**CELL BIOLOGY.** For the article “Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation,” by Jacob B. Hansen, Claus Jørgensen, Rasmus K. Petersen, Philip Hallenborg, Rita De Matteis, Hans A. Boye, Natasa Petrovic, Sven Enerbäck, Jan Nedergaard, Saverio Cinti, Hein te Riele, and Karsten Kristiansen, which appeared in issue 12, March 23, 2004, of Proc. Natl. Acad. Sci. USA (101, 4112–4117; first published March 15, 2004; 10.1073/pnas.0301964101), the authors note that the units in the last sentence of the Fig. 5 legend should be “μm” instead of “mM.” The figure and its corrected legend appear below.

**CELL BIOLOGY.** For the article “Cholesterol depletion induces PKA-mediated basolateral-to-apical transcytosis of the scavenger receptor class B type I in MDCK cells,” by Patricia V. Burgos, Carla Klattenhoff, Erwin de la Fuente, Attilio Rigotti, and Alfonso González, which appeared in issue 11, March 16, 2004, of Proc. Natl. Acad. Sci. USA (101, 3845–3850; first published March 8, 2004; 10.1073/pnas.0400295101), the authors note that the ordinate for the graphs in Fig. 5B and C should read “cAMP levels (pmol/μg)” instead of “PKA activity (pmol/min/μg).” The corrected figure and its legend appear below.

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**Fig. 5.** Differential expression of pRB during development of WAT and BAT and during transdifferentiation of white into brown adipocytes. (A) BAT anlage of an embryonic day 19 mouse fetus. No nuclear pRB immunoreactivity is observed. (Inset) Internal positive control of the same fetus showing pRB-positive nuclei of apical cells of intestinal villi (arrowheads). (B) BAT of a 10-day-old mouse. Nuclei of well differentiated brown adipocytes are pRB-positive (+), and nuclei of endothelial cells are negative (arrowheads). (C) Epididymal fat pad of a 9-day-old mouse. f, fat pad; ep, epididymus; t, testis. (D) Epididymal WAT of a 9-day-old mouse (enlargement of the squared area in C). Adipocyte precursors with lipid droplets (*) show nuclear staining. Endothelial cells (e) and adipoblasts (a) are pRB-negative. (E) Retroperitoneal WAT of a 20-week-old rat treated with the β3-adrenergic agonist CL-316243 for 7 days. Most of the transdifferentiating multilocular adipocytes exhibit pRB negative nuclei (arrows). A unilocular adipocyte with positive nucleus is visible (arrowhead). (F) Same tissue as in E. A unilocular adipocyte with positive nucleus is visible (arrowhead). (Bars: A and B = 30 μm; A Inset = 60 μm; D and F = 10 μm; C = 200 μm; E = 15 μm.)

**Fig. 5.** Acute cholesterol depletion by MjICD activates PKA without increasing intracellular cAMP levels. MDCK cells were incubated at 37°C with 10 mM MjICD or 50 μM FSK. (A) Both treatments increased PKA activity. (B) In contrast with FSK, cells treated with MjICD showed undetectable levels of cAMP. (C) In the presence of increasing concentrations of phosphodiesterase inhibitor IBMX, cAMP levels elicited by 1 h of FSK treatment increased progressively whereas, during treatment with MjICD, they remained almost undetectable. Each point represents average and SE.

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www.pnas.org/cgi/doi/10.1073/pnas.0402075101
CELL BIOLOGY. For the article “The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations,” by Sharon Cantor, Ronny Drapkin, Fan Zhang, Yafang Lin, Juliana Han, Sushmita Pamidi, and David M. Livingston, which appeared in issue 8, February 24, 2004, of Proc. Natl. Acad. Sci. USA (101, 2357–2362; first published February 17, 2004; 10.1073/pnas.0308717101), the authors note that the x axis of the left graph in Fig. 2B is numbered incorrectly. The corrected figure and its legend appear below.

Fig. 2. BACH1 is an ATP-dependent helicase. (A) Increasing amounts of WT and K52R mutant BACH1 were incubated with a DNA helicase substrate containing an annealed radiolabeled 19-nt oligomer (see Materials and Methods). Lane 1, annealed substrate (M13); lane 2, heat-denatured substrate (B, for boiled); lanes 3–5, BACH1 (60, 180, and 450 ng, respectively); lanes 6–8, K52R BACH1 (200, 400, and 600 ng, respectively); lane 9, WT with no ATP. (B) BACH1 unwinds DNA in a time- and dose-dependent manner. BACH1 protein (150 ng) was incubated with the 19-mer helicase substrate for the indicated times. Independently, increasing amounts of BACH1 (15, 30, 60, 120, 240, and 480 ng) were incubated with substrate for 30 min. (C) Increasing amounts of BACH1 (60, 180, and 450 ng) were incubated with a RNA:DNA helicase substrate and helicase activity was measured. (D) Increasing quantities of BACH1 (60, 180, and 450 ng) were incubated with DNA helicase substrates of increasing partial duplex length, as indicated. In all cases, reaction products were resolved in an 8% native polyacrylamide gel containing 15% glycerol. Results were quantitated by using a Molecular Dynamics Storm PhosphorImager.

MICROBIOLOGY. For the article “Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection,” by Frederik W. van Ginkel, Jerry R. McGhee, James M. Watt, Antonio Campos-Torres, Lindsay A. Parish, and David E. Briles, which appeared in issue 24, November 25, 2003, of Proc. Natl. Acad. Sci. USA (100, 14363–14367; first published November 10, 2003; 10.1073/pnas.2235844100), the authors note that the day 4 colonization data of strain EF3030 of the nasal washes have been omitted from Fig. 1B. The corrected figure and its legend appear below.

Fig. 1. Nasal delivery of 3 × 10⁶ cfu of either the nonencapsulated R36A strain or the virulent EF3030 strain of S. pneumoniae to xid mice. The neuronal tissues (ON/E, OB, and brain) and the lymphoid tissues (NALT, CLN, and lungs) were collected, minced, and analyzed for the presence of live pneumococci at 1 and 4 days after nasal challenge. Indicated is the mean of log₁₀ cfu ± 1 SE. The 0 value on the y-axis represents the absence of detectable cfu. Indicated are the mean cfu ± SE of five mice per group; data are representative of three different experiments.


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Mycobacterial polyketide-associated proteins are acyltransferases: Proof of principle with Mycobacterium tuberculosis PapA5

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Mycobacterium tuberculosis (Mt) produces complex virulence-enhancing lipids with scaffolds consisting of phthiocerol and phthiodiolone dimycocerosate esters (PDIMs). Sequence analysis suggested that PapA5, a so-called polyketide-associated protein (Pap) encoded in the PDIM synthesis gene cluster, as well as PapA5 homologs found in Mt and other species, are a subfamily of acyltransferases. Studies with recombinant protein confirmed that PapA5 is an acetyltransferase. Deletion analysis in Mt demonstrated that papA5 is required for PDIM synthesis. We propose that PapA5 catalyzes diesterification of phthiocerol and phthiodiolone with mycocerosate. These studies present the functional characterization of a Pap and permit inferences regarding roles of other Paps in the synthesis of complex lipids, including the antibiotic rifamycin.

Polyketides (PKs) are a family of complex lipids (1). Several secreted PKs and PK-containing compounds act as offensive, defensive, or adaptation effectors that contribute to mycobacterial virulence. These compounds include (phenolic)phthiocerol (POL) and phthiodiolone (PONE) dimycocerosate esters (PDIMs) (2, 3) described 40 years ago (20, 21), the enzyme(s) catalyzing diesterification of a Pap and permit inferences regarding roles of other Paps in the synthesis of complex lipids, including the antibiotic rifamycin.

Production of Recombinant Proteins. PapA5 and four mutated variants were produced as isopropylthiogalactoside (IPTG)-inducible N-terminal His-6-Smt3 fusions (25) in E. coli BL21(DE3) CodonPlus (Stratagene) by using TOPO-adapted pET-based Smt3 expression vector (Invitrogen). The PapA5 coding region was PCR amplified with primers 3xf (ATGTTTCCCGATCTTGATGCGGAAA) and 5xf (ACCCTACTCCATGATCCACCGATACGCGGCGGTCATCACTGATGCGGAAA) and 123Af (CTGACGCTATACCTCGCCCACTGCATGGGCATGGTGTCACTGATGCGGAAA) and 123Ar (ATGCAGCGCCATGCGGAAA) and 124Af (GCTGAACGCTATACCTCGCCCACTGCATGGGCATGGTGTCACTGATGCGGAAA) and 124Ar (ATGCAGCGCCATGCGGAAA). Cloning and site-directed mutagenesis were performed by using the QuickChange Mutagenesis Kit (Stratagene). Primer pairs 123Af (CTGACGCTATACCTCGCCCACTGCATGGGCATGGTGTCACTGATGCGGAAA) and 123Ar (ATGCAGCGCCATGCGGAAA) and 124Af (GCTGAACGCTATACCTCGCCCACTGCATGGGCATGGTGTCACTGATGCGGAAA) and 124Ar (ATGCAGCGCCATGCGGAAA). Cloning and site-directed mutagenesis were performed by using the QuickChange Mutagenesis Kit (Stratagene).

Materials and Methods

Strains, Growth Conditions, and Reagents. Escherichia coli was grown in LB media (22). Media were supplemented with kanamycin (30 µg/ml) for strains with plasmids pSMT3, p2NIL, pMV261, or their derivatives or ampicillin (100 µg/ml) for strains with pGOAL. Mt Erdman was grown in Middlebrook media supplemented with 10% oleate-aminob-dextrose-catalase (Becton Dickinson) (23) and, when needed, with antibiotics and/or sucrose as reported (24). Radiolabeled compounds were from American Radiolabeled Chemicals (St. Louis) or Sigma. Chloramphenicol acetyl transferase (CAT) was from Sigma and BSA from NEB. Other reagents were from Invitrogen, NEB, Novagen, or Sigma.

Polymyxins, phthiocerol (POL) and phthiodiolone dimycocerosate ester; PE, petroleum ether; PK, polyketide; Pap, PK-associated protein; AT, acyltransferase; MYC, mycocerosic; PCoA, palmitoyl-CoA; 1-OCL, 1-octanol; CD, condensation domain; ESI-MS, electrospray ionization MS.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Mt, Mycobacterium tuberculosis; POL, phthiocerol; PONE, phthiodiolone; PDIM, POL/PONE dimycocerosate ester; PE, petroleum ether; PK, polyketide; Pap, PK-associated protein; AT, acetyltransferase; MYC, mycocerosic; PCoA, palmityl-CoA; 1-OCL, 1-octanoyl; CAT, chloramphenicol acetyl transferase; CD, condensation domain; ESI-MS, electrospray ionization MS.

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Mt ΔpapA5 Construction. Deletion of papA5 in Mt Erdman was made with the p2NIL/pGOAL-based method (24). The papA5 deletion cassette, including a 500-bp segment upstream of papA5, papA5 start and stop codons, and a 495-bp segment downstream of papA5, was generated with gene splicing by overlap extension PCR (28). Primer pairs 5ufx and 5drx to generate the

CCCGCCGATCGGGC

and

5dfx (GGGACTGTTGAGATGTGACCTAACGAACCTGTCACACGATCC)

and

GGCAAACGCGATTCGGATC

were used to amplify the 5′ and 3′ ∼500-bp papA5 flanking regions, respectively, from genomic DNA. The resulting PCR products were used as template in a PCR with primers 5ufx and 5drx to generate the cassette.

Mt ΔpapA5 Complementation. Complementation was done with pMV261-based plasmids (29) expressing papA5 or its mutant alleles from the lacZ promoter. A fragment with papA5 flank by 18 and 9 bp at the 5′- and 3′-ends, respectively, was PCR-amplified with primers 5cf (GAATTCCGAGTTGGGAGTGTTGAGATGTGACCTAACGAACCTGTCACACGATCC) and 5cr (GCTAGCTGGTGAATGGCACACAGCGATCC) and 5fx

(GGACGTGTGTAAGTACCTACAAGAACACAGCGATCC)

and 5dx (TGTGCAATATGAGATGTGACCTAACGAACCTGTCACACGATCC) were used to amplify the 5′ and 3′ ∼500-bp papA5 flanking regions, respectively, from genomic DNA. The resulting PCR products were used as template in a PCR with primers 5ufx and 5drx to generate the cassette.

AT Assay. Unless otherwise indicated, AT reactions (25 μl) contained 75 mM Mes, pH 6.5, 100 mM NaCl, enzyme, and substrates (acyl-CoA thioester/alcohol pairs, one 14C-labeled per pair) at indicated concentrations. After incubation at 37°C for the indicated periods, reactions were stopped with CHCl3 (50 μl) and centrifuged to separate aqueous and CHCl3 layers. CHCl3 layers were analyzed by atmospheric pressure photoionization MS at the Hunter College MS Facility (New York).

Results

Sequence Analysis of Paps. Mt Paps A1–A5 are conserved proteins of unknown function (5). Homologs of each of these Paps were found in M. bovis (15), and Pap A3 and A5 homologs were identified in M. leprae (14). We performed protein–protein BLAST (BLASTP) and translated database (TBLASTN) similarity searches with default parameters via www.ncbi.nlm.nih.gov/blast by using Mt Paps A1, A2, A3, and A5 as queries (excluding N-terminally truncated A4). Analysis of BLAST hits indicated that the only consistent similarity of Paps to proteins of known function was to condensation domains (CDs) of peptide synthetases (data not shown). Searches for conserved domains via www.ncbi.nlm.nih.gov/blast showed a possible CD motif (conserved domain database: pfam00668.8) in each Pap (scores 49–60, E values 10−7 to 10−10). CDs (~450 amino acids) catalyze peptide bond formation during nonribosomal peptide synthesis (32–34) and contain an Hx3Dx14Y motif, which is also found in ATs (35).

Sequence alignment revealed a Hx3Dx14Y motif in the Mt. M. bovis, and M. leprae Paps noted above and in 15 Pap homologs identified with TBLASTN searches (Fig. 1). Pap homologs were identified in every mycobacterial genome examined, and only two homologs were found in nonmycobacterial genomes, one in Amycolatopsis mediterranei S699 and another in Streptomyces coelicolor A3 (2) (Fig. 1). A possible CD motif was found in each mycobacterial Pap and in S. coelicolor Pap (scores 45–72, E values 10−3 to 10−13). Analysis of genome regions encoding Pap homologs indicated that, except in S. coelicolor, Pap genes were in close proximity to pks or lipid metabolism genes (data not shown). These observations suggest that Paps may be a subfamily of ATs involved in lipid synthesis.

Demonstration of PapA5 AT Activity. We selected Mt PapA5 as a representative Pap to assess the suspected AT activity. As noted, PapA5 is encoded in the PDIM gene cluster. PDIMs are synthesized via diesterification of POL and PONE with MYC acid (donated as an unknown intermediate, possibly a CoA- or Mas

PDIMs were recovered by preparative 1D TLC of Mt apolar lipid extracts with CHCl3/MEOH (2:1) and analyzed by atmospheric pressure photoionization MS at the Hunter College MS Facility (New York).
synthase-bound thioester) by an as-yet-identiﬁed AT. Sequence analysis suggests that PapA5 could be such an AT. We developed a TLC-based assay to investigate PapA5 AT activity using 14C-labeled surrogate substrates (i.e., aliphatic alcohols and acyl-CoA thioesters) and recombinant PapA5 (Fig. 2). We reasoned that in this reaction setup, alcohols would replace the POL and PONE nucleophiles, whereas CoA thioesters would substitute for the mycoseryl thioester.

TLC analysis of reactions with PapA5, 1-octanol (1-OCL), and 14C-palmitoyl-CoA (PCoA) showed two products (Fig. 3A). The lower Rf product migrated like palmitate (data not shown), and ESI-MS conﬁrmed its identity (m/z 256, Fig. 3B). Palmitate was also formed, although in variable quantities, in reactions without alcohol or protein, in reactions with control proteins (Fig. 3A), and in reactions (data not shown) with mutated PapA5 variants described below. However, PCoA presence was needed for palmitate formation (Fig. 3A). These results suggest that palmitate is formed by nonspeciﬁc hydrolysis of PCoA. In contrast, the higher Rf product was formed only in reactions with PapA5 and both substrates (Fig. 3A). ESI-MS revealed a m/z [M+Na]+ of 391,2, in accordance with the mass of octyl-palmitate sodium adduct positive ion (Fig. 3C). PapA5 also catalyzed ester formation with [14C]hexadecan-1-ol (Fig. 3A). In this case, the higher Rf product comigrated with hexadecyl-palmitate (data not shown).

Ester formation was pH-dependent (Fig. 4A), with an optimum at pH 6.5. As expected, palmitate accumulation resulting from PCoA hydrolysis increased at basic pH. Ester formation was also time-dependent, reaching a plateau with 40% conversion stoichiometry relative to PCoA after 8 h. No signiﬁcant palmitate accumulation was observed during a 46-h reaction course (Fig. 4A).

PapA5 Substrate Selectivity. To gain further insight into PapA5 catalytic competence, we explored its ability to use several acyl-CoA thioesters and nucleophiles and calculated preliminary kinetic parameters with the best substrate pair. Pairs of [14C]PCoA and one of 36 nucleophiles were tested. The nucleophiles included short-, medium-, and long-chain alcohols, diols, hydroxy esters, and acids, amines, and thiols. Conversely, each of 17 acyl-CoA thioesters from a panel with short, medium, and long acyl chains was assessed in pair with [14C]hexadecanol.

Ester formation was detected with 28 of 36 nucleophiles tested (Table 1). Preference for saturated medium chain alcohols was observed, with 1-OCL displaying the highest ester formation stoichiometry (50–60% relative to [14C]PCoA). PapA5 discriminated between enantiomers; 20- and 5-fold more ester formed with (R)-(+)2-octanol and (S)-(−)1,2-decanediol, respectively, than with their corresponding enantiomers. Preference for neutral nucleophiles over closely related negatively charged ones was also observed (e.g., 3-hydroxypalmitic acid methyl ester over 3-hydroxypalmitic acid and 2-octanol or 1,2-decanediol over 2-hydroxyoctanoic acid). Although 1-OCL was the preferred nucleophile, nonhydroxy nucleophiles of comparable chain length, such as octylamine and octanethiol, were not appreciably used.

PapA5 used various acyl-CoA thioesters (Table 1). Ester formation was detected with 6 of 17 thioesters tested and was more efﬁcient with long chain acyl thioesters. The highest yield (50–60%) was seen with PCoA. Monounsaturated C18 elaidoyl- and oleoyl-CoA were more efﬁciently used than saturated C18 stearoyl-CoA. The highest yield (60–60%) was seen with PCoA. Monounsaturated C18 elaidoyl- and oleoyl-CoA were more efﬁciently used than saturated C18 stearoyl-CoA. Lauryl-CoA (C12) was readily used, whereas no ester was detected with myristoyl-CoA (C14). This is perhaps due to PapA5 inhibition by the latter compound.

Determination of kinetic parameters for ester formation with 1-OCL and PCoA, the preferred substrates among those tested, was complicated by substrate inhibition (Fig. 5). Inhibition was observed with both substrates but was particularly severe with PCoA. Inhibition was detected with other substrates as well (data not shown). Fitting of the equation

\[ v = \frac{V_{\text{max}}}{1 + K_{\text{cat}}S + S/K_i} \]

permitted calculation of apparent kinetic parameters and Ki values. Kcat, Km, and Kd values of 0.022 mm−1, 500 μM, and 3 mM, respectively, were obtained for 1-OCL. Kcat, Km, and Kd values of 0.027 mm−1, 4 μM, and 9.6 μM, respectively, were derived for PCoA.

PapA5 Hhx5DGx3Y Motif Mutagenesis. Alignment of 25 Paps revealed a fully conserved Hxx5Dxx3Y motif and an extended Hxx5DGx3Y motif in PapA5 and its close homologs (Fig. 1). Both
motifs are present in ATs, where the fully conserved His and Asp residues are proposed to act as a catalytic base and an active site conformation stabilizer, respectively (36–38). We analyzed the activity of PapA5 variants PapH124A and PapD128A, with Ala substitutions in H124 (second His in the motif) and D128, respectively. We also examined the functional relevance of motif residues H124 and Y143 by investigating the activity of variants PapH123A (Ala substituted) and PapY143F (Phe substituted). Mutated variants were purified in the same way as PapA5 (Fig. 2). Representative results of AT assays with PapA5 variants are shown in Fig. 6. No ester formation was detected in reactions with 0.2 μM PapH123A or

Table 1. Substrate specificity of PapA5

<table>
<thead>
<tr>
<th>Compound tested*</th>
<th>Product formation, %†</th>
</tr>
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<tbody>
<tr>
<td>Nucleophile</td>
<td></td>
</tr>
<tr>
<td>1-OCL</td>
<td>100.00 ± 13.08</td>
</tr>
<tr>
<td>1-Decanol</td>
<td>78.50 ± 6.70</td>
</tr>
<tr>
<td>1-Nonanol</td>
<td>43.39 ± 7.94</td>
</tr>
<tr>
<td>(S)-1-2-Octanol</td>
<td>36.86 ± 3.61</td>
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<tr>
<td>(R)-1, 2-decanediol</td>
<td>me/de 5.25 ± 5.79/1.92 ± 0.55</td>
</tr>
<tr>
<td>(R)-2-octanol</td>
<td>20.48 ± 3.71</td>
</tr>
<tr>
<td>Stearoyl alcohol</td>
<td>18.19 ± 3.56</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>12.00 ± 2.65</td>
</tr>
<tr>
<td>Myristoyl alcohol</td>
<td>9.10 ± 1.82</td>
</tr>
<tr>
<td>1-Hexadecanol</td>
<td>6.06 ± 1.59</td>
</tr>
<tr>
<td>Stearoyl alcohol</td>
<td>5.88 ± 0.58</td>
</tr>
<tr>
<td>(S)-1, 2-decanediol</td>
<td>me/de 5.61 ± 1.83/2.80 ± 0.50</td>
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<tr>
<td>Trans 2-octene-1-ol</td>
<td>4.93 ± 2.77</td>
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<tr>
<td>DL-3-OH-methyl palmitate</td>
<td>4.40 ± 1.66</td>
</tr>
<tr>
<td>(S)-1,2-Octanediol</td>
<td>me/de 4.09 ± 3.21/3.93 ± 2.19</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>10.4 ± 0.21</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>0.99 ± 0.30</td>
</tr>
<tr>
<td>1-Octylamine</td>
<td></td>
</tr>
<tr>
<td>1,2,6-Hexanetriol</td>
<td>1.04 ± 0.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.51 ± 0.19/ND</td>
</tr>
<tr>
<td>DL-3-OH-palmitic acid</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>(S)-3,3-OH-octanoic acid</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>(R)-(−)-1,3,4-butanediol, (S)-(−)-1,3-butanediol, 2-ethyl hexane-1,3-diol (mix), 1,3-cyclohexanediol (mix), isopropanol, Methanol, (R)-(−)-1,3-nonanediol, 1-octanethiol, (2R,4R)-(−)-pentanediol, (2S,4S)-(−)-pentanediol, D(-)-trehalose</td>
<td>0.32 ± 0.28</td>
</tr>
<tr>
<td>Acyl-CoA</td>
<td></td>
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<tr>
<td>Palmitoyl-CoA (C16)</td>
<td>0.21 ± 0.07</td>
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<tr>
<td>Elaidoyl-CoA (C18)</td>
<td>0.19 ± 0.02</td>
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<tr>
<td>Oleoyl-CoA (C18)</td>
<td>0.13 ± 0.10</td>
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<tr>
<td>Heptadecanoyl-CoA(C17)</td>
<td>ND</td>
</tr>
<tr>
<td>Lauroyl-CoA (C12)</td>
<td></td>
</tr>
<tr>
<td>Stearoyl-CoA (C18)</td>
<td>38.30 ± 19.81</td>
</tr>
<tr>
<td>Acetyl-CoA (C2), Methylmalonyl-CoA (C3), Isovaleryl-CoA (C4), Hexanoyl-CoA (C6), Octanoyl-CoA (C8), Decanoyl-CoA (C10), Myristoyl-CoA (C14), Arachidonyl-CoA (C20), Docosanoyl-CoA (C22), Hexacosanoyl-CoA (C26), Benzoyl-CoA</td>
<td>38.94 ± 17.09</td>
</tr>
</tbody>
</table>

*ND, not detected. Values are average of duplicates ± SE.
†Nucleophiles (180 μM) were tested in combination with [14C]PCoA (18 μM). Acyl-CoA thioesters (18 μM) were tested in combination with [14C]hexadecanol (180 μM).
‡Relative product formation of 100% in the nucleophile and acyl-CoA panels corresponds to 270 and 240 picomoles of product, respectively (maximum yields observed with compounds of the respective panels).
§Formation of monoester (me), diester (de), and triester (te) is indicated. Reactions were incubated for 12 h.

Fig. 5. Effect of 1-OCL and PCoA concentration on the rate of PapA5-catalyzed octyl-palmitate formation. (A) Reaction velocity as a function of 1-OCL concentration with [14C]PCoA at 18 μM. (B) Reaction velocity as a function of [14C]PCoA concentration with 1-OCL at 1 mM. Reactions contained substrates, 0.2 μM PapA5, 100 mM NaCl, and 75 mM Mes, pH 6.5, and were incubated for 3 h. Means of triplicates ± SEM are shown.

Fig. 6. Activity of PapA5 mutant variants. TLC analysis showing ester formed in reactions with 18 μM [14C]PCoA/1 mM 1-OCL/75 mM Mes, pH 6.5/100 mM NaCl/0.2 μM enzyme incubated 3 h (Upper) or 2 μM enzyme incubated 24 h (Lower). Picomoles of ester are shown as mean of triplicates ± SEM. ND, not detected.
Gene papA5 (1.2 kb) was PCR amplified with gene-specific primers only from WT Mt, and PCR with priming outside papA5 gave the expected 2.2- and 1-kb products for WT and ΔpapA5 Mt, respectively. Southern analysis with two independent probes also confirmed the deletion (data not shown).

Apolar lipid analysis of Mt WT and several ΔpapA5 isolates by 1D and 2D TLC showed that the deletion abrogated PDIM synthesis (Fig. 7B and C). Identity of PDIM spots corresponding to POL- and PONE-dimycocerosate was confirmed by atmospheric pressure photoionization (APPI) MS of compounds purified from TLC plates of WT Mt (Fig. 7D). APPI MS showed the characteristic PDIM mass pattern, with 14-atomic mass unit periodicity from chain length heterogeneity (31, 39). TLC analysis of polar lipid showed no apparent differences between WT and ΔpapA5 Mt (data not shown). To confirm that the deletion was responsible for PDIM deficiency, Mt ΔpapA5 was transformed with pCPapA5, expressing papA5. Lipid analysis showed that the transformant produced PDIMs at WT levels (Fig. 7B), thus ruling out the possibility that lack of PDIMs was due to polar effect. Transformation of Mt ΔpapA5 with vector pMV261 failed to restore PDIM synthesis (Fig. 7B). In agreement with the AT activity analysis, PDIM synthesis was also restored by transformation with pCPY143 (expressing PapY124F) but not with pCPH124 (expressing PapH123A) (Fig. 7B).

**Discussion**

PapA5 and four other Mt Paps are annotated as proteins of unknown function (5). Recently, some investigators speculated that PapA5 could be involved in PK transport (40), whereas others suggested that it might be an AT (35). Our sequence analysis suggests that PapA5, 3 other Mt Paps and 21 Pap homologs in other Actinomycetales are a family of ATs characterized by a Hx3Dx14Y motif and sequence and size similarity to CDs of peptide synthetases. Notably, Paps are prevalent in Mycobacterium spp and are primarily associated with PK production pathways.

We used recombinant PapA5 and surrogate alcohol/acyl-CoA substrate pairs to provide proof of principle for PapA5 AT activity by demonstrating that the protein catalyzes hydroxy-ester formation. Various alcohols and acyl-CoA thioesters were investigated as substrates. The selection of these compounds was biased by the assumption that PapA5 catalyzes diesterification of POL and PONE with MYC acid donated from a thioester intermediate. PapA5 displays AT activity with a variety of alcohol/acyl-CoA pairs; however, the observed substrate specificity indicates that the active site has clear substrate recognition determinants. PapA5 utilizes acyl-CoA thioesters of 12–16 carbons and a variety of alkanols and alkanediols as nucleophiles (21). Notably, PCoA is readily converted to ester; however, palmitic acid and palmitoyl chloride are not appreciably used. This may suggest that the neutrality and/or the structural features of the thioesterified substrate (e.g., thioester bond functionality, phosphopantetheinyl moiety, or CoA scaffold) are crucial for recognition. Overall, the hydrophobic character of the preferred surrogate substrates resembles the greasy characteristics of POL, PONE and MYC acid acyl chains.

PapA5-catalyzed ester formation is optimal with 1-OCL and PCoA, with apparent $K_m$ values of 500 and 4 μM, respectively. With these substrates, acyltransfer proceeds with an apparent $K_m$ value of = 0.025 min$^{-1}$, albeit under severe substrate inhibition. Although 1-OCL is a preferred nucleophile, neither octylamine nor octane-thiol are significantly used. This may indicate $O$-specificity, a property consistent with PapA5 predicted function in vivo. Another property consistent with PapA5 expected function is its ability to catalyze diol diesterification. PapA5 also displays enantioselectivity. Altogether, these studies demonstrate that PapA5 is an AT and present the characterization of a Pap.

We identified a fully conserved Hx3Dx14Y motif in the Paps and an extended Hx3Dx13Y motif in PapA5 and its close homologs.
Both motifs are present in ATs (32, 33). By analogy with CAT, a prototype AT, H124 of PapA5 would be expected to act as the catalytic base (41). By the same criterion, D128 in PapA5 should be required as an active site stabilizer (38, 42). Substitution of H124 or D128 of PapA5 by Ala decreased activity by 65- and 76-fold, respectively. These results are consistent with mutagenesis analysis of the H124D128Y motif of other ATs (38, 41).

Mutational analysis of the proposed PapA5 catalytic site motif is consistent with the catalytic mechanism proposed for CAT and other ATs. We have recently solved the PapA5 crystal structure (J.B. and C.D.L., unpublished work). Preliminary analysis reveals that PapA5 is a pseudodimer of two CAT domains, a property also observed in the stand-alone CD VbhH (34). The N-terminal 150 residues of PapA5 share a high degree of structural alignment to CAT, dihydroxyoamine acetyltransferase (E2p) (43), and VbhH, particularly with respect to the HxD motif conserved residues. Consistent with PapA5 activities on model substrates, we also observed a deep hydrophobic channel that links the PapA5 surface to the proposed active site. Thus, the structural data support the PapA5 AT activity in vitro and the proposed roles of H124 and D128.

The location of papA5 suggests its involvement in PDIM synthesis. We demonstrated that the ΔpapA5 mutant is deficient in PDIM synthesis, a defect corrected by complementation with papA5. In agreement with the in vitro enzymology, epimeric expression of papH123A but not of papH123A complements the mutant. These results establish PapA5 involvement in PDIM synthesis and provide a demonstration of in vivo function for a Pap. Based on the in vitro and in vivo studies, we propose that PapA5 catalyzes diesterification of POL and PONE with MYC acid. Similarly, PapA5 homologs encoded in PDIM gene loci of other mycobacteria are expected to catalyze the diesterification required for synthesis of their PDIM variants. It had been suggested that FadD28, an acyl-CoA ligase encoded in the M. bovis PDIM gene locus and required for mycoside production, may catalyze phenol-POL diesterification or synthesize acyl-CoA precursors for MYC acid synthesis (17). In view of our results, and considering that FadD28 did not have AT activity in vitro (17), it is likely that FadD28 is required for the latter function.

Mt papa1, papa2, and papa3 are associated with loci encoding Mas-like PK synthases, which are thought to use mycolamalonyl-CoA for methyl-branched PK synthesis (44). Mt papa1 and papa2 are linked to pks2, a gene believed to be required for synthesis of the methyl-branched PK found esterified to trehalose in sulfolipids (19, 45). Mt papa3 is linked to pks3/4, which are proposed to be required for synthesis of two methyl-branched PK moieties of polyacyltrehaloses (46). Our analysis suggests that papa1, papa2, and papa3 may catalyze O-esterification of trehalose with the methyl-branched PKs. Notably, these PKs and MYC acid have not been found as free acids (12, 39), a finding consistent with the lack of thioesterases in their synthesis loci. The latter observation suggests that Mt Paps may use methyl-branched PKs thioesterified to carrier protein domains of PK synthases in a manner reminiscent of the ATs involved in lipid A synthesis (47).

Last, what we have learned during efforts to deconvolute the biosynthesis of virulence-enhancing PKs of Mt provides insight regarding a yet obscure step in the biosynthesis of the antibacterial drug rifamycin. As recently noted (48), the AT required for thioesterification of rifamycin has not been identified. It is likely that Rif20, a PapA5 homolog encoded in the rifamycin gene cluster, is such an AT.

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