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Dissecting the Functional Role of Polyketide Synthases in *Dictyostelium discoideum*

BIOSYNTHESIS OF THE DIFFERENTIATION REGULATING FACTOR 4-METHYL-5-PENTYLBENZENE-1,3-DIOL[*][S]

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Dictyostelium discoideum **exhibits the largest repository of polyketide synthase (PKS) proteins of all known genomes. However, the functional relevance of these proteins in the biology of this organism remains largely obscure. On the basis of computational, biochemical, and gene expression studies, we propose that the multifunctional *Dictyostelium* PKS (DiPKS) protein DiPKS1 could be involved in the biosynthesis of the differentiation regulating factor 4-methyl-5-pentylbenzene-1,3-diol (MPBD). Our cell-free reconstitution studies of a novel acyl carrier protein Type III PKS didomain from DiPKS1 revealed a crucial role of protein-protein interactions in determining the final biosynthetic product. Whereas the Type III PKS domain by itself primarily produces acyl pyrones, the presence of the interacting acyl carrier protein domain modulates the catalytic activity to produce the alkyl resorcinol scaffold of MPBD. Furthermore, we have characterized an *O*-methyltransferase (OMT12) from *Dictyostelium* with the capability to modify this resorcinol ring to synthesize a variant of MPBD. We propose that such a modification *in vivo* could in fact provide subtle variations in biological function and specificity. In addition, we have performed systematic computational analysis of 45 multidomain PKSs, which revealed several unique features in DiPKS proteins. Our studies provide a new perspective in understanding mechanisms by which metabolic diversity could be generated by combining existing functional scaffolds.**

The soil-dwelling *Dictyostelium discoideum* genome has revealed an unanticipated variety of genes involved in the biosynthesis of natural products. Several metabolites have already been isolated, including a family of differentiation-inducing factors (DIFs),[3] 4-methyl-5-pentylbenzene-1,3-diol (MPBD), discadenine, and dictyopyrones (Fig. 1) (1–4). Aromatic amides such as brefelamide, amino sugar analog furanodictines, and dictyomedins have also been characterized from various slime molds (5–8). *Dictyostelium* is a unique organism that shows an exceptional two-stage life cycle, which alternates between unicellular ameboid and multicellular fruiting body stages (9, 10). Remarkable studies over the years have demonstrated that differentiation, spatial patterning, and morphogenesis are controlled by a combination of cell-autonomous mechanisms and intercellular signaling through a variety of chemotactic small molecules and growth-regulating metabolites (11). Although the inventory of such molecules is growing, the molecular components of the biosynthetic machinery have not been elucidated.

Dictyostelium possesses a large abundance of genes homologous to polyketide synthases (PKSs), which are known to produce an array of secondary metabolites (12, 13). Several homologs of *O*-methyltransferases (OMTs) and acyl-CoA synthetases can also be detected in the genome. PKSs are a family of multifunctional proteins that produce metabolites through sequential condensation of small carboxylic acid thioesters (14, 15). These have been categorized into Type I–III subfamilies based on protein architecture. Type I PKSs consist of multiple catalytic domains sequestered on a polypeptide chain. Modular PKSs possess many such modules that are used once during biosynthesis, whereas iterative enzymes utilize the same set of enzymes repetitively during the biosynthesis. Type II PKSs consist of discrete proteins that form a noncovalent multienzyme complex. Type III PKSs are structurally and mechanistically quite distinct from the other two subfamily members and do not possess an acyl carrier protein (ACP)-bound phosphopantetheine arm for anchoring the intermediates (16).

Recent studies have revealed tremendous versatility of PKSs in terms of their structural and functional organization as well as their ability to produce compounds other than typical secondary metabolites (17, 18). Although PKSs have been implicated in the biosynthesis of development-regulating factors in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Tables S1–S4.

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[3] The abbreviations used are: DIF, differentiation-inducing factor; MPBD, 4-methyl-5-pentylbenzene-1,3-diol; PKS, polyketide synthase; OMT, *O*-methyltransferase; ACP, acyl carrier protein; DiPKS, *Dictyostelium* polyketide synthase; AdoMet, *S*-adenosylmethionine; RT, reverse transcription; HPLC, high pressure liquid chromatography.

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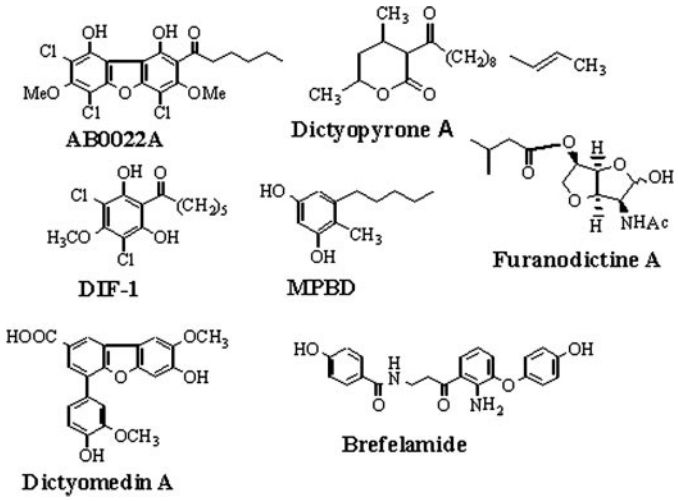

FIGURE 1. **Chemical structures of metabolites isolated from *Dictyostelium*.**

Dictyostelium, the enzymatic machinery involved in their biosynthesis has not been identified. Recent elegant studies with a Type I-Type III PKS protein showed involvement in the biosynthesis of DIF-1 by a combination of genetic and biochemical approaches (19, 20). However, the functional significance of the other 44 multifunctional PKSs in the biology of *Dictyostelium* is unclear.

In this study, we report the comprehensive analysis of *D. discoideum* PKS proteins that show distinct homology to fungal PKSs. We propose to name these *Dictyostelium* PKS (DiPKS) proteins DiPKS1–45 based on the order in which they appear in the genome. Our studies reveal several unique features associated with these large PKSs, including the presence of *C*-methyltransferase domains and novel reductase domains fused at the C-terminal ends of several multifunctional PKS proteins. The two PKS proteins DiPKS1 and DiPKS37, which were referred to previously as Steely1 and Steely2, respectively (19), contain a Type III PKS domain fused at the C-terminal end. We show that an engineered ACP Type III PKS didomain from DiPKS1 efficiently catalyzed synthesis of the demethyl analog of MPBD. This is in contrast to a previous report wherein the DiPKS1 Type III PKS domain by itself was shown to produce acyl pyrones (19). Functional characterization of the didomain emphasized the significance of protein-protein interactions in dictating biosynthesis of the final product. On the basis of the cell-free reconstitution studies and our understanding of the programming of the fungal Type I iterative PKSs, we propose that DiPKS1 may be involved in the biosynthesis of the resorcinolic metabolite MPBD. Furthermore, we have characterized an *O*-methyltransferase that methylates the hydroxyl group on the resorcinolic scaffold, which could produce new analogs of MPBD and thus generate functional diversity *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Genomic DNA from *D. discoideum* was isolated from the AX2 strain. [2-^{14}C]Malonyl-CoA (58.40 mCi/mmol) and *S*-[*methyl*-^{14}C]Adenosylmethionine (AdoMet; 51.4 mCi/mmol) was procured from PerkinElmer Life Sciences, and non-radioactive acyl-CoA starter substrates (phloroglucinol, resorcinol, olivetol, and catechol) were purchased from Sigma.

Cloning, Expression, and Purification—The Type III PKS domains of *dipks1* (GenBank^{TM} accession number DDB0234164) and *dipks37* (accession number DDB0234163) and the ACP Type III PKS didomain from DiPKS1 were amplified using gene-specific primers and cloned into pBluescript SK^{+}. For N-terminally His$_6$-tagged protein expression, these genes were cloned into pET28a and expressed in the BL21(DE3) strain of *Escherichia coli* at 18 °C using 0.5 mM isopropyl β-D-thiogalactopyranoside. The *omt12* gene (GenBank^{TM} accession number DDB0229899) was cloned into the Topo-D vector after PCR amplification and then into pET43.1a for expression as an N-terminally Nus- and His-tagged protein at 22 °C using 0.5 mM isopropyl β-D-thiogalactopyranoside. These proteins were purified by nickel-nitrilotriacetic acid affinity chromatography. However, because of very low expression, the DiPKS37 Type III PKS domain could not be purified to homogeneity. The ACP Type III PKS didomain could be eluted only from the nickel-nitrilotriacetic acid column in buffer containing 100 mM imidazole and 25% glycerol along with 10 μM β-mercaptoethanol. The identity of all three proteins was confirmed by mass spectrometry.

Enzymatic Assay and Product Characterization Using Mass Spectrometry—The enzymatic assays of the ACP Type III PKS domain of DiPKS1 and Type III PKS domains of DiPKS1 and DiPKS37 were performed as described (21), and the reaction products were resolved on reverse phase columns (Phenomenex) using a linear gradient of 30% CH_3CN in H_2O (with 2% formic acid) to 100% CH_3CN over 40 min. For the OMT assay, 100 μM substrate and 50 μM AdoMet inclusive of 15 μM [*methyl*-^{14}C]AdoMet (51.4 mCi/mmol) were used. The reaction was carried out at 22 °C for 4 h and quenched with 5% acetic acid. Products of OMT12 were extracted with 2× 300 μl of ethyl acetate and dried under vacuum before being resolved on TLC plates in chloroform/methanol/water (70:26:4, v/v/v). Reaction products of OMT12 were resolved on reverse phase columns using a linear gradient of 5% CH_3CN in H_2O (with 0.1% formic acid) to 100% CH_3CN over 30 min. All the polyketide products were characterized using electrospray ionization mass spectrometry.

Determination of Kinetic Parameters—Reactions were carried out with 44 μg of protein and varied (0.5–400 μM) starter molecule concentrations. Products were extracted with ethyl acetate and analyzed by radio-TLC. Resolved radiolabeled products were quantified using a Fuji FLA-5000 phosphoimaging system.

dipks1 Gene Expression by Reverse Transcription (RT)-PCR Analysis—*Dictyostelium* strain AX2 was grown in HL-5 medium at 22 °C. After synchronization, cells were collected starting from 0 to 24 h. RNA extraction was carried out with TRIzol (Invitrogen). RT-PCR was performed using an Invitrogen kit with an oligo(dT) primer, and PCR was performed for the *dipks1* gene using primers TCATTCGTTTTGGGTATTGGT and TTAGACAACATTTTTTAAGAAACA. Results were normalized to the transcripts of constitutive gene *IG7*.

Bioinformatic Analysis—The sequences of various PKSs of *D. discoideum* were downloaded from dictyBase (22). The catalytic domains of the PKSs were identified using the NRPS-PKS Database (23). All pairwise alignments were carried out using

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BLAST at NCBI and SEQWEB for generating multiple alignments and dendrograms. The structure coordinates were obtained from the Protein Data Bank; ACP of *E. coli* (code 1t8k) and the Type III PKS domain of DiPKS1 (code 2h84) were used to construct a theoretical model of their binary complex using the 3D-Dock program suite (24) and compared with the CoA-bound crystal structure of chalcone synthase (code 1bq6).

RESULTS

Computational Analysis of Polyketide Synthases from Dictyostelium—Comprehensive sequence analysis of the *D. discoideum* genome revealed 45 large multidomain PKS proteins with >2000 amino acids. For simplicity, we propose to annotate them as DiPKS1–45 based on the order in which they appear in the genome. Phylogenetic investigation suggested four members to be significantly different from other PKS proteins (supplemental Fig. S1). These include the two hybrid Type I-Type III PKSs DiPKS1 and DiPKS37 (referred to previously as Steely1 and Steely2, respectively) (19) and DiPKS16 and DiPKS17. The latter two are closely related proteins and are probable fatty-acid synthases.

Computational analysis of DiPKS proteins using the NRPS-PKS Database facilitated precise identification of various catalytic domains (supplemental Table S1) (23). DiPKS4, DiPKS11, DiPKS12, and DiPKS20 are probably pseudogenes with truncated domains. The other 41 proteins contain the functional units of β-ketoacyl synthase, ACP, and acyltransferase. A careful investigation of the active-site signature motif suggested that these acyltransferase domains would utilize malonate extender units (25). Methyl branching in fungal metabolites has been suggested to be derived from the *C*-methyltransferase domain (26). Systematic analyses revealed the presence of methyltransferase domains in 30 of these PKSs (supplemental Table S2), all of which show good conservation in the four sequence motifs of the methyltransferase domains (27). Dendrogram clustering studies with other functionally characterized *C*-, *N*-, and *O*-methyltransferases indeed showed that all of these domains belong to the *C*-methyltransferase family (Fig. 2).

Several PKSs show an unusually long C terminus-coding sequence after the ACP domain. Analysis of these C-terminal regions suggested the presence of a NAD-dependent reductase domain in 18 of these PKS proteins. Such domains in nonribosomal peptide synthases have been shown to catalyze reductive release of products (28–30). The C termini of DiPKS1 and DiPKS37 revealed an unprecedented Type III PKS domain. Type III PKS proteins have been characterized mostly in plants and bacteria (16, 31) and have been recently reported in fungi (32). The identification of unique features in DiPKS proteins prompted us to investigate their functional role to understand their importance in the biology of *Dictyostelium*.

Characterization of Dictyostelium Type III PKSs—As an initial approach, we decided to elucidate the functions of DiPKS1 and DiPKS37 by reconstituting C-terminal Type III PKS domains. Type III PKS proteins from bacterial systems are known to produce phloroglucinol and resorcinol rings, which are scaffolds of DIF-1 and MPBD (Fig. 3*A*). These domains were expressed with N-terminal hexahistidine affinity tags in *E. coli*,

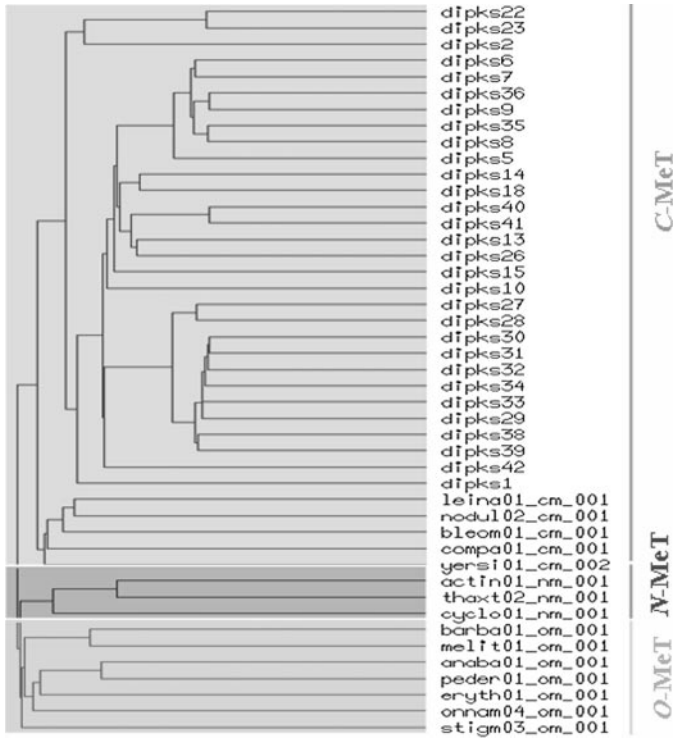

FIGURE 2. **Phylogenetic analysis of the *C*-methyltransferase domains present in the DiPKS proteins.** Shown is the dendrogram analysis of the *C*-methyltransferase domain identified in 30 of the DiPKS proteins along with other characterized *C*-, *N*-, and *O*-methyltransferases (*C-MeT*, *N-MeT*, and *O-MeT*, respectively) from bacteria and fungi. *leina*, leinamycin; *nodul*, nodularin; *bleom*, bleomycin; *compa*, compactin; *yersi*, yersiniabactin; *actin*, actinomycin; *thaxt*, thaxtomin; *cyclo*, cyclosporin; *barba*, barbamide; *melit*, melithiazol; *anaba*, anabaenopeptilide; *peder*, pederin; *eryth*, erythromycin; *onnam*, onnamide; *stigm*, stigmatellin.

and the protein identity was confirmed by mass spectrometry. Radio-TLC assays performed using [2-^{14}C]malonyl-CoA and a variety of starter units showed activity with aliphatic acyl-CoAs, but not with plant-specific acyl-CoA substrates.

Enzymatic assays with DiPKS37 and DiPKS1 Type III PKS domains showed three radioactive products with R_f values of 0.79, 0.67, and 0.88 using hexanoyl-CoA as a starter substrate (Fig. 3*B*, *lanes 1* and *2*). The product formed by the DiPKS37 Type III PKS domain was characterized by liquid chromatography/electrospray ionization mass spectrometry analysis (supplemental Fig. S2, *A* and *B*) and was determined similarly as described in the work published during the course of our study by Noel and co-workers (19). However, the products formed by Type III PKS of DiPKS1 were contrary to those in this earlier study and instead showed two distinct radioactive products (products b and c) (Fig. 3*B*, *lane 2*). The radioactive product b could be characterized by comparing the product profile with previously characterized PKS18 protein from *Mycobacterium tuberculosis* (Fig. 3*B*, *lanes 3* and *4*) (21, 33). The electrospray ionization mass spectrometry analysis confirmed this to be a triketide pyrone with a molecular ion peak of $[M - H]^{-1}$ at m/z 181.09 (Fig. 3, *C* and *D*). The other major product of DiPKS1 Type III PKS migrating with an R_f of 0.88 was subsequently purified by HPLC (product c) (Fig. 3*C*) and subjected to structural analysis. A molecular ion peak of $[M - H]^{-1}$ at m/z 179.12 was obtained, which, upon further tandem mass spectrometry analysis, yielded fragments at m/z 137.11 and 135.13 (Fig. 3*E*).

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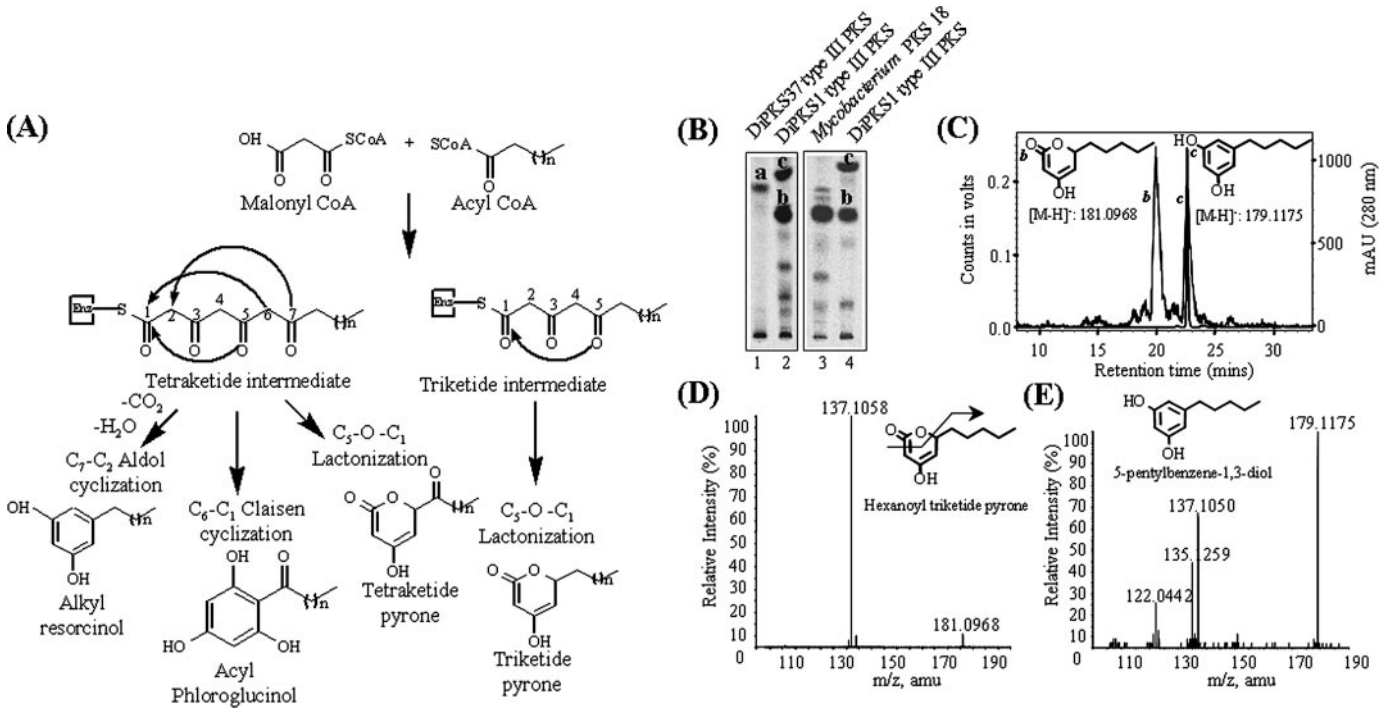

FIGURE 3. **Characterization of products catalyzed by DiPKS1 and DiPKS37.** *A*, schematic diagram showing the enzymatic reactions and products catalyzed by Type III PKS proteins following three different polyketide cyclization routes leading to formation of triketide or tetraketide acyl pyrones, acyl phloroglucinols, and alkyl resorcinols. In the starter substrates, the *n* indicates the number of carbons. *B*, TLC-based analysis of radiolabeled products of the Type III PKS domain of DiPKS37 and DiPKS1. Biosynthesis of acyl phloroglucinol by DiPKS37 Type III PKS is shown in *lane 1* (product a). Formation of acyl pyrone and alkyl resorcinol by DiPKS1 Type III PKS is shown in *lane 2* (products b and c, respectively). *Lanes 3* and *4* show the products catalyzed by *Mycobacterium* PKS18 and DiPKS1 Type III PKS protein. Reactions were carried out using [2-^{14}C]malonyl-CoA as an extender unit along with hexanoyl-CoA as a starter substrate. *C*, HPLC chromatogram for hexanoyl-primed reaction products of DiPKS1 Type III PKS (products b and c) separated using a ternary gradient system. The 280 nm absorbance of the standard demethyl-MPBD (*dotted line*) is superimposed on the radioactive measurements (*solid line*). *D* and *E*, fragmentation patterns observed by tandem mass spectrometry for hexanoyl-triketide pyrone and demethyl-MPBD, respectively. *mAU*, milliabsorbance units; *amu*, atomic mass units.

TABLE 1

Steady-state kinetic parameters for the DiPKS1 Type III PKS protein with different starter units

Results are the means (n = 3) with S.E. values <15%. ND, not determined.

Protein	Triketide pyrone			Demethyl-MPBD		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	$\times 10^{-3}\ s^{-1}$	$\times 10^{-3}\ s^{-1}\ M^{-1}$	μM	$\times 10^{-3}\ s^{-1}$	$\times 10^{-3}\ s^{-1}\ M^{-1}$
Hexanoyl-CoA	88.92	17.86	200.8	61.46	17.47	284.24
Octanoyl-CoA	40.87	13.74	336.3	34.80	14.37	412.98
Decanoyl-CoA	4.823	20.7	4291	3.641	6.5	1785.2
Lauroyl-CoA	3.929	19.5	4874	ND	ND	ND

This fragmentation pattern of M − 42 and M − 44 has been described recently for alkyl resorcinolic compounds (31). This product was unambiguously confirmed by comparison with standard 5-pentylbenzene-1,3-diol (also known as olivetol) (supplemental Fig. S2*C*). Our studies thus demonstrate that the hexanoyl-primed DiPKS1 Type III PKS can catalyze three polyketide chain extensions followed by aldol cyclization to synthesize demethyl-MPBD.

Kinetic Studies of the DiPKS1 Type III PKS Protein—The DiPKS1 Type III PKS domain showed promiscuous starter unit specificity and synthesized products in significant amounts with small-, medium-, and long-chain acyl-CoA starter substrates ranging from C_3 to C_{20} (supplemental Fig. S2*D*). Kinetic analysis showed typical saturation kinetics, and the specificity of the protein for medium- to long-chain fatty acyl-CoAs was estimated from the K_m values (Table 1). Resorcinolic products were observed for hexanoyl-, octanoyl-, and decanoyl-CoA starter units. Intriguingly, longer chain substrates could synthesize only pyrone products, albeit with favorable profiles of lower K_m and higher k_{cat} values. Because Type III PKS enzymes are known to produce acyl pyrone derailment products under non-optimal assay conditions (16), kinetic specificity for long-chain substrates may not reflect the true *in vivo* biological function. Another distinct possibility for incomplete resorcinol synthesis could be the suboptimal folding of the engineered 362-amino acid Type III PKS domain, which is fused at the end of a large DiPKS1 protein (3148 amino acids). Computational analysis suggested that the Type III PKS domain is connected to the ACP domain through a 134-amino acid intragenic linker region. Considering the fact that interdomain interaction often helps in attaining native conformation of the protein (15), we decided to characterize the ACP Type III PKS didomain protein for its capability to synthesize polyketide products.

Cell-free Reconstitution and Structural Analysis of the ACP Type III PKS Didomain of DiPKS1—The ACP Type III PKS didomain of DiPKS1 was cloned and expressed in *E. coli*. Com-

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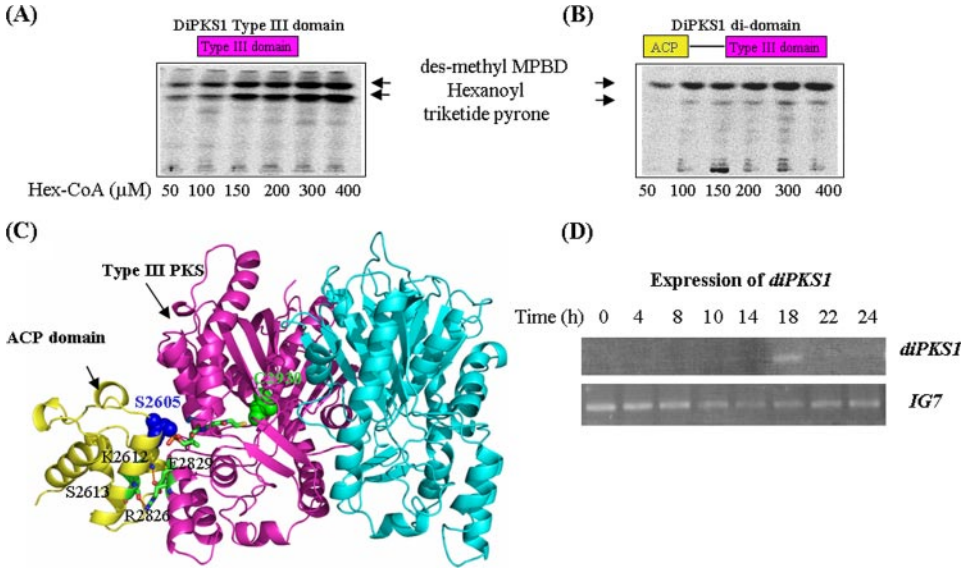

FIGURE 4. **Novel ACP Type III PKS didomain analyses.** *A* and *B*, radio-TLC of hexanoyl (*Hex*)-CoA-primed reaction products of the DiPKS1 Type III PKS domain and the DiPKS1 ACP Type III PKS didomain, respectively, at increasing concentrations of starter substrate. *C*, structural model for the ACP (*yellow*) and Type III PKS (*pink* and *green*) didomain complex of DiPKS1. Some of the crucial interacting residues involved in the interaction between the two domains (Lys^{2612} and Ser^{2613} of ACP and Glu^{2829} and Arg^{2826} of Type III PKS) along with the catalytic Cys (*green*) and Ser (*blue*) are shown. The phosphopantetheine arm of the ACP domain is shown in *ball-and-stick*. *D*, stage-specific expression of the *dipks1* gene in wild-type AX2 cells analyzed by RT-PCR. Hours of development are indicated above the panels. Expression of the constitutive *IG7* gene is shown as a control.

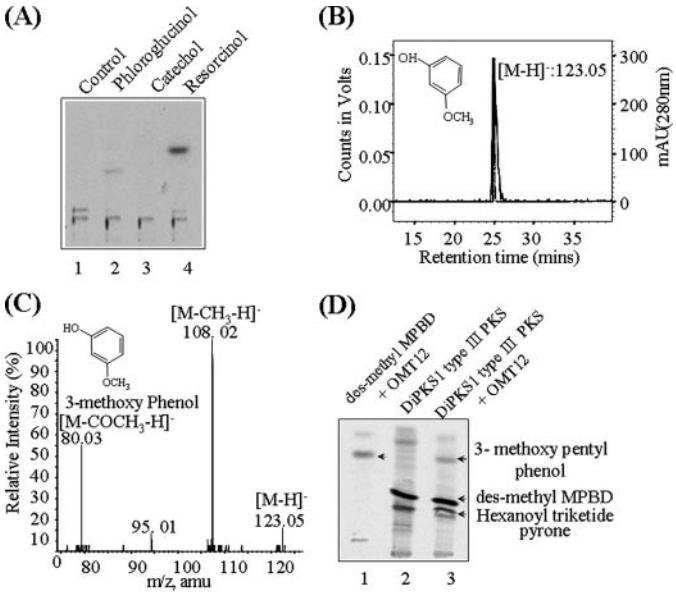

FIGURE 5. **Identification of products catalyzed OMT12.** *A*, autoradiogram of radio-TLC showing the products catalyzed by the *O*-methyltransferase OMT12 with phloroglucinol, resorcinol, and catechol as substrates. *B*, HPLC chromatogram overlaying standard methoxyphenol (*dotted line*) with the enzyme-catalyzed product of OMT12 (*solid line*). The molecular mass obtained for the peak by nanospray mass spectrometry analysis is indicated below the structure of the expected product. *C*, tandem mass spectrometry fragmentation pattern for the resorcinol-primed product 3-methoxyphenol. *D*, TLC showing the radiolabeled products of the coupled assay of OMT12 (*lane 3*) performed using [2-^{14}C]malonyl-CoA as an extender and hexanoyl-CoA as a starter unit. *Lane 2* is the control DiPKS1 Type III PKS reaction carried out in the absence of OMT12, and *lane 1* shows the catalytic activity of OMT12 with demethyl-MPBD using [^{14}C]AdoMet. *mAU*, milliabsorbance units; *amu*, atomic mass units.

parative analysis of the enzymatic activity in the didomain protein and the Type III PKS domain showed distinctive differences (Fig. 4, *A* and *B*). The didomain protein efficiently catalyzed biosynthesis of alkyl resorcinol, and almost no acyl pyrones could be detected in these *in vitro* assays (supplemental Table S3). We argued that the interactions between ACP and the Type III PKS domain may be crucial for optimal functioning of the DiPKS1 protein.

Because Type III PKSs typically exist as independent proteins, we further investigated the interactions that may be crucial during enzymatic catalysis. A homology model was built for the ACP domain using the crystal structure (code 1t8k) as a template, and the modeled ACP structure was docked onto the available crystal structure (code 2h84) of the DiPKS1 Type III PKS domain (19). In the biologically meaningful complexes of ACP Type III PKS, the phosphopantetheine group attached to the catalytic Ser must reach the catalytic Cys of Type III PKS. We therefore selected complexes in which the catalytic Ser was within 6 Å of the residues lining the mouth of the CoA-binding tunnel of Type III PKS. Fig. 5*C* shows a structural model for interaction between ACP and the Type III PKS domain of DiPKS1. Analysis of this complex suggested two potential electrostatic interactions, ACP Lys^{2612}-Type III PKS Glu^{2829} and ACP Ser^{2613}-Type III PKS Arg^{2826}, that could stabilize this complex. A similar constrained docking approach successfully identified residues involved in interaction between mycocerosic acid synthase and PapA5 (34). To examine the importance of these predicted residues, we performed site-directed mutagenesis. However, these mutant proteins could not be expressed in the soluble form and thus precluded any further functional analysis.

Stage-specific Expression of dipks1 in Dictyostelium—The *Dictyostelium* life cycle starts with the unicellular ameboid stage. Upon starvation, these solitary amebas enter a multicellular developmental program in which they start aggregating into mounds and differentiate into prespore and prestalk cells. These aggregates are enveloped in acellular sheaths, forming slugs, which finally develop into fruiting bodies. MPBD has been isolated previously from these later developmental stages (2). A recent study has suggested, however, that *dipks1* is expressed in the early stages of *Dictyostelium* development (19), which does not corroborate our *in vitro* studies. We therefore decided to probe the expression of *dipks1* at different stages of *Dictyostelium* development using RT-PCR. As shown in Fig. 5*D*, *dipks1* was found to be expressed primarily at 18 h of the developmental process. A low level of transcript could also be detected at 22 h, which provides credence to our proposition that DiPKS1 is involved in MPBD biosynthesis.

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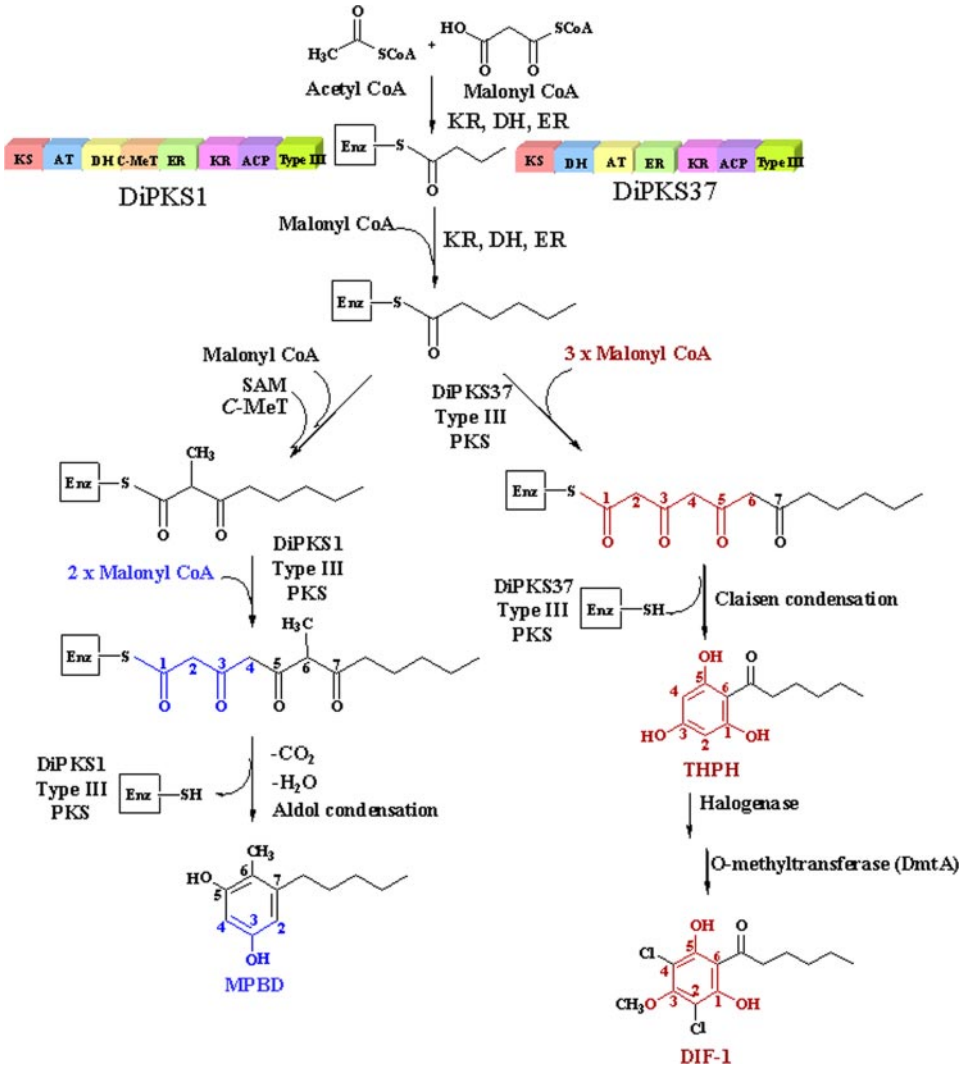

FIGURE 6. **Proposed biosynthesis of MPBD and DIF-1 catalyzed by DiPKS1 and DiPKS37.** *KS*, β-ketoacyl synthase; *AT*, acyltransferase; *C-MeT*, *C*-, methyltransferase; *Enz*, enzyme; *SAM*, *S*-adenosylmethionine; *THPH*, trihydroxyphenylhexanone; *DH*, dehydratase; *ER*, enoyl reductase; *KR*, ketoreductase.

confirming formation of 3-methoxyphenol (Fig. 5*C*). OMT12 also showed good activity with the alkyl resorcinol product of DiPKS1. *In situ* coupled assays involving DiPKS1 Type III PKS and OMT12 indeed confirmed formation of the new product, 3-methoxypentylphenol (Fig. 5*D*).

DISCUSSION

The *Dictyostelium* genome has revealed a large number of genes homologous to PKSs and post-PKS-processing enzymes. The advances in the understanding of the biochemical basis for the programming of polyketide biosynthesis provide an impetus and opportunity to dissect the roles of such enzymes from these versatile soil amoebas. In this work, we have characterized the catalytic function of the DiPKS1 protein by a combination of computational, biochemical, and gene expression studies. We propose that the DiPKS1 protein is involved in the biosynthesis of the differentiation regulating factor MPBD. In a previous study, the functional role of DiPKS1 could not be ascertained, and the Type III PKS domain was shown to produce acyl pyrones (19). In this study, we performed enzymatic assays with a novel ACP Type III PKS didomain of DiPKS1, which catalyzes three rounds of chain extensions with a hexanoyl-CoA starter unit followed by aldol cyclization to release the demethyl analog of MPBD. Our studies reveal the crucial role of protein-protein interactions in determining the final biosynthetic product.

Dictyostelium O-Methyltransferase as a Post-DiPKS1-processing Enzyme—The *Dictyostelium* genome contains 12 potential OMTs (named as OMT1–12), listed in supplemental Table S4. Because a possible modification of resorcinol rings would involve *O*-methylation, we investigated whether any of these OMTs could modify MPBD. Computational analysis of *Dictyostelium* OMTs suggested a distinct homology for the OMT12 protein to the resorcinol-modifying enzyme SbOMT3 from *Sorghum bicolor* (35, 36). OMT7 and OMT8 also showed reasonable homology to SbOMT3; however, OMT8 (referred to previously as DmtA) has been demonstrated to modify the phloroglucinol ring of DIF-1 (37). We cloned and expressed OMT12 and investigated its functional activity with substrates such as phloroglucinol, catechol, and resorcinol (Fig. 5*A*). [^{14}C]AdoMet was used as methyl donor, revealing a major radioactive product with resorcinol. This product was purified by HPLC and analyzed by tandem mass spectrometry (Fig. 5*B*). A molecular ion peak of $[M - H]^{-1}$ at m/z 123.04 was obtained, which, upon further tandem mass spectrometry analysis, yielded expected fragments at m/z 108.02, 95.01, and 80.03,

MPBD has been isolated previously from fruiting bodies in the late stages of *Dictyostelium* development and has been suggested to have a polyketide origin based on cerulenin inhibition experiments (2). Our RT-PCR analysis of *dipks1* demonstrated that this gene is expressed in the later stages of development. This is contrary to the previous study, in which DiPKS1 was instead found to be expressed at 4 h of development (19). We believe that this discrepancy in expression profile studies may have originated from the wrong choice of primers used for RT-PCR analysis. The previous study used primers from the β-ketoacyl synthase region, which is the most homologous domain among all PKS proteins. We performed these experiments using primers designed based on the Type III PKS domain. The polyketide metabolites often undergo secondary modifications by various tailoring enzymes, thereby enhancing their functionality to yield many bioactive compounds. Such post-PKS proc-

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essing has been proposed in DIF-1 in *Dictyostelium*, where the chlorinating and OMT enzymes produce a group of closely related DIF-1 analogs (4, 37). In this study, we have characterized an OMT that shows the capability to modify the alkyl resorcinol ring to synthesize a variant of MPBD. We propose that such a modification *in vivo* could in fact provide subtle variations in terms of biological functions and specificity.

Our systematic analyses of multifunctional PKS proteins provide a basis to describe the biosynthetic pathway for MPBD and DIF-1 metabolites. We propose that both DiPKS1 and DiPKS37 proteins resemble fungal Type I iterative PKSs and utilize the β-keto reductive domains in a precisely programmed mechanism (Fig. 6). The Type I PKS from both DiPKS1 and DiPKS37 would perform two rounds of iterative condensation of malonyl-CoA using acetyl-CoA as a starter unit. In these two rounds, all three β-keto reductive domains would be utilized to produce ACP/*S*-hexanoyl thioester. In the case of DiPKS37, the acyl chain is then transferred to the Type III PKS domain to catalyze three rounds of C_2 unit condensation and cyclization to produce a trihydroxyphenylhexanone intermediate. This further undergoes post-PKS halogenations and *O*-methylation to produce DIF-1. DiPKS1 instead would undergo another round of iterative nonreducing condensation followed by *C*-methylation of the polyketide chain by a *C*-methyltransferase domain. The β-ketoacyl-ACP chain would be then transferred to the Type III PKS domain, which would carry out two more condensation steps followed by aldol-type cyclization to produce MPBD. The transfer of acyl chains directly from the ACP domain to Type III PKS is uncommon. Two other examples of utilization of ACP/thioester substrates by Type III PKS were reported recently in *Streptomyces coelicolor* (38, 39). In conclusion, our study presents a new perspective of understanding the functional role of PKSs in producing signaling molecules, which may be crucial in the orchestration of the developmental stages in the life cycle of *Dictyostelium*.

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