**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 thiolysis 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.

**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 function and subcellular localization: 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 is a simple 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. The former AT gene (acat) and to determine the mechanism of its cellular localization.

Table 1. Primers used in this work

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<th>Primer</th>
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<tr>
<td>XSBf</td>
<td>5'-CCGGCCGGCTCTAGAAGCTATGGATCC-3'</td>
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**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 thiohfn). A thiohfn 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 pBluescript SKII(−) to yield pBthio-. The gfp gene, amplified using pUC118H-gfp as a template with a set of primers (gfp#1 and gfpXhor), 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 (N15thfn) 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...
were designated pDthioN15-gfp, pDthioN18-gfp, pDthioN25-gfp, pDthioN33-gfp and pDthioN50-gfp. To construct pDAN15thio-gfp, a 5'-thiolinker was annealed to 5'-thioSf with 5'-thioAsr oligonucleotides was prepared, in which the BamHI and AccI sites were introduced at its 5' and 3'-ends, respectively. After deletion of the original BamHI/AccI 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 act-5 gene, containing a 271 bp intron or a deletion of part of the first exon of the 5' region, was PCR amplified using pBSGT-3 (Tanaka et al., 2011) as a template with two sets of primers (SXBF and 1stexthiobamf as forward primers, and thiokin as a reverse primer). The 1stexthiobamf primer was designed to remove the sequence encoding the N-terminal 7 amino acids from the Ddacat 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, yielding pDgtgthio-gfp and pDAS5gthio-gfp.

To generate the site-directed mutants R15L and R15Lr and K21E primers were designed. The 5' region of the sequence was amplified with SXBF and R15Lr primers using pBSGT-3 as a template. The amplified fragment (mutated R15L) digested with BamHI and AccI was inserted between the corresponding sites of pBthio-gfp (deletion of the wild-type BamHI/AccI fragment) to make pBthio(R15L)-gfp. The fragment (mutated K21E) amplified with K21EF and thiokin primer was digested with BamHI and inserted between the corresponding sites of pBthio-gfp (deletion of the wild-type AccI/HindIII fragment) to make pBthio(K21E)-gfp. To generate K15E and R15L mutants, the KR1415ELr primer was designed. The double-mutated fragment was amplified with SXBF and KR1415ELr primers and subcloned into pBthio-gfp (deletion of the wild-type BamHI/AccI fragment) to yield pBthio(K14E,R15L)-gfp.

The BamHI/XhoI fragment (containing fusion genes of the mutated thi-15(R15L), thi-21(K21E) and thi-(K14E,R15L) with gfp) was inserted into the corresponding sites of pDNeo67 to yield pDthio(R15L)-gfp, pDthioK14E(R21)-gfp and pDthioK14E(R15L)-gfp, respectively.

To create a putative PTS-2 signal sequence, a PTS2 linker of annealed pts2linkerSf with pts2linkerAsr oligonucleotides was prepared, in which the AccI site present in the original act-5 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 AccI and HindIII sites were introduced at the 5' and 3'-ends of the linker, respectively. The linker was inserted into the corresponding sites of the AccI/HindIII-treated pBAN15thio-gfp, from which the wild-type AccI/HindIII fragment of act-5 was deleted, to yield pBpts2-gfp. pDpts2-gfp was also prepared, as described above. To add the SKL sequence to the C-terminal end of GFP, a gfsKSLXho oligonucleotide was prepared. The gfp-SKL fragment, amplified using pUC118-gfp with the primers gfpS1f and gfsKSLXho, was treated with EcoRI and XhoI and then inserted into the corresponding sites of pDNeo67 to create pDgfp-SKL.

The mutated 5' region of the Ddacat gene was PCR amplified with a pair of primers (SXBF and m16vacc1) using pBSGT3 dsDNA (Tanaka et al., 2011) as a template. After the PCR product was digested with BamHI and AccI, it was inserted into the corresponding sites of BamHI/AccI-doubl digested pBthio (deletion of the original 90 bp BamHI/AccI fragment) to yield pBthio(m16v). The fragment from pBthio(m16v) dsDNA digested with BamHI and XhoI was inserted into the corresponding sites of BamHI/XhoI-doubl digested pDNeo67 to yield pDthio(m16v).

The mutated full-length Ddacat was PCR amplified with a pair of primers (SXBF and thiokin) using pBthio(m16v) dsDNA as a template. The PCR product, digested with BamHI/HindIII, was subcloned between the corresponding sites of pB-gfp (derived from pBthio-gfp by deleting the thiokase sequence) to yield pBthio(m16v)-gfp. pDthio(m16v)-gfp was also prepared, as described above.

To compare the strength of targeting signals of ThioN18 as a mitochondrial targeting signal (MTS) and PTS2, we prepared the plasmid pBthioN18-pts2-gfp, which encodes the PTS2-GFP fusion protein connected to the ThioN18 sequence at its N-terminus, as follows: the AccI/HindIII fragment prepared from pBpts2-gfp was inserted into the corresponding sites of pBthioN18-gfp (deletion of gfp) to yield pBthioN18-pts2-gfp. The AccI/HindIII fragment of pBthioN18-pts2-gfp was inserted into the corresponding sites of pBthioN18(m16v)-gfp, in which the Met at position 16 within ThioN18 is substituted by Val. To substitute Met at positions 16 and 20 within ThioN18-PTS2 by Val, a reverse primer (m20vhinr) was generated. The 5' region of the sequence was PCR amplified with a pair of primers (SXBF and m20vhinr) using pBthio(m16v) dsDNA. The PCR product digested with BamHI/HindIII was subcloned between the corresponding sites of pBpts2-gfp (deletion of pts2) to yield pBthioN18(m16v)-pts2(m20v)-gfp. To create a additional mutant, R19I, the pts2linkerSf (for r19I) and pts2linkerAsr (for r19I) primers were designed. The mutated linker to anneal r19IS was R19AS nucleotides was prepared. AccI and HindIII sites were introduced at the 5'- and 3'-ends of the linker, respectively. The linker was inserted into the AccI/HindIII sites of pBthioN18-PTS2-gfp (deletion of pts2) to yield the plasmid pBthioN18-pts2(r19I)-gfp. The BamHI/XhoI fragments from pBthioN18-pts2-gfp and its mutated plasmid DNAs were subcloned to pDNeo67, as described above, to yield pBthioN18-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. Transfected 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 ~10⁶ 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 BamHI/XhoI fragment from pBSGT-3 was 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 400 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{Dd}Acat 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 EKLIRRYSKPISSKL, 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{Dd}Acat, 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.
(b) (iii) ThiоN15-GFP

(iv) ΔN15Thiо-GFP

(c) (i) Thiо(R15)-GFP

(ii) Thiо(K21E)-GFP

(iii) Thiо(K14E,R15L)-GFP

(iv) pPTS2-GFP

Subcellular localization of DdAcat

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)]. *Dn15Thio-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.

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

The Ddacat 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 pDA5’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 A5’gthio-gfp at the BamHI site. In cells transformed with pDA5’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 pDA5’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, Thio\textsuperscript{OE}, was

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**Fig. 3.** The second Met causes distribution of DdAcat 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 pD\textDelta5'gthio-gfp, deleting the 5' region encoding the first 7 amino acids in the first exon of the acat gene, was generated. Dictyostelium transformants harbouring pDgthio-gfp (a) and pD\textDelta5'gthio-gfp (b) were grown in HL-5 medium. Fluorescence microscope observation was performed as described in Fig. 1. Bars, 5 \mu m.
prepared. We examined whether \textit{Dd}Acat is overexpressed in the Thio\textsuperscript{OE} strain by Western blot analysis. A larger amount of \textit{Dd}Acat was expressed by the Thio\textsuperscript{OE} strain than by the AX-3 strain (Fig. 5a). We prepared the supernatant and precipitate fractions from the post-nuclear fraction of the Thio\textsuperscript{OE} strain. As shown in Fig. 5(b), larger amounts of \textit{Dd}Acat 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.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{subcellular-localization.png}
\caption{Subcellular localization of ThioN18-PTS2-GFP and its site-directed mutants. In addition to pD/thioN18-pts2-gfp, constructs 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.}
\end{figure}
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 \textit{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 \textit{DdAcat} in each subcellular compartment is important. We found that the N-terminal region of \textit{DdAcat} contains a unique nonapeptide (15RMYTTAKNL23) sequence, designated pPTS-2, which is similar to the consensus sequence (R/K)(L/V/I)X\textsubscript{3}(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 \textit{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; Rucktäschel \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 \textit{Dictyostelium}, two peroxisomal enzymes, the multifunctional enzyme MFE1 (Matsuoka \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 \textit{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{α}-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 \textit{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 \textit{Dictyostelium} \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 \textit{DdAcat} localized to the mitochondria, suggesting that the weak MTS functions to direct them to mitochondria.

Experiments for localization of \textit{DdAcat} were carried out using cells at a density below \(5 \times 10^6\) cells ml\textsuperscript{-1} in the
exponential phase. However, when cells were used at the stationary phase (cell density of over $10^7$ cells ml$^{-1}$), \textit{Dd}Acat (Thio-GFP) localized to the mitochondria (Fig. 1d). Interestingly, this result suggests that \textit{Dd}Acat 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 \textit{Dd}Acat (a) and a possible mechanism for the dual localization of \textit{Dd}Acat (b). (a) The first 30 amino acids of \textit{Dd}Acat. 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 \textit{Dd}Acat produced by alternative translation initiation are dual targeted within the \textit{Dictyostelium} cell. (i) The schematic gene structure of \textit{Dd}Acat. 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 \textit{Dd}Acat. 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 pDNS67 was in-frame with that of the truncated Thio-GFP encoded by Δ5’gthio-gfp. As the result, the longer Thio-GFP could be formed with the artificial N-terminal pre-sequence of 22 residues (MFKLARLQVDSRGVLSNSVNR), out of which the extra N-terminal 14 residues are derived from the sequence of pDNS67. 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, Dicystostelium has only a single acat gene, suggesting that Dicystostelium 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 (Kurckhin 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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