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Rapid Report

Activation of the Pacidamycin PacL Adenylation Domain by MbtH-like Proteins†

Wenjun Zhang, John R. Heemstra Jr., Christopher T. Walsh,* and Heidi J. Imker

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115, United States

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ABSTRACT: Nonribosomal peptide synthetase (NRPS) assembly lines are major avenues for the biosynthesis of a vast array of peptidyl natural products. Several hundred bacterial NRPS gene clusters contain a small (~70-residue) protein belonging to the MbtH family for which no function has been defined. Here we show that two strictly conserved Trp residues in MbtH-like proteins contribute to stimulation of amino acid adenylation in some NRPS modules. We also demonstrate that adenylation can be stimulated not only by cognate MbtH-like proteins but also by homologues from disparate natural product pathways.

In addition to the canonical NRPS domains involved in amino acid activation and peptide bond formation, many auxiliary proteins exist in NRPS gene clusters that act to tailor the peptide scaffold, enable host resistance, or provide transport mechanisms. An additional family of very small proteins (~8 kDa) has been coined "MbtH-like", based on homology to gene H in the Mbt cluster for biosynthesis of the *Mycobacterium tuberculosis* siderophore mycobactin (*1*). As of June 2010, GenBank contains >400 MbtH homologues. While not found in every NRPS system, MbtH-like proteins occur in gene clusters for diverse natural products, including glycopeptide antibiotics (e.g., vancomycin), iron chelating siderophores (e.g., enterobactin), aminocoumarins (e.g., clorobiocin), and lipopepeptides [e.g., calcium-dependent antibiotic (CDA)] (Figure S1 of the Supporting Information). Genetic evidence indicates the essentiality of MbtH homologues in several such NRPS assembly lines (*2*−*5*).

Recently, three different studies by our group have converged by virtue of the role MbtH-like proteins have played in in vitro characterizations of NRPS-mediated biosyntheses. We found the MbtH-like proteins associated with the vicibactin, glidobactin, and pacidamycin gene clusters to be crucial for obtaining and assaying specific multidomain NRPS modules VbsS, GlbF, and PacL, respectively (*6*−*8*) (Figure 1). While critical for VbsS and PacL adenylation activity, the MbH-like protein was crucial for GlbF expression, suggesting a complex multifunctional role in NRPS stability and/or activation. In all three cases, co-elution of the MbtH-like and NRPS proteins in close to stoichiometric amounts was observed after nickel affinity chromatography (*6*, *8*) (Figure S2 of the Supporting Information).

Given these experiences, first we aimed to better define the apparent role MbtH-like proteins play in NRPS module activation. We chose the pacidamycin NRPS module PacL because it

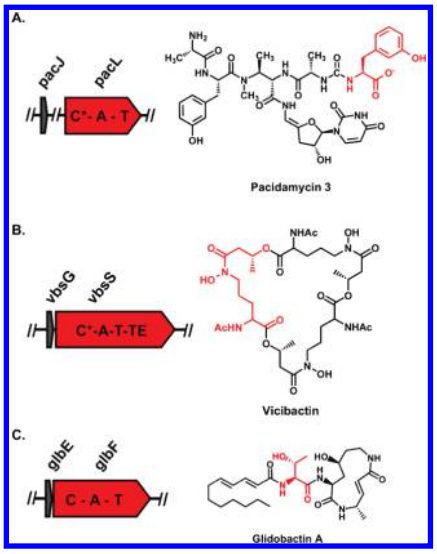

FIGURE 1: Partial operon context and natural product with the MbtH-like protein-associated amino acid highlighted for (A) PacJ/L (pacidamycin), (B) VbsG/S (vicibactin), and (C) GlbE/F (glidobactin). NRPS domain abbreviations are as follows: A, adenylation; T, thiolation; C, condensation; C*, truncated condensation; TE, thioesterase.

can be obtained without coexpression or copurification of an MbtH-like protein and the inactive adenylation (A) domain of purified PacL can be stimulated by addition of separately purified PacJ, the MbtH-like protein encoded in the *pac* gene cluster (*7*). Starting with our previous observations that MbtH-like proteins and NRPS modules form tightly associating complexes (*8*), we tested the hypothesis of Gulick and co-workers that a hydrophobic face containing strictly conserved Trp resides may be important in moderating protein−protein interactions (*3*). Indeed, our analysis of 260 unique MbtH-like protein sequences showed an extremely high level of conservation of the residues embedded in the SxWP and PxGW motifs (Figures S3 and S4 of the Supporting Information). Thus, we constructed the single W22A PacJ mutant and the double W22A/W32A PacJ mutant to examine their effects on formation of the complex and activation of adenylation activity with the NRPS PacL.

PacL activity was measured by ATP−PP_i exchange using *m*-Tyr (D/L) as the adenylation substrate. Activity is totally dependent on PacJ as indicated by no addition of PacJ at point 0 (Figure 2A). Notably, assay of PacL in either the apo (as shown here) or holo form (*7*) did not influence the rescue of activation by PacJ. The wild type and either the single or double PacJ mutant were titrated into the reaction mixture at ratios varying from 0 to 10 equiv of PacJ to PacL. The results show that the level of turnover was reduced to ~50% of that of the wild type for the W22A mutant, while the W22A/W32A mutant failed

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*To whom correspondence should be addressed. Phone: (617) 432-0930. Fax: (617) 432-0556. E-mail: christopher_walsh@hms.harvard.edu.

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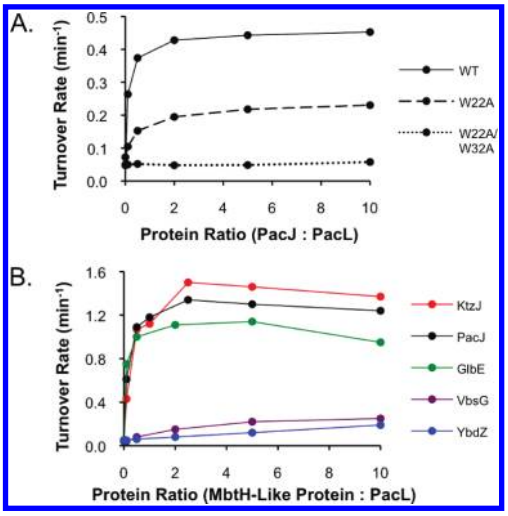

FIGURE 2: Adenylation of D/L-*m*-Tyr during titration of apo-PacL with (A) wild-type PacJ (—), W22A PacJ (---), and W22A/W32A PacJ (· · ·) and (B) MbtH homologues PacJ (black), KtzJ (red), GlbE (green), YbdZ (blue), and VbsG (purple).

to rescue PacL activity beyond baseline (Figure 2A). Furthermore, when PacL (90 kDa) and the double PacJ mutant (8 kDa) were mixed and then dialyzed against a 50000 molecular weight cutoff membrane, virtually no PacJ−PacL complex was observed as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (data not shown). It is notable that mutation of MbtH-like protein GlbE from the glidobactin system affected both solubility and complex formation during copurification with GlbF (Figure S5 of the Supporting Information). It is likely the conserved Trp residues participate in the formation of a crucial protein−protein interface, requiring future structural studies of such complexes.

Studies of the CDA, clorobiocin, and coelichelin pathways indicate in vivo that noncognate MbtH-like proteins can rescue stalled pathways (*4*, *5*). To evaluate this in vitro, we expressed and purified MbtH-like proteins PacJ, VbsG, and GlbE as well as those found the in the enterobactin (YbdZ) and kutzneride (KtzJ) gene clusters and titrated them into ATP−PP_i exchange reaction mixtures with PacL as done for the assay of the PacJ mutants described above (Figure 2B).

KtzJ [kutzneride (*9*)] and GlbE [glidobactin (*10*)] could readily stimulate PacL adenylation, while those from vicibactin and enterobactin biosynthesis were less effective. KtzJ shares the highest pairwise percent identity (39%) with PacJ; however, GlbE (31%) is comparable to YbdZ (29%) and VbsG (33%). Inspection of the five sequences did not suggest a motif or even a signal residue unique to PacJ, GlbE, and KtzJ (Figure S2 of the Supporting Information). This experiment corroborates the in vivo findings that MbtH-like proteins can cross-talk between disparate NRPS clusters and suggests that MbtH-like protein−NRPS interactions involve more extended surfaces than the conserved Trp residues. Although PacJ improves the performance of the A domain of PacL, it had no effect on Ala activation by PacO or 2,3-DABA activation by PacP (*7*) in the pacidamycin pathway, indicating a likely spectrum of interaction of MbtH members with different NRPS modules.

As this study was completed, Felnagle et al. arrived at convergent conclusions from a study of MbtH-like proteins CmnN and VioN on A domain activation of CmnO and VioO in capreomycin and viomycin assembly as well as YbdZ activation of serine recognition by EntF in enterobactin synthesis (*11*). We note in Figure 2 that YbdZ also heterologously activates the PacL adenylation activity. Both groups of investigators see stoichiometric association of MbtH members with some NRPS module partners [e.g., in glidobactin or vicibactin modules (*6*, *8*)] as well as rescue of some but not all adenylation activities when mixed after separate purification. These two studies provide evidence that investigators should consider coexpressing NRPS modules with cognate MbtH homologues to optimize adenylation activities. The in vivo essentiality of MbtH-like proteins for production of many nonribosomal peptides, including enterobactin (*11*), suggests many assembly lines have at least one vulnerable A domain in which an MbtH-like protein may function as a chaperone or regulatory subunit. Investigation of the structure of MbtH-like protein−A domain pairs should provide guidance for the mode of A domain activation and instruct MbtH-like protein-directed inhibition strategies, which may be of practical use, for example, in halting siderophore production.

SUPPORTING INFORMATION AVAILABLE

A description of experimental details, including cloning, purification, and assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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