[6] Mapping Protein–Protein Interactions with Alkaline Phosphatase Fusion Proteins

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Introduction

Mediation of protein–protein interaction through modular binding domains, such as SH2, SH3, PTB, PDZ, WW, or EH domains, is an important mechanism for intracellular signaling.[1] The physiological importance of one such module, the SH3 domain, is evidenced by its presence in both the regulators and effectors of the Ras-mediated mitogen-activated protein (MAP) kinase signaling pathway.[2,3] SH3 domain-containing proteins have also been shown to function in a variety of diverse biological events including cytoskeletal organization,[4] subcellular localization of proteins,[5] and endocytosis.[6]

SH3 domains are known to bind to short proline-rich peptide sequences within interacting proteins. Typically, the ligands have the consensus sequence, PxxP, and adopt polyproline type II (PPII) helical structures that fit into shallow hydrophobic pockets, formed by aromatic residues on the surface of SH3 domains.[7] Furthermore, SH3 domains bind the peptide ligands in one of two opposite orientations, N to C terminus or C to N terminus.[8,9] The orientation and specificity of peptide ligands for SH3 domains is heavily influenced by interaction between the nonproline residues in the PPII helix and the residues flanking the main hydrophobic binding surface of the SH3 domain.[10,11]

In this chapter, we describe a convenient and versatile method for determining the molecular recognition properties of SH3 domains, using bacterial alkaline phosphatase as a fusion protein. A variety of proteins or

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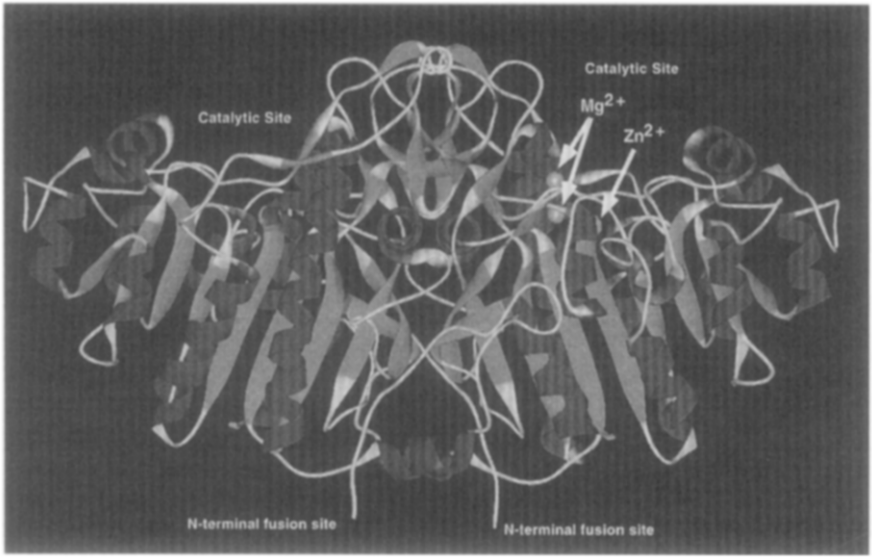

FIG. 1. Structure of bacterial alkaline phosphatase. The three-dimensional structure of a homodimer of *Escherichia coli* alkaline phosphatase is shown.[37] The structure of the protein (PDB database accession number 1ALK) is shown as modeled with the program WebLab ViewerLite 3.2 (*http://www.msi.com*). The catalytic sites with their Zn^{2+} and Mg^{2+} ions and the N-terminal fusion sites are indicated.

peptides have been fused to the N terminus of alkaline phosphatase (AP) by genetic engineering, including antibody chains (i.e., single-chain and Fab antibody segments),[12–15] epitopes,[16,17] and the extracellular domain of the Steel receptor.[18] Figure 1 highlights the three-dimensional structure of a homodimer of *E. coli* AP. Because fusions to AP occur at its N terminus, which protrudes away from the globular body of the protein, many protein segments or peptides can be fused to the AP without interfering with the

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catalytic activity of the enzyme. In our hands, we have used the culture media as one-step detection probes for detecting protein–protein interactions on a variety of surfaces including, nitrocellulose or PVDF membranes, microtiter plate wells, and plastic pins.

Description of Bacterial Alkaline Phosphatase Fusion System

Generation of Fusion Proteins

Alkaline Phosphatase Fusion Vector. The major components of the expression plasmid include (1) a bacterial signal peptide derived from OmpA that directs secretion of the fusion protein into the periplasmic space, where it can leak into the culture medium, (2) the FLAG epitope at the N terminus of the mature AP protein, (3) multiple cloning sites downstream of the epitope for insertion of coding segments, (4) a regulated promoter to permit induction of the fusion protein, and (5) an antibiotic resistance gene to maintain the plasmid by selection in bacterial cells. A map of the AP fusion vector, pMY101, is shown in Fig. 2. We have observed

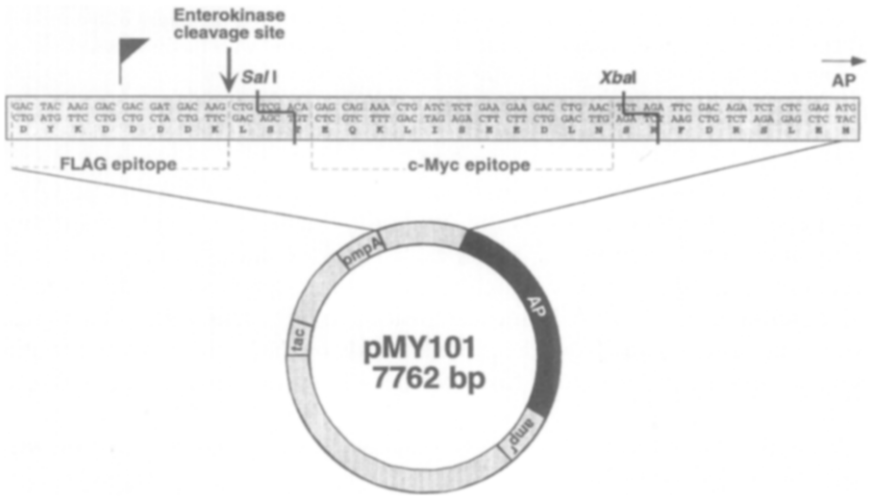

FIG. 2. Map of the bacterial alkaline phosphatase fusion vector, pMY101. The coding region of a segment of the *E. coli* alkaline phosphatase (AP) gene is shown with the FLAG and c-Myc epitope sequences below. The *Sal*I and *Xba*I restriction sites flank the c-Myc epitope in the same reading frame as cloned peptides that were displayed by a bacteriophage M13 combinatorial peptide library. The 7762-base pair (bp) vector was derived from the pFLAG-1-AP vector in which the OmpA signal/leader sequence is upstream of the FLAG and c-Myc epitopes and the mature AP-coding sequence (black). The vector also carries genes for ampicillin resistance (Amp^r) and the *lac* repressor (*lacI*), which regulates the *tac* promoter, upstream of the AP gene.

that polypeptides ranging from 8 to 300 amino acids in length can be fused to the N terminus of AP, without loss of either the phosphatase activity or binding properties of the fused segment.

Preparation of Alkaline Phosphatase Fusion Protein. After the DNA fragment encoding the peptide or protein of interest is cloned in frame into the pMY101 vector, the construct is transformed into *E. coli.* Recombinant plasmids can be identified by polymerase chain reaction (PCR) or restriction digestion analysis, and verified by DNA sequencing. Ampicillin-resistant colonies are used to inoculate 5–15 ml of LB medium (containing ampicillin at 100 μg/ml) and allowed to grow overnight at 37°. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 50–100 μM final concentration in the culture medium during log-phase growth of the bacteria increases the yield of most fusion proteins. The AP fusion protein, which is secreted into the culture media, can be separated from the bacterial cells by centrifugation (500*g* for 5 min at 4°) and stored at 4°.

The AP fusion protein should be kept at 4° for no longer than 2 weeks, as the integrity of the fused protein or the AP activity is often lost with time. Protease inhibitors such as Complete (Boehringer Mannheim, Indianapolis, IN) can be added to increase the shelf life of the fusion protein, although longer storage may require purification. To confirm that the AP fusion protein is active, transfer 50 μl of culture supernatant to an Eppendorf tube containing 50 μl of *p*-nitrophenyl phosphate (pNPP) (Sigma Fast; Sigma, St. Louis, MO); typically, the solution becomes bright yellow within 20 min. As the culture medium does not interfere with the interaction of the AP fusion protein with other proteins, no additional purification is necessary prior to its use.

Troubleshooting

If little or no AP activity is detected in the culture medium, several considerations are suggested for improving the yields of the AP fusion protein of interest.

1. *Lower the temperature of the bacterial culture:* Growing the cells at 30° sometimes leads to improved secretion of the fusion proteins into the culture medium. This is especially important if the N-terminal fusion to AP is larger than 90 amino acids.

2. *Start with a fresh transformant:* In a number of cases, we have observed that bacteria lose their ability to secrete AP fusion protein over time. Presumably this is due to the negative selective pressure on the bacteria caused by long-term overexpression of AP, which may be detrimental to growth. This problem can be solved by using bacteria from a newly transformed colony to inoculate the culture medium.

3. *Break the cell membrane:* On rare occasions, the fusion proteins are retained in the periplasmic space or cytoplasm instead of leaking out into the culture medium. In this situation, the protein can be recovered by harvesting the contents of the periplasmic space with an osmotic shock.

4. *Concentrate or purify the protein:* The fusion protein can be concentrated from the culture medium by centrifugation. Transfer 10–20 ml of culture medium containing the AP fusion protein of interest into a Centriprep YM-30 (Millipore, Bedford, MA) filtration device. Centrifuge at 1500*g* for 30–45 min at 4°, according to the manufacturer protocol. If desired, exchange the buffer of the retained liquid by adding Tris-buffered saline [TBS; 25 m*M* Tris–HCl (pH 7.5), 145 m*M* NaCl, 3 m*M* KCl] containing 0.1% (v/v) Tween 20 to the concentrate and centrifuge a second time. Conversely, the AP fusion proteins can be affinity purified with an anti-FLAG antibody (M1 monoclonal antibody; Sigma) coupled to agarose (Sigma). We have also found that a run of six histidines can be added to the N terminus of AP, thereby permitting the purification of the fusion protein by chromatography over a resin containing nickel-loaded iminodiacetic acid.[19]

Binding Strength and Specificity of Fusion Proteins

To utilize AP fusion proteins to study SH3 domain–ligand interaction, either the peptide ligand or the SH3 domain can be fused to the N terminus of the enzyme. As illustrated in Fig. 3 the AP fusion proteins have been used to study binding properties of the SH3 domains of Src and Abl. While the two SH3 domains are 52% similar in primary structure, their ligand specificities are quite different: the optimal ligand preferences for the Src SH3 domain are R(A/P)LPPLP or PPVPPR, while the Abl SH3 domain binds PPPVPLP.[20] This specificity can be demonstrated with AP fusion proteins by immobilizing either peptide ligands or SH3 domains on the surface of microtiter plate wells.

In Fig. 3A, individual wells of a 96-well high-binding microtiter plate (Costar, Cambridge, MA) are incubated with 1 μg of streptavidin (Sigma) in 100 m*M* $NaHCO_3$ (pH 8.3) for 30 min at room temperature. The wells are then washed three times with TBS containing 0.1% (v/v) Tween 20. For each triplicate set of wells, 0.1 m*M* biotinylated peptide is added and incubated for 30 min. To eliminate nonspecific binding of the wells, 10 μg of bovine serum albumin (BSA) is then added to each well and incubated for an additional 30 min.

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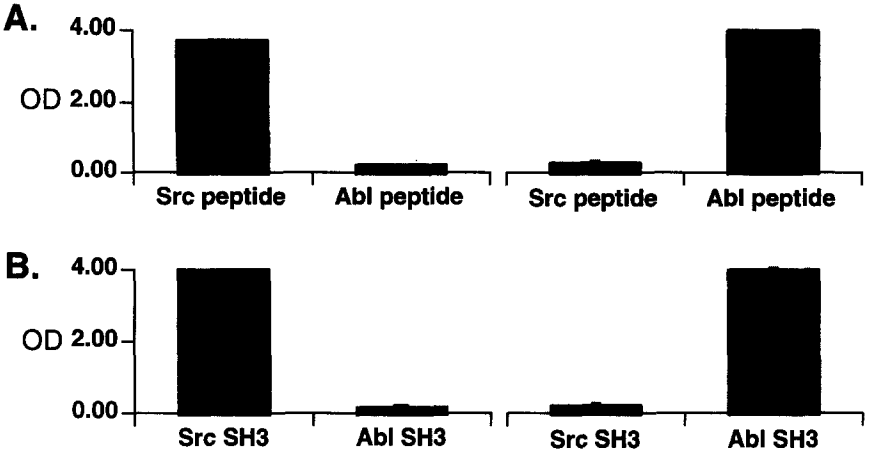

FIG. 3. Binding strength and specificity of SH3 domain–ligand interaction, using the AP fusion system. (A) Biotinylated Src and Abl SH3 domain peptide ligands were added to duplicate wells of a microtiter plate coated with streptavidin, washed, and incubated with AP fusions to either Src (*left*) or Abl (*right*) SH3 domains. (B) Binding properties of Src (*left*) or Abl (*right*) SH3 peptide ligands (fused to AP) to either Src SH3 or Abl SH3 domains (fused to GST) that were immobilized onto triplicate wells of microtiter plate. Bound AP fusion proteins were detected by incubation with pNPP; average optical density values and standard error are shown. The synthetic peptide ligands of the Src and Abl SH3 domains are SGSGVLKRPLPIPPVTR and SGSGSRPPRWSPPPVPLPTSLDSR, respectively. The peptide ligands of the Src and Abl SH3 domains that were fused to the N terminus of AP are ISQRALPPLPLMSDPA and GPRWSPPPVPLPTSLD, respectively. The enzymatic conversion of pNPP was allowed to proceed overnight to demonstrate that this type of assay yields not only a robust signal, but also a low background.

In Fig. 3B, glutathione *S*-transferase (GST)–SH3 fusion proteins are immobilized by adding 1 μg of protein in 100 μl of 0.1 m*M* $NaHCO_3$ (pH 8.3) into microtiter wells, and incubating for 1 hr at room temperature. To eliminate nonspecific binding of the wells, 10 μg of BSA is added to each well and incubated for an additional 30 min. Glutathione *S*-transferase (GST) fusion proteins are prepared according to the manufacturer protocol (Pharmacia, Piscataway, NJ).

After the peptide ligands or GST fusion proteins are immobilized on the microtiter plate wells, 100 μl of culture supernatant containing SH3–AP or peptide–AP fusions is added to the appropriate wells that have been washed and contain 50 μl of TBS. After a 1-hr incubation the wells are washed five times with TBS–0.1% (v/v) Tween 20. The amount of AP fusion protein retained in the microtiter plate wells is estimated by adding 150 μl of pNPP, incubating the solution for 20–120 min at room temperature, and quantitating the absorbance of the wells with a plate spectropho-

tometer (Molecular Devices, Sunnyvale, CA), at a wavelength of 405 nm. If necessary, the color reactions can be extended overnight at room temperature to maximize the absorbance values.

The results in Fig. 3 demonstrate that the interactions between the Src and Abl SH3 domains and their peptide are specific, whether the domain or the peptide ligand is fused to the N terminus of AP. We typically achieve a higher degree of specificity and lower background with the AP fusion protein system than with soluble synthetic or phage-displayed peptide ligands as probes.[21]

Notes

1. In every incubation step, the wells of the microtiter plates must be sealed with plastic wrap (or tape) to avoid evaporation.
2. The FLAG antibody can serve as a useful positive control to ensure that equal amounts of AP fusion protein have been added per microtiter plate well. Coat separate wells with the antibody and verify that the amounts of AP enzyme activity retained in the different wells are comparable.[21]

Types of Assays

AP fusion proteins can be used to detect SH3 domain and ligand interactions in a number of different experimental formats.

Detection of Protein–Protein Interactions on Membranes

The AP fusion system has been used to detect interacting proteins that have been immobilized on nitrocellulose or PVDF membranes (i.e., dot or Western blots). Nonspecific protein-binding sites on the membranes can be blocked by incubating them in 1.5% (w/v) BSA in TBS–0.1% (v/v) Tween 20, for 1 hr at room temperature, or overnight at 4°. The membranes are then incubated with culture supernatant containing the AP fusion protein in a small plastic bag, and after 1–2 hr of incubation at room temperature, the membranes are washed three times with agitation in TBS–0.1% (v/v) Triton X-100 for 10 min. AP fusion proteins that have been retained on the membranes are finally detected by either colorimetric or chemiluminescent reactions. For colorimetric detection of the AP fusion protein, the membranes are incubated in nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT–BCIP; Sigma Fast) solution for 10 min, washed with deionized water,

[21] M. Yamabhai and B. K. Kay, *Anal. Biochem.* **247,** 143 (1997).

and allowed to dry. For chemiluminescent detection of the fusion protein, the membranes are incubated in TBS for 10 min, drained of excess buffer, and covered with a solution of disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate (CSPD; Tropix, Bedford, MA). The chemiluminescent reaction is allowed to proceed for 5 min in the dark before the excess solution is drained away, and the membranes are exposed to X-ray film (Eastman Kodak, Rochester, NY) for 15–20 min.

Figure 4 demonstrates that peptide–AP fusion proteins can be used to probe for particular proteins present on dot or Western blots. The Src SH3 peptide ligand–AP fusion binds only to the Src SH3 domain, whereas the Abl SH3 ligand–AP fusion binds only to the Abl SH3 domain. Thus, AP fusion proteins can be used to detect specific SH3 domain-mediated protein–protein interactions on blots. SH3 domains appear to retain their three-dimensional structure (or renature efficiently) once affixed to a mem-

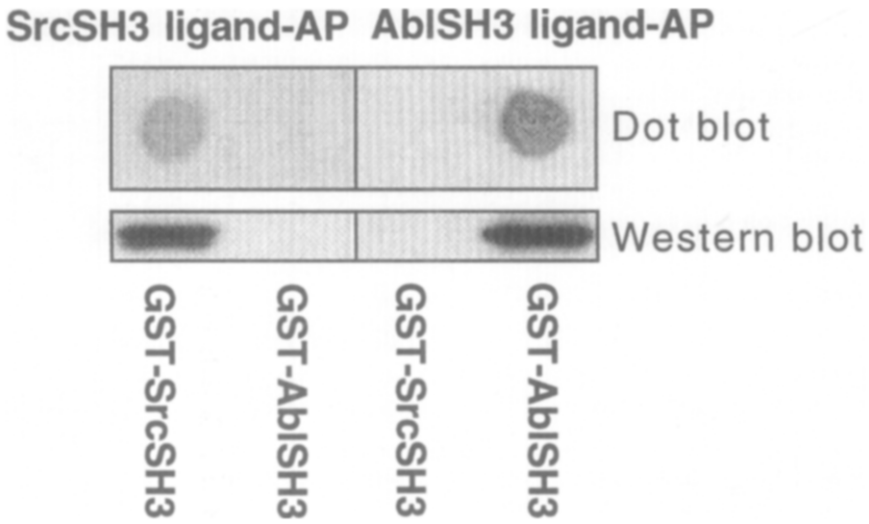

FIG. 4. Dot-blot and Western blot analyses with AP fusion proteins. One microgram each of GST–Src SH3 and GST–Abl SH3 fusion proteins were dot blotted onto nitrocellulose membrane (*top*) or fractionated by SDS–PAGE and transferred to PVDF membrane (*bottom*). The immobilized proteins were incubated either with the Src SH3 ligand–AP fusion protein (*left*) or Abl SH3 ligand–AP fusion protein (*right*). Colorimetric (NBT–BCIP) or chemiluminescent (CSPD) reagents were used for detection of the bound AP fusion protein in the dot (*top*) and Western (*bottom*) blots, respectively. The Src SH3 peptide ligand–AP fusion bound only to the GST–Src SH3 domain, whereas the Abl SH3 ligand–AP fusion bound only to the GST–Abl SH3 domain.

brane, presumably because of their innate thermal stability and lack of disulfide bonds.[22,23]

The AP fusion system can also be used to probe cDNA expression libraries in bacteriophage λ. We have used AP fusions to different protein interaction modules (i.e., SH3 domains of Src and Abl, EH domains of intersectin) to screen a mouse 16-day embryo cDNA expression library (Novagen, Madison, WI). Intersectin is a protein containing two N-terminal EH domains, an α-helical structure, and five C-terminal SH3 domains, and has been shown to function in endocytosis and cellular signaling.[24–26] The EH domain is a protein interaction module that has been shown to bind peptides containing the peptide sequence asparagine-proline-phenylalanine (NPF).[24,27,28]

In the primary screen, the library was plated at a density of 3×10^4 recombinant phage per 90-mm plate. Thirty plates were screened with each of the three AP fusion proteins. After four rounds of screening, individual plaques were isolated and plasmids bearing the cDNA inserts were rescued from the isolated λ phage by Cre-mediated excision, according to the manufacturer protocols (Novagen). Inserts were sequenced by automated fluorescent dideoxynucleotide sequencing and are summarized in Table I. All the isolated proteins contain short peptide sequences that have been previously shown to bind each domain, and in many cases have been demonstrated to be interacting partners in cells. Thus, the AP fusion system is a useful tool in identifying potential interacting proteins from a cDNA expression library.

Detection on Plastic Pins

H. M. Geysen and colleagues have devised a convenient method for synthesizing 96 peptides at a time on plastic pins, using microtiter plate wells as reaction chambers.[29] This technique can be easily duplicated in

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[23] M. C. Parrini and B. J. Mayer, *Chem. Biol.* **6,** 679 (1999).
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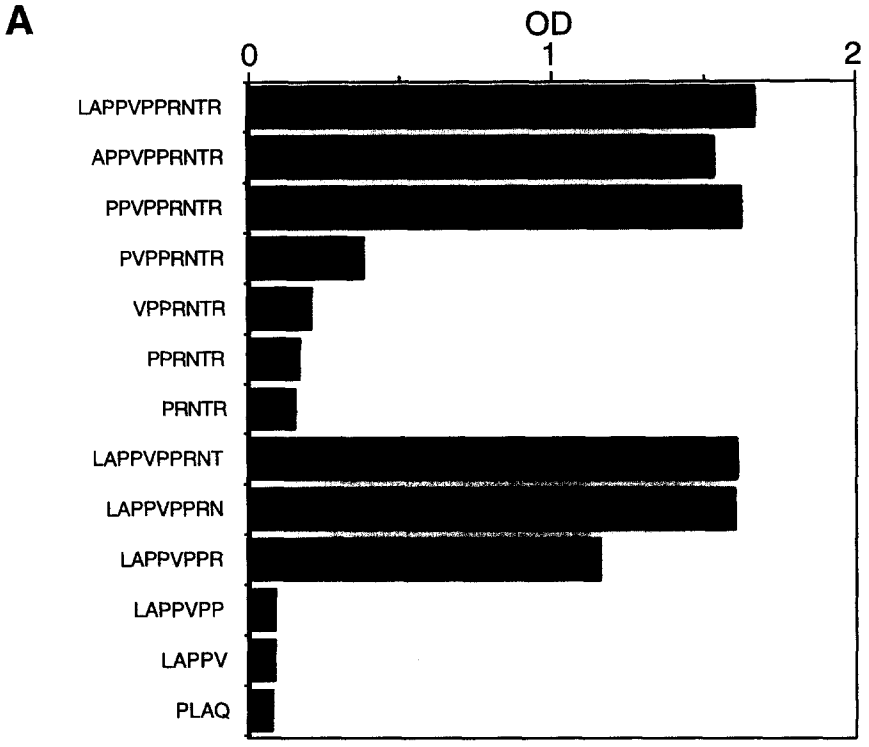
[27] A. E. Salcini, S. Confalonieri, M. Doria, E. Santolini, E. Tassi, O. Minenkova, G. Cesareni, P. G. Pelicci, and P. P. Di Fiore, *Genes Dev.* **11,** 2239 (1997).
[28] S. Paoluzi, L. Castagnoli, I. Lauro, A. E. Salcini, L. Coda, S. Fre, S. Confalonieri, P. G. Pelicci, P. P. Di Fiore, and G. Cesareni, *EMBO J.* **17,** 6541 (1998).
[29] H. M. Geysen, *Southeast Asian J. Trop. Med. Public Health* **21,** 523 (1990).

TABLE I
IDENTITY OF PROTEINS ISOLATED FROM SCREENS OF cDNA EXPRESSION LIBRARY WITH VARIOUS AP FUSION PROTEINS[a]

AP fusion partner	Amino acid sequence of cDNA clones isolated	Interacting protein	Ref.
Src SH3 domain	QHRPRLPSTESLSRRPLPALPVSEAPAPSPAPSP APGRKGSIQDRPLPPPPPCLPGYGGLKPEGD	Efs	38
	TSEAPPLPPRNAGKGPTGPPSTLPLGTQTSSGSS TLSKKRPPPPPPGHKRTLSDPPSPLPHGPPN	S19	39, 40
Abl SH3 domain	PPAPPPPPPLPSGPAYASALPPPPGPPPPPPLPS TGPPPPPPPPPPLPNQAPPPPPPPPAPPLP	Mena	41, 42
Intersectin EH domain	NPFLPSGAPPTGPSVTNPFQPAPPATLTLNQLRL SPVPPVGAPPTYISPLGGGPGLPPMMPPGPPAP NTNPFLL	Epsin 1	24, 25, 43–45
	NPFLAPGAAAPAPVNPFQVNQPQPLTLNQLRGSP VLGSSASFGSGPGVETVAPMTSVAPHSSVGASGS SLTPLGPTAMNMVGSVGIPPSAAQSTGTTNPFLL	Epsin 2	24, 25, 43–45

[a] A λ library expressing cDNA segments prepared from a 16-day-old mouse embryo was probed with various domain–AP fusions. Putative peptide ligands of the SH3 and EH domains are underlined in the isolated cDNA segments.[21,24] Additional information regarding either the identified proteins (right-hand column) or evidence of protein–protein interactions can be found in the references noted above.

most laboratories and blocks of plastic pins, software, and reagents can be purchased from Mimotopes (Clayton Victoria, Australia; *http://www.mimotypes.com*). To explore the possible utility of the pin format in dissecting the specificity of SH3 domains, we have synthesized two sets of peptides on pins and examined their binding to SH3 domain fusions to AP. After the synthesis was complete, the nonspecific binding of proteins to the pins was blocked by incubating them in TBS–0.2% (w/v) BSA at room temperature. After a 30-min incubation, the pins were washed three times by dipping them in a tray containing TBS–0.1% (v/v) Tween 20 for 10 min each time. Next, the pins were placed into wells of a 96-well microtiter plate containing 100 μl of the conditioned culture medium (containing the secreted AP fusion protein) and 50 μl of TBS to adjust the pH. After 1 hr of incubation, the pins were washed by dipping them three times (10 min each) in a tray containing TBS–0.1% (v/v) Tween 20. The amount of AP fusion protein retained on the wells was then revealed by incubating the pins in a microtiter plate containing 100 μl of pNPP solution in each well. The enzymatic reaction was allowed to proceed until the optical density (OD) of the wells reached values of 1.0 or greater. To reuse the pins, bound AP fusions can be stripped off by incubating the block of pins at 50° in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OD
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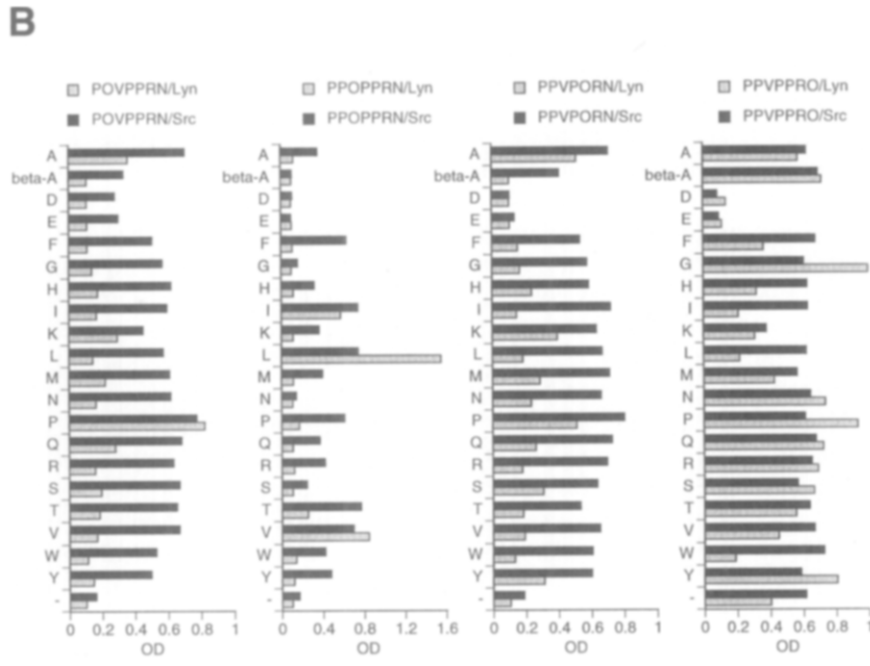
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-
0 0.2 0.4 0.6 0.8 1
0 0.4 0.8 1.2 1.6
0 0.2 0.4 0.6 0.8 1
0 0.2 0.4 0.6 0.8 1
OD

a solution of 10% (w/v) sodium dodecyl sulfate (SDS) with 30 min of constant sonication.

A series of peptides, corresponding to truncations from the N and C termini of the parent sequence LAPPVPPRNTR, were synthesized on plastic pins, to define the boundaries of a class II peptide ligand for the Src SH3 domain.[30] The pins were then incubated with the Src SH3–AP fusion and the bound AP fusion was detected as described above. As seen in Fig. 5A, the Src SH3–AP fusion protein can bind to peptides synthesized on plastic and the minimal peptide ligand of the Src SH3 domain is PPVPPR.

A second set of peptides on pins was synthesized and tested for their binding to two different SH3 domains. In this set, each residue within the minimal ligand PPVPPRN was systematically replaced with 20 different amino acids, to test the importance of each amino acid at each position (Fig. 5B). When the pins were probed with the Src SH3–AP fusion, the optimal ligand was deduced to be PxnPx′Rx′ (where x is any amino acid, n represents all but β-A, D, E, G, and N, and x′ means all but acidic amino acids). When the same set of pins was reprobed with an AP fusion to the Lyn SH3 domain, a member of the Src family of tyrosine kinases, the optimal ligand was observed to be PP(L/V/I)PoRo′ (where o represents A, H, K, M, N, P, Q, S, T and Y, and o′ represents all but D, E, I, L, and W). Thus, even though the Src and Lyn SH3 domains are 70% similar in primary structure, their binding preferences are distinct, in agreement with previously published phage display experiments.[31,32]

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[31] R. J. Rickles, M. C. Botfield, Z. Weng, J. A. Taylor, O. M. Green, J. S. Brugge, and M. J. Zoller, *EMBO J.* **13,** 5598 (1994).

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FIG. 5. Mapping the ligand preferences of SH3 domains with peptides synthesized on pins. A set of peptides on pins (*y* axis) was designed to define the boundaries of a particular peptide ligand for the Src SH3 domain that was isolated from a phage display combinatorial peptide library (A). The block of pins was incubated with the Src SH3–AP fusion protein and the bound protein was detected by incubation of the pins with pNPP. Optical density at 405 nm is shown. Binding of the two different SH3 domains to a set of pins in which a number of residues in the peptide sequence PPVPPRN were systematically replaced with different amino acids. The binding of Src SH3–AP to the pins was measured first, before sonicating and washing the pins, and testing the binding of Lyn SH3–AP. O represents the residue replaced one at a time with β-A, A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y, or nothing. All optical density signals below 0.2 are considered background.

Determination of Modular Domain–Ligand Binding Properties on Microtiter Plates

The affinity of a particular domain for its ligand is commonly measured by fluorescence polarization, equilibrium dialysis, or surface plasmon resonance. We have found that competition assays with AP fusion proteins are a less time-consuming and expensive method for estimating the binding strength between various domains and their peptide ligands. We have utilized displacement of AP fusion proteins from binding target proteins immobilized onto microtiter plate wells with soluble compounds (i.e., peptides, domains) as a convenient means of estimating their relative binding strengths.

We have utilized this assay format to evaluate the binding strength of peptide ligands for the Src SH3 domain and the EH domains of intersectin. In this type of competition assay, 1 μg of GST–SH3 or GST–EH fusion protein prepared according to standard protocols[33] is added to microtiter plate wells in 100 μl of 0.1 m*M* $NaHCO_3$ (pH 8.3). After the protein has been allowed to bind to the well surface at room temperature for 1 hr, nonspecific protein-binding sites are blocked by adding 100 μl of TBS–0.2% (w/v) BSA to the wells and incubating for 1 hr at room temperature (or overnight at 4°). The wells are then washed three times with TBS–0.1% (v/v) Tween 20 and various amounts of synthetic peptides are added to the wells along with 100 μl of peptide ligand–AP fusion protein. After 2 hr of incubation at room temperature, the wells are then washed five times, and the relative amount of AP fusion protein retained in the wells is detected by reaction with 100 μl of pNPP.

Figure 6 demonstrates a competition assay in which increased amounts of soluble Src-l–AP and EH-l–AP fusion proteins, were added to microtiter plate wells containing immobilized GST fused to EH or SH3 domain. The soluble peptides inhibited only the binding of their respective peptide–AP fusion proteins. The 50% inhibitory concentration (IC_{50}) values of the peptides for both the Src SH3 and intersectin EH domains are ~10 μM and ~3 μM, respectively. Thus, the IC_{50} value obtained from this type of assay can be used to predict the binding strength of different peptide ligands to protein interaction modules.

Discussion and Conclusions

With the ability to display a wide range of polypeptides at the N terminus of bacterial AP, it is possible to identify or map protein–protein interactions

[33] D. B. Smith and K. S. Johnson, *Gene* **67,** 31 (1988).

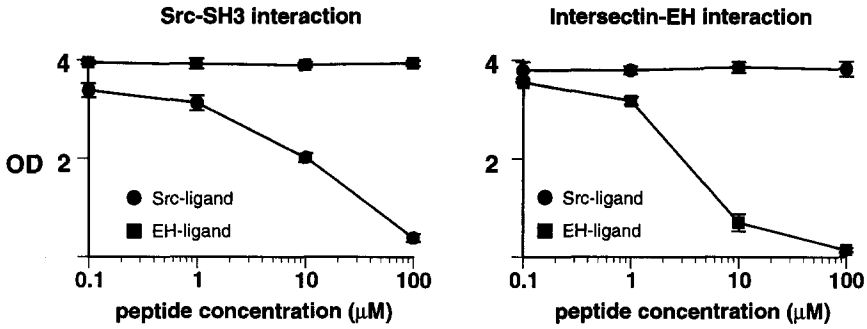

FIG. 6. Estimation of the binding strength of the Src SH3 and intersectin EH domain–ligand interactions. One microgram of GST–Src SH3 or GST–intersectin EH fusion proteins was immobilized in microtiter plate wells. AP fusions to either the Src SH3 ligand (*left*) or the EH ligand (*right*) were incubated in the presence of increased amount of either soluble Src peptide (RPLPIPPVTR) or EH peptide ligand (DCTNPFSCWR, cyclized through the cysteines). Average $OD_{405\ nm}$ values are shown for triplicate wells, along with standard error.

in a number of formats. There are several kinds of alkaline phosphatase substrates available (i.e., soluble or insoluble, chemiluminescent or colorimetric), so a particular substrate can be chosen to suit specific needs. Furthermore, the utility of this system is enhanced by the fact the AP fusion protein is secreted from *E. coli* and the culture medium can be used directly in experiments; once the appropriate fusions have been constructed, the cells are simply grown overnight, and the fusion protein is harvested with the culture supernatant. One limitation of the current vector, however, is that this system is not appropriate for studying protein–protein interactions that require C-terminal sequences, because the fusions to AP occur at its N terminus.

The successful application of the AP fusion system to examine binding properties of the SH3 domain and other protein-binding modules, as described in this chapter, suggests that this system may prove useful for the general study of protein–protein interactions. Protein interaction modules may be well suited to display as AP fusion proteins, because the modules are small, typically fold properly in *E. coli,* lack disulfide bonds, and their interaction surface is opposite the N and C termini of the module. The AP system may be useful in studying the peptide ligand preferences of protein interaction modules, because it permits the detection of modest (i.e., 10 μM) interactions by eliminating additional incubation and washing steps as a one-step detection reagent. Peptides synthesized on pins or spots[34] can

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be quickly surveyed for binding to a particular protein interaction module expressed as a fusion with AP.

One interesting application of the AP fusion system is its potential use in the drug discovery process. Libraries of natural products or small molecules can be screened for inhibitors of protein–protein interactions that either block the binding of an AP fusion protein and an interacting protein in a microtiter plate well,[35] or bind an AP fusion protein directly.[36] The identification of compounds that can specifically antagonize specific protein–protein interactions should prove useful in analyzing the physiological consequences of these molecular interactions, as well as suggest leads for drug development.

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