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Examining the Specificity of Src Homology 3 Domain–Ligand Interactions with Alkaline Phosphatase Fusion Proteins

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Sixteen-amino-acid-long peptides, corresponding to the optimal ligand preferences of the Src homology 3 (SH3) domains of Abl, Cortactin, Crk, p53BP2, and Src, were fused to the N-terminus of *Escherichia coli* alkaline phosphatase (AP). These secreted fusion proteins have been used as one-step detection probes of peptide ligand–SH3 domain interactions on microtiter plates and membranes. The binding of both the class I and II SH3 ligand–AP fusion proteins to their targets is robust and specific in comparison to chemically synthesized biotinylated peptides, used either in monovalent or tetravalent formats. p53BP2 and Cortactin SH3 ligand–AP fusions have been used to screen a mouse embryo λ cDNA expression library and resulted in the cloning of p53BP2 and several known proteins with SH3 domains similar to that of Cortactin, respectively. In addition, the ∼60-amino-acid-long SH3 domains of Src and Abl were fused to AP and the resulting fusion proteins were found to bind specifically to their respective peptide ligands in microtiter plates and proteins containing proline-rich regions in screens of a λ cDNA expression library. Thus, SH3 peptide ligand– and SH3 domain–AP fusion proteins are convenient and sensitive reagents for examining the specificity of SH3 domain–ligand interactions, identifying potentially interacting proteins, and establishing high-throughput screens of combinatorial chemical libraries. © 1997 Academic Press

Regulation of protein–protein interactions through modular binding domains (e.g., SH2, SH3, and WW domains) plays a primary role in cellular signal transduction (1, 2). Src homology 3 (SH3)[2] domain, a protein module of ∼60 amino acids, has been shown to modulate protein–protein interaction in various biological events such as, receptor protein tyrosine kinase signaling, subcellular localization (1, 2) and cytoskeletal organization (3), activation of NADPH oxidase system in the phagocyte (4), initiation of endocytosis (5), or growth of HIV (6). The three-dimensional structures of several SH3 domains complexed with peptide (7, 8) or protein (6) ligands have demonstrated that SH3 domains have specificity in their molecular interactions.

The ligand specificity of SH3 domains has been defined principally from two different methods. In the first approach, λ cDNA expression libraries have been screened with SH3 domains and the region(s) responsible for binding identified by "whittling down" the isolated cDNA clones (9, 10). In the second approach, synthetic (11) or phage displayed random peptide libraries (12–14) are screened with SH3 domains and the sequence of the binding peptides is deduced by sequencing. Most SH3 binding peptides identified to date have an arginine two residues either N-terminal (class I) or C-terminal (class II) of the motif XPpXP, where X is any amino acid and p is typically a scaffolding residue (i.e., proline) (15). Given that the overall structure of SH3 domains is similar, despite their limited amino acid similarity (<40%), it is likely that the highly variable residues in SH3 domain are responsible for specific protein–protein interaction of SH3 domain within the eukaryotic cells.

In our work with SH3 domain-binding phage isolated from M13-displayed random peptide libraries we realized that the synthetic peptides were useful tools for evaluating the ligand specificity of SH3 domains (16) and discovering new SH3 domain-containing proteins (17). However, we found the synthesis of peptides corresponding to our many phage isolated to be costly and time-consuming. To overcome these limitations, we decided to fuse the inserts from selected phage to the gene encoding *Escherichia coli* alkaline phosphatase

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[2] Abbreviations used: AP, alkaline phosphatase; SH3, Src homology 3; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

(AP) and express them as fusion proteins. The application of AP fusion proteins as one-step detection probes for the study of antibody–epitope interactions has been reported previously. Short epitopes (18–20) and antibody chains (i.e., Fab, svFv) (21–23) have either been fused at the N-terminus or inserted into a surface loop (18, 19, 24) of AP. Such AP fusion proteins generally retained full enzymatic activity and immunoreactivity in ELISA. In this paper, we report that the SH3 peptide ligand– and SH3–AP fusion proteins are released efficiently by bacterial cells into the culture supernatant and that they can be used directly for the detection of SH3–peptide ligand interactions either in microtiter plate wells or on nitrocellulose or nylon membranes without any further purification or concentration steps. These reagents are convenient, sensitive, and highly specific for examining the specificity of SH3 domain–ligand interaction, identifying potential interacting cellular proteins, and establishing high-throughput screens of combinatorial chemical libraries.

MATERIALS AND METHODS

AP Expression Vector Construction

The bacterial alkaline phosphatase (AP) fusion expression vector, pMY101, was modified from the pFLAG-1-BAP vector (Eastman Kodak Co., New Haven, CT) by replacing the DNA segment between the HindIII and SalI sites with a double-stranded oligonucleotide encoding the c-Myc epitope (i.e., EQKLISEE-DLN), which is recognized by the 9E10 monoclonal antibody (Oncogene Sciences, Long Island) (37). The epitope was flanked by SalI and XbaI sites in the same reading frame as phage (38) encoding random peptides displayed at the N-terminus of protein III. See Fig. 1 for actual sequence details. The FLAG epitope (i.e., DYK-DDDDK) is recognized by the M1 monoclonal antibody (Eastman Kodak Co.). E. coli AP is a homodimer with both N-termini facing one side of the molecule (39).

Construction of AP Fusion Recombinants and Proteins

Src class I (ISQRALPPLPLMSDPA), Abl (GPRWSP-PPVPLPTSLD), p53BP2 (WVVDSRPDIPLRRSLP), Cortactin (VTRPPLPPKPGHMADF), and Crk (IMP-FDSPPLLPPKREV) SH3 peptide ligands were fused to AP by amplifying the inserts of selected phage recombinants (14) by PCR (40), digesting the products with XhoI and XbaI, and cloning the resulting fragments between the SalI and XbaI sites of pMY101. In recombinants, the c-Myc epitope is replaced by the insert fragments. Constructs expressing calmodulin (CaM; VPRWIEDSLRGGAARAQTRLA) and Src class II (GSGGILAPPVPPRNTRP) SH3-binding peptides fused to AP were generated by ligation of synthetic oligonucleotides. Src and Abl SH3 domain–AP

fusion constructs were generated with fragments amplified by PCR from cDNA clones. All recombinants were confirmed by DNA sequencing. E. coli (DH5αF′) bearing the AP constructs were grown in Luria broth (containing 50 μg/ml ampicillin) until cultures reached an optical density (600 nm wavelength) of 0.5 and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 6–18 h continued incubation at 37°C, the conditioned medium was recovered by centrifugation at 7000g for 15 min. Activity of the AP fusion protein in the culture supernatant was typically stable at 4°C for 2–3 weeks. When particular AP fusion proteins were not secreted, the bacterial cells were recovered by centrifugation and sonicated to release the fusion protein.

Detection of AP Interactions in Microtiter Plates and on Nitrocellulose Membranes

Purified glutathione S-transferase fusion proteins of various SH3 domains (14, 17) were applied to microtiter plate (Costar, Cambridge, MA) wells as described elsewhere (41). In some cases, the well were first coated with streptavidin, washed, and incubated with biotin–SGSGVLKRPLPPLPVTR-NH₂ and biotin–SGSGSR-PPRWSPPPVPLPTSLDSR-NH₂ peptides, Src and Abl SH3 ligands, respectively. Nonspecific binding sites were blocked (SuperBlock, Pierce Chemical Co., Rockford, IL) and the wells were washed three times with Tris-buffered saline (TBS; 25 mM Tris·HCl, pH 7.5, 145 mM NaCl, 3 mM KCl) containing 0.1% Tween 20. One hundred microliters of culture supernatant containing individual AP fusion proteins was incubated in the wells at room temperature for 2 h. Later the wells were washed three times with TBS–0.1% Tween 20, and the bound AP fusion proteins were detected by colorimetric assay with 8 mM p-nitrophenyl phosphate in 100 μl buffer (1 M diethanolamine, pH 8.0, 1 mM MgCl₂, 0.04 mM ZnSO₄). The optical absorbance of the wells was read at 405 nm with a plate reader (Molecular Devices, Sunnyvale, CA).

One microgram of various GST–SH3 fusion proteins in 100 μl TBS was dot-blotted onto Immobilon P nylon membrane strips (Millipore, Bedford, MA). Membrane strips were blocked with SuperBlock for 2 h at room temperature and incubated with ~2 ml of culture supernatant containing different peptide–AP fusions. After incubation for 12–18 h at 4°C, the membrane were washed five times with TBS–0.1% Triton X-100. Bound AP fusions were detected by incubating with 100 μl of 50 mg/ml nitro blue tetrazolium chloride in 70% dimethyl formamide and 50 μl of 50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate–p-toluidine in dimethyl formamide in 15 ml alkaline phosphatase buffer; 0.1 M Tris·HCl, pH 9.4, 0.1 M NaCl, 50 mM MgCl₂.

The λEXlox mouse 16-day-embryo cDNA expression

library was obtained from Novagen (Madison, WI) and screened according to published protocols. For the primary screen, the library was plated at the density of 3 $\times$ 10^4 recombinant phage/90 mm plate. Forty plates were screened with the p53BP2 SH3–peptide ligand–AP fusion. Twenty plates were screened with either Cortactin SH3 peptide ligand–AP fusion or Src and Abl SH3–AP fusions, and the binding specificity was revealed at the fourth round of screening (i.e., the filters were cut in half and exposed separately to individual AP fusions). Plasmids bearing the cDNA inserts were rescued from the isolated λ phage by Cre-mediated excision according to the manufacturer's protocols and the inserts were sequenced by automated fluorescent dideoxynucleotide sequencing.

Detection of Bound Bacteriophage M13

Purified glutathione S-transferase fusion proteins of various SH3 domains (14, 17) were applied to microtiter plate (Costar) wells as described elsewhere (41). Nonspecific binding sites were blocked (SuperBlock, Pierce Chemical Co.) and the wells were washed three times with phosphate-buffered saline (PBS; 100 mM Tris·HCl, pH 7.5, 100 mM NaCl) containing 0.1%

Tween 20. One hundred microliters of culture supernatant containing individual bacteriophage M13 was incubated in the wells at room temperature for 2 h. Later the wells were washed five times with PBS–0.1% Tween 20, and the bound phage were detected by ELISA with an anti-M13 phage antibody conjugated to horseradish peroxidase (Pharmacia, Piscataway, NJ) as described elsewhere (42).

RESULTS

To analyze the specificity of SH3 domains, six different peptide sequences were fused near the N-terminus of mature *E. coli* AP of the vector pMY101 (Fig. 1). Sixteen-amino-acid-long peptides, optimized for the SH3 domains of Abl, Cortactin, Crk, and p53BP2 with phage display random peptide libraries (14), were expressed in bacteria as SH3–ligand–AP fusion proteins. In addition, the class I and II binding ligands of the Src SH3 domain were fused to AP. As negative controls, two non-SH3 peptide ligands, the c-*Myc* epitope and a calmodulin-binding peptide, were fused to AP. It should be noted that the AP protein has the OmpA signal sequence which directs its secretion and that the FLAG epitope is present at the N-terminus of all mature AP fusion proteins (Fig. 1).
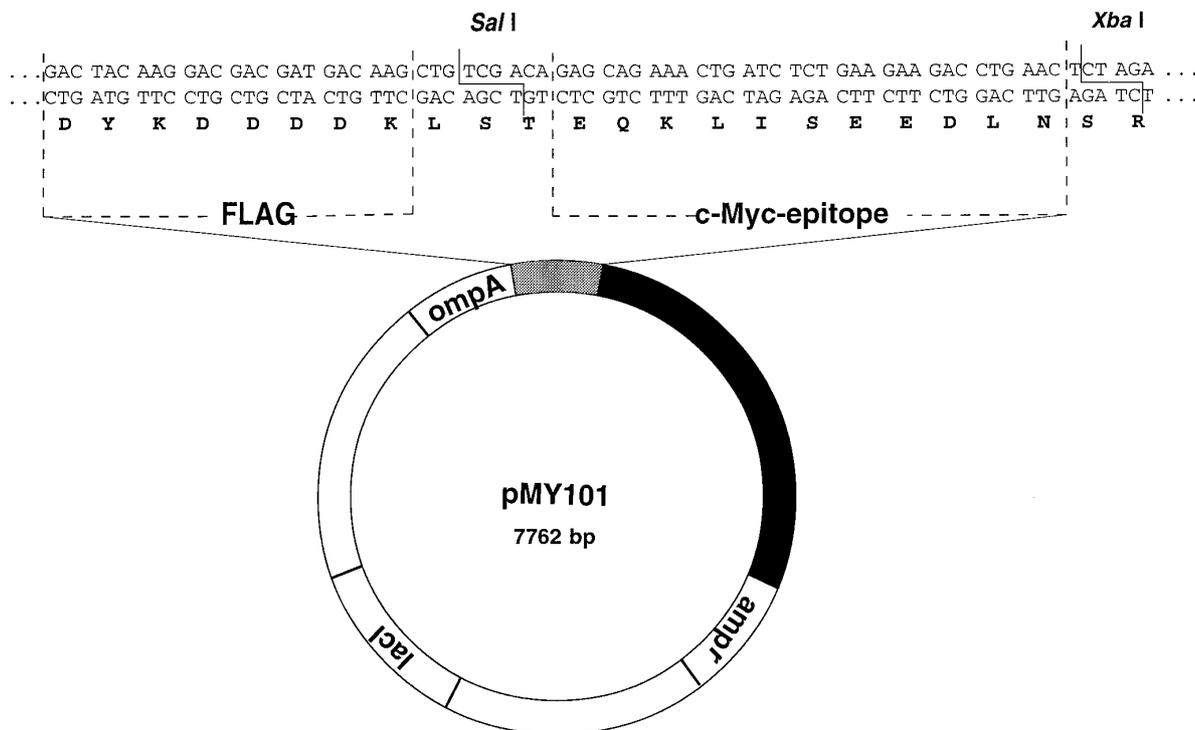

FIG. 1. Map of the bacterial alkaline phosphatase fusion vector, pMY101. The coding region of a segment of the *E. coli* alkaline phosphatase (AP) gene is shown with the FLAG and c-*Myc* epitope sequences below. The *Sal*I and *Xba*I restriction sites flank the c-*Myc* epitope in the same reading frame as cloned peptides displayed by a bacteriophage M13 random peptide library (14). The 7762-base-pair (bp) vector was derived from pFLAG-1–BAP vector (43) in which the OmpA signal/leader sequence is upstream of the FLAG and c-*Myc* epitopes (gray) 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.

The binding properties of each peptide–AP fusion protein was conveniently monitored in a microtiter plate format. Wells were coated with a variety of proteins and each peptide–AP fusion was shown to bind specifically to its respective target without any cross-reactivity to nonrelated targets (Fig. 2). The only exceptions were the Cortactin SH3–ligand–AP which bound to both Cortactin and Crk SH3 domains and the Src class II–ligand–AP which cross-reacted with p53BP2 and Crk SH3 domains; both results are in accordance with previous work which showed that the optimal ligands for these SH3 domains are very similar (14, 16). The enzyme generated signal of the peptide–AP fusions for particular SH3 domains was robust compared to unrelated targets (i.e., GST, BSA). The FLAG monoclonal antibody, M1, served as an internal positive control, demonstrating that equal amounts of AP fusion protein were added to each well during the binding reaction. Thus, the detection of peptide–protein interactions with the peptide–AP fusion system is highly specific.

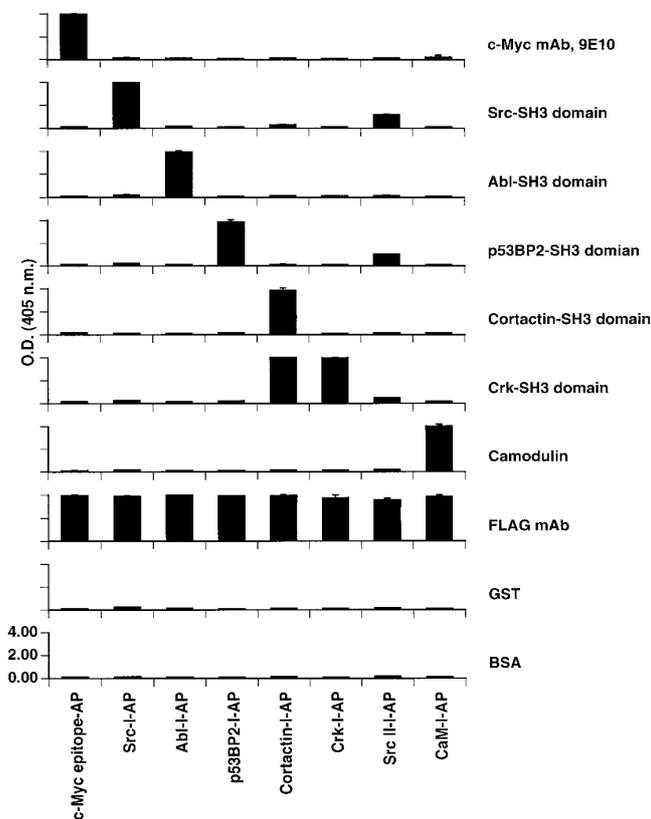The specificity of the peptide–AP fusions was con-

FIG. 2. Binding specificity of AP–fusion peptides. An array of proteins (right) was immobilized onto duplicate wells of a microtiter plate, incubated with various AP–fusion proteins (below), washed, and incubated with pNPP reagent to permit color development. The reactions were permitted to develop until they reached an optical density (OD) of ~4.0 at 405 nm wavelength. Averages are shown with error bars.
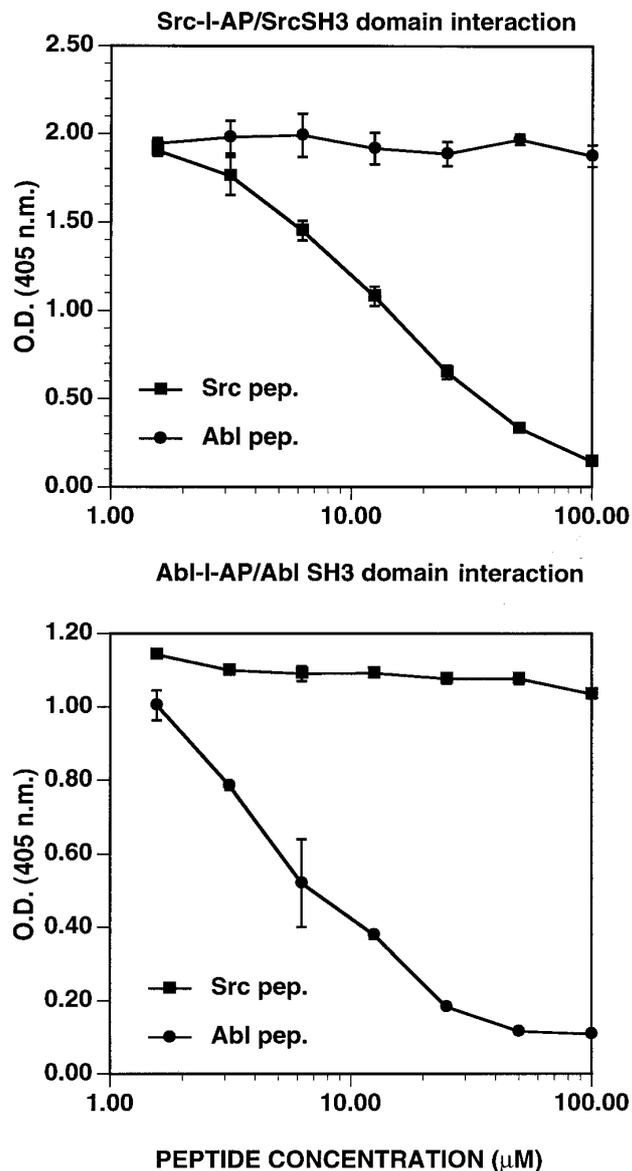

FIG. 3. Specific inhibition of AP–fusion peptide ligand–SH3 interaction by soluble peptide ligands. One microgram of GST–Src SH3 or –Abl SH3 fusion protein was immobilized in microtiter plate wells. AP–fusion Src SH3 peptide ligand (top) and AP–fusion Abl SH3 peptide ligand (bottom) were incubated in the presence of increased amount of either soluble Src or Abl SH3 domain peptide ligands. Average $OD_{405\,nm}$ values are shown for triplicate wells, along with standard error.

firmed with competition experiments. Increased amounts of soluble peptides, corresponding to the same peptide sequences fused to the Abl-l–AP and SrcI-l–AP fusion proteins, were added to microtiter plate wells containing immobilized GST–SH3 proteins. As seen in Fig. 3, the soluble peptides inhibited the binding of only the respective peptide–AP fusion protein. The IC_{50} of the peptides for the Src and Abl SH3 domains is ~10 and ~5 μM,

respectively; these values are similar to the K_d's reported for peptide ligands for these two SH3 domains (25).

We then compared the binding strength and specificity of the AP fusions to soluble and phage-displayed peptide ligands. Various GST–SH3 domains, GST, and BSA were immobilized on microtiter wells and incubated with AP fusions, phage, biotinylated peptides, or biotinylated peptides precomplexed with streptavidin. The peptide sequences were similar for each of the four formats. As seen in Fig. 4, the AP fusions had the greatest specificity for binding to the appropriate targets;

the SH3 domains of Src and Yes are highly similar (i.e., 88% identity) and have identical ligand preferences (14). The M13 phage, displaying the peptides at the N-terminus of 3–5 copies of protein III per virus particle (26), bound the appropriate proteins with less specificity. The soluble, biotinylated peptides (monovalent) bound poorly, whereas when the peptides were precomplexed with streptavidin (tetravalent), they bound much better and with less specificity. Thus, it appears the valency of the SH3-binding peptides influences their binding strength and specificity. It should be
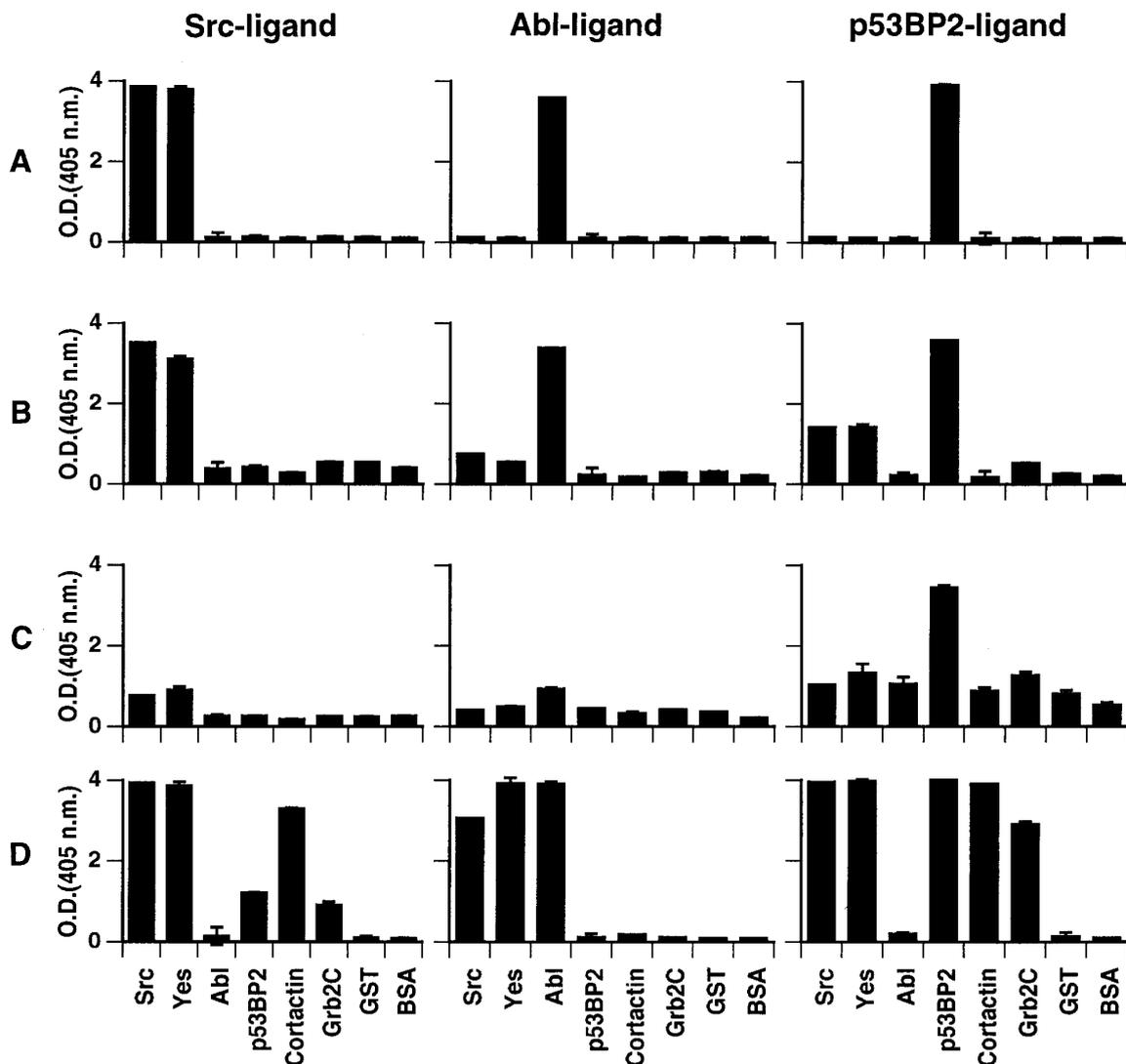

FIG. 4. Comparison of the binding of four different peptide formats. Various GST–SH3 fusion proteins were immobilized onto triplicate wells of a microtiter plate and incubated with different formats of peptide ligands (i.e., AP–fusion protein (A), N-terminal fusion to the mature protein III of bacteriophage M13 (B), monovalent biotinylated peptide (C) (uncomplexed), followed by binding with strepavidin–alkaline phosphatase (SA–AP), or mutivalent SA–AP (D) (complexed). Bound AP–fusion proteins and biotinylated peptides were detected by colorimetric assay with 8 mM p-nitrophenyl phosphate, whereas bound bacteriophage particles were detected by ELISA (42). The average $OD_{405\,nm}$ values and standard errors are shown. The synthetic peptide ligands of the Src, Abl, and p53BP2 SH3 domains are SGSGVLKRPLPIPPVTR, SGSGSRPPRWSPPPVPLPTSLDSR, and SGSGSYDASSAPQRPPLPVRKSRPGG, respectively. The peptide ligands of the Src, Abl, and p53BP2 SH3 domains that fused to the phage coat proteins and AP are ISQRALPPLPLMSDPA, GPRWSPPPVPLP-TSLD, and WVVDSRPDIPLRRSLP, respectively.

noted that AP (27) and streptavidin (28) are homodimeric and -tetrameric molecules, respectively.

The high degree of binding specificity of the peptide–AP fusions is also evident in a membrane format. Various GST–SH3 domains were dot-blotted onto nylon membranes and incubated with different peptide–AP fusions as described above. Figure 5 shows that the binding was strong and highly specific, corroborating results obtained from the microtiter plate. As a positive control, we incubated a membrane strip with an AP fusion to the C-terminal 300 amino acids of the mouse Sos protein (29). This segment is proline-rich and appears to have the capacity to interact with many different SH3 domains (Fig. 5).

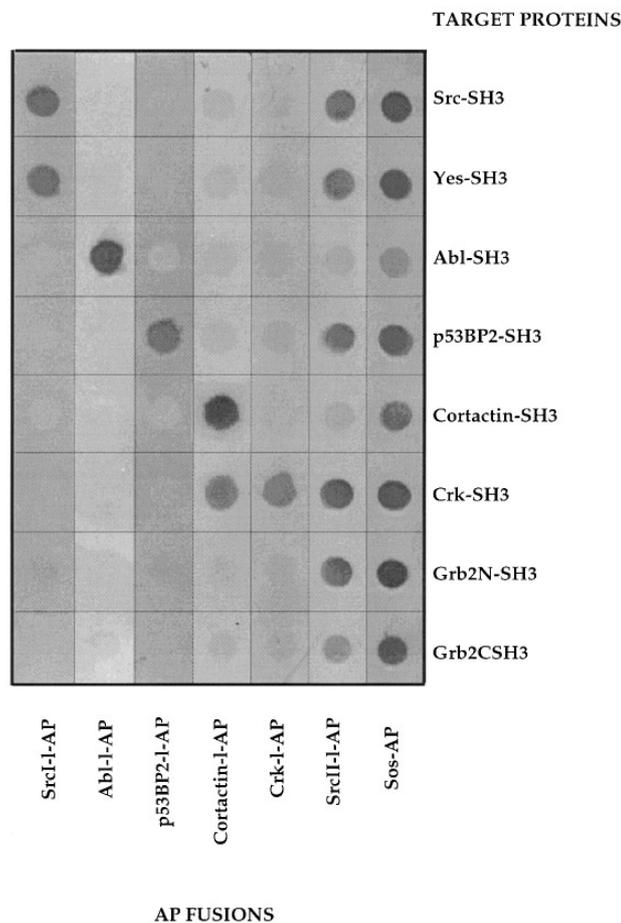Based on the successful detection of SH3–peptide

TARGET PROTEINS

FIG. 5. Binding of AP–fusion peptides to GST–SH3 fusion proteins immobilized on membrane strips. One microgram of each GST–SH3 fusion protein was immobilized onto nylon membrane using dot blot apparatus, and strips were probed with seven different AP fusions. Six of the AP fusions are fused to peptide ligands of Src, Abl, p53BP2, Cortactin, Crk SH3 domains; the class I and II ligands of Src SH3 are denoted as SrcI and SrcII, respectively. The seventh AP fusion is to the 300 C-terminal amino acids of the mouse Sos protein. The amount of AP fusion protein bound to the membrane strips was visualized with the chromogenic reagents 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

ligand interactions on solid supports, we decided to screen a λ cDNA expression library with different AP fusions (i.e., p53BP2 and Cortactin SH3 peptide ligand–AP fusions). The results of a screen of a 16-day-mouse embryo λ cDNA library are shown in Table 1. When $\sim 1.2 \times 10^6$ different plaques were screened with the p53BP2–peptide ligand–AP fusion protein, only two binding clones were identified and sequencing revealed that they both encoded mouse p53BP2. When the same peptide sequence in a biotinylated, tetravalent format (14) was used to screen the same library, a very large number (i.e., $\sim 0.005\%$) of the clones reacted (data not shown). A screen of half the number of λ recombinants with the Cortactin–peptide ligand–AP fusion yielded nine clones, which collectively encoded SH3P7 (14), HS1 (14), and Efs (30). Thus, it appears that the SH3 domains of SH3P7, HS1, and Efs bind similar peptide ligands as Cortactin, suggesting that these SH3 domains may interact with the same cellular targets. Although we failed to isolate λ cDNA clones encoding Cortactin or Crk, we found that the Cortactin–ligand–AP fusion binds well to Cortactin and Crk λ cDNA-expressing phage (14) when tested (data not shown).

Given that the SH3 peptide and protein segment ligands could be successfully displayed from AP, we decided to test the SH3 domains themselves. SH3 domains are ~ 60 amino acids in length, lack disulfide bonds, and are thermostable. Both the Src and Abl SH3 domains were fused to AP, and as seen in Fig. 6, the fusion proteins bind only to their respective peptide ligands. These two SH3–AP constructs were then used to screen the 16-day mouse embryo λ cDNA library; the results of the screen is shown in Table 2. With the SrcSH3–AP fusion we isolated two novel proteins, teg27 (GenBank X80437), and Efs (30), as well as two clones with inserts fused in the wrong reading frames of known proteins (i.e., NEDD3, BF-2) that generated proline-rich peptide sequences. Clone s19 represents a novel protein with a SH3 domain at its C-terminus; in addition, examination of the s2 clone failed to identify any matches with known protein modules. The Efs protein has two proline-rich peptide sequences (underlined) similar to the class I motif (RPLPPLP) of SrcSH3 peptide ligands. On the other hand, s2 appears to have a sequence corresponding to the class II motif (APPVPPR) of SrcSH3 peptide ligands. Interestingly, teg27 and s19 have peptides corresponding to both class I and II motifs. With the AblSH3–AP fusion, we isolated one novel protein, the mouse homolog of the *Drosophila melanogaster* Abl substrate, Enabled (31), and the 51C protein (32), which is related to inositol polyphosphate-5-phosphatase. The novel protein, s2, is the only sequence isolated which binds to both Src– and Abl–SH3 domains, most likely through its long proline-rich region. Thus, AP fusions can be used to identify potential SH3 domain-interacting proteins, comparable to

Identity of Proteins That Interact with p53BP2 and Cortactin-1–AP Fusions

Probe	Description	SH3 domain sequence	Frequency
p53BP2-1–	p53BP2	NKGTVYALWDYEAQNSDELSFHEGDAITILRRKDENETEWWWARLGDREGYVPKNLLGLY	2
Cortactin-1–	SH3P7	GQGLCARALYDYQAADDTEISFDPENLITGIEVIDEGWWRGYGPDGHFGMFPANYVELIE	6
	HS1	GAGISAIALYDYQGEGSDELSFDPDDIITDIEMVDEGWWRGQCRGHFGLFPANYVKLL	2
	Efs	TSAQLARALYDNTAESPQELSFRRGDVLRVLQREGAGGLDGWCLCSLHGQQGIVPANRVKLLPA	1

Note. SH3 domain sequences of proteins deduced from cDNA clones isolated from a λ cDNA library of 16-day-old mouse embryo with either p53BP2 or cortactin-peptide ligand–AP fusion proteins. Frequency corresponds to the number of types a particular cDNA sequence was isolated from a screen of 1.2×10^6 (p53BP2) or 6.0×10^5 (cortactin) recombinants. The amino acid similarity between the SH3 domains of Cortactin and SH3P7, HS1, and Efs is 87, 89, and 55%, respectively. The complete nucleotide and protein coding sequences of isolated clones, along with GenBank accession numbers, can be found in the Internet (http://www.unc.edu/depts/biology/Kayref.html).

what has been achieved with biotinylated or radioactive SH3–GST fusions as probes (9, 33, 34).

DISCUSSION

Fusions of the *E. coli* AP protein to 16 amino acid peptide ligands for different SH3 domains, the SH3 domains of Src and Abl, and a 300-aa C-terminal segment of Sos have been used to examine the specificity of SH3–ligand interactions. The fusion proteins were secreted in the bacterial culture medium at sufficient levels such that the crude culture supernatant could be used directly in evaluating SH3–ligand interactions in microtiter plates or on membranes. The fusion proteins were found to bind specifically and robustly to their respective SH3

FIG. 6. Binding of Abl and Src SH3–AP fusions to peptides immobilized in microtiter plate wells. Biotinylated SrcI and Abl SH3 domain peptide ligands were added to microtiter plate wells coated with streptavidin, washed, and incubated with AP fusions to either the Src or Abl SH3 domains. Bound SH3 AP fusion protein was detected by incubation with pNPP; average OD values and standard error are shown for duplicate wells.

domains or peptide ligands. In addition, the presence of the FLAG epitope in the AP fusion proteins permitted their immunochromatagraphic purification (data not shown) and quantitative normalization.

As previously published in a procedure termed *cloning of ligand targets*, COLT, biotinylated SH3 peptide ligands can be complexed with streptavidin-linked AP and used to screen λ cDNA expression libraries. Such a screen with the peptide ligands for the Src and Cortactin SH3 domains led to the isolation of 8 known (i.e., Cortactin, Crk, Fyn, H74, HS1, Lyn, MLN50, p53BP2), and 10 novel SH3-domain-containing proteins (14). Thus, COLT with synthetic peptides identified both potentially interacting proteins as well as protein family members which carry SH3 domains. However, as demonstrated here, the SH3-l–AP fusions reacted with a much less diverse set of proteins in the λ cDNA libraries than did the synthetic peptide probes; this difference in cross-reactivity is most likely due to the differences in the valencies of the probes (i.e., tetravalent versus bivalent). There appears to be a spectrum of reactivity achievable with COLT:monovalent peptides, while specific for their SH3 domains are inadequate for detection of membrane-immobilized proteins (16); AP–fusions are both specific and generate strong signals; and tetravalent peptides are cross-reactive and strong in their reaction. Thus, one can choose a different peptide format for screening a λ cDNA library, depending on the desired outcome (i.e., protein family members or potential interacting proteins).

The SH3-l– and SH3–AP fusions represent a useful set of reagents with which to screen libraries of combinatorial chemicals. One screening format can be based on competitive displacement of the enzyme fusions from binding natural ligands immobilized on microtiter plates. As demonstrated here, the SH3-l–AP fusions can be displaced selectively with the appropriate soluble peptides; soluble chemical antagonists from compound libraries should have similar activities (35). Alternatively, another screen can be formatted by examining the binding of the fusions directly to immo-
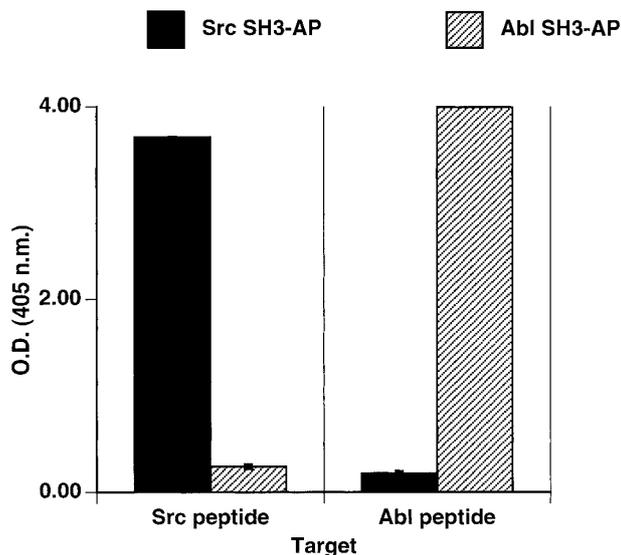
TABLE 2

Identity of Proteins That Interact with Src and Abl SH3 Domain–AP Fusions

Probe	Description	Potential interacting PXXP containing regions	Frequency
Src SH3	novel/s2	200-APGGPFGGEA<u>PPLPPPPPPPPPPPPP</u>LPP<u>P</u>APRDPPEDAREDPAAAGPEDK<u>PPPPPP</u>KGNPWTKK-265	1
	teg27	1-SNSSLGAPDSRPPA<u>R</u>PLPPPP<u>P</u>AR<u>RPPPPLPPPPP</u>L<u>PPR</u>GAQAQPPSPPSSAAAAAEHHGRRRERA-065	1
	Efs	290-QHRPRLPSTESLSR<u>R</u>PLPALPVSEAPAPSPAPSPAPGRKGSIQD<u>R</u>PL<u>PPPPPP</u>CLPGYGGLKPEGD-355	3
	novel/s19	160-TSEA<u>PPLPPR</u>NAGKGPTGPPSTLPLGTQTSSGSSTLSKK<u>RPPPPPP</u>GHKRTLSDPPSPLPHGGPPN-225	1
Abl SH3	novel/s2	200-APGGPFGGEA<u>PPLPPPPPPPPPPP</u>PPL<u>PPP</u>APRDPPEDAREDPAAAGPEDKPPPPPPKGNPWTKK-265	1
	Mena	280-G<u>PPAPPPPP</u>L<u>P</u>SGPAYASAL<u>PPPP</u>G<u>PPPPPPP</u>L<u>P</u>STG<u>PPPPPPPPPPPPP</u>L<u>P</u>NQA<u>PPPPPPPPP</u>APPL<u>P</u>-345	1
	51C	874-<u>PPPTGRPPAPP</u>RAA<u>P</u>REE<u>P</u>LT<u>P</u>RLK<u>P</u>EGA<u>P</u>EPEGVAA<u>PP</u>PKNSFNNPAYYVLEGV<u>P</u>HQLL<u>PPEPP</u>-938	1
		985-<u>PPPDFPPPPLP</u>DSAIFLPPSLDPLPGPVVRGRGGAEARG<u>PPPPP</u>KAH<u>PRPPLPP</u>GPSPASTFLGEV-1050	

Note. Conceptual translations of cDNA clones isolated from a λ cDNA library of 16-day-old mouse embryo with either SrcSH3– or AblSH3–AP fusion proteins. Proline-rich segments are underlined. Frequency corresponds to the number of types a particular cDNA sequence was isolated from a screen of 6.0×10^5 recombinants. The complete nucleotide and protein coding sequences of isolated clones can be found on the Internet as described in the note to Table 1.

bilized compounds (36). The identification of chemical antagonists that specifically disrupt particular SH3–natural ligand interactions will likely prove valuable in evaluating the contribution of certain protein–protein interactions to cellular and disease processes.

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