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PDZ Domains and Their Ligands

Protein interaction domains (PIDs) such as the SH2 and SH3 domains are known to drive protein:protein interactions by binding to linear peptides. Screens of peptide arrays and combinatorial peptide libraries have been used to identify the peptide sequences recognized by a given PID. Two reports in this issue of *Chemistry & Biology* describe a new method for the synthesis of these peptide arrays and illustrate the utility of peptide ligands.

Many eukaryotic proteins lack catalytic activity and instead exist solely to organize supramolecular complexes. Evolutionary forces have assembled these "organizer" proteins from PIDs such as the SH2, SH3, EH, and WW domains. These domains typically bind short peptide sequences with mid- to low-micromolar dissociation constants. (An excellent review detailing the large list of PIDs can be found elsewhere [1].) A wide variety of cellular processes, such as signal transduction, receptor-mediated endocytosis, formation of the cytoskeleton, and synapse organization, depend upon protein complexes that are held together with PIDs.

The specificity of PIDs can be mapped quickly by screening soluble or immobilized peptide libraries or by selecting ligands from phage or RNA displayed combinatorial peptide libraries. Typically, the peptides recovered by affinity selection share a consensus sequence that defines the ligand preferences of the domain in question. Because the selected peptides and natural proteins tend to interact with the same "hot spot" on the PID [2, 3], the peptide consensus can be used in a computer search to predict the in vivo partners of the domains. These predictions are then confirmed or disproved through a variety of cellular and biochemical techniques (e.g., synthesis and testing of peptides derived from actual proteins, coimmunoprecipitation or pull-downs from cell lysates, yeast two-hybrid screening, etc.).

One PID of great interest is the PDZ domain [4]. This 100-residue domain occurs 785 times in 436 human proteins (http://smart.embl-heidelberg.de/). Some PDZ domains have been shown to bind C-terminal peptide sequences, while others bind internal regions. Although PDZ domains share a common three-dimensional structure, they generally differ in their binding specificities. In this "proteomics era," it is of great interest to learn the specificity of PDZ domains, to discover their cellular partners, and to fashion inhibitors of their protein-protein interactions. In this way, the functional importance of particular PDZ domains can be revealed.

In this issue of *Chemistry & Biology*, two publications utilize peptide ligands of PDZ domains to define their molecular recognition properties and to generate an antagonist [12, 13]. Coincidently, both laboratories used peptide cyclization in their approach. In the publication by Boisguerin et al. [12], a library of peptides was synthesized on a solid support. Upon completion of the peptide sequence, the N termini were further modified such that they could loop back and attach to the solid support via a thioether. The C termini were then liberated from the solid support, leaving the peptides attached only by the N termini. When an array of peptides corresponding to the C termini of 6223 human proteins was screened against two PDZ domains, several new cellular partners for the PDZ domains were identified. In the publication by Piserchio et al. [13], cyclization was used to rigidify a portion of a PDZ domain's peptide ligand so that it bound 10-fold tighter to its target. The three-dimensional structure of the peptide-PDZ domain complex was solved by NMR spectroscopy, and the cyclic peptide was shown to perturb the normal activity of its target in vivo. Both of these publications and their significance are discussed in more detail below.

To understand the contributions of the first publication, it must be appreciated that phage-display vectors for C-terminal display of combinatorial peptide libraries have not been available until recently [5, 6]. Consequently, to map the specificity of most PDZ domains, only synthetic combinatorial peptide libraries were appropriate. However, unbeknownst to many molecular biologists, solid-phase peptide synthesis occurs from the C terminus to the N terminus. Consequently, the C terminus of the peptide remains attached to the solid support at the completion of the synthesis. While leaving the peptides attached permits screening with antibodies and many PIDs, this has been problematic for screening against PDZ domains that recognize C termini. Thus, synthetic peptides needed to be cleaved off the solid support prior to assaying binding to PDZ domains. Only a few soluble combinatorial peptide libraries are readily available, and while they are useful in identifying the optimal ligand preferences of PDZ domains [7], they do not permit quantitative binding comparisons of different peptide sequences to the same PDZ domain. Using the SPOT method for chemically synthesizing peptides on cellulose membranes [8], Jens Schneider-Mergener and colleagues in Berlin invented one chemical method for inverting peptides on a solid support [9]. They demonstrated its usefulness by synthesizing a library of 7-mer peptides, corresponding to 3514 human proteins, with free C termini. Screening this library with the human protein α1-syntrophin yielded 38 PDZ binding peptides. Their sequences correspond to several known α1-syntrophin-interacting proteins as well as many interesting candidates for in vivo interactions.

The work of Rudolf Volkmer-Engert and colleagues in Berlin, as reported in this issue, has expanded the size of the C-terminal library and modified the chemistry involved in SPOT synthesis [12]. A library of 6223 C termini (11-mers) from human proteins was synthesized on cellulose membranes. Eight synthetic steps, in addition to the condensation of the Fmoc-protected amino acids, were involved in generating the inverted peptides. In brief, the synthetic protocol began by attaching a mer-

captopropionyl cysteine (side-chain protected) to the N-modified cellulose amino-hydroxypropyl ether membrane. The C-terminal amino acid of the desired peptide was then attached to the mercaptopropionyl group. Following the condensation of the next ten amino acids, the completed peptides were all coupled to Fmoc-β-alanine pentafluorophenyl ester, Fmoc deprotected, and elongated with bromoacetic acid 2,4-dinitrophenyl ester. Selective deprotection of the cysteine side chain then allowed cyclization via thioether formation with the bromoacetic acid moiety. When the ester bond adjacent to the first amino acid is cleaved, the peptide becomes inverted (i.e., it is now anchored to the membrane through its N-terminal amino acid). After optimizing the peptide synthesis, positive hits against the α1-syntrophin PDZ domain provided a 20-fold increase in signal strength compared to the same peptides in the original library of Schneider-Mergener. All of the strong binders from the Schneider-Mergener library were also positive in the screen of the Volker-Engert library. This correlation served to validate the new synthetic approach. In addition, the new library revealed many positives that were not tested originally.

When the Volkmer-Engert library was screened against the PDZ domain of human Erbin, a protein identified by yeast two-hybrid screening to bind to the C terminus of the receptor tyrosine kinase ErbB2, 40 strong binders (K_d values from 8 to 110 μM) were identified. Four of the most interesting hits (two potassium channels, plasma membrane Ca^{2+} ATPase, and the breakpoint cluster region protein [BCR]) were selected for further analysis. Amino acid substitutions of the C-terminal sequences demonstrated that these four peptides bound to the PDZ domain using a canonical recognition sequence. Furthermore, the BCR protein could be successfully immunoprecipitated with Erbin from lysates of cultured cells. This work demonstrates that these arrays of C termini will have great value in defining the specificity of the large collection of human PDZ domains. A similar "proteome peptide scanning" approach has been published recently, in which the specificity of numerous SH3 domains was also defined with SPOT synthesized arrays of yeast and human proteins [10].

Once a peptide ligand has been defined for a PID, the research focus often shifts to modifying the peptide to generate antagonists. Such efforts can involve substitutions with unnatural amino acids, cyclization, or a combination of both strategies. In the publication by Piserchio et al. [13], the cyclic peptide H_2N-Tyr-Lys-c[-Lys-Thr-Glu(βAla)-]-Val-COOH was designed and synthesized. This peptide, with a β-alanine lactam side chain linker between the underlined Lys and Glu residues, was based on the six C-terminal residues (H_2N-Tyr-Lys-Gln-Thr-Ser-Val-COOH) of CRIPT, a postsynaptic protein that binds to the third PDZ domain of PSD95. Since the side chains of the P_{-3} and P_{-1} amino acids (starting from the C terminus, Val is numbered P_0) were known to point away from the surface of the PDZ domain in a crystal structure, these residues were replaced with Lys and Glu, and their side chains were linked with β-alanine [11]. The dissociation constants of the linear CRIPT peptide and the cyclic peptide variant are 10 μM and 1 μM, respectively. NMR spectroscopy of the complex formed between the cyclic peptide and the PDZ domain revealed that the peptide binds in the groove between the β2 strand and α2 helix. Similar binding has been reported for the C termini of natural protein ligands. The side chains of Lys_{-3} and Glu_{-1} can be seen to interact with several residues in the β2 and β3 strands and the lactam-containing ring projects perpendicular to the β2 strand (Protein Data Bank ID code 1RGR).

If the cyclic peptide specifically binds to the third PDZ domain of PSD-95, then it should antagonize its interactions with other proteins. The authors tested the effect of the peptide on the interaction between PSD-95 and the GluR6 subunit of the kainite receptor. Human embryonic kidney cells (HEK293) were transfected with plasmids expressing GluR6 and PSD-95, and after 24 hr the cyclic peptide and a linear peptide were transduced into the cells with Sigma Chemical's BioPORTER reagent. As monitored by coimmunoprecipitation experiments, the cyclic peptide was ~5-fold more effective in disrupting the GluR6:PSD-95 interaction than a 15-mer peptide corresponding to the C terminus of GluR6. Furthermore, the cyclic peptide was successful in preventing the clustering of the kainite receptor in cotransfected cells, as monitored by immunofluorescence staining. Compared to the linear peptide, the transduced cyclic peptide was effective over much longer periods of time (18 hr versus 5 hr), suggesting that the cyclic peptide is resistant to enzymatic degradation.

In conclusion, the "tool kit" for protein biochemists interested in PDZ domains has become larger with the two approaches introduced by the two publications in this month's issue. Spotted arrays of peptide ligands can be rapidly screened and generate hypothetical interacting partners to follow up experimentally. Once a peptide ligand has been identified, it can be used to predict in vivo binding partners, concurrently synthesized in a cyclic form, and used as an antagonist. Thus, by a combination of both approaches, the cellular role of PDZ domain-containing proteins can be explored more expeditiously in the future.

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