Selective Targeting of SH2 Domain–Phosphotyrosine Interactions of Src Family Tyrosine Kinases with Monobodies

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Abstract

The binding of Src-homology 2 (SH2) domains to phosphotyrosine (pY) sites is critical for the autoinhibition and substrate recognition of the eight Src family kinases (SFKs). The high sequence conservation of the 120 human SH2 domains poses a significant challenge to selectively perturb the interactions of even the SFK SH2 family against the rest of the SH2 domains. We have developed synthetic binding proteins, termed monobodies, for six of the SFK SH2 domains with nanomolar affinity. Most of these monobodies competed with pY ligand binding and showed strong selectivity for either the SrcA (Yes, Src, Fyn, Fgr) or SrcB subgroup (Lck, Lyn, Blk, Hck). Interactome analysis of intracellularly expressed monobodies revealed that they bind SFKs but no other SH2-containing proteins. Three crystal structures of monobody–SH2 complexes unveiled different and only partly overlapping binding modes, which rationalized the observed selectivity and enabled structure-based mutagenesis to modulate inhibition mode and selectivity. In line with the critical roles of SFK SH2 domains in kinase autoinhibition and T-cell receptor signaling, monobodies binding the Src and Hck SH2 domains selectively activated respective recombinant kinases, whereas an Lck SH2-binding monobody inhibited proximal signaling events downstream of the T-cell receptor complex. Our results show that SFK SH2 domains can be targeted with unprecedented potency and selectivity using monobodies. They are excellent tools for dissecting SFK functions in normal development and signaling and to interfere with aberrant SFK signaling networks in cancer cells.

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Introduction

Src homology 2 domains (SH2) are small modular protein–protein interaction domains. In humans, 120 SH2 domains are found in 110 signaling proteins, including kinases, phosphatases, adaptor, and scaffold proteins, and cytoskeletal and small GTPase regulators [1–3]. SH2 domains recognize tyrosine-phosphorylated peptide sequences through two adjacent binding pockets, one binds the
phosphotyrosine (pY) side chain, and a second pocket dictates selectivity by recognizing the +3 side chain downstream of the pY residue [4].

The largest class of SH2 domain-containing proteins are the Src family kinases (SFKs), which consist of eight highly homologous kinases—Src, Yes, Fyn, Fgr, Hck, Lyn, Lck, and Blk, in which the SH2 domain is located N-terminal to the kinase domain. These SH2 domains mediate an intramolecular interaction with the tyrosine phosphorylated C-terminal tails of the kinase domain that is critical for keeping the kinase in the autoinhibited state [5,6]. In contrast, active SFKs use their SH2 domain for intermolecular interactions to enable multisite processive phosphorylation of substrates [7]. N-terminal to their SH2 domain, all SFKs contain an SH3 domain that is also critical for autoinhibition and collaborates with the SH2 domain in recognizing SFK substrates [8,9]. Three SFKs (Src, Yes, and Fyn) are ubiquitously expressed; the others are only expressed in particular cell types, such as Hck in myeloid cells or Lck in T- and NK-cells [10]. Mouse knockout studies have helped identify important functions of SFKs downstream of receptor tyrosine kinases, GPCRs, and immune cell receptors in bone, neural, and hematopoietic cell development and signaling [11].

Src was the first oncoprotein that was identified, and today, SFKs are important therapeutic targets in solid tumors and hematological malignancies [12,13]. Several small molecule ATP-competitive tyrosine kinase inhibitors to SFKs are already in the clinic or in clinical trials but suffer from the rapid development of drug resistance and poor selectivity [14]. Although the SH2 domain of SFKs can be targeted with dominant-negative pY peptides, peptidomimetics, and small molecules [15] that reach up to low nanomolar affinities, these inhibitors cannot discriminate among the SFK SH2 domains and lack comprehensive selectivity data among all SH2 domains [15,16]. Certain inhibitors originally developed toward selective inhibition are used even as “pan” SH2 domain affinity probes for proteomics experiments and enable the enrichment of a few dozen SH2 proteins, consistent with the paucity of truly selective SFK SH2-selective inhibitors [17].

To accelerate the studies of biologically important aspects of SFK SH2 domains in normal signaling and target them in aberrant oncogenic signaling, we aimed at developing high-performance synthetic binding proteins, termed monobodies. Monobodies are generated from large combinatorial libraries constructed on the molecular scaffold of a fibronectin type III domain [18,19]. We have developed and characterized monobodies that target the SH2 domains of the leukemogenic Abl kinase [20–22] and the oncogenic Shp2 tyrosine phosphatase [23]. These monobodies are potent pY ligand antagonists and inhibited Abl or Shp2 signaling. These monobodies achieved very high selectivity [22,23], which, however, can be rationalized by the fact that only one human paralog (Arg and Shp1, respectively) each exists for Abl and Shp2. Thus, selective targeting of SFKs presents a much greater challenge, given the larger number of highly homologous SFK SH2 domains.

Here, we describe the development and rigorous characterization of high-affinity monobodies to six of the eight SFK SH2 domains. We performed a thorough biochemical and structural analysis to understand the high selectivity that we observed in vitro and in cells and their effects on autoinhibited and active SFKs. This work provides in-depth understanding of SFK SH2 specificity and provides the foundation for the use of these high-precision tools to dissect SFK signaling in cells and in vivo.

Results

Generation of monobodies targeting six SFK SH2 domains

We recombinantly expressed and purified the SH2 domains of the eight SFKs (Yes, Src, Fyn, Fgr, Hck, Lyn, Lck, and Blk; SI Fig. 1a). The Blk SH2 domain nonspecifically bound to the beads used for monobody selection and the Fyn SH2 domain was not stable under the selection conditions. These SH2 domains were therefore excluded. We generated monobodies using phage and yeast display from the initial “loop-only” library and the recently developed “side-and-loop” library [18,20,24]. Monobody pools enriched with high-affinity binders were identified after two to three rounds of yeast display for all of these six SH2 domains (Fgr, Lck, Src, Yes, Hck, and Lyn). We determined the amino acid sequences of eight monobody clones for each of the SFK SH2 domain targets and selected two clones that had distinct amino acid sequences for further analysis (Fig. 1). All eight of the monobody clones for the Src SH2 domain had the same sequence, representing the single clone reported [Mb(Src_2)]. All but one clone [Mb(Hck_1)] were derived from the side-and-loop-library (Fig. 1). Based on their sequences, we observed two general types of clones. Yes, Src, and Fgr SH2-binding monobodies have a CD loop corresponding to the wild-type FN3 and a diversified FG loop that contains different sequences for all the different monobodies. In contrast, the Lyn and Lck SH2-targeting monobodies, and Mb(Hck_2), showed diversified CD and FG loops (Fig. 1).

In order to determine the binding affinity of the selected monobody clones to the SH2 domain for which they were selected (“on-target SH2”) and the SH2 domains of the other SFKs (“off-target SH2”), we performed binding titrations in the yeast surface display format that allows for $K_d$ estimations [20]. Mb(Lck_1) and Mb(Lck_3) bound to Lck, and
Mb(Lyn_2) and Mb(Lyn_4) to Lyn with very high affinity ($K_d = 10–20$ nM), respectively (Fig. 1 and SI Fig. 1b). The monobodies to Src, Hck, Fgr, and Yes, which have the wild-type FN3 sequence of the CD loop, exhibited lower affinity with dissociation constants of 150–420 nM for their respective on-targets (Fig. 1 and SI Fig. 1b).

Next, we measured binding to the seven potential off-target SH2 domains across the SFK family in the yeast-display format at 250 nM SH2 concentration (Fig. 2 and SI Fig. 1c). Three important observations can be made from these experiments. All monobodies showed the strongest binding to their on-targets. The pairs of monobody clones for the same target show similar selectivity profiles. Monobodies selected for the SrcA family (Yes, Src, Fgr) SH2 domains show weak or no binding to the SrcB family (Hck, Lyn, Lck, and Blk), and SrcB-targeting monobodies exhibited the opposite tendency (Fig. 2). Additional binding titrations for selected SH2 domains to several monobody clones revealed that the binding affinities for off-target SH2 domains were 5–10-fold lower than for the on-target (SI Fig. 1). Together, these results suggest that these monobodies are highly selective to either SrcA or SrcB.

In order to more precisely determine the thermodynamic binding parameters of the selected monobodies to their off-target SH2 domains, we prepared them as purified proteins and performed binding measurements using isothermal titration calorimetry (ITC). The binding affinities of selected monobody clones to their on-target SH2 domains were in the low to mid nanomolar range (Fig. 3), generally consistent with the yeast-display measurements. All measurements suggested a monobody:SH2 domain binding stoichiometry of 1:1 (Fig. 3). In all cases, the $\Delta H$ values were in the range of $-10$ to $-20$ kcal/mol, indicating that monobody binding is strongly enthalpically driven. Mb(Lck_1) and Mb(Lck_3) bound the Lck SH2 with similar affinities (23.5 nM and 7 nM, respectively; Fig. 3). Mb(Yes_1) was found to bind the Src SH2 domain with 38 nM affinity, which appeared much higher than observed in the yeast binding assay (Fig. 3 and SI Fig. 1).

Further ITC experiments were performed to determine binding affinities to off-target SH2 domains that were identified in the yeast-display binding assay (SI Fig. 2). In particular, Mb(Lck_1) and Mb(Lck_3) showed remarkable discrimination properties. Whereas their $K_d$ values for Lck, Blk, and Hck (SrcB family) were all between 6 nM and 40 nM (Fig. 3 and SI Fig. 2), we could not detect their binding to Yes and Src SH2 domains (SrcA family) at any tested protein concentration (SI Fig. 2). These results show that the selected monobodies bind their on-target SH2 domain with high affinity and display SFK subgroup selectivity.

**SFK SH2 monobodies inhibit SH2–pY interactions**

Our goal was to generate monobodies with high binding affinity but also with the ability to compete with SH2 pY ligand binding. We first determined if the selected monobodies blocked the pY peptide binding first using a peptide containing the pYEEI motif. This peptide bound with similar $K_D$ values of 310–675 nM to the eight SFK SH2 domains (SI Fig. 3). We produced the generated monobodies as purified proteins. The monobody Mb(Lyn_2) did not show a monodispersed gel-filtration profile and was therefore excluded from further analysis (data not shown). Most of the generated monobody clones robustly competed with pY peptide binding to their respective on-target SH2 domain, whereas a non-binding control monobody (HA4-Y87A [22]) showed no competition as
expected (Fig. 4a and SI Fig. 4). Interestingly, Mb(Lck_1) and Mb(Lyn_4) did not compete with pYEEI peptide binding (Fig. 4a–b). We then tested Mb(Lck_1) and Mb(Lck_3) using two additional pY peptides with lower binding affinity for the Lck SH2 domain (pYIIP: $K_d = 955$ nM, and pYQPQ: $K_d = 2200$ nM). Unlike the results with the pYEEI peptide, both monobodies compete against these pY peptides (Fig. 4b), suggesting that both monobodies are inhibitors of the Lck SH2–pY peptide interaction.

We next analyzed SFK subgroup binding selectivity using the pY peptide competition assay. All selected monobodies were tested against all SFK SH2 domains. In this assay, the submicromolar affinity of the pYEEI peptide necessitated the SH2 concentration to be in a similar range, and we also needed to use an excess monobody concentration (fivefold higher concentration than the SH2 concentration) to ensure that SH2 was nearly fully saturated with a monobody. This setup ensures that even low affinity binding to off-targets shows inhibition. We observed that the SrcA family-targeting monobodies were better inhibitors of the SrcA than the SrcB family SH2 domains, whereas the opposite was observed for the SrcB family-targeting monobodies (SI Fig. 4). These results are in line with the binding selectivity data (Figs. 2 and 3, and SI Fig. 1c and 2) and confirm the general selectivity of the monobodies for the SrcA or SrcB subgroups.

We next sought to study the potential of the developed monobodies as functional antagonists of SH2 domain ligand binding in a cellular proteome containing full-length proteins rather than isolated pY peptides. We therefore pulled down Lck SH2-interacting proteins from T-cell receptor-stimulated Jurkat cells with the recombinant biotinylated Lck SH2 domain. Competition with recombinant Mb(Lck_3) monobody, but not the non-binding control monobody HA4-Y87A [22], strongly reduced the overall interactions with tyrosine-phosphorylated Jurkat cell proteins (SI Fig. 5a). Importantly, the interaction with Zap70, which is phosphorylated on Y319, a known interactor of the Lck SH2 domain in activated T-cells, was efficiently disrupted by Mb(Lck_3) (Fig. 4c). As expected, Mb(Lck_3) was not able to compete with known interactors of the Src SH2 domain, as Mb(Lck_3) is not able to bind to it.

In summary, we have identified several monobodies...
that are SrcA- or SrcB-selective competitors of SH2–pY peptide and protein–ligand interactions.

**SFK monobodies bind to SFK family members with high selectivity in cells**

To evaluate the specificity of selected monobodies in complex cellular proteomes, we chose the chronic myeloid leukemia cell line K562 and the T-cell acute lymphoblastic leukemia cell line Jurkat, as each expresses a different spectrum of SFKs. Members of the SrcA group (Yes, Src, and Fyn) are expressed in both cell lines, whereas SrcB members are expressed in a cell-type-specific manner in either cell line: Jurkat cells express Lck, whereas K562 cells express Lyn [25,10,26]. We stably expressed

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**Fig. 3.** ITC measurements of different monobodies with SH2 domains. All calorimetric titration of the monobodies with SH2 domain were performed at 25 °C. Each panel shows (at the top) the raw heat signal of an ITC experiment. The bottom panel shows the integrated calorimetric data of the area of each peak. The continuous line represents the best fit of the data based on a 1:1 binding model computed from the MicroCal software. All experiments were performed in 25 mM Hepes (pH 7.5) and 150 mM NaCl. A representative measurement is shown for each example with $K_d$ value and stoichiometry (N) calculated from the fit. (a) Mb(Lck_1) (70 μM) titrated to Lck (7 μM), (b) Lck (115 μM) titrated to Mb(Lck_3) (11 μM), (c) Mb(Src_2) (180 μM) titrated to Src (12 μM), (d) Blk (142 μM) titrated to Mb(Lck_1) (13 μM), (e) Mb(Lck_3) (150 μM) titrated to Blk (15 μM), and (f) Mb(Yes_1) (215 μM) titrated to Lck (15 μM).
Mb(Src_2), Mb(Yes_1), Mb(Yes_3), Mb(Lck_1), and Mb(Lck_3) as tandem affinity purification (TAP)-tagged proteins in K562 cells, and Mb(Lck_1) and Mb(Lck_3) additionally in Jurkat cells (Fig. 5a). The monobodies were expressed at comparable levels in both cell lines, and the monobodies and their on-targets were efficiently retrieved after the second affinity purification step, as confirmed by immunoblot analysis (Fig. 5b and SI Fig. 5b–d). Captured proteins were visualized by silver staining and identified by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS; Fig. 5c–d, Table 1, and SI Tables 1–3). In the Mb(Src_2), Mb(Yes_1), and Mb(Yes_3) TAP experiments, Yes, Src, and Fyn were identified among the most abundant proteins by LC–MS/MS, whereas Mb(Lck_1) and Mb(Lck_3)

bound predominantly to Lck in Jurkat cells and to Lyn in K562 cells. These results are consistent with the dominant ~58-kDa bands detected by silver staining (Fig. 5c–d, Table 1, and SI Tables 1–3). In the biological replicate of the Mb(Lck_1) and Mb(Lck_3) TAP, other SFKs were identified in K562 and Jurkat cells, but at a much lower abundance (Table 1 and SI Tables 2–3). Importantly, no other SH2 domain-containing proteins were enriched in any Mb(Src_2), Mb(Lck_1), and Mb(Lck_3) samples, despite the expression of ~90 SH2 domains in these cells [23,26]. For Mb(Yes_1) and Mb(Yes_3), low levels of Btk and Tec kinases, which both contain an SH2 domain, were detected (Table 1 and SI Table 1). The LC–MS/MS result did not reveal any other off-targets.
These results demonstrate the ability of the studied monobodies to efficiently bind their full-length target protein in complex cellular proteomes. They only bind detectably to certain other SFK SH2 domains, but no other off-targets. To the best of our knowledge, these are the most selective reagents targeting SFK SH2 domains reported to date.

Structural basis for binding specificity of SFK SH2 monobodies

To understand the structural basis for SH2 binding and the observed selectivity of the SFK SH2 monobodies, we determined the crystal structures of three monobody/SFK SH2 complexes: Mb(Yes_1)/Yes SH2, Mb(Lck_1)/Lck SH2, and Mb(Lck_3)/Lck SH2 at 1.95, 2.85, and 2.40 Å resolution, respectively (Table 2 and Fig. 6). All these monobodies bound to the surfaces of their targeted SH2 domain that overlap with the pY peptide-binding pockets, as expected from their ability to inhibit SH2–pY peptide interactions (Fig. 6a–d). Mb(Lck_1) and Mb(Lck_3) had a very similar orientation with respect to the SH2 domain, whereas that of Mb(Yes_1) was distinct (Fig. 6b–d).

Mb(Yes_1) uses its long FG loop, the most extensively diversified region in the library (Fig. 1), to present an extended segment that is positioned perpendicular to the central SH2 β-beta sheet over
the peptide-binding cleft, mimicking the canonical backbone conformation of a pY ligand peptide (Fig. 6a and b). Tyr83 (residue numbering is according to Fig. 1) in the FG loop is inserted into the pY pocket of the SH2 domain (SIFig. 6a), where it makes cation–π interactions with Arg17 and Lys65 of SH2. Interestingly, a sulfate ion, possibly from the crystallization buffer, was found at the position that is occupied by the phosphate group of the pY peptide ligand in SH2–pY peptide complexes (SI Fig. 6a). Thus, the FG loop of Mb(Yes_1) mimics the backbone conformation and the pY moiety of a pY peptide ligand. This binding mode is

Table 2. Data collection and refinement statistics (molecular replacement)

| Cell line | Bait          | Total number of proteins identified | Number of proteins meeting the selection criteria | All identified SH2 domain-containing proteins in each sample (in brackets: total spectrum counts, rank***) |
|-----------|---------------|-------------------------------------|---------------------------------------------------|---------------------------------------------------------------------------------------------------|
|           |               | 1st TAP 2nd TAP 1st TAP 2nd TAP     |                                                   | 1st TAP 2nd TAP 1st TAP 2nd TAP                                                                   |
| K562      | Mb(Lck_1)     | 68  252  6  50                      | Lyn (112, 2); Lck (7, 34)                         | Lyn (1523, 1); Hck (141, 13); Lck (133, 15); Src (44, 38)                                        |
|           | Mb(Lck_3)     | 31  335  6  31                      | Lyn (53, 1)                                      | Lyn (1614, 1); Hck (172, 9); Lck (130, 15); Src (45, 32)                                        |
| Jurkat    | Mb(Lck_1)     | 33  285  3  23                      | Lck (114, 1); Lyn (1, 22)                        | Lck (1811, 1); Src (210, 6); Lym (138, 13); Hck (151, 16)                                      |
|           | Mb(Lck_3)     | 25  275  6  19                      | Lck (109, 1); Lyn (1, 19)                        | Lck (1930, 1); Lym (177, 6); Hck (162, 7); Src (159, 9)                                       |
| K562      | Mb(Src_2)     | 39  15                           | Src (35, 7); Yes1 (31, 8); Fyn (31, 8)           | Lyn (101, 4); Fyn (76, 8); Src (61, 7); Lyn (12, 20); Tec (6, 27); Btk (1, 47)                   |
|           | Mb(Yes_1)     | 46  13                           | Yes1 (101, 4); Fyn (139, 6); Src (107, 11)      | Lyn (32, 28); Btk (4, 105)                                                                      |
|           | Mb(Yes_3)     | 259  75                          | Yes1 (235, 1); Fyn (139, 6); Src (107, 11)      | Lyn (32, 28); Btk (4, 105)                                                                      |

* Proteins were scored as identified if at least two unique peptides were detected. The complete list of identified proteins is provided in Supplementary Tables 1–3.
** Selection criteria: Proteins with a selectivity score of >0.75 (present in <25% of all TAP monobody experiments from our internal database), with a total spectrum count of >1% of the most abundant protein in the sample, excluding different isoforms of keratin and the bait protein itself.
*** All identified proteins, including common contaminants, such as Keratin, were sorted by the number of assigned spectra from highest to lowest.

the peptide-binding cleft, mimicking the canonical backbone conformation of a pY ligand peptide (Fig. 6a and b). Tyr83 (residue numbering is according to Fig. 1) in the FG loop is inserted into the pY pocket of the SH2 domain (SIFig. 6a), where it makes cation–π interactions with Arg17 and Lys65 of SH2. Interestingly, a sulfate ion, possibly from the crystallization buffer, was found at the position that is occupied by the phosphate group of the pY peptide ligand in SH2–pY peptide complexes (SI Fig. 6a). Thus, the FG loop of Mb(Yes_1) mimics the backbone conformation and the pY moiety of a pY peptide ligand. This binding mode is

Table 2. Data collection and refinement statistics (molecular replacement)

|                | Mb(Lck_3)/Lck SH2 | Mb(Lck_1)/Lck SH2 | Mb(Yes_1)/Yes SH2 |
|----------------|------------------|------------------|-------------------|
| Data collection |                  |                  |                   |
| Space group    | P4,2,2            | P3,21            | I4,22             |
| Cell dimensions|                  |                  |                   |
| α, β, γ (°)    | 81.83, 81.83, 105.96 | 91.40, 91.40, 88.78 | 101.86, 101.86, 139.62 |
| Resolution (Å) | 40.92–2.40 (2.49–2.40) | 45.70–2.85 (2.95–2.85) | 50.00–1.95 (1.98–1.95) |
| Rmerge        | 3.0 (76.6)        | 6.2 (1.0)        | 0.13 (0.00)       |
| Rfree         | 37.91 (2.90)      | 23.79 (1.63)     | 24.44 (1.50)      |
| Completeness  | 100.0 (99.00)     | 100.0 (100.0)    | 99.8 (99.7)       |
| Redundancy    | 7.2 (7.5)         | 5.5 (5.7)        | 19.1 (16.1)       |
| Refinement    |                  |                  |                   |
| Resolution (Å) | 2.40              | 2.85             | 1.95              |
| No. of reflections | 14,544          | 10,316           | 27,104 (2649)     |
| Rfree/Rfree   | 0.20/0.25         | 0.23/0.27        | 0.19/0.22         |
| No. of atoms  | 1555             | 1424             | 1652              |
| Protein       | 2                | 15               | 33                |
| Ligand/ion    | 68.40            | 79.67            | 33.18             |
| Water         | 63.77            | 115.24           | 46.73             |
| B-factors     |                  |                  |                   |
| Protein       | 62.16            |                  | 45.10             |
| Bond lengths (Å) | 0.012            | 0.008            | 0.008             |
| Bond angles (°) | 1.16             | 1.31             | 0.84              |

* One crystal for each structure.
Fig. 6. Co-crystal structures of monobodies bound to SFK SH2 domains. (a) Structure pYEEI peptide (pink) bound to LCK-SH2 (pdb: 1LKK). The lower panel shows the atoms involved in the interaction with the epitope for the indicated peptide highlighted in pink. Epitope residues are defined as those atoms within 5 Å of an atom of the pYEEI peptide. (b) The monobody Mb(Yes_1) (blue) binding to the SH2 of Yes. (c) Mb(Lck_1) binding to SH2 domain of Lck, and (d) the binding of Mb(Lck_3) to Lck SH2. (e) Surface representation of Yes–SH2 domain showing the residues specific for SrcA or SrcB family members and the residues that are all identical in all eight SH2 domains. (f) Structural alignment of Mb(Lck_1) and Mb(Lck_3) paratope bound to Lck SH2 (upper panel). Mb(Lck_1) (cyan) and Mb(Lck_3) (bright green) residues involved in binding to Lck are shown as sticks. A pY peptide is shown in yellow. A detailed view of the CD loop residues of Mb(Lck_1) (middle panel) and of Mb(Lck_3) (lower panel) shows the arrangement of the residues compared to the +1 Glu in the pYEEI peptide (yellow sticks). Residue numbering of monobodies is according to Fig. 1.
very similar to that previously observed for the HA4 monobody bound to the Abl SH2 domain with a Cα rmsd for the complex of only 1.7 Å (SI Fig. 7) [22]. Mb(Yes_1) buries 702 Å² of the solvent-exposed surface of the Yes SH2 domain, which substantially extends beyond the core pY peptide-binding interface (346 Å² for the pYEEI peptide).

Although only 10 of the 20 diversified positions are identical between Mb(Lck_1) and Mb(Lck_3) (Fig. 1), these monobodies bind to Lck SH2 in a very similar

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**Fig. 7.** EF loop residues of SrcB family dictate the subgroup specificity of Mb(Lck) monobodies. (a) Co-crystal structure of Mb(Lck_3) bound to Lck SH2 domain with the zoomed region of FG loop (monobody) binding to the EF loop of Lck SH2 domain. The sticks shown in white are critical residues, which are specific for the three members of the SrcB group: Lck, Lyn, and Blk. The blue residues (also represented as sticks) are the amino acids found in Src at the same position. (b) ITC of Mb(Lck_3) to the Src SH2 domain (left panel) and Src-S78P/Q81T/N83P mutant (right panel). Each panel shows (at top) the raw heat signal of an ITC experiment. The bottom panel shows the integrated calorimetric data of the area of each peak. The continuous line represents the best fit of the data based on a 1:1 binding model computed from the MicroCal software if the data allowed the fitting of an isotherm. Both experiments were performed in 25 mM Hepes (pH 7.5) and 150 mM NaCl. A representative measurement is shown for each example with $K_d$ value and stoichiometry (N) calculated from the fit. The titration experiments were performed at 80 μM of Mb(Lck_3) titrated to 8 μM of Src SH2 and at 95 μM of Mb(Lck_3) titrated to 9 μM of Src- S78P/Q81T/N83P.
manner (Fig. 6c–d). Interestingly, in contrast to Mb(Yes_1) and in previously reported SH2-binding monobodies, Mb(Lck_1) and Mb(Lck_3) do not bind the pY pocket. Instead, their CD loops block the +3 specificity pocket of Lck SH2, and the rest of the diversified positions including the FG loop recognize the specificity pocket of Lck SH2, and the rest of the surface. On the opposite side of the Lck SH2 domain, the CD loops block the +3 positions of a pY peptide with the Lck SH2 domain. Mb(Lck_1) and Mb(Lck_3) bury 798 Å² and 693 Å², respectively, of the solvent-exposed surface of the Lck SH2 domain, which corresponds to 14% and 11% of the total surface.

When the two structures are superimposed using the Lck SH2 domain, the similarities and differences between Mb(Lck_1) and Mb(Lck_3) became clear. 11 residues contacting the SH2 domain (defined here as those within 5 Å of an SH2 atom) that are identical between the two monobodies have nearly identical conformations (Fig. 6e). Although their CD loops are the least conserved region between them, they both would clash with the +2 and +3 residues of a pY ligand peptide based on the superposition of the Lck SH2-pYEEI structure (Fig. 6e). The two CD loops take on distinct conformations (Fig. 6e). Ser44 of Mb(Lck_3) is located closer to the backbone of the +1 residue, possibly creating an additional steric clash (Fig. 6f). In contrast, the corresponding residue in Mb(Lck_1) is Pro44 that creates a kink in the backbone conformation and moves the CD loop away from the pY moiety. These observations suggest that the CD loop of Mb(Lck_3) is more effective in interfering with the binding of the pY moiety to the pY-binding pocket than the CD loop of Mb(Lck_1), although it is clear that both monobodies block the interactions of the +1, +2, and +3 positions of a pY peptide with the Lck SH2 domain. Indeed, when we replaced the CD loop of Mb(Lck_1) with that of Mb(Lck_3), this hybrid monobody competed with the pYEEI peptide and Mb(Lck_3) and retained the SFK subgroup selectivity (SI Fig. 4). These results demonstrate that the sequence and conformation of the CD loop are critical for efficient blockade of pY ligand binding of the Lck SH2 domain by Mb(Lck_1) and Mb(Lck_3).

We next aimed at understanding the structural basis for the ability of Mb(Lck_1) and Mb(Lck_3) to discriminate SFK SH2 subgroups. Among the epitope residues (defined as Lck SH2 residues within 5 Å of a monobody atom), we identified distinct amino acids at positions in the EF loop between the SrcA and SrcB SH2 domains, which make contacts with the SYG motif of FG loop of Mb(Lck_1) and Mb(Lck_3) (Fig. 7a). Three of these residues, Pro78, Thr81, and Pro83, are identical in Lyn and Blk but are more bulky or polar residues in the SrcA family (Fig. 7a and SI Fig. 9). To test the importance of these residues for subgroup selectivity, we mutated these three positions in the EF loop of the Src SH2 domain to the respective residues of Lck (S78P/Q81T/N83P; Fig. 7a). ITC measurements showed that Mb(Lck_3) bound to the S78P/Q81T/N83P mutant of the Src SH2 domain (Kd = 64 nM), whereas Mb(Lck_3) showed no detectable binding to the wild-type Src SH2 domain (Fig. 7b). Notably, the Kd value is less than 10-fold greater than that of Mb(Lck_3) to the Lck SH2 domain. As expected, Mb(Lck_3) acted as pY peptide competitor for the Src S78P/Q81T/N83P mutant protein (data not shown). These results indicate that the ability of monobodies to bind to the pY peptide-binding site and simultaneously to recognize the EF loop sequence enables them to discriminate SrcA versus SrcB SH2 domains.

**SFK-targeting monobodies activate recombinant Src and Hck kinase activity**

The SH2 domain of SFKs has a dual role in regulating kinase activity and signaling. In the autoinhibited conformation of SFKs, the SH2 domain stabilizes the clamped conformation by an intramolecular interaction with a pY residue in the C-terminal tail emanating from the kinase domain [5,27]. This C-terminal phosphorylation event is catalyzed by the Csk kinases. In contrast, in activated SFKs, the SH2 mediates intermolecular interactions that are critical to localize SFKs to particular signaling complexes and for processive multisite phosphorylation of substrates [7].

In order to test the effect of the monobodies on SFK kinase activity, we used a continuous spectrophotometric *in vitro* kinase assay (Fig. 8a) [28]. We chose the SrcA-selective Mb(Yes_1) monobody and the SrcB-selective Mb(Lck_3) monobody. We used recombinant Src (SrcA group) and chose Hck among the SrcB group, as Lck is more difficult to express. The SH3-SH2 kinase domain units of both SFKs were purified and assayed in the absence and presence of the inhibitory Csk kinase.

Mb(Yes_1) robustly activated the activity for Src in a concentration-dependent manner (Fig. 8b). The relative increase in Src kinase activity by Mb(Yes_1) was enhanced in the presence of Csk, as Csk decreases the basal Src activity. Mb(Yes_1) also activated Hck, but less potently, in line with its lower binding affinity and lower pY competition activity to Hck as compared to Src. When testing the Mb(Lck_3) monobody, we also observed a concentration-dependent increase in Hck kinase activity in the presence of Csk, whereas no activation of Src was observed in line with the lack of binding of Mb(Lck_3) to Src (Fig. 8c). A non-binding control monobody (HA4-Y87A [22]) with no affinity for SFK SH2 domains did not result in significant changes in kinase activity (SI Fig. 10a). Likewise, Mb(Yes_1) and Mb(Lck_3) had no effect on the activity of the isolated Src or Hck kinase domains in the absence or presence of Csk (SI Fig. 10b), demonstrating that the observed effects of these monobodies on kinase activation are SH2 domain dependent. These results suggest that SFK SH2 monobodies interfere with the
autoinhibited conformation of specific SFKs and activate kinase activity.

Lck-targeting monobodies inhibit proximal T-cell receptor signaling

We finally sought to assess whether the SFK monobodies interfere with SFK-dependent signaling events in cells. Given the critical role of the Lck SH2 domain in the activation of the Zap70 kinase in the activated T-cell receptor signaling complex [29], we set out to study the effects of Mb(Lck_1) and Mb(Lck_3) on Zap70 phosphorylation. We stably expressed Myc-tagged Mb(Lck_1), Mb(Lck_3), and the non-binding control monobody HA4-Y87A in Jurkat T-cell acute lymphoblastic leukemia cells, stimulated these cells with an anti-T-cell receptor (TCR) antibody, and monitored the phosphorylation of the activation loop of Zap70 (Y493; Fig. 9). We observed a strong decrease of Zap70 phosphorylation upon TCR activation and a mild decrease of basal Zap70 phosphorylation in Mb(Lck_3) expressing cells, as compared with HA4-Y87A expressing cells. Mb(Lck_1) had milder effects in line with its weaker pY competition activity (Fig. 9). Interestingly, the protein expression levels of Mb(Lck_3) were much lower as compared to Mb(Lck_1) and HA4-Y87A, while the mRNA levels were similar (Fig. 9 and data not shown). This may indicate a possible negative regulatory mechanism limiting Mb(Lck_3) protein expression. Collectively, these results show that Mb(Lck_3) can interfere with Lck-mediated signaling in cells and inhibit the activation of Zap70.

Discussion

The identification of selective inhibitors of SH2 domains is challenged by the high structural conservation of the pY peptide binding cleft and the expression of dozens of SH2-containing proteins in any given cell. While peptidomimetics and small molecule inhibitors with high binding affinities have been developed, their selectivity was only poorly characterized, as only one or a few, in most cases, evolutionarily distant SH2 domains, were tested as potential off-targets. In contrast, the monobodies to the SFK SH2 domains that we report on here have been rigorously characterized for their binding selectivity and binding mode.

We found that 8 of the 11 monobody clones that we characterized were strong antagonists of pY ligands, even though our selection was unbiased, that is, no positive selection for a specific epitope was included. These observations underline the notion of previous studies that showed monobody binding to hotspots of protein–protein interactions in structurally diverse targets including other SH2 domains [18,22,23,30,31].

Together with the three crystal structures presented here, we now have seven crystal structures of SH2 domain–monobody complexes and thereby can distinguish at least four different binding modes that strongly differ in the way the monobody engages the SH2 domain (SI Fig. 7). In particular, the observed structural mechanisms of inhibition of pY ligand binding are amazingly different. The Mb(Yes_1) and HA4 monobodies closely mimic a pY peptide ligand by a tyrosine residue in their FG loop and an inorganic sulfate and phosphate, respectively, in the pY pocket (Fig. 6). The Shp2 monobodies NSa1 and CS1 show a pY-independent mode of interaction with the Shp2 SH2 domains, while still mimicking the bound peptide,
and can bind the same epitope in two opposite orientations [23]. Lastly, the SH13 monobody binds a surface that is not overlapping with the pY ligand binding site of the Abl SH2 domain but that is involved in a critical allosteric intramolecular interaction with the Abl kinase domain [18]. Of note, more potent inhibitors of this allosteric interface could be developed using negative selection and a decoy competitor strategy [20,21].

The lack of competition between Mb(Lck_1) and the pYEEI peptide is noteworthy but may be caused by the specific assay conditions tuned to identify pY-competing monobodies for all SFK SH2 domains and the particular binding mode of Mb(Lck_1). In contrast, it is important to note that Mb(Lck_1) inhibited the binding of two other pY peptides that have lower affinity than pYEEI (Fig. 4b), indicating that Mb(Lck_1) is a less potent inhibitor than Mb(Lck_3). In line with this view, Mb(Lck_1) inhibits T-cell activation less pronounced than Mb(Lck_3) (Fig. 9). Upon T-cell receptor activation, Mb(Lck_1) inhibits T-cell activation less pronounced than Mb(Lck_3) (Fig. 9). Upon T-cell receptor activation, Mb(Lck_1) inhibits T-cell activation less pronounced than Mb(Lck_3) (Fig. 9).

The presented work provided us with additional insight on the important issue of monobody selectivity. We observed that all monobodies can strongly discriminate between the two SFK subfamilies—SrcA and SrcB. Given that most of the surface-exposed residues within the SrcA and SrcB families are identical (Fig. 6f), we believe that we have reached a limit of binding selectivity. To obtain single SFK-selective binders, we will require the identification of new binding modes, in which less conserved surfaces on the opposite side of the pY ligand binding cleft are targeted, while still maintaining pY ligand competition, and also the extensive use of negative selection.

A TAP-mass spectrometry (TAP-MS) experiment is a rigorous method to determine monobody selectivity in the physiologically relevant cellular context and signaling pathways of the monobody target. Importantly, the monobody is expressed in the complex and competitive environment of the cellular proteome and analyzed using unbiased mass spectrometry. Still, the cell-type-specific expression of certain SFKs and their relative expression levels need to be considered to interpret selectivity among all SFKs. Therefore, it appears that a combination of TAP-MS along with binding assays (Fig. 2) focusing on important potential off-targets is a well-suited approach to comprehensively assess monobody selectivity.

In conclusion, our results provide ample evidence that the SH2 domains of SFKs can be effectively and specifically targeted with monobodies with a variety of mechanisms of action. These monobodies can now be used as excellent tools to dissect SFK signaling in normal development and signaling and to interfere with aberrant SFK signaling network in cancer cells.
Targeting Src Kinase SH2 Domains with Monobodies

Materials and Methods

Cell lines

K562 and Jurkat cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Antibodies and reagents

Antibodies were purchased from Cell Signaling Technology [Yel (#3201), Lck (#2657), Zap70 (#2705, #2709), pZap70(Y319) (#2701), pZap70(Y493) (#2704)], Thermo Scientific [V5 Tag (#MA5-15253)], Streptavidin-Dylight650 (#84547), Millipore [T-cell receptor, clone C305 (#05-919)], Sigma Aldrich [Anti-Mouse IgG FITC antibody (#F0257)], and Rockland [Myc-tag (#600-432-381)]. Streptavidin Magnesphere Paramagnetic Particles (Promega #Z5481) were used for the recombinant SH2 pull-down and during the monobody selection.

Cloning

We have cloned the eight SFK SH2 domains (complete sequences and construct boundaries; SI Fig. 8) and all reported monobodies into a pHBT vector (modified pET vector) containing a 6xHis tag, tobacco etch virus (TEV) protease cleavage site, and an Avi-tag for biotinylation. SH2 domains and monobodies were cloned into the BamHI and Xhol restriction sites of the pHBT vector.

Biotinylation of Avi-tagged proteins

In vivo biotinylation was performed by co-transforming Escherichia coli cells with a BirACm plasmid and a pHBT plasmid containing the AviTag gene fusion. After the OD_{600} of the expression culture reached 0.5–0.8, cells were induced with 0.5 mM of IPTG, and a final Biotin concentration of 50 μM from a 50 mM stock in DMSO was added. Cells were further grown overnight before purification. In vitro biotinylation was performed using recombinant BirA. We added 100 μM AviTag-fused protein in 952 μL of PBS to 5 μL 1 M magnesium chloride, 20 μL 100 mM ATP, 20 μL 50 μM glutathione S-transferase-BirA, and 3 μL 50 mM D-Biotin. The samples were incubated for 1 h at 30 °C with gentle mixing on a rocking/shaking platform. Biotinylation efficacy was tested by a streptavidin-paramagnetic bead pull-down experiment.

Monobody selection

General methods for phage and yeast-display library sorting and gene shuffling have been described [16,22]. The monobody libraries used have been reported [32]. Briefly, after three rounds of phage display, selected loops were amplified and yeast cells were transformed with enriched monobody sequences. The yeast cells were sorted in a flow cytometer fluorescence-activated cell sorting (FACS) based on positive signal for monobody display and binding of the biotinylated target. Clones were isolated, sequenced, and cloned into pHBT plasmids.

Yeast binding assay

The yeast binding assay was performed as previously described [20]. Concentrations of biotinylated targets ranged from 10 nM to 5 μM in Tris-buffered saline (TBS) with 0.1% BSA. Target protein, yeast cells, and a mouse anti-V5 antibody were incubated for 30 min at RT. After two washing steps, streptavidin-DyLight650 and a FITC-coupled anti-mouse IgG were added and incubated for 30 min at room temperature. Samples were analyzed on a Gallios (Beckman Coulter) or BD Accuri C6 (BD Bioscience) flow cytometer. FACS based on positive signal for monobody display and binding of the biotinylated target. Clones were isolated, sequenced, and cloned into pHBT plasmids.

Protein expression and purification

BL21(DE3) E. coli cells were transformed with pHBT-SH2 or pHBT-monobody constructs. The overnight culture has been added to fresh LB to reach a starting culture between 0.05 and 0.1, and the cells were grown at 37 °C with 190 rpm until the OD reached 0.5–0.7. The culture was then induced with 0.5 mM IPTG final concentration and further grown at 24–27 °C for 5 h.

Cells were pelleted at 5000 g for 15 min at 4 °C. The pellets were resuspended in Buffer A (25 mM Tris (pH 7.5), 300 mM NaCl, 5% glycerol, and depending on sample, 1 mM DTT). An Avestin Emulsiflex homogenizer was used for lysis. The soluble protein was separated from cell debris by centrifugation at 45,000 g for 30 min at 4 °C. The protein was purified by affinity- and size-exclusion chromatography using Ni-NTA resin and a Superdex 75 10/300 GL column, respectively. Kinases were expressed as described previously [33].

ITC

ITC measurements were performed on a MicroCal ITC200 (GE) instrument. The proteins were extensively dialyzed against 25 mM Hepes (pH 7.5) and 150 mM NaCl and degassed; concentration was determined by measuring UV absorbance at 280 nm. The protein in the syringe was titrated in 16 steps with 0.49 μL for the first and 2.49 μL for the other steps. Concentrations were adjusted based on assumed affinities and signal strength of the interaction. The MicroCal software was used to determine thermodynamic parameters.
Fluorescence polarization

We used three different FITC-labeled pY-peptides: EPQpYEEIYGEFAKKK-(FITC) (abbreviated “pYEET” in the figures), (FITC)-TEGQpYQCPQA (“pYQPC”), and (FITC)-ADNDpYIPLPD (“pYIPL”). We mixed 125–500 nM peptide with TBS [50 mM Tris (pH 7.5) and 150 mM NaCl] + 0.5 mM TCEP and were run on a Superdex 75 10/300 GL column to separate complex from single protein. The purified complexes were concentrated in amicon vivaspin tubes to 10–17 mg/ml. The Mb(Yes_1)/YES SH2 complex was crystallized in hanging drop plates by mixing 100 nL of protein with 100 nL of buffer conditions. Later, crystals were optimized using a 24-well format (hanging drop) by mixing 1 µL of protein with 1 µL of buffer conditions. Best resolution data were achieved when using crystals grown in 2 M ammonium sulfate, 0.2 M lithium sulfate, and 0.1 M CAPS/NaOH (pH 10.25). The Mb(Lck_1)/Lck SH2 and Mb(Lck_3)/Lck SH2 complexes were crystallized using sitting drop plates. After several days, we observed crystals in several conditions. Best diffraction data were obtained from crystals grown in 0.2 M lithium sulfate, 0.1 M Tris (pH 8.5), 10% polyethylene glycol (PEG) 8K + 10% PEG1K for Mb(Lck_1)/Lck, and 0.1 M Mes (pH 6.5) and 15% PEG 20,000 for Mb(Lck_3)/Lck. Diffraction data for Mb(Yes_1)/Yes SH2 were collected at the Advanced Lightsource (SLS, Villigen, Switzerland). Crystal structures were determined by molecular replacement (molrep, CCP4, and PHENIX) using a monobody structure excluding loop regions (PDB 3UYO and 3K2M), Lyn SH2 structure (PDB 4TZI), and Lck SH2 structures (PDB 1LKK, 1LCK, and 1BHH). Manual model building, solvent addition, and refinement of all three structures were conducted iteratively using Coot and phenix.refine. The models were of good geometry, with 94% (Mb(Lck_1)/Lck SH2), 97% (Mb(Lck_3)/Lck SH2), and 96% (Mb(Yes_1)/Yes SH2) in favored regions of the Ramachandran plot and 1.1% (Mb(Lck_1)/Lck SH2), 0.5% (Mb(Lck_3)/Lck SH2), and 0% (Mb(Yes_1)/Yes SH2) of Ramachandran outliers.

Stable cell line generation for TAP-MS

Monobodies were cloned as an N-terminal fusion to the GS-TAP tag Gateway destination vector as previously described [22]. Jurkat and K562 cells stably expressing TAP-tagged monobodies were generated by retroviral infection and FACS sorting, as previously described [22]. Monobodies Mb(Lck_1), Mb(Lck_3), and HA4-Y87A were cloned into a hPGK-ires-GFP Gateway destination vector [gift from Dr. J. Huelsken (EPFL)]. Jurkat cells stably expressing the monobodies were generated by lentiviral infection and FACS sorting and used for the T-cell signaling studies.

TAP purifications

We used 2–3 × 10^9 K562 or Jurkat cells stably expressing the SFK-targeting monobodies for TAP purifications essentially as described in Ref. [34]. Protein complexes were eluted after the two affinity purification steps using 0.1 M hydrochloric acid and immediately neutralized with 0.5 M Triethylammonium bicarbonate. The efficiency of elution was checked by boiling the beads in SDS-PAGE sample buffer. Then, 10% of the eluates were resolved by SDS-PAGE (4–20% gel; Bio-rad) and silver stained, whereas the remaining eluate were prepared for mass spectrometric analysis.

Mass spectrometric analysis

The remaining part of each TAP experiment was separated by SDS-PAGE and stained with R-250 Comassie Blue Solution. Gel lanes were cut into pieces and subjected to in-gel digestion with trypsin. Extracted peptides were separated by reversed-phase chromatography on a Dionex Ultimate 3000 RSLC nano UPLC system (Dionex) on-line connected in line with either an Orbitrap Elite or an Orbitrap Q-Exactive Mass Spectrometer (Thermo Fischer Scientific). Raw data were processed with Proteome Discoverer (v. 1.3) and searched with Mascot against a human database (Uniprot release 2012_04; 86,747 sequences). Data were further processed, inspected, and visualized using the Scaffold 3 software.

In vitro kinase assays

Monobodies Mb(Yes_1), Mb(Lck_3), and HA4-Y87A at increasing concentrations (0 µM, 0.5 µM, 2.5 µM, 10 µM, 25 µM) were tested against these kinase combinations: (1) Src + Csk, (2) Src alone, (3) Src kinase domain (Src KD) alone, (4) Src KD + Csk, and (5) Hck + Csk, 6 Hck alone. All assays were performed with or without an Src optimal substrate peptide (AEEEIYGEFAKKK). Kinase activity was measured by a continuous spectrophotometric assay [28], where
substrate phosphorylation and ATP regeneration are coupled to NADH oxidation via pyruvate kinase/lactate dehydrogenase. The NADH concentration was monitored by absorbance at 340 nm in a Molecular Devices (SPECTRA Max 340PC384) plate reader. Reactions were performed at 30 °C in 75 μL volume containing 100 mM Tris (pH 7.5), 10 mM MgCl₂, 74 U/mL pyruvate kinase, 104 U/mL lactate dehydrogenase, 1 mM phosphoenolpyruvate, 0.282 mM NADH, 0.5 mM ATP, and 0.5 mM Src optimal substrate peptide. Reactions were started by the addition of 30 nM protein kinase with the exception of Src KD and Csk, where 16 nM and 80 nM were used, respectively. All reactions were performed in triplicates. Relative kinase activity toward substrate peptide was computed by subtracting kinase background activity from reaction containing the substrate peptide.

T-cell stimulation experiments for SH2 domain pull-down and signaling studies

Jurkat cells were stimulated with anti-TCR antibody clone C305 (Millipore) at a concentration of 100 ng/ml for 5 min at 37 °C. The cells were cooled to 4 °C immediately after stimulation by the addition of ice-cold PBS and centrifugation. Cells were lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, and protease and phosphatase inhibitors), and the protein content was quantified using the Bradford reagent. We used 50–100 μg of total cell lysate for immunoblotting analysis. Magnetic beads coated with streptavidin were incubated with either biotinylated Lck or Src SH2 domain for 2 h at 4 °C, washed three times with TBS, and then incubated for 30 min with a threefold excess of Mb(Lck_3) or HA4-Y87A as a control. After washing three times with TBS, the beads were incubated for 2 h with 3 mg of lysate from stimulated Jurkat cells. After three washing steps, the beads were boiled for 5 min in SDS-PAGE loading buffer, and bound proteins were analyzed by SDS-PAGE, followed by silver staining or immunoblotting.

Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 5MTJ (Mb(Yes_1)/Yes SH2), 5MTM (Mb(Lck_3)/Lck SH2), and 5MTN (Mb(Lck_1)/Lck SH2).

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2017.03.023.

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Abbreviations used:

SH2, Src homology 2 domain; pY, phosphotyrosine; SFK, Src family kinase; ITC, isothermal titration calorimetry; TAP, tandem affinity purification; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; TAP-MS, TAP-mass spectrometry; TEV, tobacco etch virus; PEG, polyethylene glycol; TCR, T-cell receptor; FACS, Fluorescence-activated cell sorting; TBS, Tris-buffered saline.
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