCD replaced serine (S) 204 (codon TCA) with glycine (G) (codon GGA) and tyrosine (Y) 405 (codon TAC) with phenylalanine (F) (codon TTC).

3.2.2 Expression and purification of recombinant Δ39aa-hMCD in silkworm larvae and pupae

To produce a recombinant protein in silkworm, 10 μg of each recombinant BmNPV bacmid DNA was directly injected with DMRIE-C reagent (Invitrogen) into the dorsum of larvae and pupae using a syringe with a 26-gauge beveled needle (Terumo Co. Tokyo, Japan). The injected larvae and pupae were reared at 27℃ in an incubator for 6–7 days. In the case of larvae, the fat body was collected by cutting and dissection. The samples including fat body or pupae were immediately frozen at –80℃ until further analysis. Protein purification was carried out at 4℃ to minimize aggregation and protease activity. The aliquot of collected larval fat body or pupae was homogenized in 10 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.1% TritonX-100) containing an EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany) using a homogenizer (GLH-115, Yamato, Tokyo, Japan). Cell debris was removed by pelleting through centrifugation at 12,000 g for 30 min. The supernatant was filtered using a 0.45 μm syringe filter and loaded onto a 500 μL of anti-FLAG M2 antibody affinity gel (Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with equilibration buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.02% TritonX-100). The column was washed with 2.5 mL of equilibration buffer and eluted with elution buffer (100 μg/mL FLAG peptide in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4). Purified protein

concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

3.2.3 SDS-PAGE and Western blotting analysis

The integrity of purified Δ39aa-hMCD was determined by Coomassie brilliant blue (CBB)-staining and western blotting analyses (Karger et al., 2008; Park et al., 2008c). Prior to electrophoresis, purified samples were boiled for 5 min at 95℃ with protein denaturing buffer (Nacalai Tesque, Kyoto, Japan). Samples were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a Mini-protean system (Bio-Rad, Hercules, CA, USA) at 150 V for 45–60 min in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3 and 0.1% SDS). After electrophoresis, the SDS-PAGE gel was stained with Coomassie blue staining solution. For Western blotting analysis, the separated proteins on a SDS-PAGE gel were transferred to PVDF membranes (GE Healthcare) by electroblotting on a semi-dry blotter (Bio-Rad) at 15 V for 1 h. To detect the purified Δ39aa-hMCD and their phosphorylation, a mouse anti-FLAG antibody (Wako Pure Chem. Ind. Ltd., Osaka, Japan), anti-phosphoserine antibody and anti-phosphotyrosine (Sigma-Aldrich) were used as primary antibodies, respectively. An anti-mouse IgG-HRP (GE Healthcare) was used as a secondary antibody in both cases.

3.2.4 Dephosphorylation test of Δ39aa-hMCD

To evaluate the effect of phosphorylation on human MCD, we tried dephosphorylation test of purified Δ39aa-hMCD. Dephosphorylation was carried out using Lambda protein phosphatase (Lambda PP; New England Biolabs, lpswich, MA, USA). The purified Δ39aa-

hMCD was incubated with 0.5 μL of Lambda PP, 1X NEBuffer for protein metallophosphatases (PMP) and 1 mM $MnCl_2$ at 30℃ for 0, 2, 5 hr. Sterilized water instead of Lambda PP was used as negative control. Dephosphorylation was confirmed by Western blot using a monoclonal anti-phosphoserine antibody (Sigma-Aldrich) produced in mouse and an anti-mouse IgG-HRP (GE Healthcare).

3.2.5 Malonyl-CoA decarboxylase assay

The hMCD activity was measured by the production of NADH in the coupled reactions (Kim and Kolattukudy, 1978). The NADH is accumulated by the reaction of acetyl-CoA from malonyl-CoA and oxaloacetate from malate (Eq. 1–3).

$$\text{Malonyl} - \text{CoA} \xrightarrow{\text{Malnoyl-CoA decarboxylase}} \text{Acetyl} - \text{CoA} + CO_2 \qquad (1)$$

$$\text{Malate} + \text{NAD} \xleftrightarrow{\text{Malate dehydrogenase}} \text{Oxaloacetate} + \text{NADH} \qquad (2)$$

$$\text{Acetyl} - \text{CoA} + \text{Oxaloacetate} \xrightarrow{\text{Citrate synthase}} \text{Citrate} + \text{CoA} \qquad (3)$$

To measure the decarboxylase activity of recombinant human MCD, 10 μg of purified Δ39aa-hMCD was incubated with 36 μL of reaction buffer (10 mM of Tris-HCl, pH 7.5, 2 mM of malate, 2 mM of NAD, 0.8 U of malate dehydrogenase) at room temperature. After 10 min, 0.03 U of Citrate syntase was added to incubated solution. After 5 min, assay was initiated by the addition of 3 mM of malonyl-CoA and incubated at 37℃. The reaction was terminated by addition of 4 μL of 100% trichloroacetic acid. The produced NADH during reaction was detected using UV-visible spectrophotometer by measuring the absorbance at 340 nm.

3.3 Results

3.3.1 Expression and purification of soluble recombinant Δ39aa-hMCD

The human MCD (hMCD) harbors a mitochondrial targeting sequence (39 amino acids) at N-terminus and a peroxisomal targeting sequence (Ser-Lys-Leu, SKL) at C-terminus (Fig. 3.2). The peroxiomal SKL residues do not affect the protein expression levels, but the mitochondrial targeting sequence significantly diminishes the MCD expression (Voilley et al., 1999). Therefore we designed a construct without a mitochondrial targeting sequence but with retaining the peroxisomal SKL sequence to enhance the recombinant hMCD production (Fig. 3.2). The recombinant Δ39aa-hMCD with N-terminal FLAG tag was expressed in silkworm larvae and pupae, and purified using an anti-FLAG M2 affinity gel column. The purified Δ39aa-hMCD protein with more than 95% purity was migrated to ~50 kDa, an estimated molecular weight on SDS-PAGE based on comparison with molecular weight markers (Fig. 3.4A). Western blot analysis using a FLAG-specific antibody confirmed the specific expression of Δ39aa-hMCD (Fig. 3.4B). In mock-injected silkworm, however, hMCD bands were not detected in both of fat body and pupae samples (Fig. 3.4). Thus, the recombinant Δ39aa-hMCD purified here demonstrated the significant homogeneity suitable for biochemical and functional analyses.

Silkworm-based recombinant protein expression conventionally provides a high yield of purified proteins in range of 20 – 500 µg/silkworm (Table 3.1). Consistent with this, the average yields of purified Δ39aa-hMCD from ten silkworm larvae and five pupae were 119 µg/larva and 344 µg/pupa. The pupae-based protein expression provided a higher yield of purified protein than the fat body-based expression, probably due to the difference of organ

size and protease activity in the expression host. Specific decarboxylase activities of the purified Δ39aa-hMCD, measured by the production of NADH in the coupled reactions, were 59.54 ± 7.68 (n = 6) nmol/mg/min from silkworm fat body and 48.16 ± 7.89 (n = 6) nmol/mg/min from silkworm pupae (Table 3.2). These measured enzymatic activities showed no significant statistical difference ($p>0.05$), but are much higher than that of human recombinant MCD purified from E. coli (Zhou et al., 2004) indicating that the post-translational modifications implemented hMCD in silkworms regulate catalytic activity.

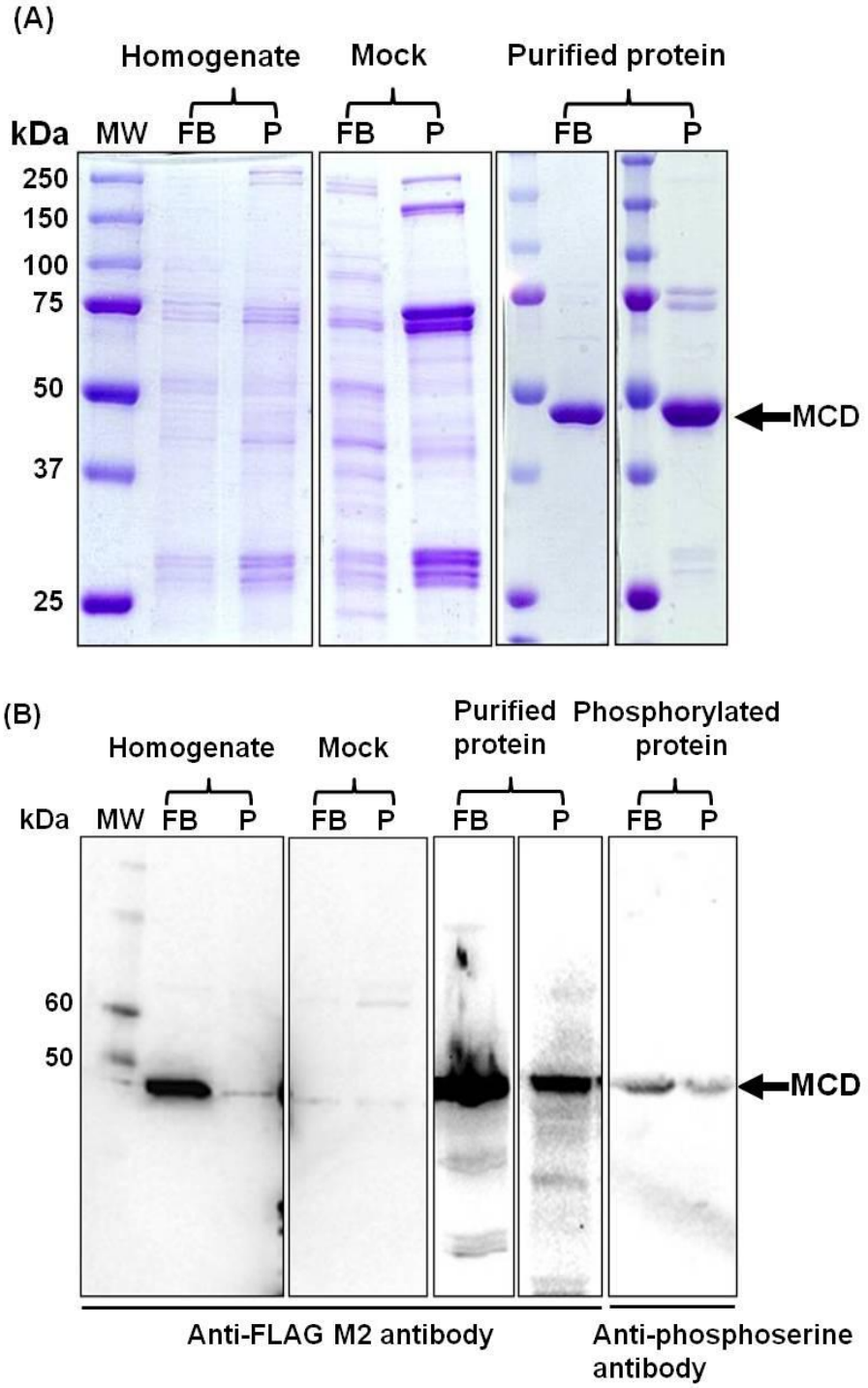

Fig. 3.4 SDS-PAGE (A) and Western blot (B) of recombinant Δ39aa-hMCD expressed in silkworm fat body and pupae. MW, molecular weight markers; FB, Δ39aa-hMCD from fat body; P, Δ39aa-hMCD from pupae. Protein in homogenate was extracted with lysis buffer

and purified by anti-FLAG M2 affinity gel column. Mock sample indicates bacmid, without hMCD gene, injected silkworm. An anti-FLAG M2 and an anti mouse IgG-HRP were used to detect Δ39aa-hMCD, and an anti-phosphoserine antibody was used to detect phosphorylation of Δ39aa-hMCD.

Table 3.1. The yields of recombinant proteins from silkworm larva or pupa

Protein	μg/larva	μg/pupa	Reference
GFPuv-β3GnT2	91		Park et al. 2007
Anti-BSA scFv	188		Ishikiriyama et al. 2009
ST6Gal1	220		Ogata et al. 2009
SAG1	170		Otsuki et al. 2013
SRS2	20		Otsuki et al. 2013
ACC2	150		Park et al. 2013
ACC2		500	Hwang et al. 2014
MCD	119	344	In this study

GFP, green fluorescent protein; β3GnT2, beta1,3-N-acetylglucosaminyl transferase 2; scFv, Human single-chain Fv fragment; ST6Gal1, rat alpha2,6-sialyltransferase; SAG1, *N. caninum* surface antigen 1; SRS2, SAG1-related sequence 2; ACC2, acetyl-CoA carboxylase 2; MCD, malonyl-CoA decarboxylase.

Table 3.2. Purification of Δ 39aa-hMCD from silkworm fat body and pupae

	Volume (mL/larva or pupa)	Protein (mg/larva or pupa)	Specific activity (mU/mg)*	Purification (Fold)
Fat body				
Homogenate	3	193	19.61 ± 3.96	1
Anti-FLAG M2 agarose	0.5	0.119	59.54 ± 7.68	3
Pupae				
Homogenate	3	232	12.81 ± 3.36	1
Anti-FLAG M2 agarose	0.5	0.344	48.16 ± 7.89	3.7

* 1 mU is defined as 1 nmol product formed per minute.

3.3.2 Dephosphorylation test of Δ39aa-hMCD using Lambda PP

Adenosine monophosphate-activated protein kinase (AMPK) regulated phosphorylation of MCD. When the MCD is phosphorylated, malonyl-CoA decarboxylation is increased. Consequently, malonyl-CoA levels are decreased and it leads to increased fat oxidation (Saha et al., 2000). MCD has several serine and threonine residues that can be potentially phosphorylated. Therefore, in order to prove the effect of phosphorylation on the human MCD, first of all, dephosphorylation test of expressed and purified Δ39aa-hMCD will need to be investigated.

We evaluated post-translational phosphorylation and dephosphorylation of recombinant Δ39aa-hMCD. The phosphorylation of Δ39aa-hMCD purified from silkworm larvae and pupae was demonstrated by western blot analysis using a monoclonal anti-phosphoserine antibody. The treatment with Lambda protein phosphatase, a Mn^{2+}-dependent dephosphorylation enzyme, showed irregular bands by incubation time (Fig. 3.5). We tried

several times this experiment by increased phosphatase concentration. Moreover, alkaline phosphatase, nonspecifically catalyzes the phosphorylation, was applied to dephosphorylate. However, the results showed that these phosphatase enzymes are difficult to act on the Δ39aa-hMCD.

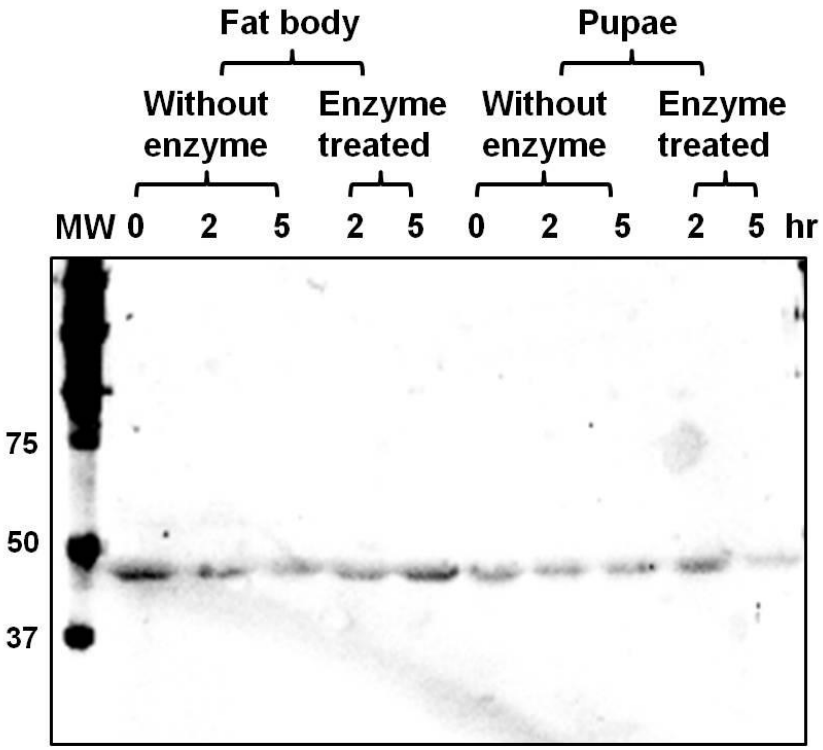

Fig. 3.5 Dephosphorylation test of purified Δ39aa-hMCD. Dephosphorylation of Δ39aa-hMCD treated with Lambda PP was assessed by Western blotting using a monoclonal anti-phosphoserine antibody produced in mouse and an anti-mouse IgG-HRP.

3.3.3 Phosphorylation-induced catalytic function of Δ39aa-hMCD

The catalytic activity of MCD associated with phosphorylation/dephosphorylation was previously evaluated (Dyck et al., 1998; Park et al., 2002; Saha et al., 2000), yet these studies

provided inconsistent results either a decrease or increase in decarboxylase activity of MCD. To clarify the phosphorylation-induced effects on MCD enzymatic function, potential phosphorylation sites of human MCD were examined. NetPhos 2.0, neural network predictions for serine, threonine and tyrosine phosphorylation in eukaryotic proteins (http://www.cbs.dtu.dk/services/NetPhos/), was applied to predict to phosphorylation sites of hMCD (Blom et al., 1999) (Fig. 3.3A). Four serine (Ser-204, Ser-275, Ser-326, Ser-380), four threonine (Thr-9, Thr-60, Thr-245, Thr-396) and one tyrosine (Tyr-468) residues were potentially identified as phosphorylation sites, which are well conserved (Fig. 3.3A & B). Based on this prediction, we also ran preliminary proteomic analysis to identify the phosphorylation sites in the recombinant hMCD and found phosphorylated serine 204 and tyrosine 406. These residues were mutated to glycine (S204G) and phenylalanine (Y405F) using site-directed mutagenesis to eliminate the hydroxyl group for phosphorylation (Fig. 3.3C).

The point mutants S204G and Y405F without hydroxyl group for phosphorylation, were purified from silkworm fat body and pupae, and analyzed by Western blotting using an anti-phosphoserine and anti-phosphotyrosine antibodies (Fig. 3.6). The hMCD mutants displayed a substantial decrease on Western blotting analysis compared with the wild type MCD, indicating that Ser-204 and Tyr-405 are indeed the residues for phosphorylation. The lack of complete absence of the phosphorylated MCD band in Western blotting, however, could be ascribed to phosphorylated amino acid residues other than Ser-204 and Tyr-405.

To evaluate the effect of phosphorylation on hMCD biological function, the decarboxylase activity of hMCD mutants were measured. The specific activities of S204G mutant purified from fat body and pupae were 30.36 ± 2.25 (n = 6) and 24.37 ± 1.99 nmol/mg/min (n = 6), respectively, which are lower by 50% than that of wild type

Δ39aa-hMCD (Table 3.3). In addition, the specific activities of Y405F mutants were 33.45 ± 3.56 (n = 6) and 31.24 ± 1.69 nmol/mg/min (n = 6) purified from fat body and pupae, respectively. Collectively, the dephosphorylation of hMCD diminishes the decarboxylase activity underlining the phosphorylation-induced regulation in catalytic function.

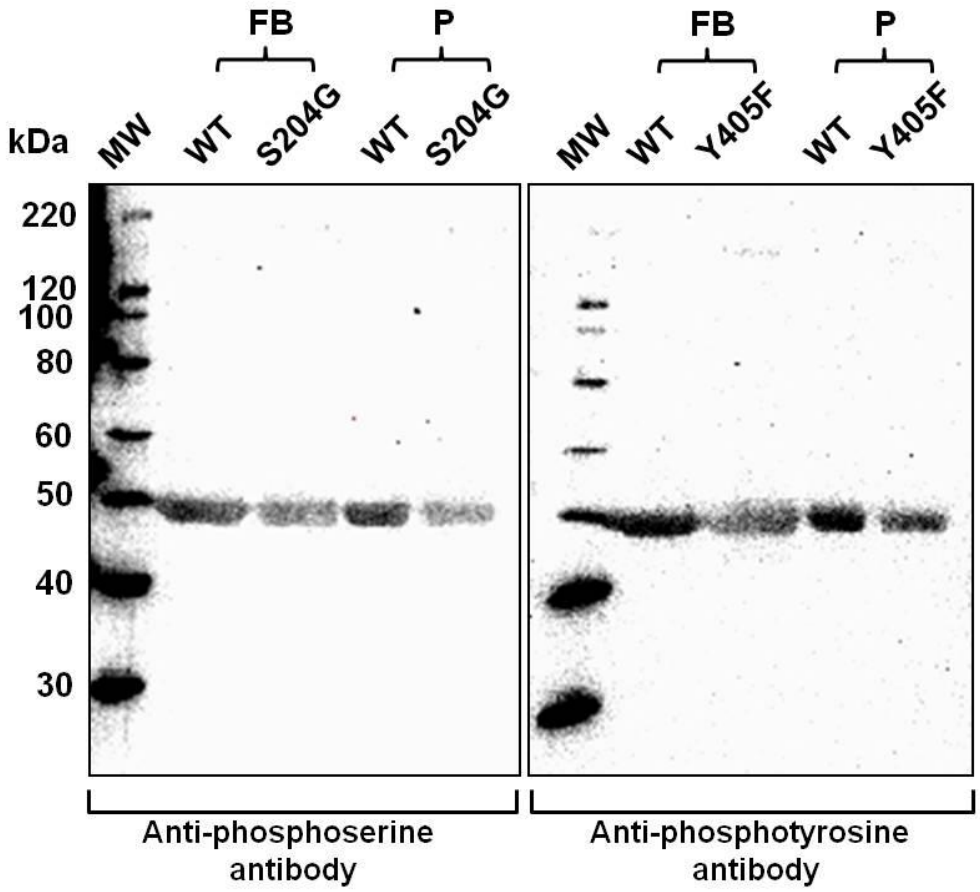

Fig. 3.6 Western blot of purified Δ39aa-hMCD and their mutants. MW, molecular weight markers; WT, wild type of Δ39aa-hMCD; S204G, mutated Δ39aa-hMCD at Serine 204; Y405F, mutated Δ39aa-hMCD at Tyrosine 405; FB and P denote proteins purified from fat body and pupae, respectively. A monoclonal anti-phosphoserine and anti-phosphotyrosine antibodies produced in mouse were used as primary antibodies, and anti-mouse IgG-HRP was used as a secondary antibody.

Table 3.3. Specific activities of each purified Δ39aa-hMCD mutants from silkworm fat body and pupae

	Specific activity (mU/mg)*	Relative comparison
Fat body		
Δ39aa-hMCD	59.54 ± 7.68	1
S204G	30.36 ± 2.25	0.51
Y405F	33.45 ± 3.56	0.56
Pupae		
Δ39aa-hMCD	48.16 ± 7.89	1
S204G	24.37 ± 1.99	0.51
Y405F	31.24 ± 1.69	0.64

* 1 mU is defined as 1 nmol product formed per minute.

3.3.4 Structural implication of hMCD phosphorylation

The resolved crystal structure of human MCD (PDB accession number: 4F0X) reveals a molecular tetramer, composed by a dimer of structural heterodimer where the two subunits show different conformations (Fig. 3.7) (Aparicio et al., 2013). The monomer of MCD has an N-terminal helical domain for oligomerization and a C-terminal domain for catalysis, and the active site of MCD is located in a prominent groove clustered with evolutionarily conserved residues (Fig. 3.7A) (Aparicio et al., 2013; Froese et al., 2013). Through the inter-subunit disulfide bonds, Cys-206—Cys-206 and Cys-243—Cys-243 (Fig. 3.7B), the four subunits of the tetramer is connected, providing the positive cooperativity to the decarboxylase catalytic function (Aparicio et al., 2013).

Ser-204 identified here for the phosphorylated residue is located in the beginning of catalytic domain. The side chain of Ser-204 is fully exposed to solvent in the monomeric

structure (Fig. 3.7A & C), and ~20 Å away from the catalytic active site, suggesting that the phosphorylation could not directly affect the structural integrity of the catalytic site. However, the quaternary structure of hMCD reveals that Ser-204 is located nearby the Cys-206, the essential residue for the inter-subunit disulfide bond interaction (Fig. 3.7C). Thus, the phosphorylation/dephosphorylation of Ser-204 might modulate the disulfide bridge formation, contributing to the catalytic function. Moreover, as Lys-210 (rat sequence; Lys-211 in human) has been reported to be an essential amino acid residue for rat MCD enzymatic function through acetylation (Nam et al., 2006), the post-translational modifications in the vicinity of Cys-206 could be a key player in regulation of MCD function.

In contrast to Ser-204, Tyr-405 is not in the vicinity of inter-subunit interactions. This residue is located near to the catalytic dyad with His-423 and Ser-329. The hydroxyl group of Tyr-405 forms a hydrogen bond with the side chain of Asn-417, which is in the same α-helix harboring catalytic His-423 (Fig. 3.7D). Based on this structural information, the phosphorylation of Tyr-405 might induce the conformational change of catalytic dyad producing the fine-tuning in decarboxylase activity.

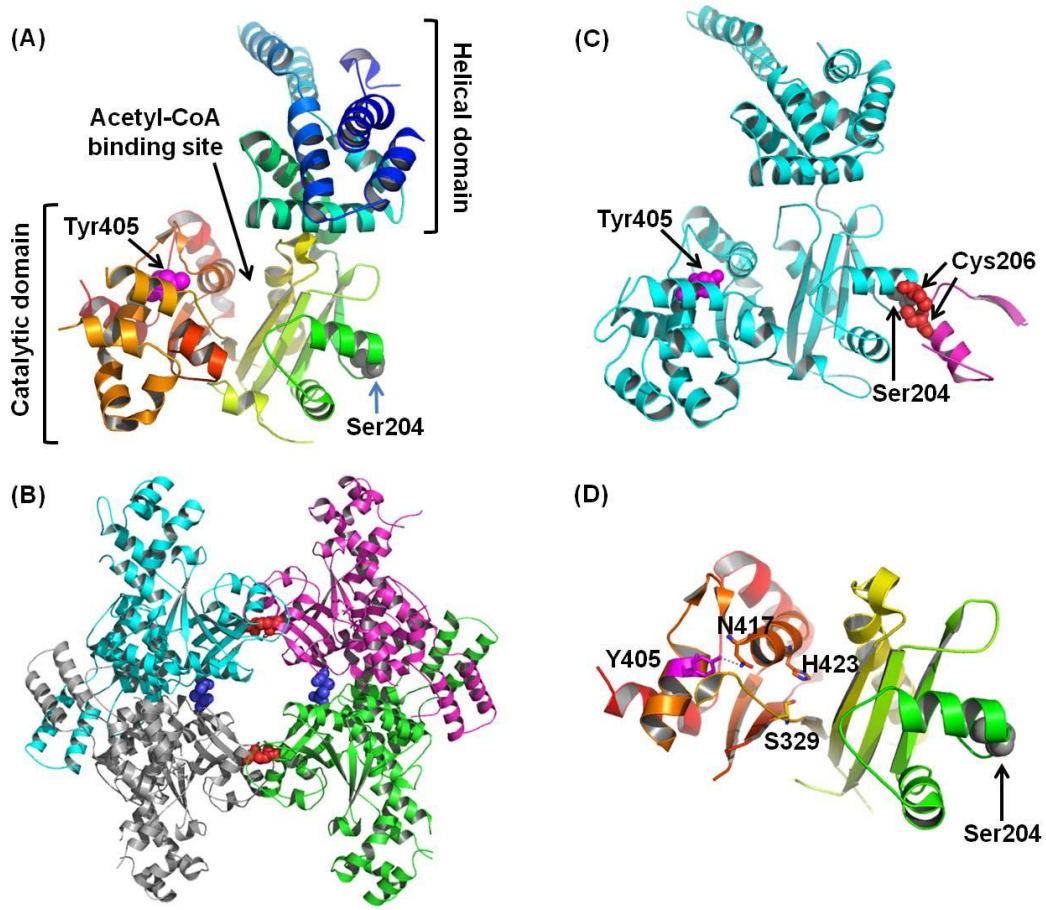

Fig. 3.7 Molecular organization of human peroxisomal MCD (PDB accession number: 2YGW). (A) A cartoon representation of MCD monomer composed of an N-terminal helical domain and a C-terminal catalytic domain where the catalytic acetyl-CoA binding site is located. (B) Structural arrangement of MCD tetramer. Inter-subunit disulfide bridges, Cys-206—Cys-206 in red spheres and Cys-243—Cys-243 in blue spheres, link the four subunits of tetramer. (C) MCD with phosphorylated residues of Ser-204 and Tyr-405. Cys-206 connects two subunits through the disulfide bond. (D) Tyr-405, located in the vicinity of the catalytic dyad of Ser-329 and His-423, forms a hydrogen bond with Asn-417. Cartoon MCD structures were generated with PyMol.

3.4 Discussion

In *de novo* long chain fatty acid metabolism, the principal role of MCD is to decarboxylate malonyl-CoA by on the rate-limiting step. This decarboxylation of MCD promotes fatty acid oxidation (Pender et al., 2006). In the study about relationship between glucose and lipid oxidation, over-expressed MCD was shown to increase fatty acid oxidation and improve whole body insulin resistance (An et al., 2004). On the other hand, reduced MCD levels provide decreased lipid oxidation with increase in glucose oxidation in human myotubes (Bouzakri et al., 2008). In addition, inhibition of MCD significantly diminished activation of palmitate oxidation and prevented increases in the ceramide level in cardiomyocytes (Samokhvalov et al., 2012). This MCD is composed of 39 amino acid mitochondria targeting sequence, 454 amino acids of MCD domain and a peroxisomal targeting sequence (SKL of C-terminus). The MCD is mainly distributed in mitochondria, cytoplasm and peroxisome (Sacksteder et al., 1999). In spite of these useful and various findings, the study about reaction mechanism of the MCD is still needed due to its biomedical importance. Here, we successfully produced the high level, purity of human MCD using silkworm expression system with post-translational phosphorylation. In protein expression study, some proteins need to folding process due to their insolubility, whereas MCD in this study was successfully expressed in a soluble form without any solubility enhance tag. Thus, the silkworm BmNPV bacmid system proved to be one of the powerful eukaryotic expression systems available. In most cases, the proteins expressed in baculovirus expression system are soluble, high levels and functionally active (Kato et al., 2010).

In order to evaluate the dephosphorylation effect on the decarboxylase activity of human MCD, we attempted the dephosphorylation using Lambda PP or alkaline phosphatase. However, the reaction of purified Δ39aa-hMCD derived from silkworm with phosphatase

revealed a poor success. According to the previous research, the structure of human MCD expressed in peroxisome showed tetrameric form and has inter-subunit disulfide bridges, Cys-206-Cys-206 and Cys-243-Cys243. It can be linked the four subunits of the tetramer (Aparicio et al., 2013). Meanwhile, our finding showed that the purified Δ39aa-hMCD from silkworm reveals a polymeric form in Native-PAGE gel. Thus, this result is supposed that this polymeric formation might be disturb the dephosphorylate reaction. Consequently, the dephosphorylation was achieved by site-directed mutagenesis with two locations of S204G and Y405F. These mutated Δ39aa-hMCDs, in common with wild-type of MCD, was successfully expressed in a soluble form and dephosphorylated.

Polymerization and phosphorylation of ACC, which catalyzes the reverse reaction of MCD, regulates its function. Therefore, polymerization of Δ39aa-hMCD and its mutants was analyzed by native PAGE. Interesting aspect of these mutants is that the mutations lead to slightly attenuated polymerization. The dephosphorylation by mutation affected the electrophoretic movement of Δ39aa-hMCD. It appeared that this changed electrophoretic movement was due to conformational changes of Δ39aa-hMCD by removal of phosphate group. Thus, this result suggests that polymerization and phosphorylation of hMCD regulated MCD function by conformational changes as well as ACC.

The phosphorylation of MCD is one of factor affecting decarboxylase activity on lipid homeostasis. Phosphorylation of MCD by AMPK promotes the enzymatic activity, leading to a decrease in the malonyl-CoA levels, whereas the dephosphorylation inhibits the catabolic function (Park et al., 2002; Saha et al., 2000). Up to date, it is estimated that MCD has several phosphorylation sites on the serine or threonine residues (Voilley et al., 1999). However, the phosphorylation effect on the human MCD activity has been much less studied. In this study, expressed human MCD in silkworm showed phosphorylated protein in western blot analysis.

Thus, the dephosphorylation of MCD was conducted by site directed mutagenesis. Consistent with previous reports, we demonstrated about 1.5–2.5-fold decreased specific activity in dephosphorylated human MCD compared to phosphorylated one. Taken together, dephosphorylation of human MCD by mutation leads to decreased polymeric MCD and decarboxylase activity. Thus, these results suggest that reduced degree of polymerization can influence enzymatic activity.

In summary, MCD is a metabolic enzyme participating in the production of acetyl-CoA from malonyl-CoA, a vital metabolite for anabolic fatty acid biosynthesis and catabolic lipid oxidation. Due to the pathophysiological relevance of MCD in many metabolic disorders, the regulation of MCD activity has been increasingly recognized as a candidate target for therapeutic interventions, essentially requiring the authentic heterologous protein production. In addition, the catalytic activity of MCD associated with phosphorylation/dephosphoryation has been previously evaluated, yet these studies provide inconsistent results (Park et al., 2002; Saha et al., 2000).

Here, we successfully produced recombinant human MCD using silkworm-based BmNPV expression equipped with a proper post-translational modification machinery. The heterologous MCD from silkworm demonstrated a consistent and higher decarboxylase activity compared to that of MCD from *E. coli*, underscoring the enzymatic activity enhancement by post-translational modification. Furthermore, we identified Ser-204 and Tyr-405 as phosphorylated residues using bioinformatics and proteomic analyses. The site-directed mutagenesis of these residues decreased enzymatic activity of MCD implying phosphorylation-induced activity regulation. Thus, the silkworm-based BmNPV expression system provides an eukaryotic protein production platform with post-translational

modification including phosphorylation, and MCD activity is significantly modulated by phosphorylation.

Chapter 4

Summary

Biotin-dependent human acetyl-CoA carboxylases (ACCs) play important roles in homeostatic lipid metabolism. By securing the post-translational biotinylation, ACCs perform its coordinated catalytic function allosterically regulated by several factors including phosphorylation/dephosphorylation and citrate. Due to the patho-physiological relevance of ACCs in lipid metabolic syndrome, the production of authentic recombinant ACCs is an emerging task to provide a reliable information and tool for drug discovery efforts by unmasking their molecular functions.

In this thesis, whether the human ACC2 (hACC2), an isoform of ACC expressed using silkworm BmNPV bacmid system, equips with proper post-translational modifications to carry out catalytic functions as silkworm harbors inherent post-translational modification machinery, was investigated. Purified hACC2 possessed biotinylation probed by biotin-specific streptavidin and biotin antibodies. In addition, phosphorylated hACC2 displayed limited catalytic activity whereas dephosphorylated hACC2 revealed 2-fold enhanced enzymatic activity. Moreover, hACC2 polymerization, analyzed by native page gel analysis and atomic force microscopy imaging, was allosterically regulated by citrate, and phosphorylation/dephosphorylation regulated citrate-induced hACC2 polymerization process.

Decarboxylation of malonyl-CoA to acetyl-CoA by malonyl-CoA decarboxylase (MCD) is an important reaction in the regulation of fatty acid metabolism. This regulation of MCD is affected by phosphorylation/dephosphorylation. Although MCD has several phosphorylation sites, whether it is inhibited or activated by phosphorylation has been not investigated. Therefore, human MCD (hMCD) without N-terminal mitochondria targeting sequence (1-39 amino acids) was over-expressed using silkworm BmNPV bacmid expression system and purified. It was proved by western blot using anti-phophoserine antibody, and native PAGE that hMCD purified silkworm fat body and pupae was phosphorylated and polymerized. The

specific activities of hMCD purified silkworm fat body and pupae were 60 and 48 nmol/mg/min, respectively. From LC-MS/MS experiment, Ser204 and Tyr405 might be phosphorylated. To make clarify and analyze the phosphorylation at each site the site-directed mutagenesis was performed. S204G and Y405F mutated proteins purified from silkworm fat body and pupa were found to be phosphorylated, but the level of phosphorylation was decreased in each mutant compared to that of wild type hMCD. The specific activities of S204G and Y405F purified from silkworm fat body were 30 and 34 nmol/mg/min and the specific activities of each mutant were lower by 40–50% than that of hMCD. The specific activity of each mutant purified from pupae was also lower by 35–50% lower than that of hMCD. Moreover, dephosphorylation by mutation leads to slightly attenuated degree of polymerization. These results suggest that dephosphorylation and polymerization of hMCD may be connected with its function. This research demonstrates that silkworm BmNPV bacmid expression system permits large potential value in recombinant eukaryotic protein production with proper post-translational modification such as phosphorylation for functional analysis.

The study might be a helpful guide for medical science or medicine industry to develop the inhibitors. Moreover, the silkworm-based BmNPV bacmid system would provide a reliable eukaryotic protein production platform for structural, functional analysis and therapeutic drug discovery applications implementing suitable post-translational biotinylation and phosphorylation.

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