Secretion of three enzymes for fatty acid synthesis into mouse milk in association with fat globules, and rapid decrease of the secreted enzymes by treatment with rapamycin

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[2] *Abbreviations used:* ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; ER, endoplasmic reticulum; FASN, fatty acid synthase; In2, involution of day 2; L10, day 10 of lactation; MFG, milk fat globule; mTOR, mammalian target of rapamycin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TOF-MS, time-of-flight mass spectrometry.

Abstract

The mammary epithelium produces numerous lipid droplets during lactation and secretes them in plasma membrane-enclosed vesicles known as milk fat globules. The biogenesis of such fat globules is considered to provide a model for clarifying the mechanisms of lipogenesis in mammals. In the present study, we identified acetyl coenzyme A carboxylase, ATP citrate lyase, and fatty acid synthase in mouse milk. Fractionation of milk showed that these three enzymes were located predominantly in milk fat globules. The three enzymes were resistant to trypsin digestion without Triton X-100, indicating that they were not located on the outer surface of the globules and thus associated with the precursors of the globules before secretion. When a low dose of rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), was injected into lactating mice, the levels of the three enzymes in milk were decreased within 3 h after injection. Since the protein levels of the three enzymes in tissues were not obviously altered by this short-term treatment, known transcriptional control by mTOR signaling was unlikely to account for this decrease in their levels in milk. Our findings suggest a new, putatively mTOR-dependent localization of the three enzymes for de novo lipogenesis.

Milk is essential for the rearing of mammalian pups. For sufficient nutritional supply, milk contains various components including milk fat, more than 95% of which is triglycerides [1]. In the mammary epithelium, triglycerides have been reported to be initially synthesized within the endoplasmic reticulum (ER)[2] membrane [2], and they are then released from the ER with a lipid monolayer covering. The lipid droplets grow in size in the cytosol and are finally secreted through a characteristic mechanism, whereby they are coated with apical plasma membrane. These secreted droplets are known as milk fat globules (MFGs). Despite many detailed studies of the formation, growth, and secretion of MFGs, which have also been reviewed [2-5], details of the processes involved remain unclear. Analysis of lipid bodies in various cells is believed to be helpful for understanding the evolutionarily conserved and divergent regulation of lipogenesis, thus contributing to various aspects of human health such as the prevention of metabolic diseases [6, 7]. Lipogenesis is increased in developing cancers, and proteins in this pathway are expected to become novel therapeutic targets [8].

After pups have been weaned, milk and lactating epithelial cells are unnecessary, and the mammary gland shifts to a regressive phase known as involution [9-11], in which most epithelial cells undergo apoptosis, and dying cells and accumulated milk are absorbed by surrounding tissues. Apoptosis reaches a peak around four days after weaning in mice, as estimated by fragmentation of nuclear DNA. Subsequent proteinase-dependent remodeling at the whole-tissue level ensures the tissue returns to a pre-pregnant state. To identify the genes involved in the initiation of involution, we have performed surveys using the mammary gland and milk of mice [12, 13]. A proteomic survey identified acetyl-CoA carboxylase (ACC, EC 6.4.1.2), ATP citrate lyase (ACL, EC 2.3.3.8, former EC 4.1.3.8), and fatty acid synthase (FASN, EC 2.3.1.85) in milk, and demonstrated that they are included in MFGs.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays an important role in cell growth [14]. As one of the mechanisms involved in promotion of cell growth, mTOR has been found to regulate lipogenesis, for example,

by activating the transcription of lipogenetic genes, including those of ACC, ACL, and FASN [3, 15]. Studies involving in vivo administration of the mTOR inhibitor, rapamycin, have suggested that the association of the three enzymes with MFGs is controlled by mTOR activity. However, this control by mTOR does not seem to be fully explained by the known transcriptional effect of the three enzymes.

Materials and methods

Mouse experiments

Details of the collection of milk and mammary gland tissue from mice have been described previously [12]. Briefly, BALB/c mice (8-9 weeks old) in mid-lactation, including day 10 of lactation (L10), were used, and the number of pups per dam was adjusted to 6 or 7. Milk was collected from the fourth mammary gland of BALB/c mice by hand after intraperitoneal injection of oxytocin. All pups were removed for 3 h before milking to collect milk from the lactating mice, as without this step, insufficient amounts of milk were collectable by hand, due to vigorous and continuous suckling by pups in mid-lactation. Since irreversible execution of mammary involution has been reported to begin around two days after weaning [9-11], it was considered that the effects of this short-term removal of pups would be negligible. Tissue was collected from the inguinal mammary gland, and involution was induced at L10 by removing the pups.

For rapamycin (LC Laboratories, Woburn, MA, USA) treatment, the drug was dissolved in dimethyl sulfoxide, kept at -20^{o}C, and diluted in sterile phosphate-buffered saline just before injection. BALB/c mice at L10 were injected intraperitoneally once with 0.2 mg/kg body weight rapamycin. Control mice were injected in the same way with the same volume of dimethyl sulfoxide diluted in saline. Milk was collected from each mother at 3 h after injection, and then the mammary tissue was collected. The

mothers were kept separated from the pups after the injection, in order to collect a sufficient amount of milk, as described above.

The animal experiments in this study were performed using protocols approved by the Animal Research Committee of Nagoya University.

Fractionation and triglyceride content of milk

Fresh whole milk (about 0.2 ml) was centrifuged at 25^{o}C for 15 min at 2,500 x *g*. The skim milk was then collected carefully using a pipette to prevent contamination of the cream on top of the milk. The cream containing milk fat was then washed once with isotonic buffer (10 mM Tris-HCl, pH 6.8, containing 0.25 M sucrose and 10 mM KCl). The skim milk was mixed with an equal volume of the isotonic buffer and subjected to centrifugation at 10,000 x *g* for 15 min at 25^{o}C to separate the whey (supernatants) and micellar caseins (precipitates). The caseins were washed once with distilled water before use. Total triglyceride contents of whole milk samples were determined enzymatically using a commercial kit (L-type Wako TG-M, Wako Pure Chemicals, Osaka, Japan).

Preparation of protein samples from milk and mammary gland

Proteins in whole milk and milk fat fractions were suspended in a modified sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), containing 10% SDS, and incubated at 60^{o}C for 6 h to solubilize MFG proteins effectively [16]. Proteins in the whey and casein fractions were dissolved in conventional SDS-PAGE sample buffer, containing 1% SDS, and boiled for 10 min. For protein extraction from the mammary gland, the tissue was minced with scissors, homogenized in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μm/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 50 μM proteasome inhibitor I, using a Potter-type homogenizer with a Teflon pestle, and centrifuged twice at 14,000 x *g* for 10 min at 2^{o}C. The supernatants

thus obtained were used as the protein samples of the tissue.

Protein electrophoresis and immunoblotting

SDS-PAGE, gel staining with Coomassie brilliant blue R-250, electroblotting, immunodetection, and measurement of the band intensities of the immunoblots were performed as described previously [17]. Rabbit polyclonal antibodies against mouse MFG-E8 and caseins were those described previously [18]. The following antibodies were purchased: rabbit polyclonal antibodies against ACC, ACL, ribosomal protein S6, and phospho-S6 ribosomal protein (Ser240/244) from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal anti-FASN antibody from BD Biosciences (San Jose, CA, USA); rabbit polyclonal anti-mouse transferrin antibody from Inter-Cell Technologies (Jupiter, FL, USA); mouse monoclonal antibody against α-tubulin from Sigma; goat polyclonal anti-xanthine oxidoreductase antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mass spectrometry (MS) and protein identification

Details of MS, including in-gel digestion with MS-grade trypsin (Promega, Madison, WI, USA), desalting with ZipTip C_{18} resin (Millipore), matrix-assisted laser-desorption time-of-flight (TOF) MS using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), and data analysis with Mascot (http://www.matrixscience.com), were performed as described previously [12, 13, 19]. The TOF-MS data were searched against NCBI-nr protein sequence databases using the Mascot peptide mass fingerprint search program. Protein identification was considered significant if the Mascot score was higher than that of a random match at $P < 0.05$.

Tryptic digestion and microscopic observation of MFGs

For tryptic digestion, the milk fat fractions containing MFGs were suspended

in the isotonic buffer described above and divided equally into three. Triton X-100 and trypsin (Sigma, St Louis, MO, USA) were added to appropriate MFG samples at concentrations of 1.0% (v/v) and 1.0 mg/ml, respectively. The three MFG samples were then kept at 25^{o}C for 20 min. To terminate trypsin digestion, phenylmethanesulfonyl fluoride was added at 1.5 mM.

As the control experiments, pure ACC, ACL, and FASN were digested with trypsin in the absence of Triton X-100 as follows. Recombinant human ACC and ACL, which were produced in insect cells, affinity-purified, and enzymatically active, were purchased from BPS Bioscience (San Diego, CA, USA). Each enzyme (1 μg) was subjected to tryptic digestion without Triton X-100, under the same conditions as described above, and then to immunoblotting with antibody against the enzyme. For purification of cytosolic FASN, the mammary gland at L10 was minced, homogenized in the isotonic buffer described above using the Potter-type homogenizer, and centrifuged at 700 g for 10 min at 4^{o}C. The post-nuclear supernatant was centrifuged at 20,000 g for 10 min at 4^{o}C and then ultracentrifuged at 100,000 g for 60 min at 4^{o}C. The cytosolic supernatant, containing 1.5 mg proteins, was pre-treated with 20 μl of protein G coupled to magnetic beads (New England BioLabs, Ipswich, MA, USA) at 4^{o}C for 15 h to remove material non-specifically bound to the beads. The pre-treated supernatant was incubated with the mouse anti-FASN antibody or control mouse IgG (Chemicon, Temecula, CA, USA) at 4^{o}C for 120 min. After the addition of 20 μl protein G-magnetic beads, each sample was incubated with rotation at 4^{o}C for 14 h. Protein G-bound immune complexes were washed four times with the isotonic buffer and subjected to tryptic digestion without Triton X-100 and then to immunoblotting with anti-FASN antibody.

For observation of MFGs, fresh whole milk was diluted 1:100 with phosphate-buffered saline and observed using a phase-contrast microscope (IX71, Olympus, Tokyo, Japan).

Results

Identification of ACC, ACL, and FASN in mouse milk

Figure 1A shows milk proteins in mid-lactation (L10) and early involution (48 h after forced weaning, or day 2 of involution, In2), which were separated by SDS-PAGE and then visualized with Coomassie blue. One of the obvious differences between the two profiles was the presence of ceruloplasmin and lactoferrin at In2, as reported previously [12]. In addition, two bands (a and b in this figure) were present at L10, but faintly visible at In2. The bands a and b from the L10 samples were subjected to proteomic identification by MS and found to be FASN and ACL, respectively (Table 1; the detailed MS data are presented in Supplementary Tables 1 and 2).

These two enzymes are related to de novo fatty acid synthesis. In this synthetic pathway, ACC is positioned between ACL and FASN. An acetyl group activated by ACL is converted by ACC to malonyl-CoA, and acetyl- and malonyl-CoAs are substrates for FASN. To detect these three enzymes in milk using another method, milk proteins were analyzed by immunoblotting (Fig. 1B). This analysis using an antibody against each of the three enzymes showed specific detection of cross-reactive materials in milk at L10. ACL and FASN detected by immunoblotting decreased from L10 to In2, as suggested in Fig. 1A. In addition to these two enzymes, ACC also decreased. The content of transferrin in milk was constant between L10 and In2, as reported previously [12] and shown in Fig. 1A. Because ACC and FASN have similar molecular masses [20], ACC was assumed to be included in the FASN bands (for example, band a in Fig. 1A) in SDS-PAGE under the conditions we employed. However, for unknown reasons, FASN was the only significant protein identified from band a by MS (Table 1).

ACC, ACL, and FASN are known to be located in the cytosol, and have been shown to be abundant in the lactating mammary gland [3, 20], suggesting that these

enzymes in milk originate from contaminating mammary tissue cells, which may become detached spontaneously, or during manual milk collection. To test this possibility, immunoblots from milk and mammary tissue (both at L10) were compared (Fig. 2). Under conditions where the three enzymes were clearly visible on the blots of milk and tissue, proteins of the cytoskeleton (α-tubulin) or ribosome (ribosomal protein S6), both of which are also abundant in the cytoplasm, were scarcely visible on the blots obtained from milk. This indicated that contaminating tissue cells were not the major source of ACC, ACL, or FASN in these milk samples

Localization of ACC, ACL, and FASN within MFGs

Milk can be separated into three different fractions that differ in their physicochemical properties: milk fat, whey (milk serum) including various soluble proteins, and micellar caseins [1]. Therefore we investigated the fractions that contained ACC, ACL, and FASN. Milk from mice at L10 were fractionated by low- and high-speed centrifugation (see Materials and Methods for details), and the three fractions were subjected to immunoblotting (Fig. 3A). Successful fractionation was confirmed by detection of a marker protein of each fraction. The three enzymes were detected from the milk fat fraction, there being virtually none in the whey or casein fraction. At the molecular level, milk fat is composed of MFGs. This fractionation showed that the three enzymes in milk were located predominantly in MFGs. Because cells including leukocytes and tissue debris, if present at detectable levels, were sedimented by the initial slow centrifugation, this also suggested that the enzymes did not originate from contaminating cells.

MFGs have a triglyceride-rich core covered with a lipid monolayer derived mainly from the ER membrane. The lipid bilayer derived from the apical plasma membrane surrounds the monolayer-coated core [1, 2]. Using trypsin digestion, we then examined whether ACC, ACL, and FASN were located on the outer surface of the MFG. Xanthine oxidoreductase, which is associated with cytosolic precursors of

MFGs [2, 3] and thus present inside MFGs, was completely resistant to tryptic digestion in the absence of detergent (Fig. 3B). This oxidoreductase was slowly digested in the presence of Triton X-100, perhaps because a non-denaturing form of the oxidoreductase was relatively resistant to tryptic attack [21]. On the other hand, MFG-E8, which is a secretory protein associated with the outer surface of the MFG [18, 22], was susceptible to trypsin with or without detergent. ACC, ACL, and FASN were resistant to tryptic digestion without detergent and susceptible in its presence. Pure ACC, ACL, and FASN were degraded with trypsin in the absence of Triton X-100 (Figure 3, C and D). Hence, it was unlikely that the detergent sensitized these enzymes to the protease irrespective of where they were loaded. These results indicated that the three enzymes in milk were located inside MFGs.

Inhibition of mTOR kinase causes a rapid decrease of ACC, ACL, and FASN in milk

Secretion of lipid from the mammary epithelium is unnecessary after weaning. A significant decrease in the protein levels of ACC, ACL, and FASN in the mammary gland was observed from L10 to In2 (Fig. 4A). Epithelial cells become a major population in mammary tissue during mid-lactation, and no obvious change in the cell population is evident at early involution, including In2 [9]. Thus the decrease in the whole tissue reflects that in the epithelium. These facts suggest that the observed decrease of the three enzymes in milk at In2 (Fig. 1B) results from a lack of cytosolic enzymes available for secretion, or is a secondary effect of cessation of MFG secretion during involution. To examine these possibilities, we tested the involvement of mTOR, for two reasons: (i) Manipulation of Akt kinase activity has been shown to alter milk fat secretion in mice [3], and one of the prominent targets of this enzyme is mTOR [14, 15]. (ii) mTOR inactivation was observed in early involution (Fig. 4B), as monitored by downstream S6 phosphorylation [23]. Hypophosphorylation of S6 at In2 has been demonstrated in a previous analysis of the cytoplasmic ribosome in the mouse mammary gland [19].

Rapamycin, an inhibitor of mTOR, was injected once intraperitoneally into lactating mice. The dose in this study (0.2 mg/kg) was far lower than the lethal dose in mice (LD_{50}, 600 mg/kg by intraperitoneal injection) [24]. Samples of milk and mammary gland were collected from these mice at 3 h after injection. Because continuous suckling by pups made it difficult to obtain a sufficient amount of milk for subsequent analyses (see also Materials and Methods), the dams were kept separately from their pups after the injection. mTOR activity in the mammary gland was significantly inhibited within 3 h after injection (Fig. 5A). By this time point, the levels of the three enzymes in milk were decreased (Fig. 5, B and C), as observed at In2 (Fig. 1). This alteration appeared to be selective because no change in the other main proteins in milk, such as albumin and caseins, was observed. No significant decrease of the three enzymes in mammary tissue was detected at the protein level as a result of this short-term treatment, although ACL and FASN in the tissue tended to decrease after the treatment (Fig. 5, D and E). Because no morphologic change was evident in MFGs by phase-contrast microscopy (Fig. 6A), and because the concentration of triglycerides in milk was not altered significantly (Fig. 6B), it seemed very unlikely that the decrease of the three enzymes in milk was the result of a decline in the milk MFG content. Taken together, the present findings suggested that the association of the three enzymes with MFGs was sensitively influenced by mTOR inhibition.

Discussion

The findings of the present study suggested that ACC, ACL, and FASN were secreted into milk (Figs. 1 and 2 and Table 1), in association with MFGs (Fig. 3). Association of FASN with MFGs in rodents and ruminants has been reported by several research groups [25-27]. ACC has been found to be present in bovine MFGs [28]. The presence of ACC, ACL, and FASN in the same compartment, and the synchronized

decrease of the three enzymes after weaning (Fig. 1B) and rapamycin treatment (Fig. 5C), might reflect their functional cooperation. Since the three enzymes are not secreted as free protein in milk (whey) (Fig. 3A) and are present inside the globules (Fig. 3, B-D), they are likely to be associated with fat droplets before secretion. Electron microscopy has shown that FASN in mammary epithelial cells is located on the ER surface and on cytoplasmic lipid droplets [25]. The ER possesses serial membrane enzymes that synthesize triglycerides from activated fatty acids, including palmitoyl-CoA, and glycerol 3-phosphate [2, 6, 7]. Palmitoyl-CoA is one of the major products of FASN, and ACC, ACL, and FASN are directly connected in the pathway of fatty acid synthesis. Hence, it can be hypothesized that these three enzymes are involved in triglyceride synthesis in intracellular MFG precursors. Assembling a set of enzymes for triglyceride synthesis, including those for fatty acid synthesis, on the ER membrane and in cytosolic MFG precursors is considered to be advantageous in terms of speed and efficiency. However, cytosolic lipid droplets prepared from mammary epithelial cells have been reported to show little ability to incorporate palmitoyl-CoA or glycerol 3-phosphate into triglycerides [29]. ACC in bovine MFGs is enzymatically inactive [28]. From the viewpoint of lipogenesis, it appears questionable whether particular enzymes should be so abundant as to be clearly detectable in unfractionated milk by SDS-PAGE (Fig. 1A). The protein coat of cytoplasmic lipid droplets changes dynamically in order to adapt to different functions, including formation, growth, and secretion [2, 3, 7]. Another scenario is that these enzymes are included mainly as structural constituents for budding from the ER, or fusion or secretion of cytosolic droplets. For example, knockdown of FASN has been reported to affect the morphology of cytoplasmic lipid droplets in *Drosophila* cells supplemented with oleate [30]. Xanthine oxidoreductase in MFGs is involved mechanically in their secretion [4]. Taken together, our findings appear to underscore the intriguing, but apparently complex roles of the enzymes in MFGs.

We also found a potential link between ACC, ACL, and FASN in milk and

mTOR (Figs. 5 and 6). Data linking mTOR to lipogenesis are accumulating. For example, mTOR has recently been reported to regulate the transcription of lipogenetic enzymes, including ACC, ACL, and FASN, via a transcription factor, SREBP-1 [3, 15]. The observed decrease of the three enzymes in milk was so rapid that their protein levels in mammary tissue were not obviously altered. Therefore, although association of the three enzymes with MFGs seems to be mTOR-dependent, transcriptional control is unlikely to be a primary mechanism for this association. The three enzymes have not been reported to have transmembrane domains or domains bound directly to lipid membranes. It can be speculated that these three enzymes localize to the ER membrane and/or cytosolic lipid droplets via some membrane protein, whose binding to these enzymes might be modulated by a signaling pathway including mTOR. The identification of mechanisms responsible for sorting specific proteins to the surface of intracellular lipid droplets is just beginning [7]. Our findings suggest a novel, spatial control of lipogenetic enzymes by mTOR. Further analysis of this putatively mTOR-dependent process in the mammary epithelium is expected to clarify the molecular aspects of both known and unforeseen regulatory mechanisms of protein localization in lipid droplets and also lipid droplet synthesis in mammalian cells.

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Figure legends

Figure 1. Identification of ACC, ACL, and FASN in mouse milk. (A) SDS-PAGE of proteins in whole milk. Proteins in milk (corresponding to 0.25 μl/lane) collected from mice at L10 and In2 were separated on 7.5% gels and stained with Coomassie brilliant blue. The bands a and b, which are indicated by the open arrowheads, were subjected to MS (Table 1). (B) Immunoblotting of whole milk with antibodies against ACC, ACL, FASN, and transferrin. These results are representative of at least three independent experiments. The migration positions of protein molecular mass markers are indicated on the left of each panel. Al, albumin; Cα1, α1-casein; Cα2, α2-casein; Cp, ceruloplasmin; Ltf, lactoferrin; Trf, transferrin.

Figure 2. ACC, ACL, and FASN in milk were not originated from contaminated cells of the mammary gland. Proteins from whole milk and the homogenates of the mammary gland (both at L10) were subjected to immunoblotting. The protein amount applied to each well of SDS-PAGE gels was adjusted so that the antigen recognized by antibody against ACC, ACL, or FASN was clearly detected from the samples of milk and the tissue. Under these electrophoretic conditions, immunodetection was performed using antibodies against α-tubulin and ribosomal protein S6. The migration positions of protein molecular mass markers are indicated on the left.

Figure 3. Localization of ACC, ACL, and FASN in milk. (A) Milk fractionation. Milk at L10 was fractionated into milk fat, whey, and caseins by serial centrifugation. There fractions were subjected to immunoblotting with the antibodies indicated on the right. Xanthine oxidoreductase and transferrin were used as the markers for milk fat and whey, respectively. (B) Treatment of MFGs with trypsin. The milk fat fraction including MGFs was subjected to trypsin digestion with or without Triton X-100 and then to immunoblotting. Xanthine oxidoreductase and MFG-E8 have been reported to

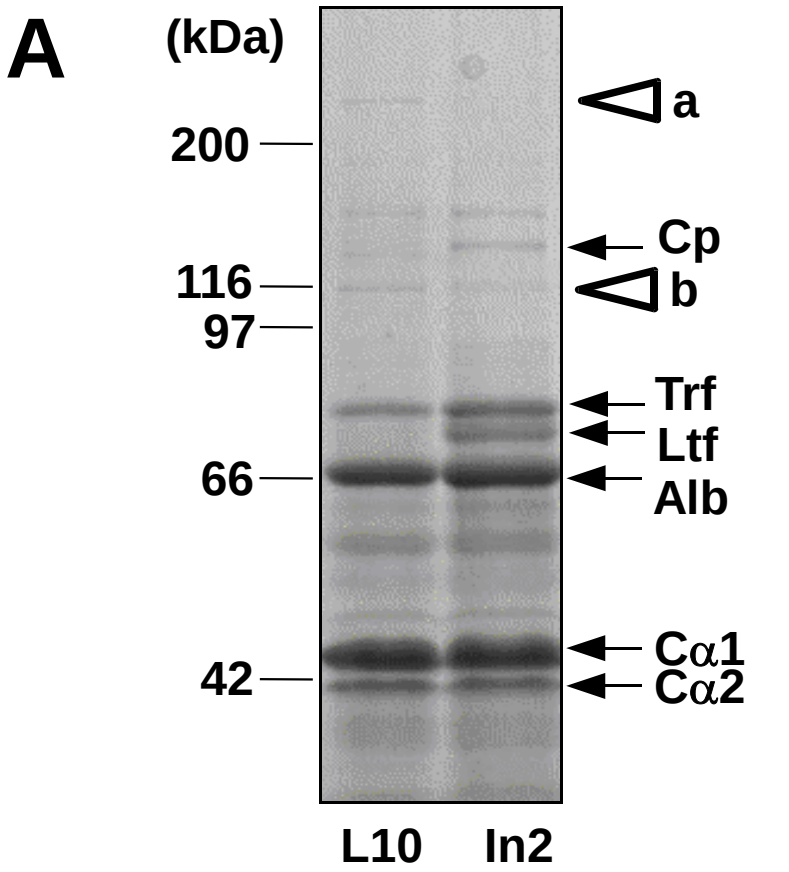
be present inside and outside MFGs, respectively. Trf, transferrin; XO, xanthine oxidoreductase. (C) Treatment of pure ACC and ACL with trypsin. Each enzyme was subjected to tryptic digestion without Triton X-100 and then to immunoblotting. (D) Treatment of immunoaffinity-purified FASN with trypsin. Cytosolic FASN was purified from the mammary gland by immunoprecipitation with anti-FASN antibody. This enzyme was subjected to tryptic digestion without Triton X-100 and then to immunoblotting with the same antibody.

Figure 4. Decrease in the protein levels of ACC, ACL, and FASN (A) and hypophosphorylation of ribosomal protein S6 (B) in the mammary gland during early involution. Proteins extracted from the tissue samples at L10 and In2 were subjected to immunoblotting with antibodies against ACC, ACL, FASN, S6, phosphorylated S6, and α-tubulin. Band intensities of the protein bands were measured by the image analyzer and estimated using tubulin as an internal control and are shown relative to that of the control (L10). The data represent mean ± SD (n = 3). Asterisks, $P < 0.05$.

Figure 5. Effect of rapamycin on association of ACC, ACL, and FASN with MFGs. Rapamycin or vehicle was injected intraperitoneally into mice at L10. Milk and the mammary gland were collected from these mice 3 h after injection and subjected to the following electrophoretic and microscopic analyses. (A) Phosphorylation of ribosomal protein S6 in the tissue. (B) SDS-PAGE (7.5% gel) of whole milk followed by Coomassie blue staining. The open arrowheads indicate the putative migration positions of FASN and ACC (upper) and ACL (lower). The migration positions of protein molecular mass markers are indicated on the left. Al, albumin; Cα1, α1-casein; Cα2, α2-casein; Trf, transferrin. (C) Immunoblotting of the three enzymes in whole milk. (D) Immunoblotting of the three enzymes and α-tubulin in the tissue. The results in (A) to (D) are representative of three independent experiments. (E) Band intensities of the protein bands in (D) were measured by the image analyzer and

estimated using tubulin as an internal control and are shown relative to that of the control (- rapamycin). The data represent mean ± SD (n = 3).

Figure 6. No obvious change in the morphology of MFGs or triglyceride content in milk after the rapamycin treatment. (A) MFGs in whole milk observed under a phase-contrast microscope. Bar, 100 μm. (B) The concentrations of triglycerides in whole milk, which were determined enzymatically and are shown relative to that of the control (- rapamycin). The data represent mean ± SD (n = 3).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L10
In2

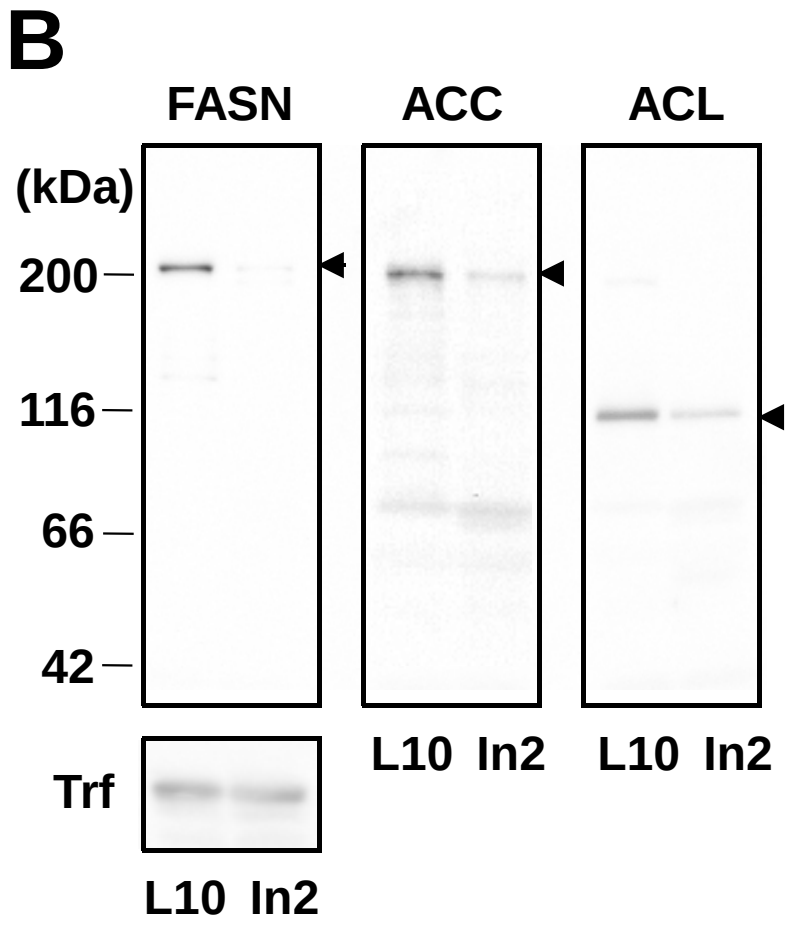

B
FASN
ACC
ACL
(kDa)
200
116
66
42
Trf
L10
In2
L10
In2
L10
In2
L10
In2

Figure 1

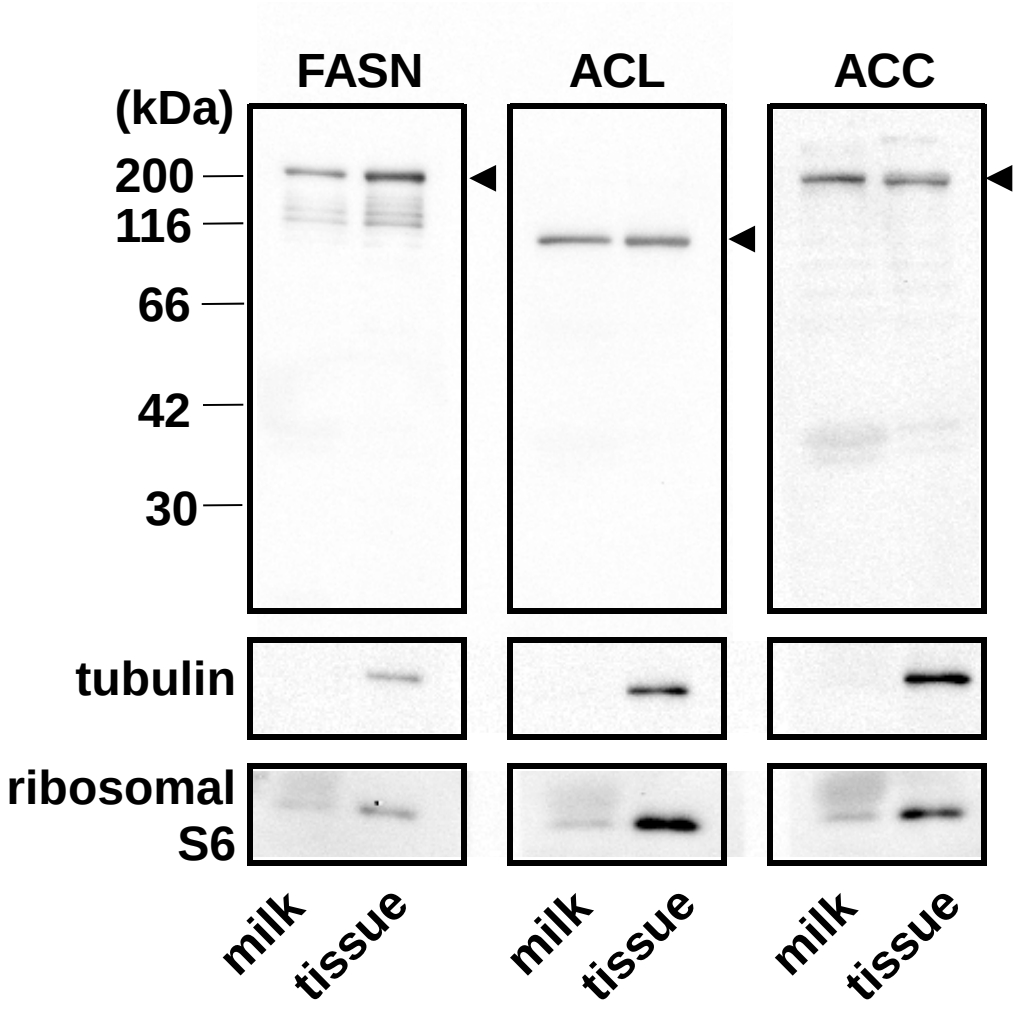

FASN
ACL
ACC
(kDa)
200
116
66
42
30
tubulin
ribosomal S6
milk
tissue
milk
tissue
milk
tissue

Figure 2

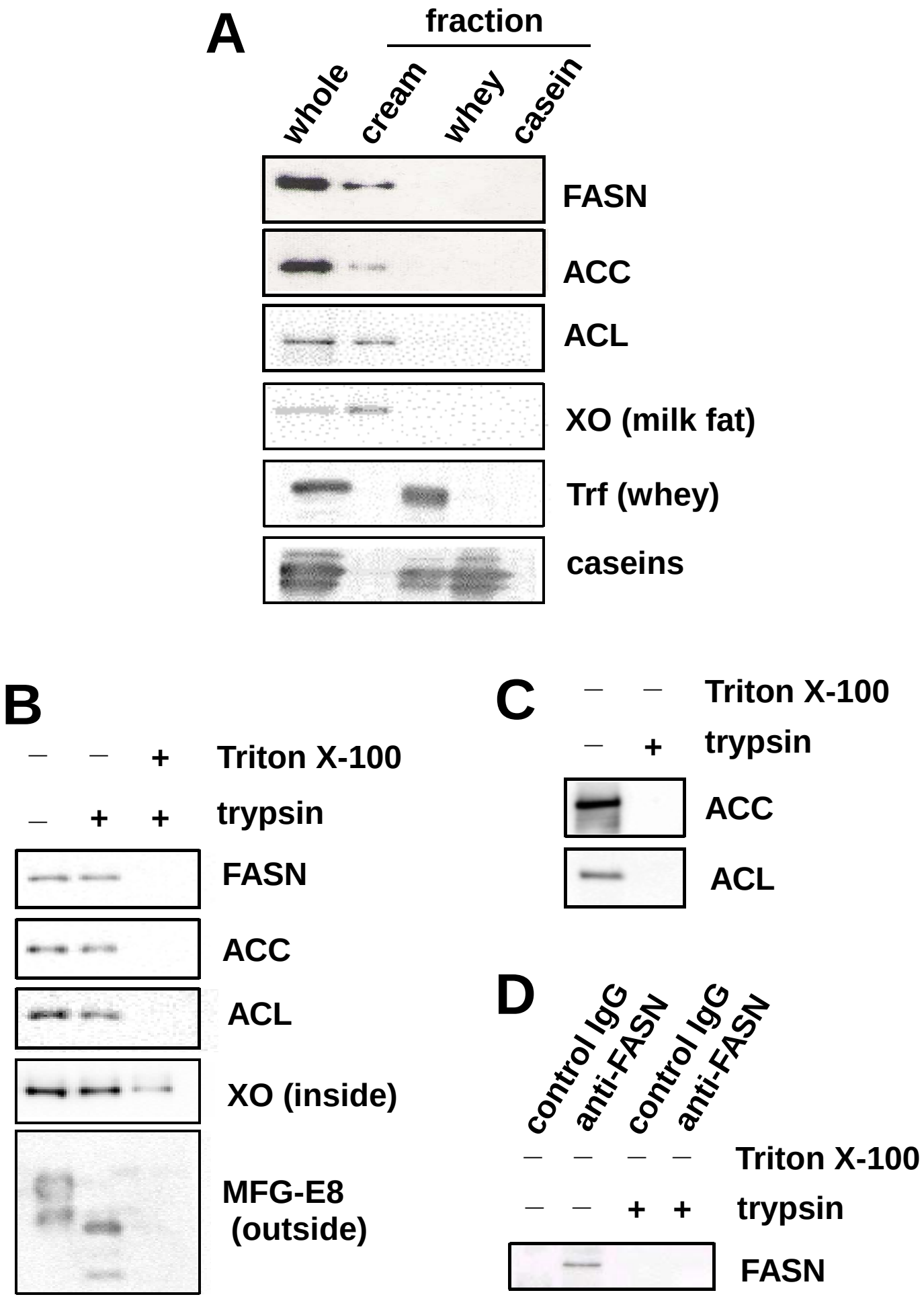
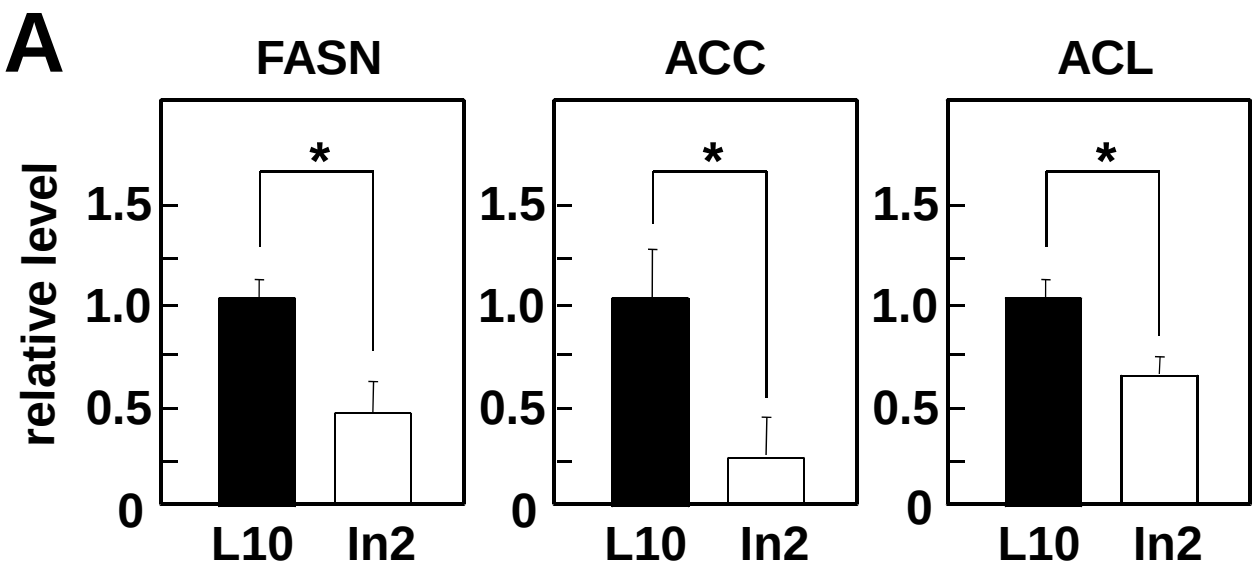

A
fraction
whole
cream
whey
casein
FASN
ACC
ACL
XO (milk fat)
Trf (whey)
caseins
B
– – + Triton X-100
– + + trypsin
FASN
ACC
ACL
XO (inside)
MFG-E8
(outside)
C
– – Triton X-100
– + trypsin
ACC
ACL
D
control IgG
anti-FASN
control IgG
anti-FASN
– – – – Triton X-100
– – + + trypsin
FASN

Figure 3


A
FASN
ACC
ACL
relative level
1.5
1.0
0.5
0
*
L10
In2

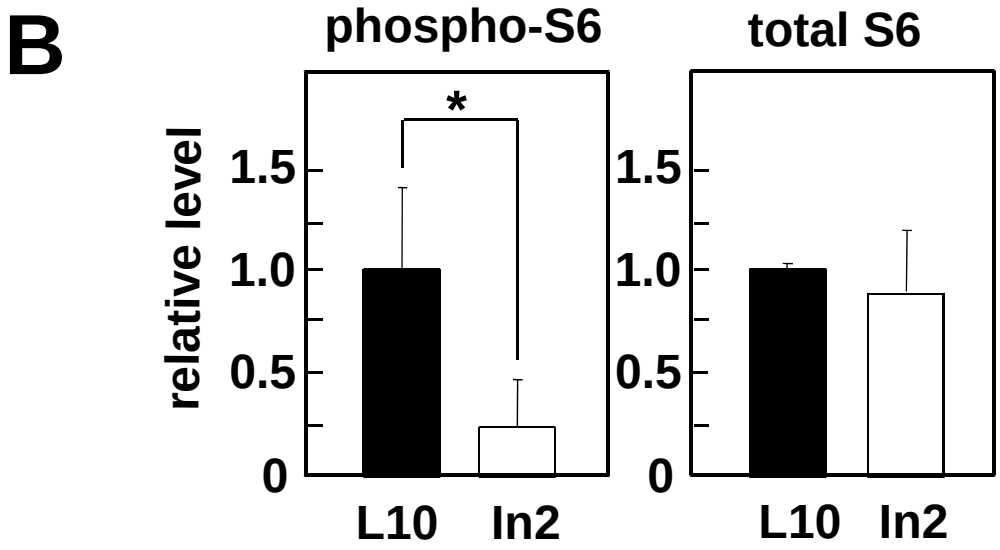

B
phospho-S6
total S6
relative level
1.5
1.0
0.5
0
*
L10
In2

Figure 4

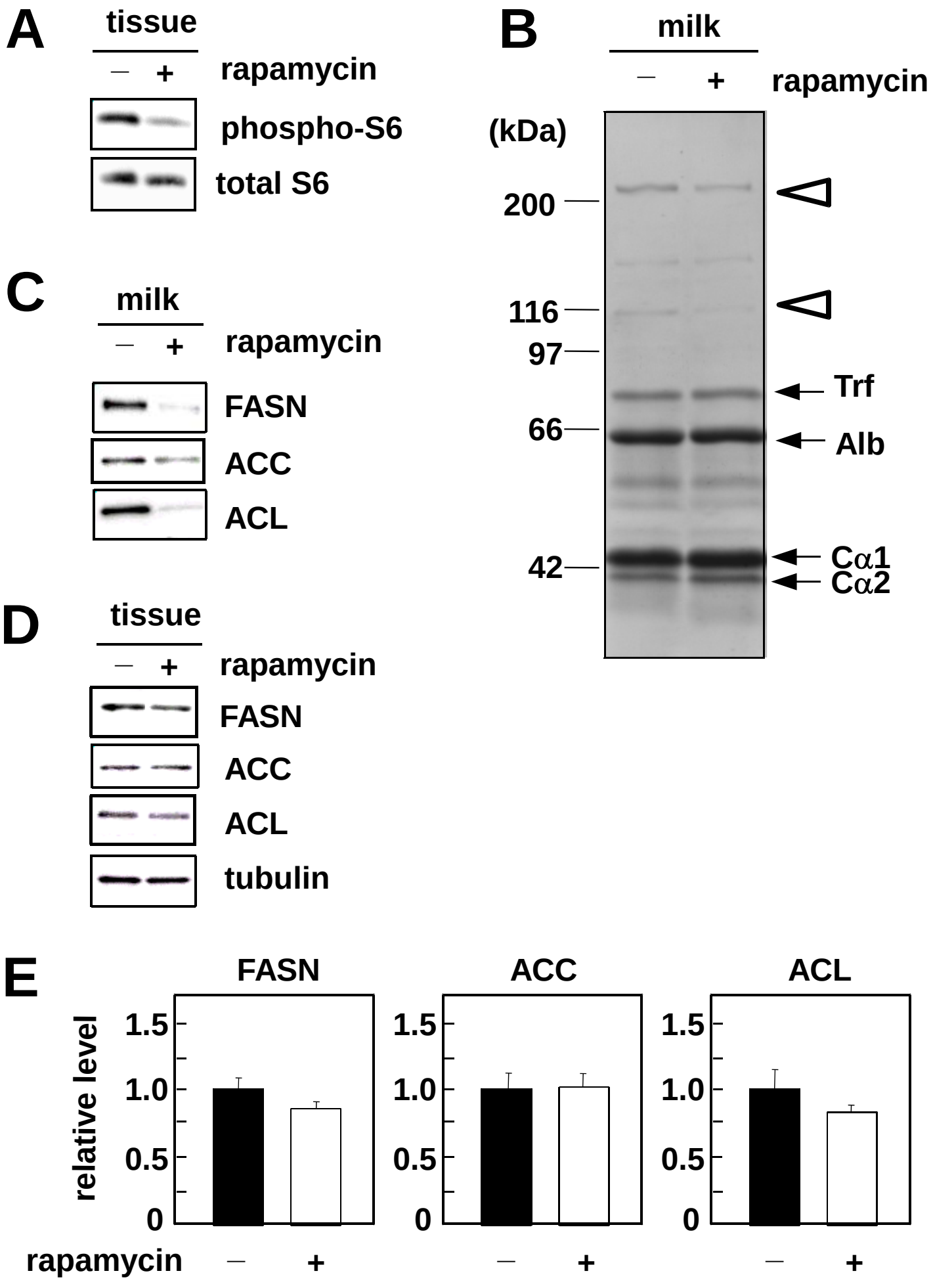

A
tissue
– +
rapamycin
phospho-S6
total S6
B
milk
– +
rapamycin
(kDa)
200
116
97
66
42
Trf
Alb
Cα1
Cα2
C
milk
– +
rapamycin
FASN
ACC
ACL
D
tissue
– +
rapamycin
FASN
ACC
ACL
tubulin
E
FASN
ACC
ACL
relative level
1.5
1.0
0.5
0
rapamycin
– +

Figure 5

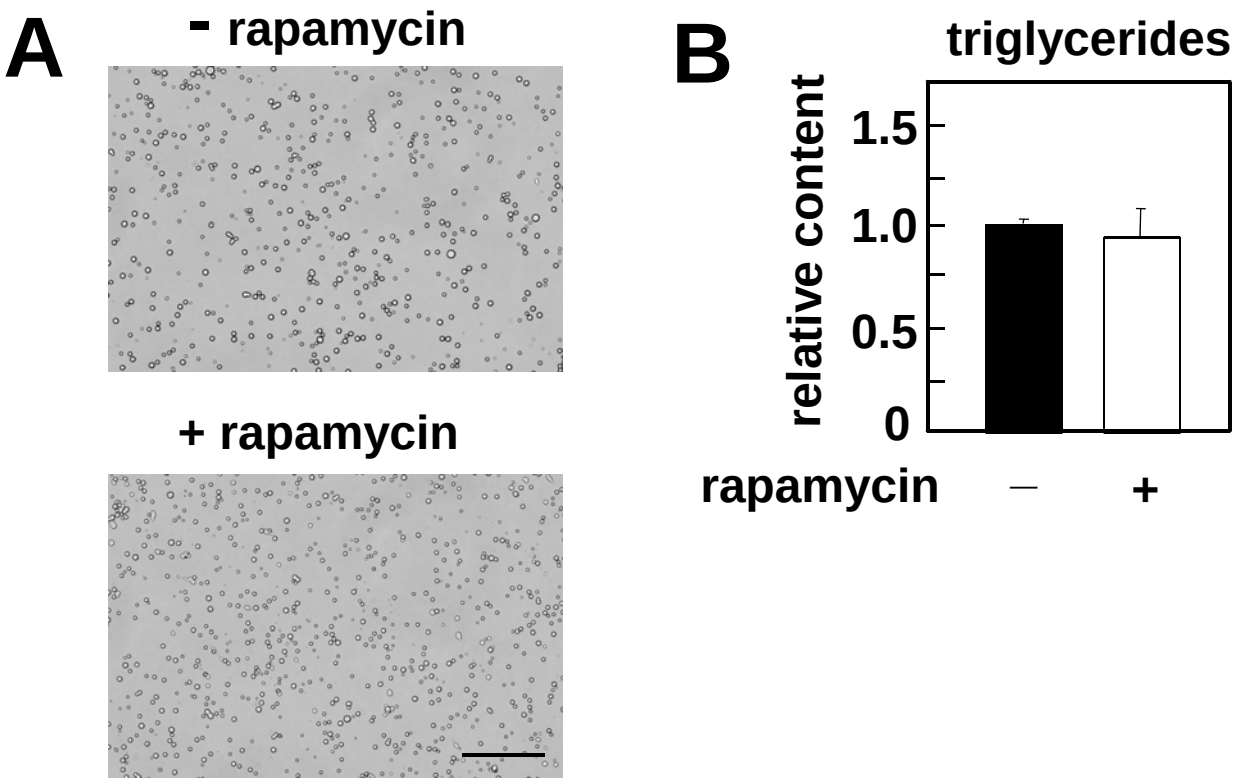

A
- rapamycin
+ rapamycin
B
triglycerides
relative content
1.5
1.0
0.5
0
rapamycin
–
+

Figure 6

Table 1. Protein identification from the bands in Fig. 1A by TOF-MS[a]

Band	Protein	Accession number	Calculated molecular mass	Score	Peptides matched
a	FASN[b]	gi|93102409	275667	127	16
b	ACL[b]	gi|29293809	120788	109	12

[a] Details of the TOF-MS data are listed in Supplementary Tables 1 and 2.

[b] Only one protein identified significantly as a *Mus musculus* protein from the band.

Supplementary Table 1. Detailed TOF-MS data on the protein band a (FASN) in Fig. 1A

Residue #	m/z (obs.)[a]	Mr (expt.)	Mr (calc.)	Delta	Miss[b]	Sequence
60–70	1251.6627	1250.6554	1250.6084	0.0470	0	K.FDASFFGVHPK.Q
214–224	1264.5472	1263.5399	1263.4826	0.0573	0	R.SFDDSGSGYCR.S
374–384	1291.8475	1290.8402	1290.7772	0.0630	0	R.LQVVDRPLPVR.G
380–409	2541.3806	2540.3733	2540.2885	0.0849	0	R.GGNVGINSFGFGGSNVHVILQPNTR.Q
430–442	1471.8452	1470.8379	1470.7678	0.0701	0	R.TLEAVQDLLEQGR.Q
469–478	1050.5959	1049.5886	1049.5506	0.0381	0	R.GYTVLGVEGR.V
598–606	1078.6182	1077.6109	1077.5607	0.0502	0	R.EAVLAAYWR.G
712–728	1960.0736	1959.0663	1958.9850	0.0813	0	R.WLSTSIPEAQWQSSLAR.T
800–825	2499.4285	2498.4212	2498.3434	0.0778	0	K.VHLTGINVNPNALFPPVEFPAPR.G
968–985	1965.0503	1964.0430	1963.9640	0.0791	0	K.LFDHPEVPTPPESASVSR.L
1464–1478	1668.9166	1667.9093	1667.8665	0.0429	0	R.CILLSNLSNTSHAPK.L
1519–1531	1485.8552	1484.8479	1484.7736	0.0743	0	K.EQTAHAFVNVLTR.G
1705–1717	1497.7635	1496.7562	1496.6896	0.0666	0	R.FPQLDDTSFANSR.D
1879–1895	1724.0071	1722.9998	1722.9305	0.0694	0	K.SYIITGGLGGFGLELAR.W
1955–1969	1558.9375	1557.9302	1557.8701	0.0601	0	K.LGPVGGVFNLAMVLR.D (including M oxidation)
2408–2419	1398.7649	1397.7576	1397.6979	0.0597	0	R.ELSFAAVSFYHK.L

[a] The observed m/z values in TOF-MS correspond to singly charged peptides (MH^+). Each experimental m/z value was transformed to a relative molecular mass (M_r) by the Mascot program. The M_r values are shown as “Mr (expt.)” in this table.

[b] One missed cleavage was allowed in a Mascot search.

Supplementary Table 2. Detailed TOF-MS data on the protein band b (ACL) in Fig. 1A

Residue #	m/z (obs.)[a]	Mr (expt.)	Mr (calc.)	Delta	Miss[b]	Sequence
5–17	1479.8864	1478.8791	1478.7980	0.0811	1	K.AISEQTGKELLYK.Y
233–244	1417.7690	1416.7617	1416.6826	0.0791	0	K.WGDIEFPPPFGR.E
337–355	1950.2052	1949.1979	1949.0986	0.0993	0	K.ILIIGGSIANFTNVAATFK.G
370–378	1129.6694	1128.6621	1128.5928	0.0694	0	K.EHEVTIFVR.R
379–389	1246.7037	1245.6964	1245.6214	0.0750	1	R.RGGPNYQEGLR.V
489–497	1061.6218	1060.6145	1060.5488	0.0657	0	K.AIVWGMQTR.A
552–566	1738.0555	1737.0482	1736.9573	0.0909	1	K.KHPEVDVLINFASLR.S
553–566	1609.9369	1608.9296	1608.8624	0.0672	0	K.HPEVDVLINFASLR.S
583–597	1567.9897	1566.9824	1566.8981	0.0844	0	R.TIAIIAEGIPEALTR.K
641–652	1367.8196	1366.8123	1366.7357	0.0766	0	K.LYRPGSVAYVSR.S
682–693	1422.7672	1421.7599	1421.6762	0.0837	0	R.YPGSTFMDHVLR.Y
909–924	1646.9840	1645.9767	1645.8887	0.0881	0	K.DLVSSLTSGLLTIGDR.F

[a] The observed m/z values in TOF-MS correspond to singly charged peptides (MH^+). Each experimental m/z value was transformed to a relative molecular mass (M_r) by the Mascot program. The M_r values are shown as “Mr (expt.)” in this table.

[b] One missed cleavage was allowed in a Mascot search.