A Single-Chain Antibody/Epitope System for Functional Analysis of Protein−Protein Interactions[†]

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ABSTRACT: Protein−protein interactions play a critical role in cellular processes such as signal transduction. Although many methods for identifying the binding partners of a protein of interest are available, it is currently difficult or impossible to assess the functional consequences of a specific interaction in vivo. To address this issue, we propose to modify proteins by addition of an artificial protein binding interface, thereby forcing them to interact in the cell in a pairwise fashion and allowing the functional consequences to be determined. For this purpose, we have developed an artificial binding interface consisting of a anti-Myc single-chain antibody (ScFv) and its peptide epitope. We found that the binding of an ScFv derived from anti-Myc monoclonal antibody 9E10 was relatively weak in vivo, so we selected an improved clone, 3DX, by in vitro mutagenesis and phage display. 3DX bound well to its epitope in a yeast two-hybrid system, and GST-fused 3DX also bound to several Myc-tagged proteins in mammalian cells. In vivo binding was relatively insensitive to the position of the ScFv in a fusion protein, but was improved by including multiple tandem copies of the Myc epitope in the binding partner. To test the system, we successfully replaced the SH3 domain-mediated interaction between the Abl tyrosine kinase and adaptor proteins Crk and Nck with an engineered interaction between 3DX and multiple Myc tags. We expect that this approach, which we term a functional interaction trap, will be a powerful proteomic tool for investigating protein−protein interactions.

In cellular signal transduction, protein−protein interactions play a critical role in regulating processes such as proliferation, adhesion, and differentiation. Signaling proteins often contain one or more modular protein−protein binding domains such as Src homology 2 (SH2)[1] or Src homology 3 (SH3) domains (*1*). Although such protein-binding domains have considerable binding specificity, in almost all cases several partners can bind with similar affinity. This redundancy leads to complex networks of interactions; a given stimulus can lead to multiple outputs, or different stimuli can generate the same output, due to the binding of overlapping sets of partners. To truly understand the mechanisms of intracellular signaling, therefore, it is necessary to tease out which specific interaction or set of interactions is responsible for a particular biological output.

A variety of methods for analyzing protein−protein interactions are available, including pull-down assays and the yeast two-hybrid system, which are now being applied to define protein interactions on a proteome-wide scale (*2*−*7*). While such screens provide insight into what complexes can exist in the cell, they do not address the functional consequences of those interactions. We propose an approach, called the functional interaction trap (FIT), for assessing and validating the functional role of specific interactions in the context of an intact cell. In this method, the normal binding interface between two proteins of interest is replaced by an artificial, highly specific protein binding interface. By expressing the two modified proteins in the cell, we can therefore force them to interact in a pairwise fashion and determine the functional consequences of that interaction, for example, whether a biological response of interest can be rescued (Figure 1).

For the FIT approach to work, a robust artificial protein interaction interface will be required. Many protein−protein interactions are mediated by small, globular domains that bind to short, linear peptide ligands (*8*), and we aimed to develop an interface with similar properties. Other important considerations for the interface are that it have no endogenous binding partners, that its affinity be comparable to that of authentic protein−protein interactions, and that it be amenable to large-scale screening. Others have reported the development of homodimerization or heterodimerization interfaces that can be inducibly cross-linked by addition of small-molecule chemical inducers of dimerization (CIDs) (*9*−*15*). These systems have some disadvantages, however,

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[1] Abbreviations: SH2, Src homology 2; SH3, Src homology 3; FIT, functional interaction trap; CID, chemical inducer of dimerization; ScFv, single-chain antibody; V_H and V_L, variable regions of immunoglobulin heavy and light chains, respectively; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; 3-AT, 3-amino-1,2,4-tetrazole; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CDRs, complementarity determining regions.

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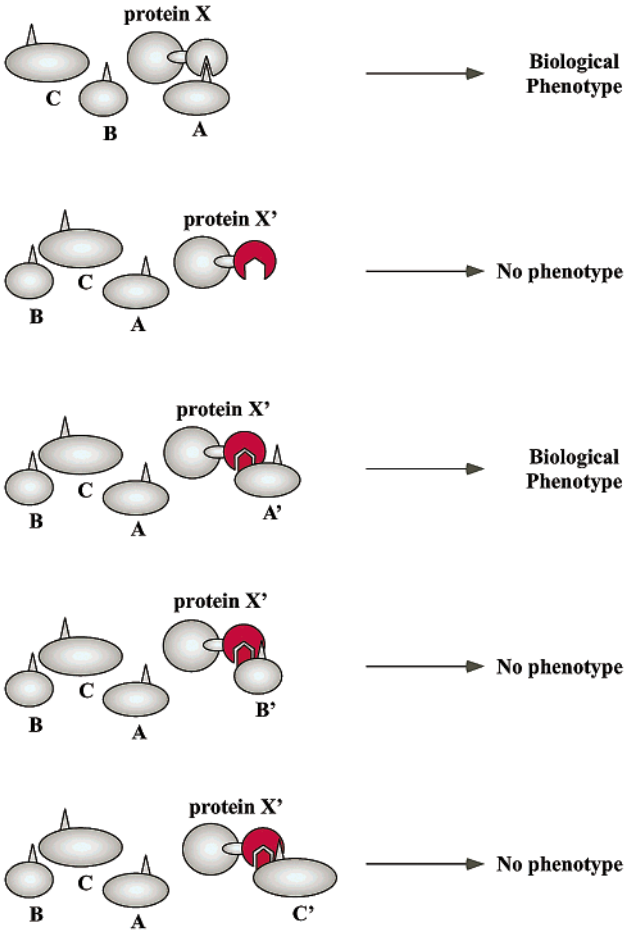

FIGURE 1: Schematic representation of the FIT strategy. In this example, the binding of protein X to protein A leads to a biological output of interest; however, protein X can also be shown to bind to proteins B and C (top). In the FIT approach, protein X and its candidate binding partners are modified by addition of an artificial protein binding interface which can mediate their specific, pairwise interaction, in the absence of other competing interactions that might normally also occur. When the protein binding domain of protein X is replaced with an engineered interaction interface (red), no biological output is seen in cells because the modified protein (protein X′) no longer binds to A, B, or C. Candidate partners are then assayed by fusing them to a complementary binding interface (red) and coexpressing them with protein X′. The engineered interface mediates specific, pairwise interaction between X′ and A′, X′ and B′, and X′ and C′. In this example, the biological output is observed only in cells coexpressing proteins X′ and A′, providing very strong evidence that the specific binding of proteins X and A is sufficient to induce this phenotype.

including the requirement for relatively bulky fusion partners, competing homodimerization reactions, the need for costly CIDs, and the potential effects of CIDs on endogenous proteins. Here we report the development of a modified single-chain antibody (ScFv) and its peptide epitope for use as the artificial protein binding interface for FIT.

An ScFv consists of the heavy and light chain variable regions (V_H and V_L, respectively) of an antibody connected by a flexible peptide linker (*16*). ScFvs have been adapted for expression in the phage-display system, which allows genetic selection of mutants with desirable properties (*17*). Since ScFvs can be expressed as a single 25 kDa peptide, they have been proposed as tools for therapeutic purposes and functional genomics (*18*, *19*). For example, several studies have described ScFvs directed against Her2/Neu, a receptor-type tyrosine kinase that is amplified in many breast cancers (*20*). To date, most studies have used ScFvs that target cell surface proteins, while a few successes have also been reported using ScFvs intracellularly. Besides therapeutic purposes, intracellular ScFvs, so-called "intrabodies", have also been proposed as tools for functional genomics, as a means of specifically knocking out the function of targeted proteins (*21*, *22*).

In this report, we demonstrate the feasibility of the FIT approach by reconstituting the SH3 domain-mediated interaction between the Abl tyrosine kinase and the SH2/SH3 adaptors Crk and Nck. The kinase activity of c-Abl is tightly regulated; for example, truncation or point mutation in the SH3 domain stimulates Abl kinase activity and renders it oncogenic (reviewed in ref *23*). Furthermore, Abl can be regulated by several adaptor proteins, including Crk and Nck, which bind via their SH3 domains to a proline-rich region located in the C-terminal tail of c-Abl (*24*−*26*). Adaptor proteins play an important role in many signaling processes by mediating the assembly of multiprotein complexes (*27*). Although molecular mechanisms of Abl regulation by adaptor proteins have been investigated biochemically, the biological outputs from each specific interaction in vivo are still unclear. In this study, we demonstrate that the ScFv system can be used to induce the association of Abl with specific adaptor proteins, as a first step in assessing the biological role of specific Abl−adaptor complexes in signaling.

EXPERIMENTAL PROCEDURES

Plasmids. For bait and prey vectors in the yeast two-hybrid assay, we used pGBK-BN and pGAD-PA, which are modified plasmids derived from pGBKT7 and pGADT7 (Clontech), respectively. To make pGBK-BN, a fragment of the multicloning site of pGBKT7 from the *Nco*I site to the *Not*I site, which contains the Myc epitope tag, was replaced with a fragment containing *Bam*HI, *Asp*718, and *Not*I sites, thus deleting the Myc tag. Five tandemly repeated Myc tags were amplified from pCS2-MTBN (*28*) by polymerase chain reaction (PCR) using Pfu polymerase and inserted into the *Bam*HI and *Not*I sites of pGBK-BN to generate pGBK-BN-5Myc. To make pGAD-PA, the fragment of pGADT7 from the *Nco*I to *Xho*I sites was deleted to remove the HA epitope. A DNA fragment derived from the hinge domain of *Staphylococcus aureus* protein A encoding 13 amino acids (AKKLNDAQAPKSD) (*29*) was inserted as a linker followed by *Bam*HI, *Asp*718, and *Not*I sites. *Bgl*II−*Not*I fragments encoding anti-Myc ScFv cDNAs were inserted between the *Bam*HI and *Not*I sites of pGAD-PA.

Constructs expressing ScFvs fused with glutathione *S*-transferase (GST) were made in pEBG (*30*) and pEBGC vectors. To make pEBGC, the GST fragment of pEBG was amplified by PCR using a 5′ primer containing *Bam*HI and *Asp*718 sites preceding the initiation codon of GST, and a 3′ primer containing a termination codon followed by a *Not*I site. The resulting PCR fragment was inserted into the *Bam*HI and *Not*I sites of pEBB (*30*). Using pEBG, a cDNA of interest can be inserted to the C-terminal side of GST, while using pEBGC, the cDNA can be inserted to the N-terminal side of GST. *Bgl*II−*Asp*718 fragments of the anti-Myc ScFv

were inserted between the *Bam*HI and *Asp*718 sites of pEBG and pEBGC to make GST−ScFv and ScFv−GST proteins, respectively. Expression vectors for p130Cas, HS-1, and CD19 were made as follows. A synthetic oligonucleotide fragment encoding the *Bgl*II site−FLAG epitope−*Bam*HI site motif was inserted into the *Bam*HI site of pEBB to make pEBB-FL. To make pEBB-FL5MT, a fragment containing five tandemly repeated Myc tags with a 5′ *Bgl*II site and a 3′ *Bam*HI site was PCR-amplified from pCS2-MTBN and inserted into the *Bam*HI site of pEBB-FL. cDNAs of rat p130Cas, mouse HS-1, and the cytoplasmic domain of human CD19 were cloned by PCR and inserted into the *Bam*HI and *Not*I sites of either pEBB-FL or pEBB-FL5MT. The Myc-tagged N-WASP expression vector was previously described (*31*).

pGDN-Abl, encoding mouse type IV c-Abl, was reported previously (*32*). pGDN-AblΔPro was made by deleting the proline-rich region from Ala^{538} to Asp^{600} and replacing it with a fragment containing *Nsi*I and *Sac*II sites. A fragment consisting of six tandemly repeated Myc tags which was PCR-amplified from pCS2-MTBN was then inserted between the *Nsi*I and *Sac*II sites to make pGDN-AblΔPro-6Myc. To make pEBB-Crk-3DX, the region from the SH3 domain to the C-terminus of Crk-I (from Asn^{132} to Arg^{205}) was replaced with 3DX coding sequences by two-step PCR, generating a 5′-*Bgl*II−Crk−*Mlu*I−3DX−*Asp*718-3′ product; similarly, pEBB-Nck-3DX was made by replacing the two N-terminal SH3 domains (from Met^{1} to Leu^{168}) with 3DX, generating a 5′-*Bgl*II−3DX−*Mlu*I−Nck−*Asp*718-3′ fragment. These fragments were cloned between the *Bam*HI and *Asp*718 sites of pEBB-HA (*33*); the resulting proteins encode an HA epitope tag at their C-termini.

Cloning and Selection of ScFv cDNAs and Their Mutants. Total RNA was extracted from hybridomas 9E10 (*34*; anti-Myc) and 12CA5 (*35*; anti-HA) and reverse-transcribed into cDNA using random primers according to standard procedures. Primer sets (*36*) were used to PCR-amplify the variable heavy (V_H) and light (V_L) chain regions of the immunoglobulins expressed in these hybridomas. These cDNA fragments were combined with a $(Gly_4Ser)_3$ linker to generate ScFv and inserted into phage-display vector pCANTAB5E (Amersham Pharmacia Biotech). In some cases, these cDNA fragments were subjected to random mutagenesis by error-prone PCR amplification using Taq polymerase (*37*) before recloning into pCANTAB5E to construct phagemid libraries of ScFv derived from 9E10 and 12CA5. To select ScFv clones with improved antigen binding properties, typically 10^{11} pfu of recombinant phages was added to six-well plates coated with GST-fused peptide epitopes (EQKLISEEDL for Myc and YPYDVPDYA for HA). Phage binding was carried out for 2 h at room temperature in PBS supplemented with 5% nonfat milk and 0.05% Tween 20, followed by extensive washes with the same buffer. Bound phage were eluted either with an excess of GST-fused antigen or with a low-pH buffer.

Yeast Two-Hybrid Assay. Experimental procedures were performed as suggested in the *Yeast Protocol Handbook* (Clontech) unless otherwise mentioned. Yeast cells (strain Y190) were grown in YPD medium and transformed with both bait and prey plasmids by the LiAc/PEG method. Transformed cells were plated onto −leucine/−tryptophan plates and −leucine/−tryptophan/−histidine plates, supplemented with 20 mM 3-amino-1,2,4-tetrazole (3-AT) to inhibit leaky basal HIS3 expression. For the β-galactosidase assay, transformants which were selected on −leucine/−tryptophan/−histidine plates with 20 mM 3-AT were inoculated into −leucine/−tryptophan medium and grown overnight. Transformants that were not viable on triply deprived plates were selected from doubly deprived plates, and grown overnight in doubly deprived medium as described above. One milliliter of these overnight cultures was resuspended in 700 μL of Z buffer [100 mM phosphate buffer (pH 7.0), 10 mM KCl, and 1 mM $MgSO_4$] containing 4 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG) and lysed by adding 25 μL of 0.1% SDS and 20 μL of chloroform. Lysates were incubated at 30 °C and occasionally monitored for a change of color. To stop the reaction, 300 μL of 1 M sodium carbonate was added to the lysates. The absorbance of the supernatants was measured at 420 nm. β-Galactosidase activities were calculated with the equation 1 unit = $1000OD_{420}$/[min(elapsed time of incubation) × OD_{600}(A_{600} of 1 mL of culture)]. Assays were performed in triplicate, and the standard error was calculated.

Cell Culture and Transfection. Human embryonic kidney cell line 293T was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Cells were transfected by calcium phosphate coprecipitation as described previously (*30*). For the GST pull-down assay, 5 μg of plasmid encoding GST or GST-fused ScFv and 2 μg of plasmid encoding FLAG-tagged proteins were cotransfected per 100 mm dish. One day post-transfection, cells were lysed with 300 μL of TXB [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitors]. For immunoprecipitation of Abl mutants and adaptor proteins, 2 μg of plasmid encoding Abl mutants and 12 μg of plasmid encoding Crk-3DX or Nck-3DX (or 1 μg of wild-type Crk or Nck plasmid) were cotransfected and treated as described above except the lysis buffer was KLB [25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and protease inhibitors].

GST Pull-Down Assay, Immunoprecipitation, and Immunoblotting. For the GST pull-down assay, equal amounts of protein lysate in TXB were added to 10 μL of a 50% slurry of glutathione−Sepharose 4B (Amersham Pharmacia Biotech). Lysates were incubated with rotation at 4 °C for 30 min and beads washed four times with ice-cold TXB. For immunoprecipitation, 1 μg of the anti-HA polyclonal antibody (Y11, Santa Cruz) was added to lysates, and immune complexes were collected on 10 μL of protein A-conjugated beads. Precipitated proteins were separated by SDS−PAGE and electrophoretically transferred to nitrocellulose or PVDF membranes. Membranes were then subjected to immunoblotting. The anti-GST polyclonal antibody (Santa Cruz), the anti-Myc monoclonal antibody (9E10, Santa Cruz), the anti-FLAG monoclonal antibody (M2, Sigma), and the anti-HA monoclonal antibody (HA11) were used as probes, and detection was performed either by the colorimetric method with alkaline phosphatase-conjugated secondary antibody or by chemiluminescence (ECL, Amersham Pharmacia Biotech) with a horseradish peroxidase-conjugated secondary antibody.

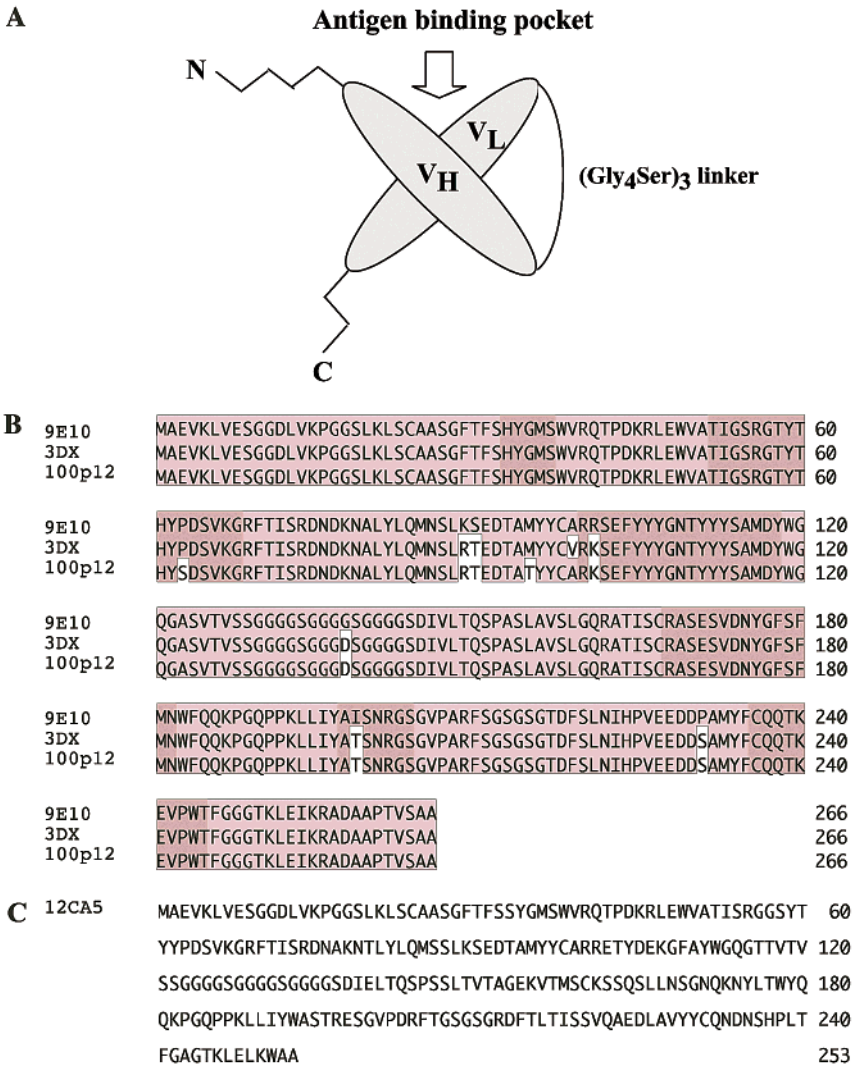

FIGURE 2: Structure and peptide sequences of ScFvs used in this report. (A) The schematic structure of an ScFv, consisting of V_H and V_L fragments derived from the variable regions of immunoglobulin connected by a $(Gly_4Ser)_3$ linker. (B) The amino acid sequences of two mutant anti-Myc ScFvs (3DX and 100p12) derived from 9E10. Mutants were aligned with the parental 9E10 sequence, and residues identical to those of 9E10 are shaded pink. CDR regions (*62*) are shaded light gray. Amino acid numbers on the right correspond to the numbering of the mutants in text. (C) Amino acid sequence of the anti-HA ScFv derived from 12CA5.

RESULTS

Selection of ScFvs. To construct ScFvs, we cloned cDNA fragments for the variable regions of the Myc-specific monoclonal antibody 9E10 and the HA-specific monoclonal antibody 12CA5 from the corresponding hybridoma mRNA. The V_H cDNA fragment was linked to the V_L portion with a 15-amino acid $(Gly_4Ser)_3$ linker. Figure 2 shows the deduced amino acid sequences of the ScFvs derived from 9E10 and 12CA5. We found that these ScFvs had relatively low binding affinities for their peptide epitopes (data not shown); therefore, to obtain clones with improved binding properties, we mutagenized the ScFv coding sequences by error-prone PCR. Using a phage-display system, we selected mutant clones for 9E10 from a library of approximately 10^5 independent recombinant phages. After four rounds of PCR mutagenesis and phage display, we selected two clones, 100p12 and 3DX, which showed significantly improved binding compared with the original 9E10 ScFv. The sequences of these two clones revealed several point mutations. 100p12 and 3DX shared $Lys^{89} \rightarrow Arg$, $Ser^{90} \rightarrow Thr$, $Arg^{101} \rightarrow Lys$, $Gly^{138} \rightarrow Asp$, $Ile^{199} \rightarrow Thr$, and $Pro^{231} \rightarrow Ser$ mutations. Among these, the last three mutations are the most drastic, likely contributing to the improvement in binding. 3DX and 100p12 each have additional unique mutations not present in the other, an $Ala^{99} \rightarrow Val$ mutation for 3DX and $Pro^{62} \rightarrow Ser$ and $Met^{95} \rightarrow Thr$ mutations for 100p12.

Both V_H and V_L possess hypervariable regions termed complementarity determining regions (CDRs), the positions of which are indicated in Figure 2. Interestingly, only one of the major amino acid differences between 3DX and the parental 9E10 ScFv was found in a CDR ($Ile^{199} \rightarrow Thr$), while the other mutations in 3DX were located in relatively conserved framework regions. Since 3DX had the highest apparent affinity among anti-Myc ScFvs in the yeast two-hybrid assay (see below), it is likely that those mutations contributed to the improvement in affinity. We also obtained several mutant clones for 12CA5 by the phage-display system, but the improvement in their binding properties was not as pronounced as in the case of the anti-Myc ScFvs (data not shown), leading us to focus on the latter for further analysis.

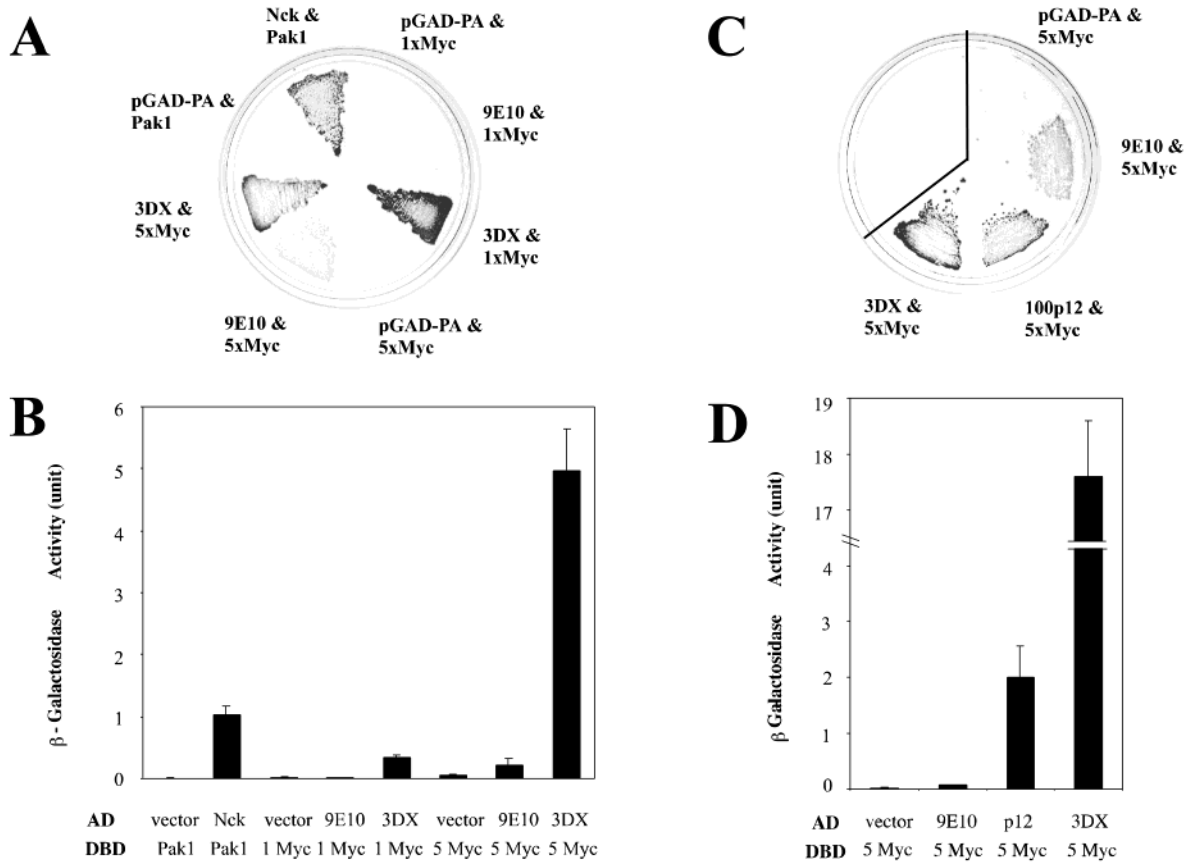

FIGURE 3: Binding of the anti-Myc ScFv to its epitope in the yeast two-hybrid system. Either pGBKT7, encoding a single Myc tag (1xMyc), or pGBK-BN-5Myc, encoding five tandem Myc tags (5xMyc), was used as a bait plasmid, while 9E10 ScFv or its mutants, inserted into pGAD-PA, were used as prey. Bait and prey plasmids were cotransformed into yeast strain Y190. The interaction between the Nck SH3-2 domain (Nck) and the Pak1 N-terminus (Pak1) serves as a positive control. (A and C) Transformants were first selected on −Leu/−Trp plates, and then viable clones were spread on −Leu/−Trp/−His plates containing 20 mM 3-AT. (B and D) Binding activities were quantitated with the liquid β-galactosidase assay as described in Experimental Procedures. AD is the pGAD-derived prey vector; DBD is the pGBK-derived bait plasmid.

Binding of ScFvs to the Myc Epitope in Vivo. We next used the yeast two-hybrid assay to test whether improvements in binding could also be seen in the intracellular milieu. Using this system, reconstitution of a known biologically relevant interaction [between the N-terminus of Pak1 and the second SH3 domain of Nck (*33*, *38*, *39*)] allowed yeast cells to grow on selective media as expected (Figure 3A). Under the same conditions using a single Myc tag as bait, growth of yeast transformed with the 9E10 ScFv prey plasmid could hardly be detected, while cells transformed with a 3DX prey plasmid showed significant growth. We then tested five tandemly repeated Myc tags in the bait plasmid instead of a single Myc tag. In this case, the growth of cells cotransformed with the 9E10 prey was greatly stimulated compared with that of the bait plasmid alone. These results were then extended using a quantitative β-galactosidase assay (Figure 3B). In this assay, 3DX exhibited a much stronger apparent interaction with the Myc epitope than 9E10, while 100p12 had an intermediate activity (Figure 3D). These results indicate that the ScFv could bind its peptide epitope in the cell, that the binding affinities of mutants derived from 9E10 were improved relative to that of the parental ScFv, and that the apparent affinities were increased when multiple copies of the epitope tag were used. It also suggests that the affinity for binding between 3DX and five tandem Myc tags was at least as high as that of the interaction between the Nck SH3 domain and Pak1.

To examine the binding between the ScFv and its epitope in mammalian cells, we used transient expression in human 293T cells followed by a GST pull-down assay. Plasmids encoding GST-fused 9E10 or 3DX were cotransfected with either untagged or Myc_5-tagged proteins, including p130Cas (*40*, *41*), HS-1 (*42*), or the cytoplasmic domain of CD19 (*43*). Because all the Myc-tagged proteins also encoded a FLAG epitope tag at their N-termini, they could all be detected by anti-FLAG antibodies. GST−ScFv fusion proteins were collected with immobilized glutathione beads, and coprecipitating Myc-tagged proteins were detected by immunoblotting. As can be seen in Figure 4, Myc-tagged proteins coprecipitated with the GST−3DX protein, suggesting that 3DX bound to its epitope in mammalian cells. Binding was much weaker in the case of the parental 9E10 fusion, where a faint band could be seen in only the lane where Myc_5-tagged HS-1 was cotransfected (Figure 4, anti-Myc).

Next we examined whether the position in which the ScFv is inserted affects its ability to bind to the Myc epitope. We fused the ScFv to either the N-terminal or C-terminal side of GST, and each construct was cotransfected into 293T cells along with a plasmid encoding Myc_6-tagged N-WASP (*44*). The resulting lysates were subjected to the GST pull-down assay to detect differences in binding affinity. As shown in Figure 5, we did not detect any significant difference between constructs where 3DX was inserted to the N-terminal or

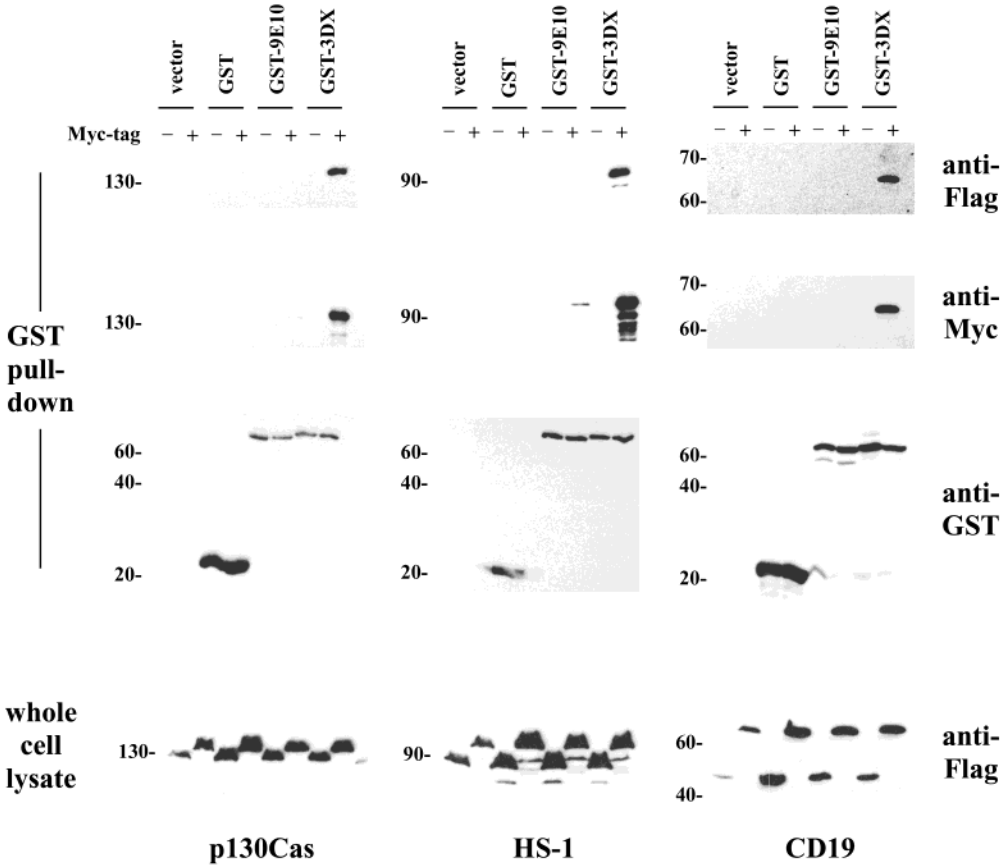

FIGURE 4: Binding between GST-fused anti-Myc ScFv and Myc-tagged proteins in mammalian cells. The plasmid expressing GST-fused 9E10 or 3DX was cotransfected into 293T cells with vectors encoding p130Cas, HS-1, or the cytoplasmic domain of CD19, tagged with the FLAG epitope with or without five tandem Myc tags. Cells were lysed 24 h post-transfection, and GST fusion proteins collected on glutathione beads. Bead-bound proteins or whole cell lysates were separated by 10% SDS−PAGE, transferred to membranes, and probed with an anti-FLAG or anti-Myc monoclonal antibody or an anti-GST polyclonal antibody. The positions of prestained molecular mass markers are indicated at the left (in kilodaltons).

C-terminal side of GST. Once again, coprecipitation of Myc-tagged N-WASP with the parental 9E10 fusion could barely be detected, irrespective of the orientation of the ScFv. These results suggest that binding between the ScFv and the Myc tag is relatively insensitive to the position of the ScFv in a fusion protein. We also tested the effect of inserting a linker peptide (consisting of 13 amino acids derived from the hinge domain of *S. aureus* protein A) between GST and the ScFv, with the aim of increasing the flexibility of the fusion proteins (*29*). No increase in the extent of binding was apparent (Figure 5), implying that there is no significant steric interference with binding, at least in the context of GST fusions. Taken together, these results demonstrate that the 3DX ScFv and multiple Myc epitopes can stably interact in the cytosol of mammalian cells, suggesting this would be a suitable artificial protein binding interface for FIT purposes.

ScFv-Mediated Association of Signaling Proteins. We next examined whether we could use 3DX and its epitope to direct the binding of biologically relevant target proteins, in the absence of their normal intrinsic binding interface. Since SH2/SH3 adaptor proteins, including Crk and Nck, are known to bind via their SH3 domains to the proline-rich region of the c-Abl tyrosine kinase (*45*, *46*), we constructed a Crk-I mutant in which the SH3 domain was replaced with 3DX (Crk-3DX), a Nck mutant in which the two N-terminal SH3 domains were replaced with 3DX (Nck-3DX), an Abl variant with the proline-rich SH3-binding domain deleted (AblΔPro), and an Abl variant in which the proline-rich region was replaced with six tandem Myc tags (AblΔPro-6Myc) (depicted in Figure 6). As shown in Figure 7, wild-type (wt) c-Abl co-immunoprecipitated with wt Crk-I or Nck when coexpressed in 293T cells. When Crk-3DX or Nck-3DX was coexpressed with wt Abl, AblΔPro, or AblΔPro-6Myc, only AblΔPro-6Myc specifically co-immunoprecipitated with Crk-3DX or Nck-3DX. These data demonstrate that AblΔPro-6Myc bound Crk-3DX or Nck-3DX in vivo via the ScFv and Myc tag. This strongly suggests that these mutants will be useful and novel tools for investigating the biological consequences of specific binding between Abl and Crk or Abl and Nck, and that this general approach could be used to direct the specific association of other proteins in the cell.

DISCUSSION

As the genomes of major experimental organisms are completed, there is a new emphasis on the functional characterization of gene products, including the construction of global interaction maps (*47*). Several groups have generated interaction maps for yeast or *Caenorhabditis elegans* by the yeast two-hybrid system (*4*, *5*, *7*). Although this is a well-characterized method for analyzing protein−protein interactions (*48*), it can only identify potential binding partners for a protein of interest; no information is provided

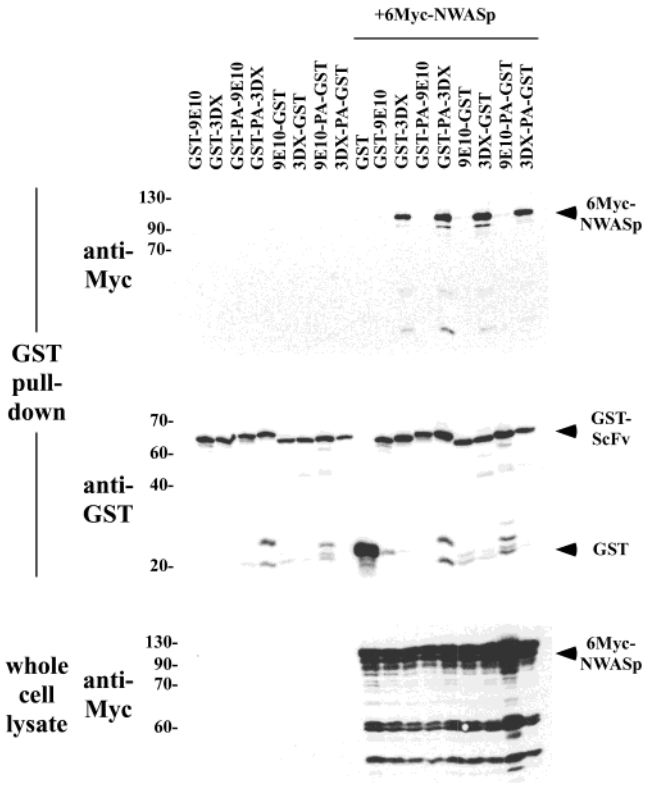

FIGURE 5: Effect of the position of the inserted ScFv on binding to Myc-tagged proteins in vivo. 9E10 and 3DX were inserted to either the N-terminal or C-terminal side of GST. In the nomenclature of GST-fused ScFv constructs, PA denotes the insertion of a linker peptide (derived from *S. aureus* protein A) between GST and the ScFv. ScFv-encoding plasmids were cotransfected into 293T cells with the plasmid encoding N-WASP bearing six tandem Myc tags (6Myc-NWASp). Lysates were analyzed with the GST pull-down assay and immunoblotting as described in the legend of Figure 4. The positions of prestained molecular mass markers are indicated at the left (in kilodaltons).

about whether an interaction actually occurs, or the consequences of binding. Pull-down approaches (*2*, *3*) can reveal protein associations that occur in a cell of interest, but still cannot address the functional consequences of those interactions. Thus, it remains difficult or impossible to identify those specific protein interactions that are functionally relevant to a biological activity of interest. Here we describe an approach, termed the functional interaction trap (FIT), that can complement binding-based methods. In this method, two proteins are forced to interact specifically via an artificial binding interface, such as an ScFv and its epitope, and the biological output resulting from this interaction can be determined.

We chose to adapt ScFvs for FIT applications because of their relatively high specificity and small size (~25 kDa), and because their binding partner (the epitope) is a short, linear peptide. The latter is important because it might allow the epitope to be inserted into various positions in a protein of interest, for example, in the solvent-exposed loops of a globular domain, with minimal disruption of the overall structure. The linear nature of the epitope also mimics the peptide ligands for naturally occurring modular protein interaction domains (*8*). In contrast, CID-based heterodimerization schemes require fusion to bulky drug binding domains (*9*, *12*, *14*). We derived our ScFvs from the well-characterized monoclonal antibodies 9E10 and 12CA5, directed against the Myc and HA epitopes, respectively, as these antibodies and their corresponding epitope tags are widely used and interact with high specificity. In most cases, addition of these epitope tags has no apparent effect on the function of proteins to which they are fused, and because the HA epitope is from a viral protein and the Myc epitope is not recognized by 9E10 in the native (nondenatured) Myc protein (*34*), expression of the ScFv is not expected to interfere with endogenous functions. Finally, many proteins are already available as Myc- or HA-tagged fusions; thus, the FIT system could take advantage of existing epitope-tagged constructs.

We found that ScFvs constructed from monoclonal antibodies 9E10 and 12CA5 bound relatively weakly to their epitopes in yeast and mammalian cells, but that after several rounds of mutagenesis and phage display we could isolate mutants such as 3DX with significantly improved in vivo binding (Figures 3 and 4). In a previous report (*49*), Schier et al. succeeded in generating picomolar affinity ScFvs using targeted mutagenesis of the CDR of V_H and V_L. In contrast, we mutagenized the entire ScFv cDNA randomly, and surprisingly, almost all of the mutations in 3DX were located outside the CDR. Since the CDRs determine antigen specificity, this result suggests that mutations in 3DX may affect the overall conformation or stability of the ScFv, as others have observed (*50*, *51*). Interestingly, one mutation was located in the $(Gly_4Ser)_3$ linker segment of 100p12 and 3DX. It is possible that this mutation may affect the structural flexibility between V_H and V_L, allowing the ScFv to bind to the epitope more tightly.

Despite the potential usefulness of intrabodies, one major problem must be overcome, their relative instability and insolubility in the reducing conditions encountered in the cytosol, where the formation of conserved intrapeptide disulfide bonds is not favored (*52*, *53*). We found that when ScFvs were fused to GST and expressed in human cells, the steady-state expression levels of the ScFv fusions were considerably lower than that of GST alone (see Figure 4). Furthermore, when we estimated the fraction of GST-fused 3DX bound to Myc_6-tagged N-WASP in transfected cells (using purified recombinant N-WASP and GST as standards), we found that only a relatively small fraction of the total GST−3DX protein was stably bound when N-WASP was in excess (data not shown). This suggests that a large percentage of the ScFv fusion may not be active in vivo. To address this issue, efforts have been made to directly screen for ScFvs that are soluble and stable in vivo by the yeast two-hybrid system (*54*−*56*). We are currently working to obtain improved anti-Myc ScFvs by selection in yeast under very stringent two-hybrid conditions. Of course, other types of protein interaction interfaces could also be adapted for FIT, such as amphipathic coiled-coil segments (*57*). We have found that engineered coiled coils can mediate specific pairwise interaction of protein kinases with candidate substrates in cells, leading to phosphorylation of those substrates (A. Sharma, S. Antoku, K. Fujiwara, and B. J. Mayer, manuscript in preparation).

We tested the FIT approach by reconstituting an interaction between the c-Abl tyrosine kinase and SH2/SH3 adaptors. In contrast to other nonreceptor tyrosine kinases such as those of the Src family, c-Abl has a long unique C-terminal

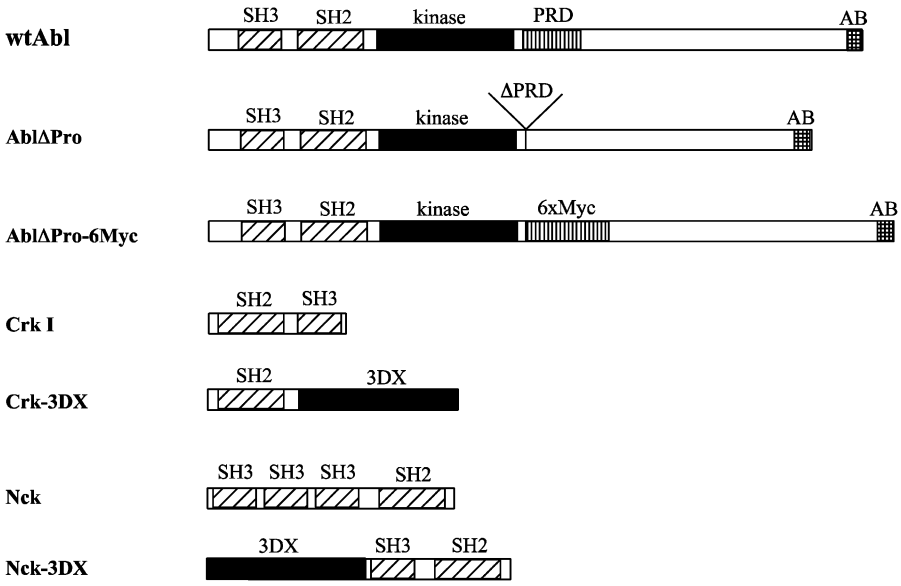

FIGURE 6: Structures of the Abl, Crk, and Nck mutants used in this report. AblΔPro has a deletion in the proline-rich region encompassing amino acids 540−645. In AblΔPro-6Myc, the proline-rich region is replaced with six tandemly repeated Myc epitopes. PRD and AB denote the proline-rich segment and actin binding domain, respectively. Crk-3DX and Nck-3DX were made by replacing a single SH3 domain in Crk-I or the two N-terminal SH3 domains in Nck with 3DX.

segment with which a number of proteins have been reported to interact. The biological consequences of most of these interactions are not known, however. A proline-rich region in the Abl C-terminus contains multiple PxxP SH3-binding motifs, which bind adaptor proteins, including Crk, Nck, and Grb2 (*45*, *46*). Overexpression of Crk can lead to cellular transformation (*58*), while Nck is thought to play an important role in actin organization (*25*). There are numerous reports also implicating Abl in both biological phenomena (*59*), and binding of Crk to Abl increases the processivity of Abl toward some substrates in vitro (*60*). For these reasons, it would be of great interest to know what specific biological outputs result from the interaction between Abl and Crk, or between Abl and Nck.

The FIT approach is ideal for addressing these issues, and we demonstrate in this report that the specific in vivo binding between Abl and modified adaptor proteins can be mediated by an ScFv and its epitope (Figure 7). These results demonstrate that 3DX and its epitope can bind when they are inserted into full-length, biologically relevant proteins, and that they can be used to substitute for the intrinsic binding modules of proteins of interest. We are now investigating the biological consequences which result from each of these specific pairwise interactions. Any biological output that occurs when both the modified Abl and modified adaptor are expressed (but does not occur when one of the two proteins lacks the ScFv or Myc epitope required for specific association) must be due to the specific pairwise interaction of the two modified proteins.

The FIT approach should have wide applications in validating the biological role of suspected protein−protein interactions; a few general considerations for this approach are outlined here. First, because the aim of the FIT approach is to replace intrinsic protein binding interactions, which vary in their affinity, it would be useful to have available a series of artificial interaction domains with various binding affinities. In this context, PCR-mutagenized variants of ScFvs, and epitopes with different numbers of tandem repeats, may provide the means for generating such a range of affinities. Second, some biological interactions may require a specific relative orientation of the interacting molecules. Although we have shown that the ScFv and epitope tag can be inserted in different positions in a protein, a negative result (lack of biological output) from an FIT experiment will always be less informative than a positive result. Finally, for the FIT approach, the region of a target protein that normally mediates its interaction with candidate binding partners must be disrupted so that only those interactions mediated by ScFv−epitope binding are possible. This can be accomplished by replacing the existing binding site with the ScFv or epitope. On the other hand, it is not necessary to disrupt the native binding site on the candidate binding partners to be tested by FIT; thus, full-length N-terminal or C-terminal fusions can be used for this purpose.

The FIT approach may ultimately be most useful in identifying novel, biologically relevant interactions from cell-based screens based on reconstituting a biological activity of interest. We anticipate that full-length fusion libraries will be available for all human cDNAs in the relatively near future (*61*), thus allowing truly proteome-wide screens for functionally important interactions with any protein of interest. For example, a library of Myc-tagged full-length cDNAs could be introduced into cells expressing a target protein fused to the 3DX domain, to screen for interactions that lead to biological phenotypes of interest in those cells. As currently described, the FIT approach can only be used to screen for dominant biological activities, as the corresponding wt proteins are still present in cells. However, in the future, FIT assays could be performed in cells where the endogenous target protein is absent, allowing screening for recessive phenotypes. This approach is straightforward in yeast, where the gene for a FIT-compatible fusion protein can be efficiently recombined into the endogenous locus, and will become more practical in vertebrate systems as more genetic

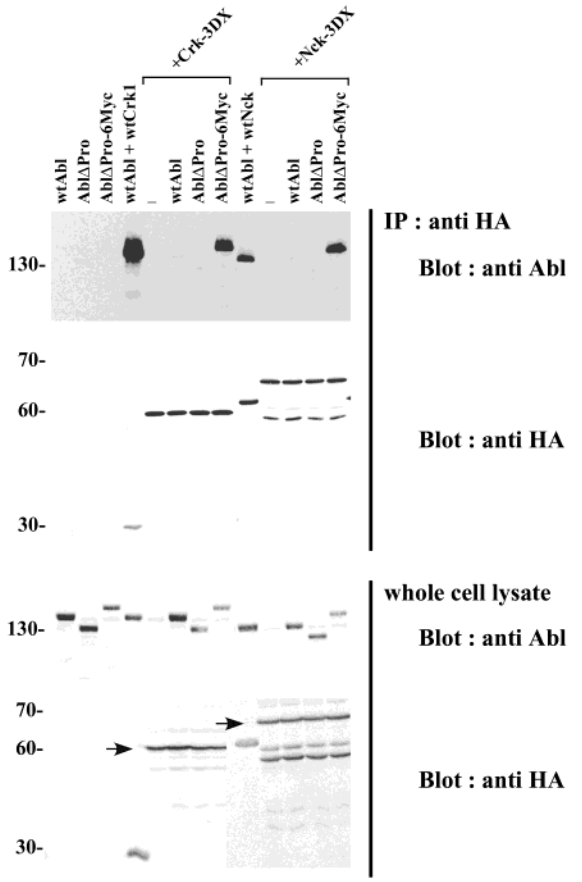

FIGURE 7: Reconstitution of binding between Abl and Crk or Nck by the FIT approach. The plasmid encoding wt Abl, AblΔPro, or AblΔPro-6Myc was cotransfected with the plasmid encoding wt Crk1 or Nck, or a 12-fold greater amount of plasmid encoding Crk-3DX or Nck-3DX. All Crk and Nck derivatives were HA-tagged. Lysates were immunoprecipitated with an anti-HA polyclonal antibody followed by immunoblotting with an anti-Abl or anti-HA monoclonal antibody. In whole cell lysates probed with anti-HA, arrows denote the positions of full-length Crk-3DX and Nck-3DX proteins. The positions of prestained molecular mass markers are indicated at the left (in kilodaltons).

knockout cell lines become available and as new methods of downregulating endogenous proteins are more widely applied.

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