Dictyostelium acetoacetyl-CoA thiolase is a dual-localizing enzyme that localizes to peroxisomes, mitochondria and the cytosol

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Acetoacetyl-CoA thiolase is an enzyme that catalyses both the CoA-dependent thiolytic cleavage of acetoacetyl-CoA and the reverse condensation reaction. In Dictyostelium discoideum, acetoacetyl-CoA thiolase (DdAcat) is encoded by a single acat gene. The aim of this study was to assess the localization of DdAcat and to determine the mechanism of its cellular localization. Subcellular localization of DdAcat was investigated using a fusion protein with GFP, and it was found to be localized to peroxisomes. The findings showed that the targeting signal of DdAcat to peroxisomes is a unique nonapeptide sequence (15RMYTTAKNL23) similar to the conserved peroxisomal targeting signal-2 (PTS-2). Cell fractionation experiments revealed that DdAcat also exists in the cytosol. Distribution to the cytosol was caused by translational initiation from the second Met codon at position 16. The first 18 N-terminal residues also exhibited function as a mitochondrial targeting signal (MTS). These results indicate that DdAcat is a dual-localizing enzyme that localizes to peroxisomes, mitochondria and the cytosol using both PTS-2 and MTS signals, which overlap each other near the N-terminus, and the alternative utilization of start codons.

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INTRODUCTION

Thiolases are known to exist as various functional forms in both prokaryotes and eukaryotes. They are divided into two groups: acetoacetyl-CoA thiolases (ATs; EC 2.3.1.9) and β-ketoacyl-CoA thiolases (KTs; EC 2.3.1.16). The former group are biosynthetic thiolases that catalyse both the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA and the CoA-dependent thiolysis of acetoacetyl-CoA. This type of thiolase is involved in ketone body metabolism, steroid biogenesis and poly-β-hydroxybutyrate biosynthesis. In contrast, KTs are degradative thiolases that play a key role in fatty acid β-oxidative degradation. In eukaryotes, there are five types of thiolases that are distinguished by their function and subcellular localization, that is, mitochondrial and peroxisomal ATs, mitochondrial and peroxisomal KTs, and cytosolic AT. The mitochondrial AT is considered to regulate the levels of acetoacetyl-CoA and acetyl-CoA in mitochondria, and is also involved in ketone body metabolism. This enzyme is encoded by the nuclear genome, synthesized on free polysomes in the cytosol as a precursor protein with the N-terminal pre-sequence, imported into mitochondria, and processed into mature enzyme by mitochondrial processing peptidase (MPP). Peroxisomal ATs have been found in yeast (Kurihara et al., 1992) and the rat liver (Antonenkov et al., 2000). It is believed that the peroxisomal AT catalyses the first reaction of peroxisomal cholesterol and dolichol syntheses (Antonenkov et al., 2000). The cytosolic AT catalyses the first reaction of the mevalonate biosynthesis pathway, and the acetoacetyl-CoA that is formed enters the steroid biosynthesis pathway in peroxisomes (Olivier et al., 2000).

Peroxisomal proteins have a peroxisomal targeting signal (PTS) sequence necessary for import of those proteins into peroxisomes. Two types of PTS are well known: PTS-1 is a tripeptide motif of SKL sequence, which is located at the C-terminal end (Gould et al., 1987; Elgersma et al., 1996); and PTS-2 is a nonapeptide motif of the consensus sequence (R/K)(L/V/I)X₅(H/Q)(L/A) within the N-terminal region, where X indicates any amino acid (Swinkels et al., 1991). The majority of peroxisomal proteins have a PTS-1 sequence.
at the C-terminus, although a few peroxisomal proteins have a PTS-2 sequence near the N-terminus.

The cellular slime mould Dictyostelium discoideum is a simple eukaryote that has two distinct stages of growth and development. In its growth phase, this organism grows vegetatively as a unicellular amoeba undergoing cell division with food supply. Development can be synchronized and separated from the vegetative growth phase. When nutrients are depleted, growing amoebae aggregate and eventually form a fruiting body containing spores that are supported on a stalk. Because of its unique life cycle, D. discoideum has been used as a model organism for studying developmental processes, and the Dictyostelium Genome Project has been published (Eichinger et al., 2005). Searches of the Dictyostelium database (http://dictybase.org) have revealed that the Dictyostelium genome includes three single genes for AT, KT and a putative thiolase, respectively (Dicty gene ID: DDB_G0271544, DDB_G0274339 and DDB_G0269588).

The latter two thiolases have not yet been characterized. DDB_G0271544, DDB_G0274339 and DDB_G0269588).

Methods

Growth of Dictyostelium. D. discoideum (AX-3 strain) was grown axenically in HL5 medium (Sussman & Sussman, 1967) supplemented with 100 μg streptomycin ml⁻¹, at 22 °C on a reciprocal shaker (150 r.p.m.) up to a density of ~ 5.0 × 10⁶ cells ml⁻¹.

Construction of fusion genes of gfp with the full-length, truncated and mutated acat genes. Specific primers used for PCR amplification are listed in Table 1. The acat gene was PCR amplified using pBSGT-3 cDNA (Tanaka et al., 2011; renamed as pBthio) as a template with a pair of primers (XSBf and thiohir). A thiohir reverse primer was generated to create a HindIII site instead of the stop codon. The PCR product was inserted between the BamHI and HindIII sites of plBluescript SKII(+) to yield pBthio-. The gfp gene, amplified using pUC118H-gfp as a template with a set of primers (gfp1f and gfpxhr), was fused in-frame at the 5’-end, to the 3’-end of the acat gene in pBthio-, to yield pBthio-gfp. The BamHI/XhoI fragment (containing the thio-gfp fusion gene) from pBthio-gfp was subcloned between the corresponding sites of the Dictyostelium expression vector pDNeo67 to yield pDthio-gfp.

The various lengths of the acat N-terminal coding sequence, which encodes the first 15, 18, 25, 33 and 50 residues, were PCR amplified with acat gene-specific primers using pBSGT-3 cDNA as a template (Table 1). The PCR products digested with BamHI and HindIII were subcloned between the corresponding sites of pB-gfp (derived from pBthio-gfp by deleting the full-length acat gene) to generate the plasmids pBthioN15–gfp, pBthioN18–gfp, pBthioN25–gfp, pBthioN33–gfp and pBthioN50–gfp. In the case of the pBthioN15–gfp construct, a mutated primer (N15thim) was used as a reverse primer, in which base changes were introduced to create a HindIII site in the sequence encoding K14 and R15 (described later). The BamHI/XhoI fragments (containing the fusion genes of gfp with various lengths of the acat N-terminal coding sequence) from these plasmid DNAs were subcloned between the corresponding sites of pDNeo67. The resulting constructs

Table 1. Primers used in this work

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<th>Primer</th>
<th>Sequence</th>
<th>Enzyme sites introduced</th>
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<tr>
<td>XSBf</td>
<td>5'-GCCGCCCGCTCTAGAACTGATGATCCG-3'</td>
<td>XhoI, SpeI, BamHI</td>
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<tr>
<td>thiohir</td>
<td>5'-GCCAGCTTTGCTAAAAACTAAAGGAG-3'</td>
<td>HindIII</td>
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<td>N50hir</td>
<td>5'-GCCAGCTTTGCTAAGCTGGTATTGTTG-3'</td>
<td>HindIII</td>
</tr>
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<td>N33thim</td>
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<td>HindIII</td>
</tr>
<tr>
<td>N25thim</td>
<td>5'-GCCAGCTTTGCTAAGCTGGTATTGTTG-3'</td>
<td>HindIII</td>
</tr>
<tr>
<td>N18thim</td>
<td>5'-GCCAGCTTTGCTAAGCTGGTATTGTTG-3'</td>
<td>HindIII</td>
</tr>
<tr>
<td>N15thim</td>
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</tr>
<tr>
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<td>HindIII</td>
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were designated pDthioN15-gfp, pDthioN18-gfp, pDthioN25-gfp, pDthioN33-gfp and pDthioN50-gfp. To construct pDAN15thio-gfp, a 5'-thiolinker to anneal 5'-thioSF with 5'-thioASr oligonucleotides was prepared, in which the BamH I and Acc I sites were introduced at its 5'- and 3'-ends, respectively. After deletion of the original BamH I/Acc I fragment from pBthio-gfp, this linker was inserted into the corresponding sites of pBthio-gfp to yield pBAN15thio-gfp. pDAN15thio-gfp was also constructed, as described above.

The acat gene, containing a 271 bp intron or a deletion of part of the first exon of the 5' region, was PCR amplified using pBSGT-23 (Tanaka et al., 2011) as a template with two sets of primers (XS6F and 1stexthiobamf as forward primers, and thiohirn as a reverse primer). The 1stexthiobamf primer was designed to remove the sequence encoding the N-terminal 7 amino acids from the Dlacat gene in pBSGT-23. According to the construction procedure for pDthio-gfp, described above, each amplified fragment was joined in-frame to the 5'-end of the gfp sequence to yield pDgthio-gfp and pDA5'thio-gfp.

To generate the site-directed mutants R15L and K21E, R15Lr and K21Ef primers were designed. The 5'- region of the sequence was amplified with XS6F and R15Lr primers using pBSGT-3 as a template. The amplified fragment (mutated R15L) digested with BamH I and Acc I was inserted between the corresponding sites of pBthio-gfp (deletion of the wild-type BamH I/Acc I fragment) to make pBthio(R15L)gfp. The fragment (mutated K21Ef) amplified using K21Ef and thiohirn primers was digested with Acc I and Hind III, and inserted into the corresponding sites of pBthio-gfp (deletion of the wild-type Acc I/Hind III fragment) to make pBthio(K21E)gfp. To generate K15E and R15L mutants, the KR1415ELr primer was designed. The double-mutated fragment was amplified with XS6F and KR1415ELr primers and subcloned into pBthio-gfp (deletion of the wild-type BamH I/Acc I fragment) to yield pBthio(K14E,R15L)gfp. The BamH I/Xho I fragments containing fusion genes of the mutated thio(R15L), thio(K21E) and thio(K14E,R15L) with gfp were inserted between the corresponding sites of pDNeo67 to yield pDthio(R15L)-gfp, pDthio(K21E)-gfp and pDthio(K14E,R15L)-gfp, respectively.

To create a putative PTS-2 signal sequence, a PTS2 linker of annealed r19iS with r19iASr oligonucleotides was prepared, in which the Acc I site presented in the original acat putative PTS-2 sequence was eliminated by mutating the third base of the Y16 codon (TAT—TAC), with no change in the encoded amino acid. The Acc I and Hind III sites were introduced at the 5'- and 3'-ends of the linker, respectively. The linker was inserted into the corresponding sites of the Acc I/Hind III-treated pBAN15thio-gfp, from which the wild-type Acc I/Hind III fragment of acat was deleted, to yield pBppts2-gfp. The PCR product, digested with Acc I/Hind III sites were introduced at the 5'- and 3'-ends of the linker, respectively. The linker was inserted into the corresponding sites of pBAN15thio-gfp (deletion of gfp) to make pBppts2(r19i)gfp. To yield the plasmid pBthioN18-pts2(r19i)-gfp, the BamH I/Xho I fragments from pBthioN18-pts2-gfp and its mutated plasmid DNAs were subcloned to pDNeo67, as described above, to yield pDthioN18-pts2-gfps, pDthioN18(m16v)-pts2-gfps, pDthioN18(m16v)-pts2(m20v)-gfp and pDthioN18-pts2(r19i)-gfp.

The constructed plasmid DNAs were introduced into AX-3 cells by electroporation using a method described elsewhere (Howard et al., 1988), and transformed cells were selected using 20 μg G418 ml−1 as described previously (Nagayama et al., 2008). The expression of the fusion gene is regulated under the Dictyostelium actin-6 promoter and actin-8 terminator.

Fluorescence microscopy. Transformed cells expressing the full-length, truncated and mutated Thio-GFP fusion proteins were grown in HL-5 medium with shaking. Cells at a density of ~10^6 cells ml^-1 were harvested, and then mitochondria were labelled with MitoTracker Red (Invitrogen Molecular Probes) at a concentration of 100 nM in HL-5 medium. Observation of the stained cells was performed as described previously (Nagayama & Ohmachi, 2010).

Preparation of DdAcat-expressing strain ThioOE. To construct plasmid DNA for overexpression of DdAcat, the BamH I/Xho I fragments from pBSGT-3 were subcloned between the same sites of pDNeo67 to create pDthio. The construct was introduced into AX-3 cells, and transformed cells were selected by the procedures described above to obtain the DdAcat-overexpressing ThioOE.

Subcellular fractionation of Dictyostelium cells. Vegetatively growing cells of wild-type AX-3 and DdAcat-overexpressing strains were harvested and washed twice in ice-cold 12 mM sodium phosphate buffer (pH 6.7). After the cells were homogenized in sucrose buffer [250 mM sucrose in 30 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA and 1 mM PMSF] with a Potter–Elvehjem glass homogenizer using a Teflon pestle, the homogenate was centrifuged at 40 g for 10 min to remove cell debris. After the supernatant was centrifuged at 900 g for 10 min to remove nuclei, the resulting supernatant was then centrifuged at 10 000 g for 15 min to obtain the precipitate fraction (consisting mainly of peroxisomes and mitochondria) as a pellet. The supernatant was collected as the cytosol fraction.
Preparation of antibodies. The anti-\textit{DdAcat} and anti-\(\beta\)-MPP (anti-\(\beta\) subunit of MPP) antibodies were prepared previously (Nagayama et al., 2008; Tanaka et al., 2011). The antibody against \textit{Dictyostelium} peroxisomal HMGS-B (3-hydroxy-methylglutaryl-CoA synthase B) was raised by immunizing rabbits with synthetic peptide EKLIRRYKSKPISSKL, corresponding to amino acids 452–468 of HMGS-B (unpublished data). The antiserum was tested by Western blot analysis. Anti-actin antibody from Sigma-Aldrich was used.

Western blot analysis. The crude extracts (20 \(\mu\)g protein) from vegetatively growing cells and developing cells were separated on 12.5 \% SDS-polyacrylamide gels and then transferred to PVDF membranes. Western blot analysis was performed according to methods described previously (Nagayama et al., 2008), using anti-\textit{DdAcat}, anti-\(\beta\)-MPP or anti-HMGS-B antibodies as the primary antibodies. Anti-rabbit IgG-alkaline phosphatase (Sigma) or anti-mouse IgG-alkaline phosphatase (Dako) was used as the secondary antibody.
Subcellular localization of DdAcat

(b) (iii) ThiO(R15)-GFP

(iv) ΔN15Thio-GFP

(c) (i) ThiO(N15)-GFP

(ii) ThiO(K21E)-GFP

(iii) ThiO(K14E,R15L)-GFP

(iv) pPTS2-GFP

GFP MitoTracker Merger
RESULTS

**DdAcat localizes to peroxisomes**

To determine the subcellular localization of DdAcat, we constructed pDthio-gfp, in which pBSGT-3 cDNA is fused in-frame at the 3′-end with the 5′-end of gfp. Thio-GFP localized largely to punctate organelles [Fig. 1a(i)]. In cells transformed with pDgfp encoding GFP alone [prepared previously (Nagayama & Ohmachi, 2010)] as a control, GFP was distributed throughout the cytosol [Fig. 1a(iii)]. In the positive control, GFP-SKL, encoding GFP tagged with the SKL sequence of PTS-1 at the C-terminus, the fusion protein localized specifically to peroxisomes [Fig. 1a(ii)]. These results indicate that DdAcat localizes to peroxisomes.

**DdAcat contains signals for targeting to both peroxisomes and mitochondria within its N-terminus**

To identify the signal for targeting to peroxisomes, we examined the effects of the N-terminal region of DdAcat on its import to peroxisomes. At first, we prepared five constructs, which encoded various lengths of the N-terminal region (the first 15, 18, 25, 33 and 50 residues) fused in-frame to the N-terminus of GFP. ThioN50-GFP, ThioN33-GFP (data not shown) and ThioN25-GFP were localized to peroxisomes [Fig. 1b(i)]. ThioN15-GFP was distributed throughout the cytosol [Fig. 1b(iii)]. D(N15)Thio-GFP, which has the first 15 N-terminal residues deleted and is translated from the second start codon at position 16, was distributed throughout the cytosol [Fig. 1b(iv)]. These results suggest that the amino acids from positions 15 to 25 provide a sufficient signal for targeting to peroxisomes. However, ThioN18-GFP localized to the mitochondria [Fig. 1b(ii)], suggesting that the first 18 amino acids provide a sufficient signal for targeting to mitochondria.

**Targeting signal for import to peroxisomes is RMYTTAKNL**

The N-terminal sequence search revealed that DdAcat possesses a unique nonapeptide sequence (RMYTTAKNL) from positions 15 to 23, which is similar but not identical to the consensus sequence of PTS-2. To elucidate the specificity of this nonapeptide (referred to as a putative PTS-2-like sequence, pPTS-2), two site-directed mutants (R15L and K21E) were prepared. The mutated Thio(K21E)-GFP mislocalized to mitochondria [Fig. 1c(ii)], whereas the mutated Thio(R15L)-GFP was located in peroxisomes [Fig. 1c(i)]. The R15L substitution caused the formation of
a new nonapeptide sequence (KLMYTTAKN) with a single amino acid shift toward the N-terminus, which fits the consensus PTS-2 sequence. The results indicate that this nonapeptide exhibits a PTS-like function, causing the mutated protein to direct to peroxisomes. Next, we constructed pDthio(K14E, R15L)-gfp, which contains both K14E and R15L mutations. The double-mutated Thio(K14E, R15L)-GFP localized completely to the mitochondria [Fig. 1c(iii)]. pPTS2-GFP, containing the pPTS-2 sequence at the N-terminus of GFP produced from pDppts2-gfp, localized to peroxisomes [Fig. 1c(iv)]. From these results, we conclude that the RMYTTAKNL sequence of pPTS-2 is a PTS functioning as a type 2 PTS.

**DdAcat is also present in the cytosol**

Careful observation of the Thio-GFP distribution revealed that DdAcat may be present in the cytosol in addition to the peroxisomes. Using the cell fractionation procedure, we examined whether DdAcat is detected in the cytosol by preparing the precipitate and the supernatant from the post-nuclear fraction of Dictyostelium cells. DdAcat was strongly detected in the precipitate, but also detected slightly in the supernatant (Fig. 2a). Its amount in the supernatant was less than 10 %, compared to that in the precipitate. As a control, localization of Dictyostelium β-MPP (Nagayama et al., 2008) and HMGS-B (containing the C-terminal PTS-1; unpublished data), which are mitochondrial and peroxisomal enzymes, respectively, were analysed. These two enzymes were detected in the precipitate containing mainly peroxisomes and mitochondria, but not in the supernatant (Fig. 2b, c), showing that the cytosolic fraction was not contaminated with mitochondrial and/or peroxisomal proteins. As a cytosolic control, actin was detected in the supernatant (Fig. 2d). These results indicate that DdAcat is present both in peroxisomes and in the cytosol.

**Fig. 2.** DdAcat is also present in the cytosol. The precipitate (Ppt) and supernatant (Sup) fractions were prepared from the post-nuclear fraction of AX-3 cells by differential centrifugation, as described in Methods. Twenty micrograms of protein from each fraction was separated on 12.5 % SDS-PAGE gels, followed by Western blotting/immunostaining. DdAcat (a), β-MPP (b), HMGS-B (c) and actin (d) were detected with anti-DdAcat, anti-β-MPP, anti-HMGS-B and anti-actin antibodies, respectively. Lane M shows the protein marker, and the arrowheads indicate the 48 kDa protein band.

**Translation initiation from the second Met codon produces the cytosolic form of DdAcat**

The Ddcat is present as a single copy in the Dictyostelium genome, which has a single 271 bp intron with high A + T content (Tanaka et al., 2011). The intron disrupts the ORF between the N12 (AAT) and V13 (GAT) codons. DdAcat has a second Met at position 16. To examine the localization of the DdAcat produced from the gene, we constructed pDgthio-gfp carrying the full-length gene fused in-frame to the gfp gene. Most Thio-GFP produced from pDgthio-gfp localized in peroxisomes and partially in the cytosol (Fig. 3a). To avoid translation from the first Met codon, we constructed pD Δ5′gthio-gfp, in which the 5′ region encoding the first 7 N-terminal residues of the first exon was deleted, a 271 bp intron was retained and the sequence downstream of the act6 promoter in pDNeo67 was bound to Δ5′gthio-gfp at the BamHI site. In cells transformed with pD Δ5′gthio-gfp, the truncated Thio-GFP was distributed largely in the cytosol (Fig. 3b), suggesting that translation initiation from the second Met codon causes production of the cytosolic form. The fusion protein derived from pD Δ5′gthio-gfp also exhibited significant localization to the mitochondria.

To examine whether translation initiation from the second Met codon, M16, might give rise to the cytosolic form of the protein, we constructed a plasmid pDthio(m16V)-gfp, in which the M16V mutation was incorporated. Mutated Thio(M16V)-GFP produced from pDthio(m16V)-gfp was completely localized to peroxisomes and not in the cytosol [Fig. 1c(v)]. These results indicate that translation initiation from the second Met codon produces the cytosolic form of DdAcat.
Distal MTS signal at the N-terminus has priority over the proximal signal

Although the N-terminal 15 residues alone do not function for targeting to organelles [ThioN15-GFP, Fig. 1b(iii)], the first 18 residues function as an MTS [ThioN18-GFP, Fig. 1b(ii)]. However, ThioN25-GFP containing PTS-2 connected downstream of the first 14 residues is targeted to the peroxisomes [Fig. 1b(i)]. We prepared the plasmid pDthioN18-pts2-gfp, encoding the fusion protein ThioN18-PTS2-GFP, in which PTS2 is connected downstream of the first 18 residues. This fusion protein was targeted largely to mitochondria and partially to peroxisomes (Fig. 4a). A small amount of the fusion protein was also distributed in the cytosol. In this fusion protein, the second and third Met at positions 16 and 20 within its N-terminal region are present. Two plasmids were then prepared, pDthioN18(m16v)-pts2-gfp and pDthioN18(m16v)-pts2(m20v)-gfp, in which Met at positions 16 and 20 was substituted to Val, respectively. Cytosolic distribution of ThioN18 (M16V)-PTS2-GFP, derived from pDthioN18(m16v)-pts2-gfp, was observed to be similar to that of ThioN18-PTS2-GFP (Fig. 4b). However, ThioN18 (M16V)-PTS2(M20V)-GFP, derived from pDthioN18(m16v)-pts2(m20v)-gfp, was not localized in the cytosol (Fig. 4c). These results suggest that the second or third Met existing within the N-terminus of ThioN18-PTS2-GFP is able to serve as an initiation site to generate the cytosolic form of the protein. Furthermore, these fusion proteins were localized in part to the peroxisomes, as described above. Next, a defect in the PTS2 was created, that is, pDthioN18-pts2(r19i)-gfp in which the first Arg of the PTS2 signal was substituted to Ile was created. ThioN18-PTS2(R19I)-GFP was expressed in cells, but was not localized to the peroxisomes (Fig. 4d).

When MTS and PTS2 were arranged in tandem and the MTS sequence contained the 18 residues of ThioN18-PTS2, the protein was predominantly localized to the mitochondria, even with the presence of PTS2 downstream of MTS. ThioN18-PTS2 was also localized to peroxisomes, using the PTS2 signal, but its amount was low. These results suggest that the distal signal at the N-terminus is preferred over the proximal signal.

DdAcat precursor is processed by processing peptidase

To investigate the processing of de novo synthesized DdAcat in Dictyostelium, the DdAcat-expressing strain, ThioOE, was

![Fig. 3](image-url). The second Met causes distribution of DdAcet in the cytosol. The construct pDgthio-gfp, in which the full-length acat gene containing a 271 bp intron is fused to the gfp gene, was generated as described in Methods. The construct pDΔ5′gthio-gfp (a) and pDΔ5’gthio-gfp (b) were grown in HL-5 medium. Fluorescence microscope observation was performed as described in Fig. 1. Bars, 5 μm.
We examined whether DdAcat is overexpressed in the Thio\textsuperscript{OE} strain by Western blot analysis. A larger amount of DdAcat was expressed by the Thio\textsuperscript{OE} strain than by the AX-3 strain (Fig. 5a). We prepared the super-
natant and precipitate fractions from the post-nuclear frac-
tion of the Thio\textsuperscript{OE} strain. As shown in Fig. 5(b), larger
amounts of DdAcat were detected in the precipitate and
smaller amounts in the supernatant of the Thio\textsuperscript{OE} strain,
and they were expressed at higher levels in those fractions
relative to the AX-3 strain. Interestingly, two bands, the
long and short forms (Fig. 5b, indicated by an arrowhead
and a circle, respectively), were detected in the supernatant

\textbf{Fig. 4.} Subcellular localization of ThioN18-PTS2-GFP and its site-directed mutants. In addition to pD/thioN18-pts2-gfp, con-
structs were generated with M16V and/or M20V mutations and the R19I mutation. (a) ThioN18-PTS2-GFP, (b) ThioN18
(M16V)-PTS2-GFP, (c) ThioN18 (M16V)-PTS2(M20V)-GFP and (d) ThioN18-PTS2(R19I)-GFP. Fluorescence microscope
observation was performed as described in Fig. 1. Bars, 5 \( \mu \)m.
of the Thio\textsuperscript{OE} strain, although the short form was only detected in the supernatant of the AX-3 strain. When the supernatant of the Thio\textsuperscript{OE} strain was incubated at 25 °C for 1 h, these two bands were not changed in size. When incubated with the precipitate prepared from the AX-3 strain, the long form of the protein was processed to the smaller protein (Fig. 5c), suggesting that the precipitate fraction might contain some processing peptidase(s) that changes the long form to the short form. Taken together, we conclude that the short form is a cytosolic DdAcat and that the long form detected in the supernatant is a precursor, which has not yet been imported into the peroxisomes.

**DISCUSSION**

Eukaryotic AT has been reported to be present in the cytosol, mitochondria and peroxisomes (Thompson & Krisans, 1990; Hovik \textit{et al.}, 1991; Olivier \textit{et al.}, 2000).

Mitochondrial AT is involved in leucine and ketone body metabolism, whereas peroxisomal AT catalyses the first reaction of the mevalonate pathway leading to cholesterol biosynthesis (Antonenkov \textit{et al.}, 2000). The localization of DdAcat in each subcellular compartment is important. We found that the N-terminal region of DdAcat contains a unique nonapeptide (15RMYTAKNL23) sequence, designated pPTS-2, which is similar to the consensus sequence (R/K)(L/V/I)X5(H/Q)(L/A) of PTS-2 for import of proteins into peroxisomes (Swinkels \textit{et al.}, 1991). This pPTS-2 functions as a targeting signal to direct DdAcat to peroxisomes.

It is well known that many peroxisomal proteins have PTS-1 at the C-terminus, and a few peroxisomal enzymes have PTS-2 at the N-terminus (Heiland & Erdmann, 2005; Ruktäschel \textit{et al.}, 2011). These proteins are imported into peroxisomes using targeting signals by receptors Pex5 or Pex7 (Gatto \textit{et al.}, 2000; Lazarow, 2006). In Dictyostelium, two peroxisomal enzymes, the multifunctional enzyme MFE1 (Matsuo \textit{et al.}, 2003) and citrate synthase CshA (Huang \textit{et al.}, 2004), have been well characterized, which have a C-terminal PTS-1 and an N-terminal PTS-2, respectively. Recently, Nuttall \textit{et al.} (2012) reported that Dictyostelium farnesyl diphosphate synthase possesses a nonapeptide closely related to that of PTS-2 and that it is a peroxisomal enzyme imported through the PTS-2 pathway. It has also been reported that fatty-acyl-CoA synthase B (FcsB) is transported to peroxisomes by mPTS (the targeting signal for peroxisomal membrane proteins) through the Pex19 machinery via the endoplasmic reticulum (ER) (Paschke \textit{et al.}, 2012).

The majority of mitochondrial proteins are synthesized in the cytosol as a precursor protein with an N-terminal MTS sequence, which is removed by MPP to form the mature protein. The MTS pre-sequences are different in length and in sequence, but tend to be rich in positively charged, hydroxylated and hydrophobic residues in order to form an amphiphilic \textit{\textalpha}-helix structure, which is necessary for the import of mitochondrial proteins into the mitochondria (von Heijne, 1986; Roise & Schatz, 1988; Roise \textit{et al.}, 1988; Dinur-Mills \textit{et al.}, 2008). In DdAcat, as shown in Fig. 1b(iv), the relatively short sequence of N-terminal 18 residues could function as an MTS. Judging from the low number of positively charged residues within this sequence, it appears to be a weak MTS for targeting to mitochondria, compared to that of exclusively mitochondrial proteins, such as Dictyostelium \textit{\textbeta}-MPP (Nagayama \textit{et al.}, 2008). Recently, it was reported that dual-localizing mitochondrial proteins tend to have a weaker MTS sequence than exclusively mitochondrial proteins (Yoge & Pines, 2011). When the function of pPTS-2 was disrupted by site-directed mutagenesis, the mutated DdAcat localized to the mitochondria, suggesting that the weak MTS functions to direct them to mitochondria.

Experiments for localization of DdAcat were carried out using cells at a density below $\sim 5 \times 10^6$ cells ml$^{-1}$ in the

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**Fig. 5.** DdAcat precursor is processed by processing peptidase. (a) Crude extracts (30 \(\mu\)g protein) from the vegetatively growing cells of the wild-type AX-3 and Thio\textsuperscript{OE} strains were separated on 12.5 % SDS-PAGE gels, followed by Western blotting/immunostaining using anti-DdAcat antibody as described in Fig. 2. (b) The precipitate (Ppt; 20 \(\mu\)g protein) and supernatant (Sup; 20 \(\mu\)g protein) fractions prepared from the post-nuclear fraction of the AX-3 and Thio\textsuperscript{OE} strains were subjected to analysis by SDS-PAGE and Western blotting/immunostaining. The small arrowhead and black circle indicate the long and short forms, respectively, of DdAcat. (c) Sup fractions (5 \(\mu\)g protein) of the Thio\textsuperscript{OE} strain were mixed with Ppt fractions (5 and 10 \(\mu\)g protein) prepared from AX-3, and incubated at 25 °C for 1 h, followed by SDS-PAGE and Western blotting/immunostaining. The large arrowhead indicates the short form contained in the Ppt fraction of AX-3.
exponential phase. However, when cells were used at the stationary phase (cell density of over 10^7 cells ml\(^{-1}\)), DdAcat (Thio-GFP) localized to the mitochondria (Fig. 1d). Interestingly, this result suggests that DdAcat can be localized to mitochondria using the MTS sequence overlapping with pPTS-2 within the N-terminal region under different growth conditions based on cell density. Czarna et al. (2010) reported that the TNR receptor-associated protein 1 (Dd-TRAP1), which belongs to the Hsp90 family, is largely localized in the cell membrane/cortex in vegetatively growing cells at low cell density (below 2 \times 10^6 cells ml\(^{-1}\)), but is translocated to mitochondria

Fig. 6. Characteristics of the N-terminal region of DdAcat (a) and a possible mechanism for the dual localization of DdAcat (b). (a) The first 30 amino acids of DdAcat. Positively charged and hydroxylated residues are indicated in red and blue, respectively, in the first 23 residues. Hydrophobic residues are underlined. A nonapeptide sequence of pPTS-2 is underlined with dashes. MTS and pPTS-2 are indicated. (b). A possible dual-localization mechanism. Two isoforms of DdAcat produced by alternative translation initiation are dual targeted within the Dictyostelium cell. (i) The schematic gene structure of DdAcat. Grey boxes indicate the exons of the acat gene, and the white box between the grey ones indicates an intron of 271 bp. (ii) A single, long mRNA transcribed from its gene. The mRNA has two in-frame AUG codons. (iii) Two forms of DdAcat. The long form translated from the first AUG possesses two targeting signals of MTS and pPTS-2, whereas the short form translated from the second AUG lacks both signals.
at high cell density (over 2–3 × 10^6 cells ml\(^{-1}\)). It is interesting that growth conditions give rise to different subcellular localizations of protein. The mechanism by which subcellular distribution of DdAcat is changed from peroxisomes to mitochondria remains unknown.

The presence of the second Met suggests the possibility of an alternative translation initiation site. The distribution pattern of Thio-GFP within cells shows a small amount of Thio-GFP distributed in the cytosol, although it is largely localized to peroxisomes [Fig. 1a(i)]. In subcellular fractionation experiments, DdAcat was detected in the supernatant as well as in the precipitate (Fig. 2). Considering the distribution of ΔN15Thio in the cytosol, DdAcat detected in the supernatant was found to be a cytosolic form translated from the second AUG codon. There are other examples of enzymes containing two in-frame Met residues. In watermelon, a 70 kDa heat-shock protein has two isoforms, the longer and shorter forms, which are produced from the first and second Met codons, respectively. The longer form carrying the N-terminal signal pre-sequence is imported to plastids, and the shorter form lacking the pre-sequence is localized to peroxisomes using PTS-2 (Wimmer et al., 1997). It was also reported that Lingulodinium iron-containing superoxide dismutase has two isoforms, one that resides in plastids/mitochondria and one in peroxisomes; the two forms are produced by alternative initiation codons (Bodył & Mackiewicz, 2007).

In pDA5'gthio-gfp, we expected that the N-terminally truncated Thio-GFP translated from the second AUG codon would distribute in the cytosol; however, Thio-GFP significantly localized to mitochondria instead of the cytosol (Fig. 3b). Investigation of the sequence adjacent to the cloning site revealed that the ORF start codon in the sequence upstream of the BamHI site in the multi-cloning site of pDNAeo67 was in-frame with that of the truncated Thio-GFP encoded by ΔS'gthio-gfp. As the result, the longer Thio-GFP could be formed with the artificial N-terminal pre-sequence of 22 residues (MFKLARLQVDSRGSVLNSNVKR), out of which the extra N-terminal 14 residues are derived from the sequence of pDNAeo67. The pre-sequence is 7 residues longer than that of wild-type and appears to have characteristics for forming α-helical structure, due to its 5 positively charged, 3 hydroxylated and 8 hydrophobic residues. Its localization to mitochondria revealed that this pre-sequence functions as an MTS of superior signal to that of the overlapping PTS-2 within the N-terminal region. These results indicate that subcellular localization is based on the strength of the signals. That is, the stronger signal has precedence in directing protein to the suitable compartment for localization.

It is well known that dual-targeting proteins can be located in two or more compartments using different targeting signals within the molecule (YogeV & Pines, 2011; Ast et al., 2013). As a simple example for dual localization, several proteins, such as rat mitochondrial AT (Olivier et al., 2000), dienoyl-CoA isomerase (Filppula et al., 1998), malonyl-CoA decarboxylase (Voilley et al., 1999) and 3-hydroxy-methylglutaryl-CoA lyase (Ashmarina et al., 1999), are each dictated by two targeting signals, an N-terminal MTS and a C-terminal PTS-1 within their molecules. A unique example is CYP1A1, which harbours both an ER targeting signal and an MTS near its N-terminus. The majority of CYP1A1 is translocated to the ER by its signal, and the remainder is targeted to mitochondria using the MTS exposed after removal of the ER signal by a cytosolic protease (Addya et al., 1997). In the case of DdAcat, besides the MTS and PTS-2 signals overlapping each other near the N-terminus (Fig. 6a), it has the second Met at position 16. Although DdAcat is able to occupy three different compartments, peroxisomes, the cytosol and mitochondria, Dicyostelium has only a single acat gene, suggesting that Dicyostelium produces the respective isoforms functioning in these compartments from its single gene. From the data obtained, we propose the possible dual-localization mechanism of DdAcat summarized in Fig. 6(b). The acat gene is transcribed to generate a single mRNA in which two in-frame AUG codons are present. The long form of DdAcat, having the N-terminal pre-sequence with both MTS and PTS-2, is translated from the first AUG and localizes to either peroxisomes or mitochondria under different conditions. However, the short form lacking the N-terminal pre-sequence is translated from the second AUG through alternative AUG utilization, resulting in the cytosolic enzyme.

The long form of DdAcat, carrying the MTS/PTS-2 sequence, is processed to the mature form, as shown in Fig. 5(c). When imported to mitochondria, DdAcat is processed by MPP. It is estimated that the site cleavage by MPP is the position between Y17 and T18, according to the R-2/3 rule (Gavel & von Heijne, 1990; Gakh et al., 2002). Peroxisomal proteins carrying the PTS-2 sequence in higher eukaryotes are processed by peroxisomal processing peptidase, as has also been reported for Tysnd1 (Kurochkin et al., 2007) and DEG15 (Schuhmann et al., 2008). Since DdAcat containing the PTS-2 sequence is imported to peroxisomes, it might be processed by peroxisomal processing peptidase as well; however, this has not yet been proven.

In this study, we showed that DdAcat is a dual-localizing enzyme, which enables its localization to three compartments. It is of interest to understand the dual-localization mechanism by which DdAcat, produced from a single gene, localizes to peroxisomes and mitochondria or remains in the cytosol. It is well known that peroxisomal, mitochondrial and cytosolic ATs are involved in metabolic processes within their respective compartments; however, the function of each form of DdAcat is not known in detail. The functions of DdAcat during development remain to be investigated and should be the focus of further studies.
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REFERENCES


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