Functional genomics of intracellular peptide recognition domains with combinatorial biology methods

Sachdev S Sidhu[*], Gary D Bader[†§] and Charles Boone[‡]

Phage-displayed peptide libraries have been used to identify specific ligands for peptide-binding domains that mediate intracellular protein–protein interactions. These studies have provided significant insights into the specificities of particular domains. For PDZ domains that recognize C-terminal sequences, the information has proven useful in identifying natural binding partners from genomic databases. For SH3 domains that recognize internal proline-rich motifs, the results of database searches with phage-derived ligands have been compared with the results of yeast-two-hybrid experiments to produce overlap networks that reliably predict natural protein–protein interactions. In addition, libraries of phage-displayed PDZ and SH3 domains have been used to identify the residues responsible for ligand recognition, and also to engineer domains with altered specificities.

Addresses
[*]Department of Protein Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA
e-mail: sidhu@gene.com
[†]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5S 1A8 and Department of Biochemistry, University of Toronto, Ontario, Canada M5G 1L6
[‡]Banting and Best Department of Medical Research and Department of Molecular and Medical Genetics, University of Toronto, Ontario, Canada M5G 1L6
[§]Current address: Computational Biology Center, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

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Abbreviations

Erbin	ErbB2 interacting protein
NMDA	*N*-methyl-D-aspartate
PDZ	PSD95-Discs large-ZO1
PSD-95	postsynaptic density protein-95
SH3	Src-homology-3

Introduction

Many intracellular signaling complexes are assembled by specialized peptide-binding modules that recognize small, continuous sequence motifs within large proteins [1,2]. There are many structurally distinct families of peptide recognition domains and each family typically recognizes a common core motif; specificity amongst family members is determined by differing preferences for residues that flank the core [3,4•]. It has become apparent that eukaryotic cells contain numerous intracellular scaffolding proteins that consist of various peptide recognition modules embedded within the primary sequence and, often, also contain distinct catalytic domains such as kinases and phosphatases. The binding of various proteins to these scaffolding proteins serves to bring together particular cellular components within specific spatial arrangements, and thus modulates intracellular architecture and signal transduction. A detailed understanding of the binding specificities and natural ligands recognized by different peptide-binding modules would provide significant insights into cell biology and could also lead to the development of novel therapeutics.

Phage display is a powerful technology that has been especially fruitful in the study of peptide recognition domains [5,6]. The technology permits the display of extremely diverse libraries ($>10^{11}$) of peptides or proteins on the surfaces of phage particles that also contain the DNA that encodes the displayed polypeptide [7]. Library members with particular binding characteristics can be isolated by binding to an immobilized receptor *in vitro*, and polypeptide sequences can be decoded by sequencing the encapsulated DNA.

This review covers the use of phage display in the study of peptide recognition domains, with particular emphasis on developments over the past year. In particular, we focus on two domain families with which the technology has been particularly effective, namely, PSD95-Discs large-ZO1 (PDZ) domains and Src-homology-3 (SH3) domains. In these cases, phage-derived peptide ligands have been used to explore in detail the molecular interactions responsible for binding affinity and specificity. These ligands have also been used as leads for database mining to identify potential natural binding partners. In some cases, peptide ligands have been introduced into living cells to act as disruptors of natural protein–protein interactions. Phage display has also been used to explore the other side of the binding interface, that is, libraries of phage-displayed peptide recognition domains have been used to identify residues that participate in peptide-binding interactions. Taken together, these studies have provided considerable insight into the relationships between structure and function in various peptide-binding modules, and they have helped to define the cellular processes mediated by these simple yet diverse protein–protein interactions.

PDZ domains

PDZ domains are 80–100 amino acid modules that predominantly recognize the C-termini of various intracellular and cell-surface proteins and, in so doing, assemble multiprotein complexes at specific subcellular sites [8,9]. It has been proposed that the human genome contains as many as 440 PDZ domains in 259 different proteins involved in numerous aspects of cellular function [10]. Structural studies have revealed that peptide ligands intercalate in a groove between a β-sheet and an α-helix in an antiparallel fashion with respect to the PDZ domain strand [11,12], and a detailed understanding of these interactions can be expected to contribute significant insights into the cellular functions of PDZ-containing proteins.

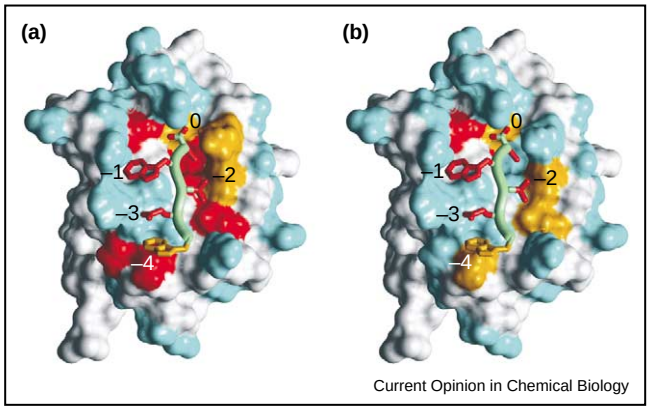
PDZ domains have been broadly grouped into two main classes: type I, with a binding consensus of X[S/T)]X[V/I/L]$_{COOH}$; and type II, with a binding consensus of XΦXΦ$_{COOH}$ (where Φ is a hydrophobic residue) [13–15]. However, studies with combinatorial peptide libraries have been instrumental in further defining the fine specificity of individual domains and revealing binding contributions from as many as six C-terminal residues. Early studies used chemically synthesized libraries [16] or a 'peptides-on-plasmids' system [17]; phage display was not initially utilized because of the presumed difficulty of displaying peptides with free C-termini using conventional M13 phage display systems. Recently, however, C-terminally displayed peptide libraries with M13 [18,19•] and lambda phage [20] have been used successfully to explore PDZ domain specificity.

Fusion of peptides to the C-terminus of the D protein of lamda phage resulted in extremely high valency display on the capsid, thus enabling the selection of even low-affinity binders [20]. Using such a system, peptide ligands were selected for the seven PDZ domains of the human INADL protein and different degrees of consensus were observed, with different PDZ domains selecting ligands with consensus at either two, three or four C-terminal residues. Although each PDZ domain displayed a distinct specificity, there was some cross-reactivity between some PDZ domains and ligands selected against other PDZ domains, at least as judged on the basis of qualitative solid phase immunoassays [20]. By contrast, peptide libraries fused to the C-terminus of the M13 major coat protein appear to be displayed at lower valencies and thus result in more stringent selection on the basis of intrinsic affinity. When such a library was used to isolate ligands for two PDZ domains from a human membrane-associated guanylate kinase with inverted orientation (MAGI-3), one of the domains yielded only a single binding sequence whereas the other yielded multiple binding sequences that showed a clear consensus in all four C-terminal positions [18]. Synthetic peptides based on the phage sequences bound their cognate PDZ domains with affinities in the submicromolar range while exhibiting no detectable binding to noncognate PDZ domains. The differing selection stringencies afforded by high or low valency display may provide complementary sets of data with each set being useful for different purposes. Low stringency selections provide a broad range of binders that can be used to characterize the full range of potential PDZ domain ligands (including low affinity binders), whereas high stringency selections can be used to identify the optimal ligand for each domain.

Recently, M13 C-terminal phage display was used to study the binding specificity of the single PDZ domain of ErbB2 interacting protein (Erbin) [19•]. While Erbin had originally been identified as a putative ligand for ErbB2 in a yeast-two-hybrid screen, phage display revealed a binding consensus for the Erbin PDZ domain ([D/E][T/S]WV$_{COOH}$) that differed significantly from the C-terminus of ErbB2 (DVPV$_{COOH}$). Because PDZ domain binding interactions require a ligand with a free C-terminus, the number of potential ligands in genomic databases is greatly reduced. Indeed, database mining with the phage-derived consensus uncovered only three unique matches and these were all p120-related catenins (δ-catenin, ARVCF and p0071), which all terminated in an identical sequence (DSWV$_{COOH}$). Subsequent *in vitro* and *in vivo* studies showed that the Erbin PDZ domain binds to these catenins with high affinity and specificity, whereas affinity for ErbB2 is much lower [19•]. These studies demonstrated that, although yeast-two-hybrid is a powerful tool for discovering putative protein–protein interactions, the method can be highly sensitive to even low-affinity interactions and, thus, it is worthwhile corroborating yeast-two-hybrid results with other methods.

In a follow up study, the structure and function of the Erbin PDZ domain have been studied in detail using a combination of NMR spectroscopy, phage display and affinity assays with synthetic peptides [21••]. The NMR structure of the Erbin PDZ domain complexed with a phage-optimized peptide revealed five distinct binding sites that accommodated the five C-terminal ligand side-chains (Figure 1). Statistical analysis of a large population of phage-derived peptide ligands (~150) enabled the calculation of the relative frequency of each natural amino acid binding at each site, thus defining not only the optimal ligand but also the range of side-chains accommodated at each site. Assays with synthetic peptides designed on the basis of these data were used to quantitatively assess the binding contributions of each side-chain, and it was found that the five C-terminal side-chains each contribute to binding, with the last two side-chains and C-terminal carboxylate providing the majority of binding energy. The Erbin PDZ domain was also displayed on phage and a combinatorial shotgun scanning strategy was used to assess the binding contributions of 44 protein side-chains in and around the peptide-binding site; two separate scans were used to assess the effects of

Figure 1



Structural and functional analysis of the Erbin PDZ domain bound to a high affinity peptide [21••]. The NMR structure of the Erbin PDZ domain is represented with a Connolly surface and the phage-derived peptide ligand ($TGWETWV_{COOH}$) is shown in tube form. Only the last five peptide residues (numbered 0, −1, etc. from the C-terminus) are visible in the structure. The side-chains are coloured according to the results of an alanine-scan of the peptide and the results of **(a)** a combinatorial alanine-scan or **(b)** homologue-scan of the PDZ domain; the colour code shows the fold reduction in binding due to each substitution, as follows: red > 100-fold, yellow > 10-fold, cyan < 10-fold, grey residues were not scanned. Peptide residues 0, −2 and −4 make critical binding contacts with PDZ side-chain atoms, while residues −1 and −3 interact mainly with PDZ backbone atoms. In general, the effects of homologous substitutions are attenuated with respect to those of alanine substitutions, as might be expected from the subtle nature of the homologous mutations.

alanine substitutions or subtle homologous substitutions (e.g. Asp for Glu, Arg for Lys, etc.). The combined structural and functional data provided extremely comprehensive views of the interactions that mediate ligand binding to the Erbin PDZ domain (Figure 1). Detailed analyses of this type, together with sequence alignments and homology modeling, should be useful for understanding the general principles that determine ligand specificity and affinity within the PDZ domain family.

In another application, peptide ligands have also been used to disrupt natural PDZ–ligand interactions *in vivo*. When cells co-transfected with ARVCF and the Erbin PDZ domain were treated with a phage-optimized peptide ligand (conjugated to an antennapedia carrier peptide that allowed entry into the cell), co-precipitation of the co-transfected proteins was severely inhibited [19•]. In an impressive example, peptides were used to disrupt the interaction between the second PDZ domain of postsynaptic density protein-95 (PSD-95) and the *N*-methyl-D-aspartate (NMDA) receptors, either in cultured cortical neurons or in live rat brains. In a rat stroke model, disruption of this interaction greatly reduced ischaemic brain damage following transient middle cerebral artery occlusion [22••]. These results demonstrate that peptide ligands can act as *in vivo* inhibitors of natural PDZ–ligand interactions and, thus, may have utility in the validation of protein–protein interactions as therapeutic targets.

SH3 domains

SH3 domains are small modules (~60 residues) that are found in many membrane-associated and cytoskeletal proteins, and also in adaptor proteins [23]. The domain fold is extremely widespread, as over 1500 different SH3 domains have been identified in the protein databases of eukaryotic organisms [24]. Most SH3 domains recognize a core motif of XPXXPX, and the specificity of each domain is determined by preferences for different residues around and between the two proline residues. Binding to proline-rich sequences is a common molecular recognition mechanism used not only by SH3 domains but also by the structurally distinct families of WW domains [23] and EVH1 domains [25].

Several studies with peptide–phage libraries have provided significant insights into the mechanisms responsible for the recognition of proline-rich sequences by SH3 domains [26–32]. These analyses have produced proline-rich ligands that resemble natural ligands but, in many cases, phage-derived ligands have exhibited higher affinities than natural interactions. In addition, some studies have identified binding motifs that differ from the 'classical' SH3 binding motifs, suggesting that the range of SH3 ligand specificities may be more diverse than previously appreciated [28–30]. Studies with WW domains have also yielded high-affinity ligands that have been useful in defining the binding specificities that characterize this family [33,34]. While, in theory, the consensus motifs defined by phage display could be used to search for natural binding partners in genomic databases, such searches usually yield an excessively large number of false positives. This is because, unlike PDZ domains, which bind predominantly to C-terminal sequences, SH3 and WW domain recognize motifs that can occur anywhere within a protein sequence.

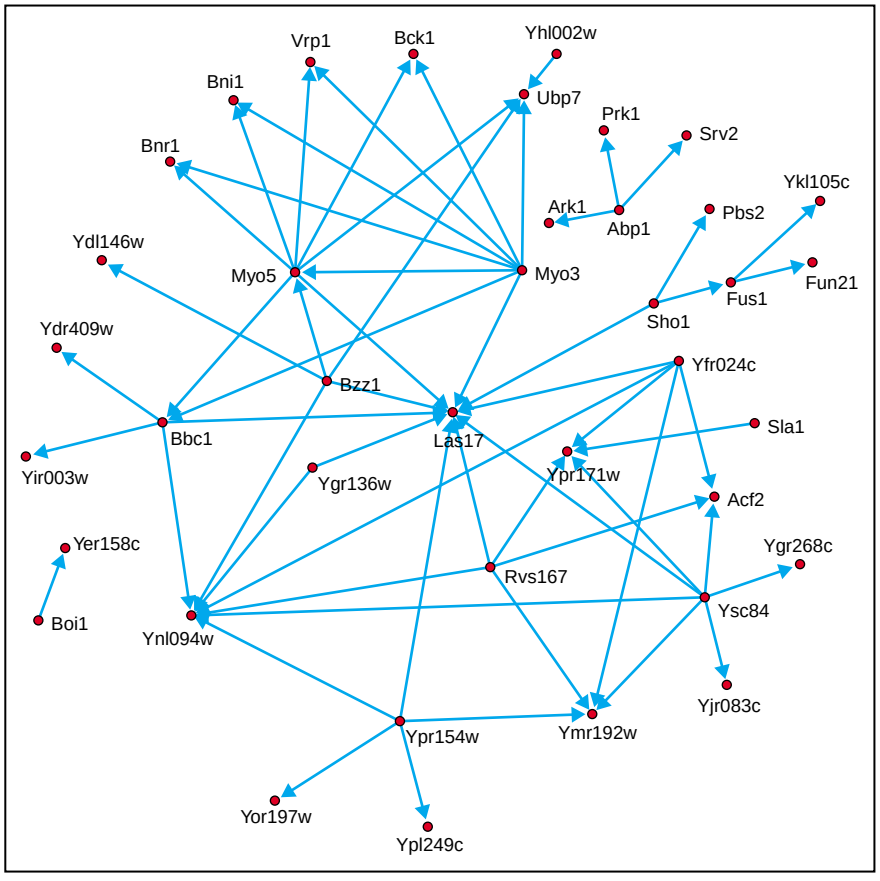
To address this problem of false positives, Tong *et al.* [35••] have recently developed an approach that combines data from phage display with data from yeast-two-hybrid experiments. As a first step, phage display was used to define the binding consensus sequences for 20 of the 28 SH3 domains in the yeast genome. This data was analysed computationally using a position-specific scoring matrix to identify potential natural binding partners within the yeast proteome. This search produced a highly connected network containing 206 proteins with 394 putative interactions. Next, 18 SH3 domain baits were screened against conventional yeast-two-hybrid libraries and an ordered genome-wide array of open reading frames. These experiments generated a second, independent interaction map that contained 233 interactions between 145 proteins. In the final step, the data from the two methods were compared with each other to reveal an overlap network

of 59 interactions between 39 proteins that was common to both putative networks (Figure 2). The overlap network was enriched for interactions validated in the literature, over fivefold compared with the phage display network and over threefold compared with the two-hybrid network. Furthermore, the predicted interactions with a central member of the overlap network (Las17) were confirmed with further experimental analyses.

The work of Tong *et al.* is one of the first to combine large-scale experimental analyses with computational sequence analyses to generate putative natural interaction networks. Although SH3 domains are an ideal model system for this type of approach, it is likely that the general concepts can be extended to the study of other peptide-binding modules. A key strategic point was the filtering of two large, orthogonal datasets against each other to obtain a smaller overlap network enriched for biologically relevant protein–protein interactions. While this step was necessary to generate a tractable overlap network depleted of most false positives, the filtering process also removed interactions that were only represented in one dataset but, nevertheless, may be biologically relevant. The development of more sophisticated algorithms that assign probabilities to each prediction may improve the filtering process. In addition, the incorporation of data from other experimental methods and biologically validated interactions may further improve the completeness and reliability of such networks [35••,36].

Considerable progress has also been made in the use of phage-displayed SH3 domain libraries to engineer novel binding specificities and to identify residues that determine peptide-binding specificity. HIV-1 Nef binds to the SH3 domain of the tyrosine kinase Hck, and Hiipakka *et al.* [37] were able to select variant SH3 domains with about 40-fold improved affinity for HIV-1 Nef from a library based on the Hck-SH3 domain. Panni *et al.* [38•] have constructed an SH3 library that attempts to represent the binding surfaces of natural SH3 domains on a phage-displayed scaffold of the human Abl-SH3 domain. This was accomplished by randomizing 12 positions on the

Figure 2



The overlap network for yeast SH3-mediated protein–protein interactions [35••]. The network represents the overlap of interaction networks derived from phage display and two-hybrid experiments, and it contains 59 interactions between 39 proteins. All of the putative interactions are mediated by SH3 domains; the arrows point from SH3 domain proteins to putative ligands. Reprinted with permission from [35••]. Copyright 2002 American Association for the Advancement of Science.

binding surface of the Abl-SH3 domain and preferentially allowing only those residues that commonly occur in the alignment of 560 different SH3 domains. The repertoire was successful in generating SH3 domains that bound preferentially to either a peptide ligand for Abl-SH3 domain or to a peptide ligand for Src-SH3 domain. Surprisingly, the novel Src-SH3-like specificity was dependent on an unexpected cysteine mutation that formed a disulfide bond with another cysteine within the Abl-SH3 domain. Thus, this mutant used an unusual mechanism for ligand binding that does not occur in natural SH3 domain–ligand interactions. Nonetheless, the repertoire represents a promising means for generating information that is complementary to the results of peptide–phage experiments with SH3 domains as binding targets, because, together, the two approaches should enable exploration of the specificity determinants on both sides of the binding interface.

Conclusions

Phage display has proven invaluable in the study of the structure and function of peptide-binding modules. In addition, the binding specificity information gained from peptide ligands has been useful in predicting natural binding partners. Combinatorial strategies have been most effective when combined with structural studies and detailed mutagenesis and affinity experiments. In terms of mapping natural interaction networks, phage display works best when combined with other approaches such as yeast-two-hybrid and rigorous cell biology methods. In the future, further refinements in the technology and data processing should enable more accurate and rapid analyses. In fact, it is conceivable that specificity profiles for all or most peptide-binding domains within the human proteome may soon be available. Coupled with other large-scale protein–protein interaction mapping methods, these databases may prove instrumental in deciphering the complex processes mediated by the numerous protein–protein interactions within the proteome.

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The authors produced an SH3 domain library designed to resemble the binding surfaces of natural SH3 domains. Such repertoires should be useful in generating not only binding domains for peptides of interest but, perhaps more importantly, they may help to identify natural SH3 domains that bind particular natural proline-rich ligands. In combination with data from peptide–phage libraries, these data may reveal general recognition rules for SH3 domain–ligand interactions.