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Paper:


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Intrinsic acyl-CoA thioesterase activity of a peroxisomal ABC transporter is required for transport and metabolism of fatty acids.

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Peroxisomes are organelles that perform diverse metabolic functions in different organisms, but a common function is β-oxidation of a variety of long chain aliphatic, branched and aromatic carboxylic acids. Import of substrates into peroxisomes for β-oxidation is mediated by ABC transporter proteins of subfamily D, which includes the human ALDP protein defective in X-linked adrenoleukodystrophy. Whether substrates are transported as CoA esters or free acids has been a matter of debate. Using COMA TOSE (CTS), a plant representative of the ABCD family, we demonstrate that there is a functional and physical interaction between the ABC transporter and the peroxisomal long chain acyl-CoA synthetases LAC5 and 6. We expressed recombinant CTS in insect cells and showed that membranes from infected cells possess fatty acyl-CoA thioesterase activity which is stimulated by ATP. A mutant, S810N, which is defective in fatty acid degradation in vivo retains ATPase activity but has strongly reduced thioesterase activity, providing strong evidence for the physiological relevance of this activity. Thus CTS, and most likely the other ABCD family members, represent rare examples of polytopic membrane proteins with an intrinsic additional enzymatic function that may regulate the entry of substrates into the β-oxidation pathway. The regulation of CTS by CoA raises questions about the side of the membrane where this occurs and this is discussed in the context of the peroxisomal CoA budget.

ABC transporter | beta oxidation | COMATOSE | peroxisomes | thioesterase

Introduction

The peroxisome is the sole site of β-oxidation of fatty acids and related molecules in plants and fungi and is essential in metabolism of very long chain fatty acids and bioactive lipid molecules in mammals. ATP Binding Cassette (ABC) proteins of subfamily D are required for the transport of these substrates across the peroxisome membrane (1). These are Pxa1P/Pxa2P in yeast, ALDP (ABCD1), ALDPR (ABCD2) and PMP70 (ABCD3) in mammals and Comatose (CTS; also known as PED3, PXA1 and AtABCD1) in plants. Defects in ALDP result in X-linked adrenoleukodystrophy, a neurological disorder in which very long chain fatty acids accumulate (2). Similarly, cts mutants are defective in germination and mobilisation of stored triacylglycerol (3).

Activation by formation of a Coenzyme A (CoA) thioester is a pre-requisite for entry of substrates into β-oxidation (4) but whether ABCD proteins accept free fatty acids or acyl-CoAs has been contentious. Arabidopsis mutants lacking CTS accumulate acyl-CoAs (5) and the basal ATPase activity of the protein is stimulated by acyl-CoAs rather than free fatty acids (6). Studies with yeast cells and trypanosomes suggest that Pxa1P/Pxa2P and the trypanosome ABCD protein GAT1 transport acyl-CoAs (7,8) and human ALDP, ALDAR and Arabidopsis CTS are all able to complement the Saccharomyces cerevisiae pxa1/pxa2Δ mutant (6,9,10). Conversely, in Arabidopsis, genetic evidence indicates that CTS and the peroxisomal long chain acyl-CoA synthetases LAC5 and 7 act in the same pathway, suggesting that free fatty acids are transported (11). Moreover, knock down of the Arabidopsis peroxisomal adenylic nucleotide translocators inhibits fatty acid degradation, consistent with a need for intra peroxisomal ATP for fatty acid activation (12).

To reconcile these differences, a model was proposed in which CTS transports fatty acyl-CoA but the CoA moiety is removed either by the transporter or by a peroxisomal thioesterase (11). Recently, complementation of the S. cerevisiae pxa1/pxa2Δ mutant by human ALDP was shown to be dependent upon the presence of the peroxisomal fatty acyl-CoA synthetase, Faa2p (13), indicating that CoA is cleaved during or directly after transport. CoA cleavage was demonstrated by isotopic labelling but the protein responsible for the acyl-CoA thioesterase activity was not identified (13). Peroxisomes of mammals, yeast and plants contain acyl-CoA thioesterases which catalyse the hydrolysis of fatty acyl-CoAs to free fatty acids and CoASH but their physiological roles are not well characterised. It has been suggested that these enzymes are important for maintaining CoASH at optimal levels during periods of increased fatty acid oxidation (14).

In this study we demonstrate that CTS requires Faa2p or the targeting of the Arabidopsis synthetases LAC5 or LAC7 to yeast peroxisomes to support β-oxidation of oleate in S. cerevisiae. This functional interaction between transporter and synthetase is also reflected in a physical interaction in planta. We expressed CTS in insect cells and demonstrated that the protein has intrinsic thioesterase activity which is stimulated by ATP and is essential for fatty acid degradation in yeast and in planta. This resolves the long standing debate about the transport substrate for ABCD transporters and provides a rare example of a transporter which also possesses an intrinsic accessory enzyme activity.

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β-wild type activity (Fig. 1B), in marked contrast to the expression of wild type CTS oxidase in yeast (Fig 1A), despite the protein being correctly targeted to peroxisomes (Fig. S1). Expression of wild type CTS in plants (15,16) also resulted in an inability to support oleate oxidation in planta. A mutation, S810N, that compromises CTS-dependent fatty acid β-oxidation approximately 90% of the wild type level (Fig. 1A) and (6). A mutation, αr1/αr2/fαa2-oxidation result in only 12% of the wild type level. Expression of either synthetase in the absence of CTS gave a much smaller rescue of oleate β-oxidation showing that the effect of CTS plus LACS is additive (Fig. 1B). This reflects the ability of yeast to import free fatty acids via an ABCD-independent route that requires Fαa2p and which accounts for roughly 10% of the wild type (Fig 1A). In Arabidopsis two peroxisomal acyl-CoA synthetases with a broad substrate range are involved in fatty acid β-oxidation (11,17). When either of these synthetases was co-expressed with CTS in the triple mutant background, β-oxidation activity was restored to approximately 40% (LACS6) and 80% (LACS7) of the wild type level. Expression of wild type CTS in the αl1/αl2αfα2 triple mutant resulted in only 4% of the wild type activity (Fig. 1B), in marked contrast to the β-oxidation activity of cells where CTS was expressed in the Δαl1Δαl2Δ background (Fig 1A), showing dependence of CTS on Faa2p.

In Arabidopsis two peroxisomal acyl-CoA synthetases with a broad substrate range are involved in β-oxidation of fatty acids (11,17). When either of these synthetases was co-expressed with CTS in the triple mutant background, β-oxidation activity was restored to approximately 40% (LACS6) and 80% (LACS7) of the wild type level. Expression of either synthetase in the absence of CTS gave a much smaller rescue of oleate β-oxidation showing that the effect of CTS plus LACS is additive (Fig. 1B). This reflects the ability of yeast to import free fatty acids via an ABCD-independent route that requires Faa2p and which accounts for

Fig. 1. Peroxisomal acyl-CoA synthetase is required for CTS-dependent transport. The Saccharomyces cerevisiae pxa1/pxa2Δ and pxa1/pxa2/ffa2Δ mutants were transformed as indicated. A, B: yeast cells grown on oleate medium were incubated with [1-14C] oleic acid (C18:1) and β-oxidation rates were measured. Genotypes are shown to the left of the graphs. The fox1-1 mutant lacks acyl-CoA oxidase (the first enzyme of β-oxidation) and serves as a negative control. The activity in wild type cells was taken as a reference (100 %) and values are means ± SD of 3 independent experiments. C-E: 17,000 g pellets of oleate-grown pxa1/pxa2/ffa2Δ cells expressing LACS6 (C), LACS7 (D) or mis-targeted LACS7 (LACS7-PTS; E) were subjected to Nycodenze equilibrium density gradient centrifugation and C8:0-CoA synthetase activity was measured in the different fractions. 3-hydroxy-CoA dehydrogenase (3-HAD) activity was used as a peroxisomal marker. Activity in each fraction is expressed as a percentage of the total activity measured throughout the gradient.

Fig. 2. CTS forms a complex with peroxisomal acyl-CoA synthetase in planta. Membrane proteins from Arabidopsis thaliana suspension cultured cells were solubilised with β-dodecyl maltoside. A: solubilised membrane proteins were subjected to blue-native PAGE (in first dimension) followed by denaturing SDS-PAGE and immunoblotting for CTS. B: solubilised membrane proteins were separated in a sucrose step gradient. Fractions were collected from the top of the gradient and subjected to immunoblotting with a range of antibodies as indicated. T: total membranes. Positions of molecular weight markers (sizes in kDa) are shown above (first dimension or sucrose gradient) and to the left (second dimension) of panels A and B. C: Solubilised membrane proteins were subjected to immunoprecipitation using anti-CTS beads, separated by SDS PAGE and immunoblotted with anti-LACS and anti-CTS antibody. Lane 1, mock-immunoprecipitation without protein extract; lane 3, immunoblot of protein extract probed with anti-LACS and anti-CTS antibody, to show positions of LACS and CTS. Figure to the left of the panel indicate positions of molecular weight markers (sizes in kDa).

Results

Peroxisomal acyl-CoA synthetase is essential for CTS-dependent fatty acid β-oxidation

Expression of CTS in the S. cerevisiae pxa1/pxa2Δ mutant permitted this strain to metabolise oleate (C18:1) at approximately 90% of the wild type level (Fig 1A) and (6). A mutation, S810N, that compromises CTS-dependent fatty acid β-oxidation in plants (15,16) also resulted in an inability to support oleate β-oxidation in yeast (Fig 1A), despite the protein being correctly targeted to peroxisomes (Fig. S1). Expression of wild type CTS in the pxa1/pxa2/ffa2Δ triple mutant resulted in only 12% of the wild type activity (Fig. 1B), in marked contrast to the β-oxidation activity of cells where CTS was expressed in the pxa1/pxa2Δ background (Fig 1A), showing dependence of CTS on Faa2p.
that LACS7 has a much higher ratio of C18:1- to C8:0-CoA synthetase activity. This revealed that the residual growth of the pxa1/pxa2Δ mutant on oleate (Fig. 1A (9,18)).

Complementation was dependent on the correct peroxisomal targeting of the synthetase. The localisation and function of LACS6 and 7 were assessed by measuring octanoyl-CoA (C8:0) synthetase activity in different subcellular fractions, since this activity is absent from the cytosol of yeast (18). In pxa1/pxa2Δ cells transformed with LACS6 or 7, C8:0-CoA synthetase activity was detectable in cell lysates, organelle pellets, and purified peroxisomes but was absent from cells transformed with vector lacking insert (Fig. 1C-E; S2A-B). Removal of the PTS1 and 2 translocators, PNC1 and PNC2 (not shown for clarity). Alternatively, in model B, CoA is released on the cytosolic side of the peroxisome membrane and must enter peroxisomes via the PXN carrier, probably in exchange for AMP, thereby explaining the more efficient rescue of oleate β-oxidation by LAC7 compared to LAC6 (Fig. 1B).

**CTS is present in a complex with LACS6/7 in planta**

These results indicate a functional interaction between CTS and the peroxisomal acyl-CoA synthetases LACS6, LACS7 and pxa2Δ, therefore evidence for a physical interaction was sought. Membranes were prepared from Arabidopsis thaliana suspension culture cells, solubilised with β-dodecylmaltoside (β-DDM), separated on a blue native gel followed by a denaturing (SDS-PAGE) second dimension and then immunoblotted with an antibody against CTS. The transporter (MW 150 kDa) was found predominantly in a complex of ca. 700 kDa (Fig 2A). The experiment was repeated using membranes from seedlings that express a CTS-GFP fusion from the native CTS promoter. This construct is functional, as judged by complementation of the cts-1 mutant (Fig. S3A). On a 2D gel, a similar complex of ca. 700 kDa was observed when probed with anti-GFP antibody, although the apparent size of the denatured CTS-GFP species was approximately 170kDa, as expected from the addition of GFP (28 kDa) (Fig. S3B). The complex is quite stable, since it was also detected in the presence of Triton X-100 (Fig. S3C). When this complex was probed with both anti-CTS and anti-LACS6/7 antibodies (19), two pools of LACS immuno-reactivity were seen (Fig. S3C). The most intense migrated at around 150 kDa, the expected size for the dimeric LACS proteins, but a significant immuno-reactivity was also detectable in the higher molecular weight fractions between 700 and 1100 kDa that also contained CTS (Fig. S3C).

Detergent solubilised membranes were fractionated on a sucrose gradient followed by SDS-PAGE and immunoblotting. CTS sedimented in a broad peak with maximum intensity in fractions 6-9, corresponding to complexes >700 kDa (Fig. 2B). The same fractions were probed with antibodies against LACS6/7 and the β-oxidation enzymes acyl-CoA oxidase 1 (ACX1), multifunctional protein 2 (MFP2) and 3-ketoacyl-CoA thiolase (KAT2). MFP2 and KAT2 sedimented in the lightest fractions of the gradient, and showed no co-migration with CTS, as would be expected for soluble proteins. However both LACS and ACX1 showed a distinct minor peak in fraction 8 which also contained CTS.

While co-migration of LACS proteins with CTS is suggestive of their being part of the same complex, this does not provide evidence for a physical interaction between the two. Therefore solubilised membranes were immunoprecipitated with anti-CTS antibodies, and the immunoprecipitates probed with anti-LACS6/7 and anti-CTS antibodies (Fig. 2C). Since the antibody chains from the immunoprecipitation (IP) react with the secondary antibody in the blot, a mock IP was carried out without extract but with anti-CTS antibodies (lane 1) and processed alongside the extract.
409 containing sample (lane 2). Bands present in lane 2 but not lane
410 1 represent proteins specifically immunoprecipitated by the anti-
411 CTS antibody. The ca. 70 kDa band that is absent from control
412 lane 1 but is present in lane 2 co-migrates with native LACS as
413 detected by immunoblotting of the extract without prior IP (Fig.
414 2C, lane 3).
415 CTS exhibits intrinsic thioesterase activity
416 CTS was expressed in insect cells to investigate its biochemical
417 properties. Expression of the active, full-length protein was
418 demonstrated by the presence in membrane preparations of
419 immuno-reactive protein of MW ca. 150 kDa and detection of
420 ATPase activity that was much higher than uninfected controls
421 (Fig. 3A,F), and was inhibited by AIF, as previously described
422 (6). Insect cell membranes containing CTS exhibited thioesterase
423 activity towards 18:0-CoA which was absent from membranes
424 prepared from uninfected cells (Fig. 3B). ATP stimulated C18:0-
425 CoA thioesterase activity 2 fold. C14:0-CoA thioesterase ex-
426 hibited about 60% of the activity of C18:0-CoA (Fig. 3B). In
427 the presence of ATP, acyl-CoA thioesterase activity exhibited
428 Michaelis-Menten behaviour; the Km for C18:0-CoA was 1.05 ±
429 0.16 mM, Vmax 38.15 ± 7.27 nmol mg CTS−1 s−1 and quantification
430 of CTS levels by immunoblotting permitted estimation of km at
431 5.7 ± 3 s−1 (Fig. 3C).
432 A mutant, ped3-4, has been described which is a cis missense
433 mutation, S810N. In planta this mutant germinates but cannot
434 break down fatty acids and establish (15,16) and it failed to
435 complement the S. cerevisiae pxa1/pxa2 Δ mutant for oleate β-
436 oxidation (Fig. 1A). Membranes from insect cells expressing
437 CTS581SN exhibited ca. 70% of WT CTS-dependent ATPase active-
438 ity but only 40% thioesterase activity (Fig. 3D,E). No thioesterase
439 activity was detected in the absence of ATP, indicating that this
440 activity retained nucleotide sensitivity, similar to the wild type
441 (Fig. 3D).
442 Discussion
443 Previous studies have provided indirect evidence in favour of both
444 acyl-CoAs and free fatty acids as transport substrates of ABCD
445 proteins. It was proposed that the solution to this paradox is that
446 the CoA moiety is cleaved during transport (11), and indirect
447 evidence based on isotope labelling patterns of yeast cells is con-
448 sistent with this (13). The current study provides direct evidence
449 that CTS has an ATP-stimulated thioesterase activity although
450 the protein has no obvious homology with known thioesterases.
451 Previously described acyl-CoA thioesterases are not stimulated by
452 ATP, thus this appears to be a novel type of activity. By analogy
453 with thioesterases of known structure and mechanism, catalytic
454 residues could potentially include either conserved acidic, hy-
455 drrolyl, histidine and amide side chains, as in the "hot-dog fold"
456 thioesterases, or the triad of a nucleophilic residue, histidine
457 and acidic residue found in a β-hydrolyse-type thioesterases (20).
458 Members of the clade of plant, yeast and metazoan ABCD trans-
459 porters known to be involved in peroxisomal fatty acid import (21)
460 contain highly conserved residues of these types at a number of
461 positions. In human ALDP some of these have been implicated in
462 X-linked adrenoleukodystrophy, attesting to their functional
463 importance. Future work will systematically explore the role of
464 these CTS levels by CTS, in the context of our structural model of
465 a lumen-facing conformation of the protein (16).
466 A functional and physical interaction between the peroxiso-
467 mal ABC transporter CTS and the peroxisomal acyl-CoA syn-
468 thetases LACS6 and LACS7 could also be demonstrated. Thus
469 CTS (and probably the other peroxisomal ABCD transporters)
470 cleaves acyl-CoA as part of the transport cycle, with the free
471 fatty acid requiring subsequent reactivation by peroxisomal acyl-
472 CoA synthetases (Fig. 4). Vorticel acylation is a common feature
473 of fatty acid transport across membranes. In mammals, fatty
474 acids are transported into mitocondria by conversion to acyl
475 carnitine by carnitine acyl transferases and re-esterification to
476 CoA in the matrix, thus the mitochondrion maintains a separate
477 CoA pool from the cytosol. Uptake of fatty acids into yeast cells
478 and adipocytes requires co-operation of a long chain acyl CoA
479 synthetase and a fatty acyl transport protein (FATP) that may
480 itself also possess acyl-CoA synthetase activity (22). Recently
481 S. cerevisiae Fatlp1 was shown to be dually targeted to both the
482 plasma membrane and the peroxisomal membrane, and to form
483 a functional interaction with Pxa1p/Pxa2p that is required for the
484 stability of the former (13). The activity of Fatlp1 explains
485 the ability of faa2Δ cells to metabolise LCFA in the presence of
486 Pxa1p/Pxa2p (Fig. 1B). CTS cannot substitute for Pxa1p/Pxa2p
487 when Faa2p is also absent, suggesting that it is unable to interact
488 functionally with Fatlp1 (Fig. 1B). HsABC1D1 and HsABC2D2
489 are similarly unable to rescue the pxa1/pxa2/ffa2 triple mutant (13).
490
491 The S810N mutant has markedly reduced thioesterase activity
492 and is unable to support oleate β-oxidation when expressed in
493 the S. cerevisiae pxa1/pxa2 Δ mutant, demonstrating that the
494 thioesterase activity is important for biological function in vivo.
495 Ser810 is predicted to be located in the second transmembrane
496 (TM) helix of the second TM domain (i.e. TM helix 8 of the
497 complete CTS molecule, which is a fused heterodimer) and the
498 equivalent residue in ALDP (Ala141) is mutated in some X-ALD
499 patients (www.x-ald.nl), supporting its functional importance. In
500 plants, the S810N mutant (originally described as ped3-4; ref. 15)
501 germinates slowly but cannot degrade its oil bodies or establish
502 (15,16). The germination defect of mutants with a severe block
503 in β-oxidation has been ascribed to an inability to metabolise
504 the jasmonic acid precursor, 12-oxophytodienoic acid (OPDA;
505 23), a plant hormone which is thought to be transported into
506 the peroxisome by CTS for β-oxidation (24). It is possible that
507 cleavage of activated OPDA is less severely affected by the S810N
508 mutation compared to fatty acyl-CoAs and that the residual
509 thioesterase activity in ped3-4 may be sufficient to reduce OPDA
510 concentrations below the physiological threshold for germina-
511 tion, or alternatively that free OPDA can be accepted by the
512 transporter.
513 CoA cleavage by CTS might appear to be an energetically
514 wasteful process, since an additional two high energy phosphate
515 bonds are expended for every fatty acid converted to a CoA
516 thioester following import into the peroxisome. However, energy
517 expenditure is frequently utilised as a means of regulation or
518 control, e.g. GTP-dependent proof reading in protein synthesis.
519 Which side of the membrane CoA is released is unknown but
520 has important implications for the CoA budget. Release on the
521 cytosolic side would prevent the peroxisome acting as a sink
522 for CoA, especially during periods of high flux though β-
523 oxidation, but would imply an independent uptake route for this cofactor
524 (Fig. 4). The Arabidopsis peroxisome membrane protein PXN
525 was originally identified as an NAD+ transporter (25) but has re-
526 cently been shown to be able to transport CoA (26). Surprisingly,
527 paxn knockout mutants were still able to degrade fatty acids, albeit
528 at a reduced rate (25), suggesting the existence of more than one
529 route for CoA import. Thus it is possible that the thioesterase
530 activity of CTS releases the CoA moiety on the luminal side of
531 the peroxisome membrane (Fig. 4). This scenario implies that a
532 mechanism for CoA removal exists, such as degradation via
533 peroxisomal nuxid hydrolases (27).
534 Succrose gradients and native gels revealed that the majority
535 of CTS resides in a complex of >700 kDa. Because of the effect
536 of the detergent on protein migration it is impossible to size
537 accurately but is indicative of an oligomer of three or more CTS
538 molecules. This is consistent with other ABC proteins including
539 PMP70 and ALDP, which form homo-oligomers (28). A sub-
540 population of LACS and ACX1 proteins was associated with the
541 CTS complex, which is in accordance with the previously
542 reported membrane association of LACS6/7 (19). Although the
Solubilised membrane proteins were diluted at 1 mg/ml in IP buffer (10 mM Tris- HCl pH 7.4, 150 mM NaCl, 0.3% (w/v) DDM) and incubated with pre-equilibrated protein A beads (Thermo Scientific Pierce) for 1 h at 4 °C. The beads were removed by centrifugation for 1 min at 1,000 g and the supernatant was diluted in IP buffer. 10 μl affinity-purified CTS antibody was added and the mix was incubated at 4 °C overnight. 50 μl of protein A beads were then added and incubated for a further 3 h at 4 °C. The beads were pelleted at 10,000 g for 1 min and washed four times with IP buffer. The immunoprecipitated proteins were eluted with SDS PAGE sample buffer.

**Insect cell expression**

Recombinant bacmids encoding CTS or its S810N mutant were prepared using the Bac-to-Bac system (Invitrogen), according to the manufacturer’s instructions. Sf9 insect cells (Spodoptera frugiperda) were infected with virus containing bacmids and grown in Sf900 II SFM at 27 °C for 72 h. The cells were collected by centrifugation at 1,000 g for 10 min and incubated in a hypo-osmotic buffer (5 mM Tris- HCl pH 7.4, containing Mini complete protease inhibitor-EDTA (Roche) for 15 min at 4 °C. 3 mM DTT was then added and cells were further disrupted using 30 strokes of a tight-fitting Dounce homogeniser. The extract was diluted in homogenisation buffer (5 mM Tris- HCl pH 7.4, 0.5 mM MgCl₂, 5 mM EGTA, 1 mM PMSF) and centrifuged at 2,000 g for 20 min at 4 °C. Membranes were pelleted at 100,000 g for 1 h at 4 °C. The pellet resuspended in resuspension buffer (20 mM Tris- HCl pH 7.4, 200 mM NaCl, 1.5 mM MgCl₂, 20 % (v/v) glycerol, 2 mM DTT, plant protease inhibitor mix (Sigma), 2 μM Pefabloc, homogenised in a small Dounce homogeniser and aliquots were flash frozen in liquid nitrogen before storing at -80 °C. CTS expression levels were estimated by quantitative immunoblotting.

**Thioesterase assay**

ATPase activity was assayed according to (35). Briefly, insect cell membranes expressing wild-type CTS or S810N mutant were mixed with buffer containing 10 mM Tris- HCl pH 7.4, 0.1 mM EDTA, 1 mM MgSO₄, 2 μM ouabain and 10 mM sodium azide (final concentrations) in the presence of 10 mM AIF₃, when indicated and incubated for 3 min at 37 °C (total volume, 50 μl). The reaction was started by the addition of ATP and incubated for 30 min at 37 °C.

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**Funding Source**

4. L2 cells.

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Conflict of Interest
None declared.