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Exploring Protein Interactions on a Minimal Type II Polyketide Synthase Using a Yeast Two-Hybrid System**

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Summary

Interactions between proteins that form the 'minimal' type II polyketide synthase in the doxorubicin producing biosynthetic pathway from *Streptomyces peucetius* were investigated using a yeast two-hybrid system (Y2H). Proteins that function as the so called 'chain length factor' (DpsB) and putative transacylase (DpsD) were found to interact with the ketosynthase subunit (DpsA), which can also interact with itself. On the basis of these results we propose a head-to-tail homodimeric structure, which is consistent with previously published *in vivo* mutagenesis studies. No interactions were found between the acyl-carrier protein (DpsG) and any of the other constituents of the complex, however, transient interactions, not detectable using the Y2H system, cannot be discounted and warrant further investigation.

Key words: *Streptomyces*, aromatic polyketides, protein interactions, doxorubicin

Introduction

Type II polyketide synthases (PKSs) are dissociable multienzyme complexes consisting of several largely monofunctional proteins. The active sites of these proteins are used in an iterative manner, typically producing polycyclic aromatic compounds (*1*). Many of these are biologically active, including the clinically significant antitumor drug doxorubicin (DNR). Genes designated *dpsA*, *B*, *C*, *D*, *G*, *E*, *F* and *Y* are responsible for the synthesis of aklanonic acid, the first enzyme-free intermediate in the biosynthetic pathway leading to the formation of DNR by the filamentous bacterium *Streptomyces peucetius* (Fig. 1). The *dpsA* gene encodes a ketoacyl-ACP synthase, *dpsB* encodes a putative decarboxylase and *dpsG* encodes for an acyl carrier protein. Acetate is commonly the starter unit for biosynthesis on type II PKSs, but the PKS leading to the formation of DNR is unusual because a propionate primer is preferentially incorporated in the presence of *dpsC*, which is a homologue of the KAS III enzyme from fatty acid biosynthesis. The role of the gene product encoded by *dpsD* is obscure. Although this protein, DpsD, is a homologue of malonyl-CoA acyltransferase (MCAT) involved in fatty acid biosynthesis, transacylase activity by DpsD is not essential for DNR biosynthesis (*2*). There is tremendous interest in manipulating type II PKSs systems to generate products with novel or improved biological activity (*3*). An appreciation of how proteins interact to make up these complexes will be essential if this goal is to be achieved, but to date only structural information pertaining to some of the individual components is available (*4–8*). We give the first report describing how individual proteins might interact to produce an enzyme complex.

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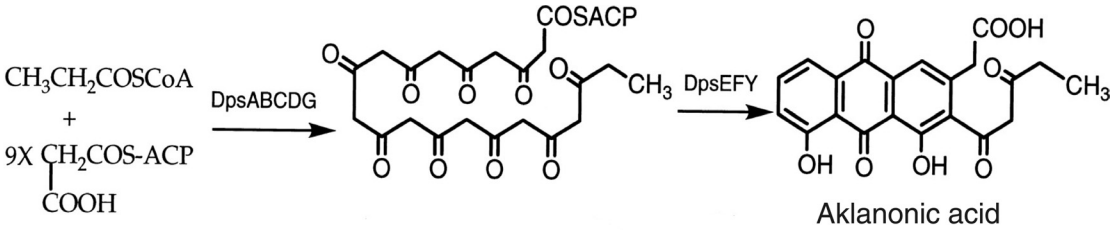

Fig. 1. Biosynthetic pathway leading to the formation of aklanonic acid, the first enzyme free intermediate in the biosynthetic pathway leading to the formation of doxorubicin in *Streptomyces peucetius*

Material and Methods

A cosmid designated pWHM1012 encoding the genes *dpsA, B, C, D, G, E, F* and *Y* (*9*) was obtained from Prof. B. Shen, University of Wisconsin, Madison WI, USA; courtesy of Prof. C. R. Hutchinson. Subcloning of these genes into vectors for use in the Y2H system followed standard procedures (*10*). Protein interactions after 14 days were identified using the yeast *Saccharomyces cerevisiae* strain AH109 by nutritional selection and β-galactosidase activity following protocols previously described by the manufacturer (BD Biosciences Clonetech, Palo Alto CA, USA). Each gene was cloned as both prey and bait in the unlikely event of a polar effect that fusion to either of the domains might adversely affect target gene activation. Also, to discount auto-activation, each gene was transformed individually into the *S. cerevisiae* host. If no interaction between prey and bait could be observed, protein expression was checked by Western blotting using anti-myc and anti-HA fusion-tag IgG monoclonal antibodies obtained from Abcam Ltd (Cambridge, UK). All interactions were performed in triplicate.

Results and Discussion

Protein interactions were defined when growth of *S. cerevisiae* was observed on the dropout medium SD-4 (adenine and histidine deficient) after 14 days of incubation at 30 °C, and when the *lac*Z assay for β-galactosidase activity was positive. Of the 15 possible interactions (Table 1), only 3 were positive. These were between DpsA – DpsA, DpsA – DpsB and DpsA – DpsD. Controls for auto-activation were all negative. Western blotting confirmed that proteins of the expected molecular mass were expressed by all of the clones, even when an interaction between proteins could not be detected.

Table 1. Matrix showing all possible protein interactions that were tested in the yeast two-hybrid system using proteins encoded as both prey and bait from the biosynthetic pathway leading to aklanonic acid in *Streptomyces peucetius*

prey \ bait	A	B	C	D
A	*AA*	*AB*	AC	AD
B	BA	BB	BC	BD
G	GA	GB	GC	GD
C	CA	CB	CC	CD
D	*DA*	DB	DC	DD

Positive interactions are shown in italics

The Y2H system (*11*) is a powerful genetic tool for detecting protein-protein interactions based upon eucaryotic transcriptional activation. Briefly, in yeast the Gal4 transcription factor is composed of physically separable and functionally independent domains. This transcription factor contains a DNA binding domain (BD) that binds to an upstream activated sequence (UAS) present in the promoter sequence of inducible genes, and an activation domain (AD). The BD must be in close association with an activation domain (AD) to direct the RNA polymerase II complex to transcribe the reporter gene. If cloned on separate vectors and expressed in the same host cell, neither of the peptide domains interact directly so the reporter genes are not activated and the yeast cells cannot grow using the nutritional selection markers. However, if the BD and AD are brought into close physical proximity, then transcriptional activation is restored. This can be achieved by construction of fusion partners between the domains and proteins under investigation. In this study, the proteins from the DNR biosynthetic pathway must interact for GAL4 activation to occur.

From our results, we propose a homodimeric head-to-tail model for the DNR producing 'minimal' PKS, which also takes into consideration results from previous *in vivo* mutagenesis studies of aklanonic acid biosynthesis (*12*). When a *dpsCD* deletion mutant of *Streptomyces* strain C5VR5 was constructed (*12*), desmethylaklanonic acid derived from an acetate starter unit was the main product formed, while aklanonic acid derived from a propionate starter unit was only detected in trace amounts. From our model (Fig. 2), we suggest that in the absence

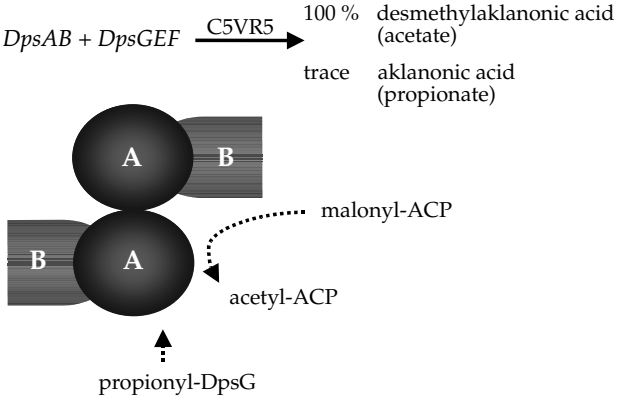

Fig. 2. Possible model based on protein interactions obtained using a yeast two-hybrid system in this study to account for chain initiation using an acetate starter unit from *in vivo* mutagenesis results (see text and reference *12* for details)

of DpsCD, malonyl-DpsG (or an alternative ACP species) could become decarboxylated by DpsB, and the resulting acetate unit then transferred to the ketoacyl-ACP synthase, DpsA, as the starter unit (although the mechanism of transfer is as yet unknown for any type II PKS). Such a mechanism for chain initiation has been suggested for the actinorhodin producing type II PKS (*13*). Decarboxylase activity by DpsB has also been suspected from *in vitro* protein reconstitution experiments (*14*). Aklanonic biosynthesis might be supported by the direct loading of a propionate starter unit onto DpsA, again this has also been observed on the actinorhodin producing PKS (*13*). Note that the proteins in our model lie symmetrically, head-to-tail, offering the attractive possibility that the synthase could support two catalytic centres.

Deletion of *dpsC*, but not *dpsD*, from *Streptomyces* strain C5VR5 resulted in the formation of desmethylaklanonic acid and aklanonic acid in a 60:40 ratio (*12*). A head-to-tail model could also explain this finding (Fig. 3). We suggest that the physical position of DpsD within the complex could block, but not completely prevent, acyl-transfer either to the active site of DpsB, or acyl--transfer of the decarboxylated product from DpsB to DpsA. Direct loading of a propionate starter unit onto DpsA (or even via DpsD, since this protein does have enough structural homology to be a putative transacylase) would then become the more kinetically favourable reaction.

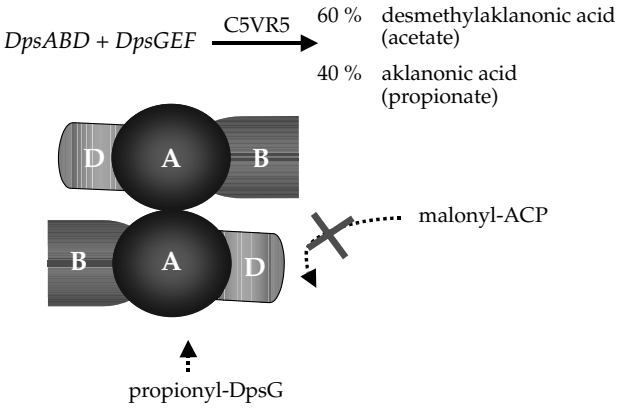

Fig. 3. Possible model based on protein interactions obtained using a yeast two-hybrid system in this study to account for chain initiation using promiscuous starter units from *in vivo* mutagenesis results (see text and reference *12* for details)

Expression of DpsC, irrespective of the presence of DpsD in *Streptomyces* strain C5VR5, is sufficient to restore aklanonic acid biosynthesis to wild type levels (*12*). This result cannot be explained from our model since DpsC was not found to interact with any of the other proteins tested in the Y2H system. We can only speculate that DpsC must interact with the complex in a transient way, making DpsA less likely to utilise a starter unit other than propionate. Our model also strongly suggests that the ACP, DpsG, does not remain bound as an integral structural component of the complex, but rather this protein only forms a transient interaction to sustain acyl-transfer during polyketide biosynthesis.

Conclusions

We propose, for the first time, a structural model for protein interactions that can account for biosynthesis on a type II polyketide synthase. This model suggests a head-to-tail configuration of proteins within the complex, and is supported by experimental data of others reported in the literature (*12*). More recently, x-ray crystallography studies have also confirmed that the KS and CLF from the actinorhodin producing PKS also interact with each other (*15*). The influence of downstream proteins and proteins that form only transient interactions have yet to be accounted for, nor has stoichiometry within the system been addressed. However, these initial results provide an exciting starting point for further investigations and future rational engineering strategies using these clinically significant protein complexes.

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Istraživanje interakcije proteina i minimalne poliketid sintetaze tipa II upotrebom kvaščeva dvohibridnoga sustava

Sažetak

Primjenom kvaščeva »sustava dvaju hibrida« (Y2H) istražene su interakcije proteina što oblikuju minimalnu poliketid sintetazu tipa II u metaboličnom putu biosinteze doksorubicina s pomoću bakterije *Streptomyces peucetius*. Utvrđeno je da su proteini, koji djeluju kao tzv. »čimbenik što određuje duljinu ugljikova lanca« (DpsB), i pretpostavljena transacilaza (DpsD) u interakciji s podjedinicom ketosintetaze (DpsA), koja može biti i u interakciji sama sa sobom. Na temelju tih rezultata predlažemo njihovu homodimeričnu struktura »glava-do-repa«, koja je u suglasju s prethodno objavljenim studijama mutageneze *in vivo*. Nije utvrđeno nikakvo djelovanje između proteina nosača acila (DpsG) i bilo kojega drugog sastavnog dijela enzimskog kompleksa, ali bi bilo potrebno provesti dodatna istraživanja kako bi se isključila mogućnost stvaranja slabijih interakcija koje se ne mogu ustanoviti primjenom sustava Y2H.