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Semisynthetic Src SH2 Domains Demonstrate Altered Phosphopeptide Specificity Induced by Incorporation of Unnatural Lysine Derivatives

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SUMMARY

Site-directed mutagenesis to the 20 natural amino acids becomes a limitation when evaluating subtle perturbations of an amino acid side chain within a protein. To further the study of Src homology 2 (SH2) domain ligand binding, we have developed a system allowing its semisynthesis from three fragments by native chemical ligation. We have replaced a key lysine residue with lysyl derivatives possessing progressively shorter aliphatic side chains. Biophysical characterization of these SH2 domain analogs has allowed for the first time a systematic dissection of the side chain length contribution from a lysine residue to ligand binding. We show that the specificity of the SH2 domain of the Src kinase can be altered by incorporation of such lysyl derivatives, thereby demonstrating the potential of the technique for the development of SH2 domain-based research tools and therapeutics.

INTRODUCTION

SH2 domains are modular interaction domains of ~100 amino acids that play fundamental roles in eukaryotic cell signaling (Kuriyan and Cowburn, 1997; Pawson, 1994; Pawson and Nash, 2003). The human genome consists of 120 different SH2 domains residing within 110 distinct proteins (Liu et al., 2006). They are dedicated to the recognition of tyrosine phosphorylated sequence motifs and regulate a number of essential cellular processes. Many signaling pathways intersect with SH2 domains, making them potential therapeutic targets, and mutations directly within SH2 domains are being progressively identified as disease associated (Lappalainen et al., 2008; Waksman et al., 2004). The Src SH2 domain resides within the protein tyrosine kinase Src and is involved in its recruitment and autoregulation of catalytic activity, thereby playing a pivotal role in its function (Cooper et al., 1986; Luttrell et al., 1988; Xu et al., 1997). Tyrosine phosphorylation by Src leads to cell signal propagation, and elevated levels of Src kinase activity have been directly implicated with cancer (Frame, 2002; Yeatman, 2004). It has also been shown that the isolated Src SH2 domain alone can elicit significant cellular responses such as halting of the cell cycle and enlargement of focal adhesions (Twamley-Stein et al., 1993; Avizienyte et al., 2002). The latter is a consequence of the SH2 domain's role in forming signaling complexes at the sites of focal adhesion proteins (Burnham et al., 2000; McLean et al., 2005). However, the precise role of Src during the various stages of tumorigenesis is still poorly defined (Yeatman, 2004). This is likely to be a result of the many different biological Src SH2 domain ligands that all bind with high affinity. Interestingly, Src knockout mice demonstrate only one phenotype, that being osteopetrosis (Boyce et al., 1992). This is due to a defect in the formation of the so-called ruffled border on osteoclasts rendering them ineffective at bone resorption. The significance of the noncatalytic regions of Src on this phenotype has been highlighted because the normal phenotype can be rescued by transgenic expression of kinase-deficient Src (Schwartzberg et al., 1997). These findings have provided impetus for the development of Src SH2 domain binding inhibitors that could be used to treat numerous degenerative bone disorders such as osteoporosis, Paget's disease, osteolytic bone metastasis, and hypocalcaemia associated with malignancy. Despite considerable investments into developing Src SH2 domain inhibitors, the broad specificity of Src family SH2 domains coupled with the strict requirement for a highly charged phosphomimetic has hindered progress and has largely been abandoned by pharmaceutical companies (Bradshaw and Waksman, 2002). However, inhibitors with therapeutic promise have been developed, and some even confer bone-targeting properties ensuring concentrations are localized at the osteoclast-bone interface (Shakespeare et al., 2000; Violette et al., 2000).

Previous attempts to engineer SH2 domains with altered or enhanced specificity have been met with unpredictable enthalpy-entropy compensation effects (Lubman and Waksman, 2002; Taylor et al., 2008), and library-based approaches only identified variants that had dual specificity, thus retaining affinity for the original ligand scaffold (Malabarba et al., 2001). Therefore, there remains a need for an unequivocal understanding of the Src SH2 domain's mode of ligand recognition. These points gave us impetus to attempt to rationally engineer SH2 domain analogs, by unnatural amino acid incorporation, that had genuine altered specificity and that could find application as research tools. Furthermore, protein therapeutics are becoming increasingly attractive (Leader et al., 2008), and such SH2 domain analogs may find application here, particularly when

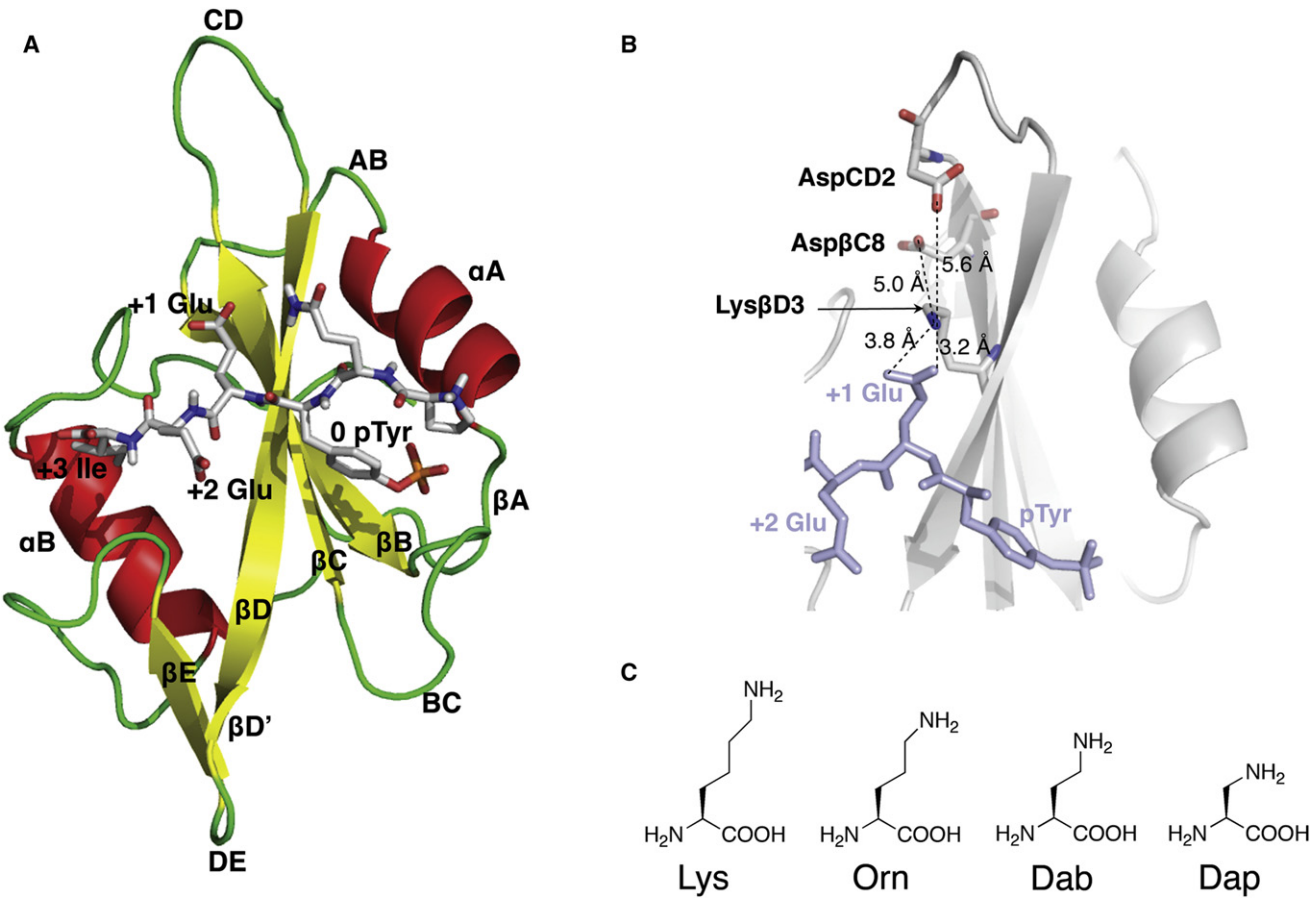

Figure 1. Src SH2 Domain Structure, Phosphopeptide Binding Site, and Incorporated Unnatural Lysine Derivatives

(A) Cartoon representation of the Src SH2 domain bound to a high-affinity pYEEI peptide (stick notation). The various secondary structural elements of the protein are labeled in accordance with Eck et al. (1993).

(B) Cartoon representation of the binding site of the Src SH2 domain. Key protein residues are depicted in stick notation. The phosphopeptide is in stick notation and colored blue. The +1 Glu residue of the phosphopeptide lies almost parallel with the side chain of LysBD3 providing the potential for hydrophobic and electrostatic contacts with each other.

(C) Lysine and lysyl derivatives: Orn, Dab, and Dap.

their pharmacokinetic profile can be improved by polymer attachment (Kochendoerfer, 2005), which could help to address the shortcomings of Src SH2 domain inhibitor development.

The Src SH2 domain consists of a central antiparallel β sheet flanked by two α helices (Figure 1A). The binding site consists of a region responsible for high-affinity phosphotyrosine (pTyr or pY) binding and an extended region, termed "specificity determining region" (Waksman et al., 1993). Specificity is achieved by cumulative interactions with the 3 residues C-terminal to the ligand's pTyr (termed "+1, +2, and +3 residues") (Songyang et al., 1993; Waksman et al., 1993). Songyang et al. revealed that the pYEEI phosphopeptide motif binds with the highest affinity to the Src SH2 domain. One of the most significant protein residues involved in specificity is LysβD3 (Figure 1B) (Bradshaw et al., 2000). In the structure of the Src SH2 domain-pYEEI complex, this residue lies within close proximity of the +1 glutamate (+1-Glu) of the pYEEI motif, suggesting that binding of +1-Glu is due to electrostatic interactions with LysβD3 (see Figure 1B, where the epsilon amino group of the Lys side chain is within 3.2 to 3.8 Å of the carboxylic group of the ligand's +1 Glu). However, this explanation turned out to be oversimplistic when it was realized that a mutation of +1-Glu to Ala in the pYEEI ligand had a negligible effect on binding to the wild-type SH2 domain (Bradshaw and Waksman, 1999), whereas a LysβD3Ala SH2 domain mutant exhibited a significantly decreased affinity for the pYEEI peptide (Bradshaw et al., 2000). Subsequently, it was shown that the decreased affinity displayed by the LysβD3Ala mutant was caused by a network of acidic residues further afield (AspβC8 and AspCD2) that exert a repulsive force on the +1-Glu in the absence of LysβD3 in the LysβD3Ala mutant (Figure 1B) (Lubman and Waksman, 2002). Thus, in the wild-type, the role of LysβD3 is to counter the negative electrostatic potential emanating from AspβC8 and AspCD2 rather than neutralizing the charge of +1-Glu. This also explained why little change in binding affinity was observed when mutating the +1-Glu to a series of residues including Asp and Ala (Bradshaw and Waksman, 1999).

The complexity of the role of LysβD3 in +1 residue binding led us to consider the βD3, βC8, and CD2 positions in the SH2 domain as potential sites for engineering altered binding affinity

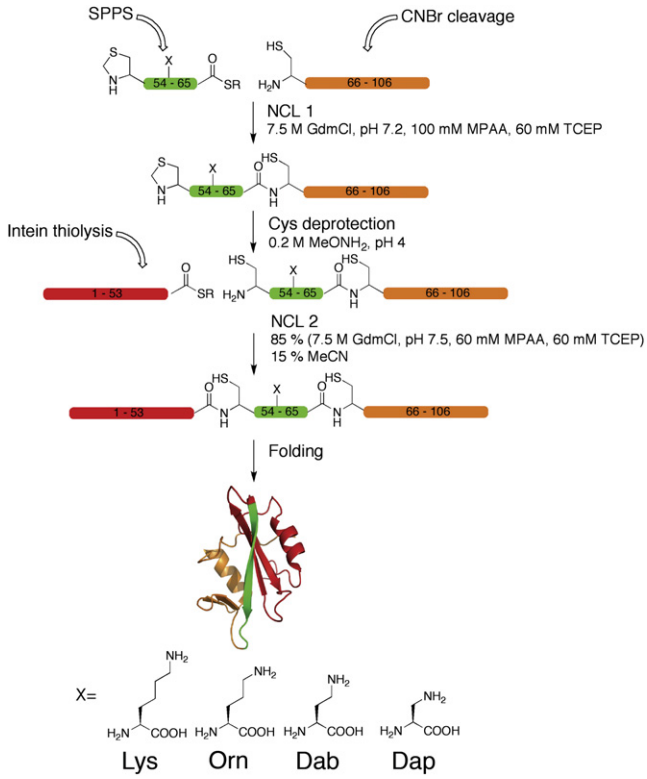

Figure 2. Three-Fragment Approach to Semisynthesis of the Src SH2 Domain

The small size of the synthetic cassette (green) allowed rapid and facile solid-phase synthesis of peptides which could be used to incorporate unnatural amino acids into the βD strand of the "specificity determining" region. The C-terminal αCys peptide (orange) was prepared by CNBr cleavage of a biosynthetic precursor and the N-terminal thioester peptide (red) was prepared by intein-mediated thiolysis of a biosynthetic fusion protein.

and specificity. Thus, AspβC8 and AspCD2 were mutated to Ala, resulting in a 2-fold increase in pYEEI binding affinity, possibly due to an increased electrostatic interaction of +1-Glu with LysβD3 (Lubman and Waksman, 2002). We also hypothesized that reduction of the aliphatic portion of LysβD3 residue would result in a weaker interaction with AspβC8 and AspCD2, thereby allowing the positive charge at the tip of the shorter aliphatic chain to be more easily directed toward and interact with a negatively charged residue at the +1 position of a phosphopeptide ligand. We further hypothesized that, because the tip of +1-Glu reaches out slightly beyond LysβD3 in the Src SH2 domain-pYEEI complex crystal structure (see Figure 1B), a shorter LysβD3 side chain would more easily be directed to a shorter acid at the +1 position, Asp for example. This would result in a change of binding specificity.

Here we report the engineering and binding thermodynamics of Src SH2 domains with progressively shorter lysine side chains at the βD3 position. Generation of such mutants was only possible using a semisynthetic route. Thus, in this report, we first present the semisynthesis of the Src SH2 domain. We also show that a three fragment semisynthesis of a relatively small domain can be achieved without extensive synthetic peptide synthesis. We then describe the preparation and characterization of semisynthetic Src SH2 domain analogs containing the unnatural amino acids ornithine (Orn), diaminobutyric acid (Dab), and diaminopropionic acid (Dap) (Figure 1C) at the βD3 position. This allows us to report for the first time the significance of the length of the aliphatic side chain of a lysine residue in protein-ligand specificity, and Src SH2 domain phosphopeptide recognition in particular. We show that fine-tuning of the specificity-determining region of the Src SH2 domain by incorporation of Dab enables an altered specificity and 5-fold enhancement in binding affinity for a peptide that possesses Asp at the +1 position. This affinity also surpasses that of the wild-type SH2 domain interaction with the pYEEI motif by 2-fold. This study also resolves an outstanding question regarding the hydrophobic contribution of the aliphatic chain of the Lys residues to the free energy of macromolecular interactions in general, and shows that the electrostatic interaction between lysyl derivatives and an acidic +1 ligand residue seems to be of primary importance in Src SH2 domain binding because affinity is retained when the nonpolar surface complementarity is reduced.

RESULTS

Semisynthetic Strategy

The semisynthesis relied on a three-fragments strategy where chemoselective peptide ligations were carried out by native chemical ligation (NCL) (Dawson and Kent, 2000) (Figure 2; see Figure S1 available online). The C-terminal αCys peptide SrcSH2$^{Cys66-106}$ was prepared by cyanogen bromide (CNBr) cleavage of a recombinantly derived precursor polypeptide. The middle peptide (SrcSH2$^{Thz54-65}$-Bn (benzyl) thioester) was a 12-mer representing the βD strand of the protein that harbored residue LysβD3 and was prepared by 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS). The N-terminal thioester fragment (SrcSH2$^{1-53}$-MES [mercaptoethanesulfonic acid] thioester) was prepared by thiolysis of a recombinant intein fusion (Muir et al., 1998). To assemble these peptides, we used a strategy based on that first adopted by Kent and coworkers (Bang and Kent, 2004).

By design, and also to solve some of the issues raised while setting up the protocol, four mutations were introduced (indicated in green in Figure S1). The native N-terminal Leu residue (residue 54 or LeuCD7; Figure S1) of SrcSH2$^{Thz54-65}$-Bn thioester was mutated to Cys and was protected during the first ligation reaction as the 1,3-thiazolidine-4-carboxo group (Thz) (Bang and Kent, 2004). The C-terminal residue of this peptide (residue 65 or AspDE1; Figure S1) was mutated from Asp to Gln because it has been shown that acidic residues can mediate the migration of the thioester moiety (Villain et al., 2003). Residue 66 (or SerDE2; Figure S1) of SrcSH2$^{Cys66-106}$ was also mutated from Ser to Cys to allow NCL. All of these sites resided in solvent-exposed CD and DE loop regions of the domain (Figure 1A) and had no observable influence on the binding energetics as determined by calorimetric characterization of a recombinantly prepared triple mutant Src SH2 domain (data not shown). The native peptide sequence of SrcSH2$^{1-53}$-MES thioester demonstrated very poor ligation efficiency due to formation of an internal thioester (thiolactone) with CysβC3 (residue 42; Figure S1). Consequently this residue was mutated to Ser. A SerβC3 mutant has previously been shown to result in a 8-fold increase in

affinity to the high-affinity pYEEI reference peptide (Bradshaw et al., 1999). Control experiments verified that this effect on the binding energetics was reproducible even within the context of the other Cys and Gln mutations mentioned above.

Chemical Synthesis of SrcSH2$^{Thz54-65}$ Thioester Peptides

SrcSH2$^{Thz54-65}$-Bn thioester harbored the LysβD3 residue (residue 57) that we wished to modify to unnatural lysyl derivatives and was prepared by Fmoc-SPPS employing *O*-(Benzotriazol-1-yl)-*N,N,N,N′*-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) coupling in conjunction with a sulfamylbutyryl safety-catch linker (Shin et al., 1999). Alkylation of the linker with iodoacetonitrile followed by treatment with benzyl mercaptan allowed concomitant thioester generation and cleavage from the resin. Syntheses were carried out with Fmoc-Lys(Boc)-OH, Fmoc-Orn(Boc)-OH, Fmoc-Dab (Boc)-OH, or Fmoc-Dap(Boc)-OH to generate the four SrcSH2$^{Thz54-65}$-Bn (benzyl) thioester variants at the βD3 position. All four SrcSH2$^{Thz54-65}$-Bn (benzyl) thioester variants contained the 1,3-thiazolidine-4-carboxo group to avoid cyclization. Peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography (RP-HPLC) (Figure S2A) and characterized by electrospray ionization mass spectrometry (ESI-MS) (Figure S2B).

Preparation of SrcSH2$^{Cys66-106}$ Peptide

As the length of fragments SrcSH2$^{1-53}$ thioester and SrcSH2$^{Cys66-106}$ was near the limit of efficient SPPS (Schnolzer et al., 1992), we chose to prepare these biosynthetically. This would also ensure the strategy could be readily adopted by biological researchers without expertise in chemical peptide synthesis. We sought to prepare SrcSH2$^{Cys66-106}$ by treatment of His$_6$-SrcSH2$^{54-106}$ (an available albeit insoluble construct containing residues 54 to 106 and an N-terminal His$_6$ tag) with CNBr (which cleaves C-terminal to Met residues) because we have successfully demonstrated its applicability for the preparation of insoluble Cys peptides of biosynthetic origin (Macmillan and Arham, 2004). This necessitated the mutation of the residue preceding Cys66 to Met. The precursor was purified by denaturing Ni^{2+} affinity chromatography and eluted at pH 4.5. Fractions corresponding to the precursor were further purified by semipreparative RP-HPLC employing a gradient of 10%–65% buffer B over 30 min with buffer A containing 0.1% trifluoroacetic acid (TFA) in H_2O and buffer B containing 0.1% TFA in MeCN. The product was lyophilized and dissolved in 80% formic acid at a concentration of 2.5 mg ml^{-1}. CNBr (100 Eq.) was then added and cleavage allowed to proceed for 18 hr at ambient temperature (Figure S3A). The solvent was removed under reduced pressure and the residue dissolved in 7.5 M guanidinium chloride (GdmCl). Then 50 mM NaOH and 50 mM DTT were added, and after 1 min the solution was buffered to pH 8 by addition of 100 mM Na_2HPO_4 followed by titration with HCl. This procedure disaggregated the solution and hydrolyzed undesirable formyl oxyester modifications. To achieve satisfactory separation of the Cys peptide, a RP-HPLC gradient of 30% aqueous 1:1 isopropanol/MeCN to 60% aqueous 1:1 isopropanol/MeCN was required (Figure S3B). Furthermore, this gradient system also allowed SrcSH2$^{Cys66-106}$ to be purified from a persistent formyl modification. The peptide could be prepared in satisfactory yield (37%) and of excellent purity (Figure S3C) and its identity was confirmed by ESI-MS (Figure S3D).

Preparation of SrcSH2$^{1-53}$ Thioester

The N-terminal peptide, SrcSH2$^{1-53}$ thioester, was prepared by bacterial expression of the first 53 residues of the Src SH2 domain fused to a splicing defective mutant of the GyrA intein from *Mycobacterium xenopi*. For that purpose, the SrcSH2$^{1-53}$ gene fragment was cloned in the pTXB1 vector (NEB). Chitin-affinity purification followed by sodium mercaptoethanesulfonate (MESNa) treatment in phosphate buffered 2 M urea at pH 6 enabled cleavage and generation of SrcSH2$^{1-53}$ thioester with excellent efficiency (>95%) as evaluated by SDS-PAGE (Figure S4A). Elution of the peptide was carried out in phosphate buffered 6 M urea at pH 6. Solid urea was added to the pooled fractions to a final concentration of 8 M followed by concentration. Disaggregation of the peptide required the addition of organic cosolvent (30% MeCN) and acidification with 1% TFA. The peptide was then further purified by RP-HPLC using standard conditions (Figure S4B) and its identity verified by ESI-MS (Figure S4C). The SrcSH2$^{1-53}$ thioester peptide was obtained in good yield of 2–2.2 mg per 1 l culture medium.

Native Chemical Ligation 1

Once peptides were in hand, the SrcSH2$^{Cys66-106}$ thioester was coupled to the synthetic SrcSH2$^{Thz54-65}$-Bn thioester containing the desired lysyl derivative by NCL in a ratio of 1:1.2, and monitored to completion by analytical RP-HPLC (Figure 3). Ligation under denaturing conditions (sodium phosphate buffer at pH 6.9–7.2 containing 7.5 M guanidinium chloride [GdmCl], 100 mM mercaptophenylacetic acid [MPAA] and 60 mM tris [2-carboxyethyl] phosphine [TCEP]) was 95% complete after 3 hr and conversion of the N-terminal 1,3-thiazolidine-4-carboxo group to Cys was achieved by direct addition of 200 mM methoxylamine hydrochloride ($MeONH_2$·HCl) (Figure 3). The pH was adjusted to 4 and deprotection left to proceed overnight. The SrcSH2$^{Cys54-106}$ product was purified in 61% yield by semipreparative RP-HPLC (23%–48% buffer B; buffer B = 0.1% TFA in MeCN). Fractions containing the peptide were verified by ESI-MS.

Native Chemical Ligation 2 and Protein Folding

Native chemical ligation between SrcSH2$^{Cys54-106}$ and SrcSH2$^{1-53}$-MES thioester was also carried out under denaturing conditions in a buffer (300 mM HEPES [pH 7.5], 7.5 M GdmCl, 60 mM MPAA, 60 mM TCEP) containing 15% acetonitrile as organic cosolvent. Cosolvent was necessary otherwise SrcSH2$^{1-53}$-MES thioester peptide had a high propensity to aggregate, presumably due to β sheet formation as the peptide encompasses a β sheet-rich region of the SH2 domain. However, when ligations were carried out in this buffer, over time the mass of unreacted SrcSH2$^{Cys54-106}$ was found to increase by 42 Da as detected by ESI-MS and the ligation would stall. When the reaction was treated with a higher concentration of thiol (200 mM MESNa), the increased mass of SrcSH2$^{Cys54-106}$ was still observed. From these observations we deduced that SrcSH2$^{Cys54-106}$ was becoming irreversibly modified at the N terminus. An explanation is that reaction with isocyanic acid

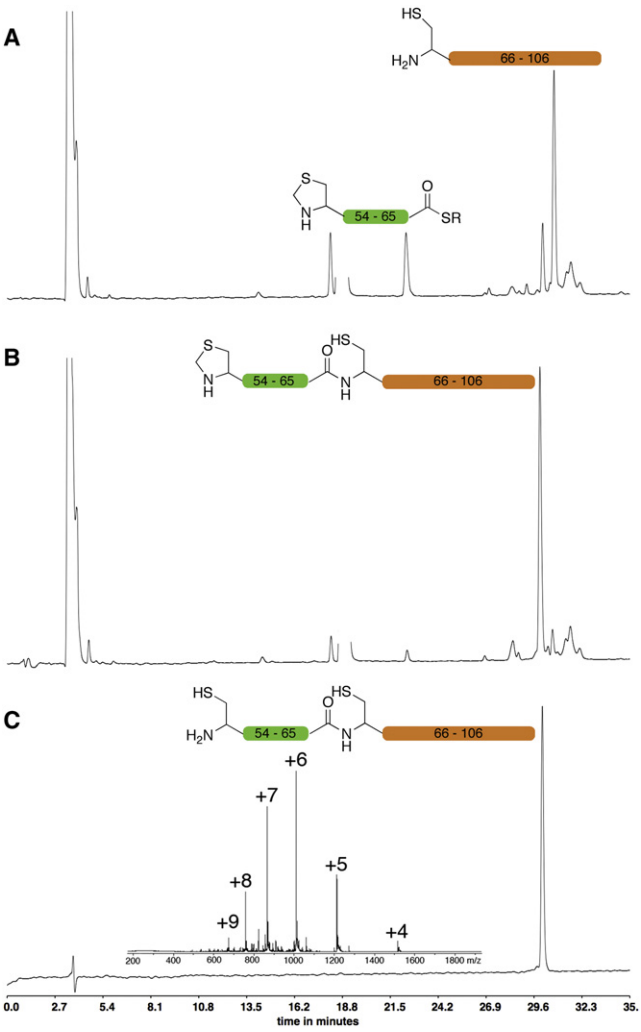

Figure 3. Representative Ligation between SrcSH2$^{Cys66-106}$ and SrcSH2$^{Thz54-65}$-Bn Thioester Monitored by Analytical RP-HPLC

(A) Reaction at t = 5 min. Two peaks at t_R = 18.2 and 22.4 min correspond to SrcSH2$^{Thz54-65}$-Bn thioester and in situ generated MPAA thioester, respectively. Peak at t_R = 30.9 min corresponds to SrcSH2$^{Cys66-106}$.
(B) Reaction at t = 3 hr. Peak at t_R = 30 min corresponds to SrcSH2$^{Thz54-106}$.
(C) The purified and deprotected ligation product SrcSH2$^{Cys54-106}$. Inset is an ESI-MS spectrum confirming deprotection to Cys for the OrnβD3 incorporation. Observed mass = 6093.8 Da; theoretical = 6094.1 Da.

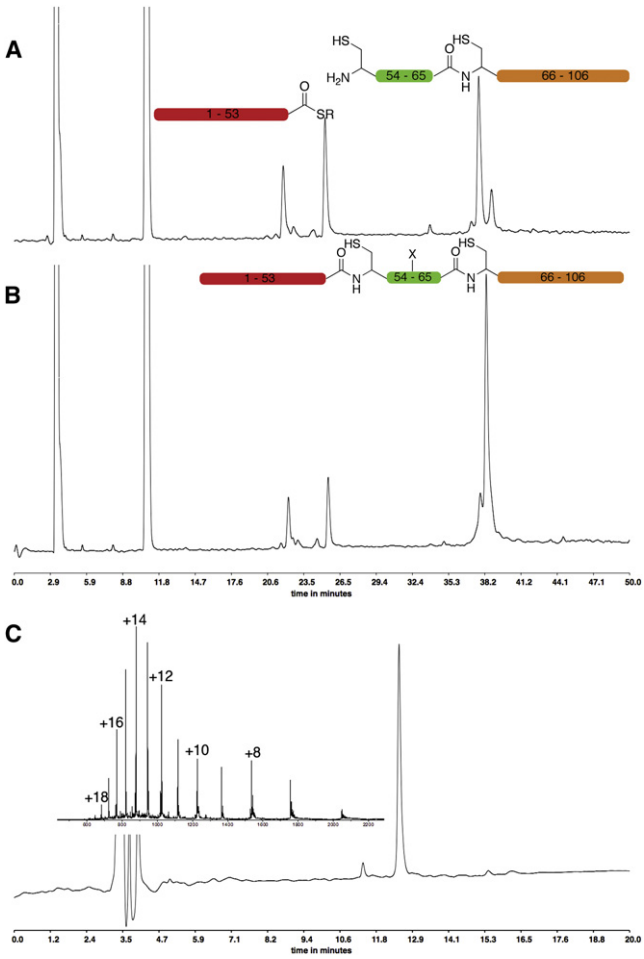

Figure 4. Representative Ligation between SrcSH2$^{Cys54-106}$ (OrnβD3) and SrcSH2$^{1-53}$-MES Thioester Monitored by Analytical RP-HPLC

(A) Reaction at t = 5 min. Two peaks at t_R = 22.5 and 26.0 min correspond to SrcSH2$^{1-53}$-MES thioester and in situ generated MPAA thioester, respectively. Peak at t_R = 37.6 min corresponds to SrcSH2$^{Cys54-106}$.
(B) Reaction at t = 90 min. Peak at t_R = 38.6 min corresponds to SrcSH2$^{1-106}$.
(C) RP-HPLC purified SrcSH2$^{1-106}$ and ESI-MS characterization (inset). Observed mass = 12,288.0 Da; expected = 12,287.9 Da.

was giving rise to carbamylation (+43 Da). Isocyanic acid is a product of urea degradation, and because the SrcSH2$^{1-53}$ thioester was treated with urea for prolonged periods, such a side reaction seems plausible. Analysis of the isolated SrcSH2$^{Cys54-106}$ peptide in the MeCN-containing buffer by RP-HPLC showed that the modification was occurring at a rate comparable to that of MPAA catalyzed chemical ligation. In subsequent reactions we simply used SrcSH2$^{1-53}$-MES thioester in a ~1.5 fold excess and incubated it for 1 hr in ligation buffer prior to addition of the Cys peptide. This ensured the majority of the thioester peptide had undergone exchange with MPAA, which has been shown to be rate limiting in a NCL reaction (Johnson and Kent, 2006). This allowed ligations to be carried out to ~80% completion in 1.5 hr with minor loss of SrcSH2$^{Cys54-106}$ through carbamylation (Figure 4). Importantly, none of the purified full-length SrcSH2$^{1-53}$ polypeptide was found to be modified.

The full-length SrcSH2$^{1-106}$ polypeptide was purified by semi-preparative RP-HPLC and lyophilized with a yield of 21% for the NCL reaction. Masses for the syntheses of the 4 SH2 domain derivatives (termed "Src SH2 Lysβ3, OrnβD3, DabβD3, or DapβD3 domains") were determined by ESI-MS (Figure 5; Figures S5A and S5B). The proteins were folded by slow drop-wise addition while stirring into 10 volume equivalents of ITC buffer (see details in Experimental Procedures). Folded proteins were further analyzed by SDS-PAGE (Figure S6). The yield of the refolding process was 70% and the total yield of folded material based on the starting peptide (SrcSH2$^{Cys66-106}$) was ~9%. The raw calorimetric data and binding isotherms for all the interaction studies are presented in Figure 6. The thermodynamic parameters are summarized in Table 1.

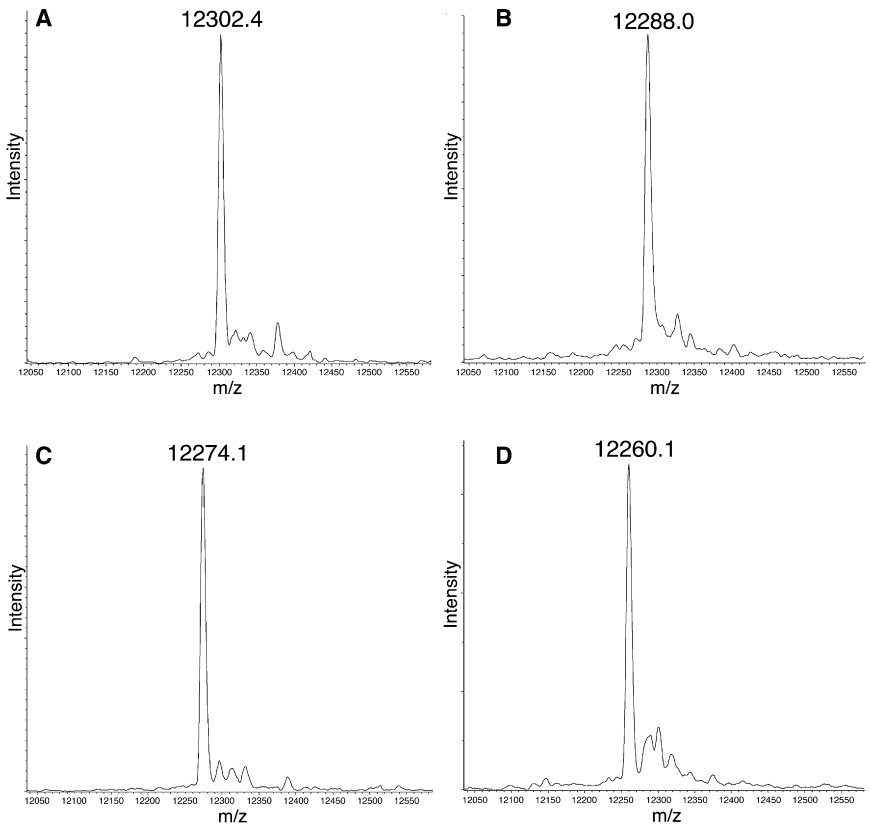

Figure 5. Deconvoluted ESI-MS Spectra of the Purified Src SH2 Variants

(A) Src SH2(LysβD3), expected mass = 12,301.9 Da.
(B) Src SH2(OrnβD3), expected mass = 12,287.9 Da.
(C) Src SH2(DabβD3), expected mass = 12,273.9 Da.
(D) Src SH2(DapβD3), expected mass = 12,259.9 Da.

Isothermal Titration Calorimetry

The calorimetric data for the reference pYEEI peptide binding to the semisynthetic Src SH2 LysβD3 domain (which, as indicated above, contains four mutations [indicated in green in Figure S1]) were in excellent agreement with those obtained for the recombinant CysβC3Ser Src SH2 domain (Bradshaw et al., 1999), thus indicating that the methodology produced a correctly folded protein with full binding potential. Raw calorimetric data and integrated binding isotherms are shown in Figure 6. As expected, conservative replacement of LysβD3 with OrnβD3 produced a Src SH2 domain with similar affinity and thermodynamic signature. However, there was a modest 1.2-fold improvement in affinity attributable to an increase in $T\Delta S$ compensating for a reduced ΔH relative to LysβD3. This trend was more pronounced for pYEEI binding to the Src SH2 DabβD3 domain. Binding affinity was reduced 2-fold as a result of a reduction in ΔH from -7.5 to -5.3 kcal mol^{-1} despite an associated increase in $T\Delta S$ from 2.3 kcal mol^{-1} to 4.0 kcal mol^{-1}. A possible explanation for this observation is that the reduced side-chain length of the βD3 residue compromises the geometry of the electrostatic interaction of the γ-amino group with the carboxylate of +1-Glu. Such observations are difficult to assign, but because the carboxylate of +1 Glu is now presumably forming a weaker interaction, and the γ-amino group would now be positioned such that it is in proximity of the aliphatic component of the +1 Glu residue, it may increase mobility. Intrigued by the capacity of the DabβD3 to reduce the binding free energy for the pYEEI peptide, we characterized the binding event of the Src SH2 DabβD3 domain to the pYDEI peptide. Gratifyingly, we observed a 5-fold enhancement in binding affinity compared with Src SH2 DabβD3 binding to pYEEI and rescue of the thermodynamic signature obtained with Src SH2 LysβD3 domain binding to the pYEEI peptide ($T\Delta S$ reduced to 2.5 kcal mol^{-1} but a much more favorable ΔH of -7.6 kcal mol^{-1}). In fact, affinity was ~2-fold higher than semisynthetic Src SH2 LysβD3 binding to the pYEEI peptide, making this interaction the tightest binding Src SH2 domain interaction with a singly phosphorylated peptide to date. To verify that the enhanced affinity for pYDEI was directly attributable to DabβD3, we characterized pYDEI binding to a recombinant Src SH2 LysβD3 domain containing all four "ligation" mutations (indicated in green in Figure S1; these are the mutations referred to above that were required for the design of the synthetic Src SH2 domains described here). We have shown that this quadruple mutant recapitulates the binding properties of the synthetic LysβD3 SH2 domain and thus constitutes a valid source of material for the experiment we wished to conduct. Binding affinities of this quadruple Src SH2 domain mutant for the pYDEI or pYEEI peptides were found to be similar (Figure S7; Table 1), in accordance with previous data obtained on the wild-type SH2 domain where no difference in affinity was observed between pYEEI and pYDEI binding (Bradshaw and Waksman, 1999). Thus, enhanced affinity of the Src SH2 DabβD3 domain for the pYDEI peptide is indeed due to the presence of Dab at the βD3 position of the SH2 domain. Mechanistically it is difficult to explain the reduced affinity for pYEEI but retained affinity for pYDEI. However, as the DabβD3:pYDEI interaction is strongly enthalpic, we hypothesize that the γ-amino group of DabβD3 forms a more idealized interaction geometry with the carboxylate of +1 Asp relative to +1 Glu present in pYEEI. This may be augmented by the fact that the γ-amino group is too short to interact with AspCD2 and as such is not conformationally dictated by this residue. Because the +1 Asp side chain is shorter, the γ-amino group would be less likely to clash with the aliphatic component of +1 Asp, which would help to ascribe the reduced $T\Delta S$ observed with pYDEI relative to pYEEI. Other factors such as facilitation of solvent coordination may also be at play. The binding of pYEEI and pYDEI to semisynthetic SH2 domains incorporating DapβD3 were also characterized. The Src SH2 DapβD3 domain had a striking 16-fold reduction in affinity for the pYEEI peptide attributable to a reduced ΔH and no significant compensatory $T\Delta S$ as

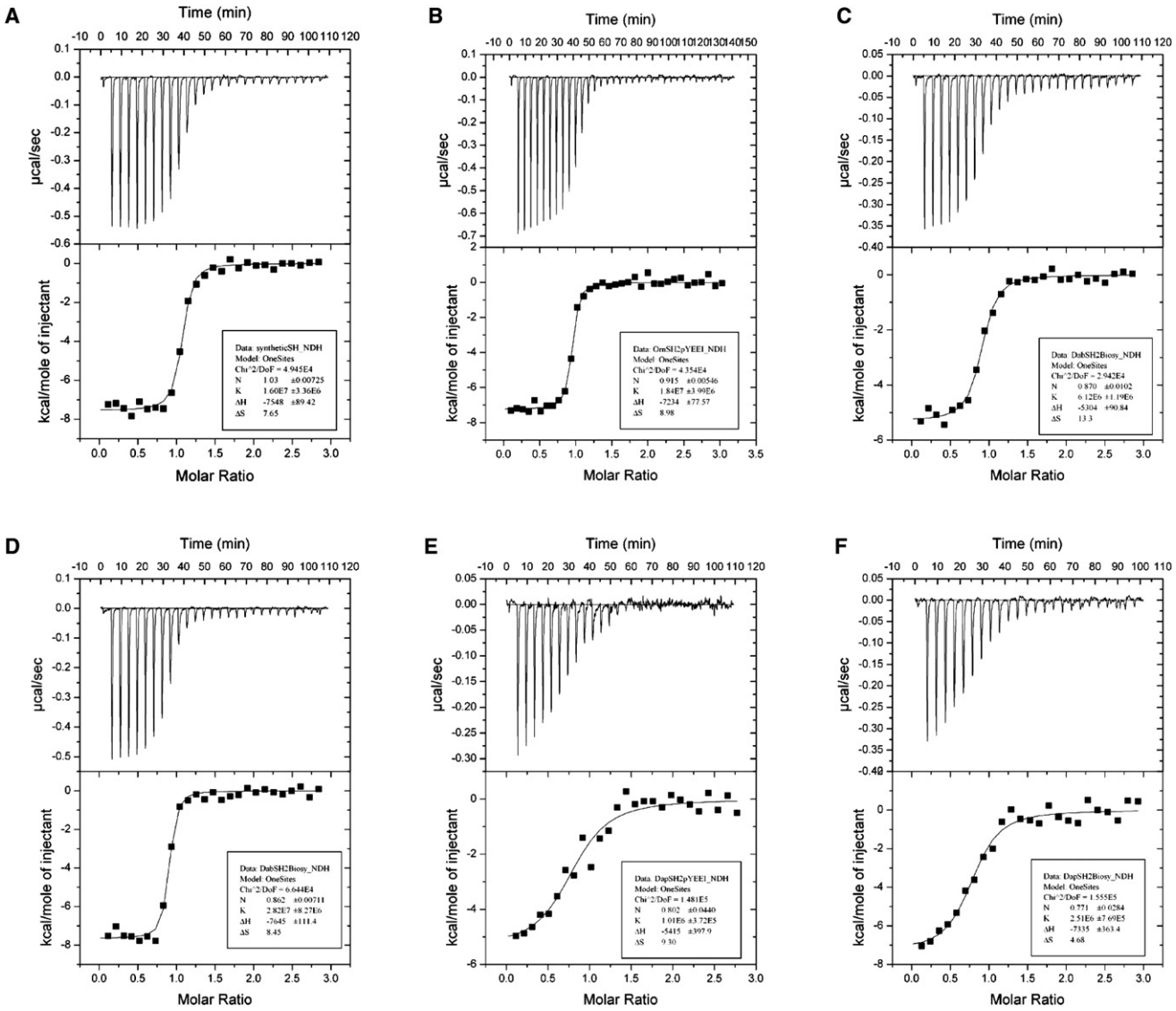

Figure 6. ITC Binding Isotherms for pYEEI and pYDEI Peptide Binding to Semisynthetic SH2 Domains

(A) Semisynthetic Src SH2(LysβD3) domain titration against pYEEI reference peptide.
(B) Semisynthetic Src SH2(OrnβD3) domain titration against pYEEI reference peptide.
(C) Semisynthetic Src SH2(DabβD3) domain titration against pYEEI reference peptide.
(D) Semisynthetic Src SH2(DabβD3) domain titration against pYDEI peptide.
(E) Semisynthetic Src SH2(DapβD3) domain titration against pYEEI reference peptide.
(F) Semisynthetic Src SH2(DapβD3) domain titration against pYDEI peptide.

observed for DabβD3. Based on theoretical calculations, the pK_a of the β-amino group of Dap would be 8.1 such that only ~80% of the amine would be protonated at the experimental pH of 7.5. This is in contrast to the other derivatives, which would be > 99% protonated. Therefore, reduced affinity for the DapβD3 analog may also be attributable to partial amine protonation reducing the capacity to form electrostatic interactions. It should also be noted that short diamino acids such as Dap are prone to N-N acyl shifts, which would perturb the protein backbone and may account for the observed low affinity. However, based on the sharp peaks we obtained after HPLC purification of all the synthetic peptides (Figure S2A), we conclude that such rearrangements were not occurring. Binding of the Src SH2 DapβD3 domain to pYDEI is improved compared with pYEEI, due to a more favorable ΔH of binding. However, $T\Delta S$ was now the lowest of all of interactions characterized. This may be due to the DapβD3 side chain being too short to influence the conformational flexibility of the phosphopeptide residue.

DISCUSSION

A system has been developed allowing the semisynthesis of the Src SH2 domain from three peptide fragments. The methodology as described herein allows unnatural amino acid

Table 1. Thermodynamic Data for pYEEI and pYDEI Binding to the Semisynthetic SH2 Domains

SH2 Domain	Ligand	K_d (nM)	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	$T\Delta S^\circ$ (kcal mol^{-1})
LysβD3	pYEEI	63	−9.8	−7.5	2.3
LysβD3[a]	pYDEI	70	−9.7	−7.7	2.1
OrnβD3	pYEEI	54	−9.9	−7.2	2.7
DabβD3	pYEEI	163	−9.2	−5.3	4.0
DabβD3	pYDEI	35	−10.2	−7.6	2.5
DapβD3	pYEEI	990	−8.2	−5.4	2.8
DapβD3	pYDEI	398	−8.7	−7.3	1.4

[a] Data collected for a *recombinant* quadruple "ligation" mutant Src SH2 domain.

incorporation into the βD strand of the protein as the peptide representing this component was synthesized chemically. However, further reliance on SPPS could in principal be adopted to incorporate unnatural amino acids at any site of the protein. To resolve ambiguities associated with the LysβD3 residues role in phosphopeptide specificity, and to try and prepare Src SH2 domain analogs with altered specificity, we incorporated unnatural lysyl derivatives with progressively shorter side chains at this position. Following a hypothesis-driven line of investigation, we incorporated the lysyl derivatives Orn, Dab, and Dap at the βD3 position. The Src SH2 DabβD3 and DapβD3 domains bound to the pYEEI reference peptide with a 2.5- and 16-fold reduction in affinity, respectively. However, the DabβD3 analog demonstrated a 5-fold preference for pYDEI versus pYEEI. Also, DabβD3:pYDEI interaction is twice as strong as the reference LysβD3:pYEEI interaction. It is thus the first time that a change in specificity resulting in an affinity higher than the one observed for the reference interaction is observed. The change is admittedly small but all perturbations (except for dephosphorylation) brought to the Src SH2 domain have been shown to have only small incremental effects. The data illustrate how high-affinity interaction at the +1 interface of the Src SH2 domain seems to be governed by the geometry of the electrostatic interaction between the lysyl βD3 residue and the +1 phosphopeptide residue. Owing to the various biological tyrosine phosphorylated substrates that have Glu and Asp at the +1 position (Table S1), the semisynthetic Src SH2 DabβD3 domain prepared as described herein may have application as a research tool by competing favorably with +1 Asp-containing phosphorylated Src SH2 domain substrates. Although not tested due to difficulties in preparing sufficient material, altered and desirable specificity changes may also exist with other +1 residues, such as hydrophobic amino acids that exist in biologically relevant Src SH2 domain targets. Further optimization may provide access to higher affinity Src SH2 domains with altered specificities, which could be further modified thus improving the pharmacokinetic profile and yielding protein therapeutics.

SIGNIFICANCE

Presented herein are to our knowledge the first examples of biophysically characterizing the significance of the length of the aliphatic component of the lysine side chain in macromolecular recognition, and reengineering of the prototype interaction module, the Src SH2 domain, such that it demonstrates altered specificity. The methodology adopted is within reach of biologists and shows that challenging peptide sequences, compatible with chemical ligation, can be prepared with recombinant DNA technology. Studies have been conducted with the lysyl derivatives discussed within, but only with regard to their involvement in intrahelical interactions (Cheng et al., 2007). It was found that intrahelical Lys-Glu pairing resulted in a low-energy conformer that could not be adopted with Orn-, Dab-, or Dap-Glu interactions, and these observations were taken as partial reasoning for the natural selection of the lysine amino acid. Within the Src SH2 domain, LysβD3 does not partake in intrahelical interaction so the role of the lysyl derivatives in this study has been elucidated with regard to protein-ligand recognition only. Because it has been demonstrated that the affinity of an interaction involving Lys can be enhanced by replacing it with Dab, it seems as though nature's choice for this residue is more convoluted. The lysine side chain is subject to a myriad of posttranslational modifications such as ubiquitination, SUMOylation, acetylation, and mono-, di-, and tri-methylation, to name but a few. The Lys side chain ensures the amino group is positioned further from the protein backbone than the other derivatives and also confers flexibility. The significance of these traits in the ability of posttranslational modifications to be performed by ubiquitin ligases, acetylases, and deacetylases, for example (Seet et al., 2006), may facilitate dynamic posttranslational modification as the amino nucleophile is less hindered and can adopt more conformations for recognition.

EXPERIMENTAL PROCEDURES

Preparation of SrcSH2$^{Cys66-106}$

The cDNA corresponding to residues 197-249 (Swiss-Prot ID P63185) was amplified by polymerase chain reaction (PCR) using the forward 5′-C ACC GAG AAT TTA TAC TTC CAA TGC AAT GTG AAG CAC TAC AAG ATC CGC AAG CTG-3′ primer and the reverse 5′-TTA CGT GGG GCA GAC GTT GGT CAG GCG-3′ primer. This allowed directional cloning into the pET151-D vector (Invitrogen) in accordance with the manufacturers protocol. Residues 208 and 209 were subsequently mutated to Met and Cys, respectively, by QuikChange mutagenesis using the forward 5′-C TAC AAG ATC CGC AAG CTG ATG TGC GGC GGC TTC TAC ATC ACC-3′ primer and the reverse 5′-GGT GAT GTA GAA GCC GCC GCA CAT CAG CTT GCG GAT CTT GTA G-3′ primer. Correct cloning was confirmed by DNA sequencing. Plasmid DNA was transformed into BL21-AI cells (Invitrogen) and a transformant used to inoculate an overnight Luria-Bertani (LB) starter culture supplemented with ampicillin (100 μg ml^{-1}). The culture was diluted 3:100 with fresh LB media (1 l) supplemented with ampicillin (100 μg ml^{-1}). Cells were grown to mid-log phase at 37°C and induced with L-arabinose (0.2%) and IPTG (0.5 mM). After 3 hr the cells were harvested, suspended in lysis buffer (20 mM Tris [pH 7.4], 500 mM NaCl), and lysed with a cell disruptor (Avestin). The lysate was clarified by centrifugation (39,000 × g, 30 min) and the pellet dissolved in binding buffer (100 mM Tris, 100 mM NaH_2PO_4 [pH 8], 6 M GdmCl) supplemented with 2 mM DTT and stirred for 2 hr. The suspension was then clarified by centrifugation (45,000 × g, 30 min) and the supernatant loaded onto a 5 ml Ni-NTA column (QIAGEN). The column was washed with 10 volumes of binding buffer and the polypeptide eluted with elution buffer (100 mM Tris, 100 mM NaH_2PO_4 [pH 4.5], 6 M GdmCl). Fractions corresponding to the polypeptide were brown in color and were pooled and desalted by multiple semipreparative RP-HPLC

runs. A gradient of 10%–65% B, with an initial 5 min isocratic stage, was applied over 25 min at a flow rate of 4.7 ml min^{-1}. Buffer A = 0.1% TFA in H_2O, Buffer B = 0.1% TFA in MeCN. Fractions were combined and analyzed by ESI-MS (observed mass = 10,661.2; expected mass = 10,660.2). The pool was then lyophilized yielding ~25 mg l^{-1} culture medium. The peptide was dissolved in nitrogen saturated 80% aqueous formic acid at a concentration of 2.5 mg ml^{-1}. In a fume hood, 100 molar equivalents of solid CNBr were added and the reaction left to stir under nitrogen with the exclusion of light for 18 hr. Solvent was then removed under reduced pressure and the residue dissolved in 7.5 M GdmCl at a concentration of 2.5 mg ml^{-1}. The solution was basicified by addition of NaOH (50 mM) and stirred for 1 min. Solid DTT (50 mM) was then added and the solution mixed 10:1 with 200 mM Na_2HPO_4, 7.5 M GdmCl. The solution was titrated to ~pH 8 by careful addition of 5 M HCl. The target peptide was then purified by multiple semipreparative RP-HPLC runs using a gradient of 30%–60% buffer B, with an initial 5 min isocratic phase, over 20 min at a flow rate of 4 ml min^{-1}. Buffer B = 0.1% TFA in 1:1 MeCN:isopropanol. Fractions corresponding to $SrcSH2^{Cys66\text{-}106}$ were determined by ESI-MS and lyophilized (observed mass = 4581.9; expected mass = 4582.2). The yield of peptide was 4 mg l^{-1} culture and the yield from the cleavage reaction was 37%.

Preparation of $SrcSH2^{1\text{-}53}$-MES Thioester

The cDNA corresponding to residues 144–196 (Swiss-Prot ID P63185) were amplified and PCR cloned using the forward 5′-G GAA TTC CAT ATG CAG GCT GAG GAG TGG TAC TTT GGG-3′ primer and the reverse 5′-GGT GGT TGC TCT TCC GCA CCC CTT GGC GTT GTC AAA GTC-3′ primer and the reaction was then treated with DpnI (20 U) for 1 hr at 37°C. The insert DNA and pTXB1 vector were double digested with NdeI and SapI restriction enzymes. The digested vector was subsequently treated with calf intestinal phosphatase. Both insert and vector were spin column purified and ligated using T4-Ready-to-go DNA ligase (Invitrogen). Colony PCR and DNA sequencing verified correct cloning. Plasmid DNA was then used to transform ER2566 cells (NEB) and a transformant used to inoculate an overnight LB starter culture supplemented with ampicillin (100 μg ml^{-1}). The culture was then diluted 3:100 with fresh Terrific broth medium (2 l) supplemented with glycerol (0.8% v/v) and ampicillin (100 μg ml^{-1}). Cells were grown to mid-log phase and induced with IPTG (0.1 mM) and expression left to commence overnight at 16°C. Cells were harvested and suspended in 100 ml ice-cold buffer (50 mM Na_2HPO_4/NaH_2PO_4 [pH 7.5], 500 mM NaCl, 5 mM EDTA, 0.1% v/v Triton X-100, 0.5 mM PMSF, protease inhibitor, Roche). Cells were lysed with a cell disruptor and clarified by centrifugation (39,000 × g, 30 min). The clarified lysate was then incubated with chitin beads (20 ml) pre-equilibrated with lysis buffer at 4°C on a shaker for 2–3 hr. The slurry was then centrifuged (100 × g, 1 min), supernatant discarded and the beads transferred to an empty XK-26 column (GE Life Sciences). The column was washed with 10 volumes of wash buffer (Na_2HPO_4/NaH_2PO_4 pH 7.5, 500 mM NaCl, 1 mM EDTA) and then with 1 column volume of 20 mM Na_2HPO_4 pH 6, 6 M urea, 500 mM NaCl, 1 mM EDTA. The column was equilibrated with 1 column volume of 20 mM Na_2HPO_4 pH 6, 2 M urea, 500 mM NaCl, 1 mM EDTA followed by 3 column volumes of the same buffer supplemented with MESNa (100 mM). The flow was stopped and the column left to incubate for 3 nights. Alternatively, the buffer was supplemented with MESNa (200 mM) and left for 1 night. The thioester peptide was then eluted with 20 mM Na_2HPO_4 pH 6, 6 M urea, 500 mM NaCl, 1 mM EDTA. Fractions containing the polypeptide and cleavage efficiency (>95%) were determined by SDS-PAGE. Solid urea was then added to the combined fractions to a final concentration of ~8 M. The peptide was concentrated with an Amicon centrifugal filter unit, 3 kDa cutoff (Millipore) to 5 ml. Acetonitrile was then added to a final concentration of 30% followed by 1% TFA. The solution was filtered and purified by semipreparative RP-HPLC using a gradient of 20 - 60% buffer B over 20 min, with a 5 min isocratic phase and a flow rate of 4 ml min^{-1}. Buffer A = 0.1% TFA in H_2O, Buffer B = 0.1% TFA in MeCN. Fractions corresponding to $SrcSH2^{1\text{-}53}$-MES thioester were determined by ESI-MS (observed mass = 6350.2; expected mass = 6350.1). The peptide was then lyophilized yielding ~4 mg from 2 l culture.

Chemical Synthesis of $SrcSH2^{Thz54\text{-}65}$-Bn Thioester

Peptide synthesis was conducted on a 0.1 mmol scale using an Applied Biosystems 433A peptide synthesizer operating the Fastmoc protocol (HBTU/HOBt, DIPEA) using NMP as solvent. 4-sulfamylbutyryl Novasyn® TG resin (loading = 0.23 mmol g^{-1}) preloaded with NH_2-Gln(Trt)-OH (Novabiochem) was used to prepare the sequence NH_2-NVXHYKIRKLQ-resin, where X = Lys, Orn, Dab, or Dap. Amino acids were coupled for 0.5 hr and V and I were double coupled. Following automated synthesis the resin was transferred to a manual synthesis vessel. Boc-Thz-OH (117 mg, 0.5 mmol) was weighed into a vial and dissolved in anhydrous DMF (1.0 ml). To this solution was added a 0.45 M solution of HBTU/HOBt in anhydrous DMF (1.1 ml) followed by DIPEA (0.15 ml) while stirring. This solution was then transferred to the manual synthesis vessel which was subsequently capped and shaken (250 rpm) at room temperature for 2 hr. The reaction vessel was washed extensively with DMF and DCM. The dry resin was transferred to a vial and anhydrous DMF (4.0 ml) was added followed by DIPEA (300 μl) and iodoacetonitrile (300 μl). The reaction was stirred for 36 hr at room temperature with the exclusion of light. The alkylated resin was washed with DMF then DCM and resuspended in anhydrous DMF (4.0 ml). Benzylmercaptan (300 μl) was added followed by benzenethiol, sodium salt (5 mg). The resin was stirred at room temperature for 24 hr after which time the resin was filtered off and washed extensively with DMF then DCM. The solvent was removed under reduced pressure and the residue exposed to 95% v/v TFA, 2.5% v/v ethanedithiol, 2.5% v/v H_2O (4.0 ml) for 5 hr. The crude product was then precipitated and washed with cold diethylether. The crude peptide was dissolved in 20% aqueous MeCN and lyophilized yielding ~40 mg of crude peptide per synthesis, which was of >75% purity and stored at −20°C. Peptide was purified as required by semipreparative RP-HPLC by applying a gradient of 5%–45% Buffer B over 30 min. Buffer A = 0.1% TFA in H_2O, Buffer B = 0.1% TFA in MeCN.

Native Chemical Ligation 1

$SrcSH2^{Cys66\text{-}106}$ (4 mg, 0.87 μmol) was dissolved in a 1.14 ml degassed ligation buffer (200 mM Na_2HPO_4 [pH 6.9], 7.5 M GdmCl, 100 mM MPAA, 60 mM TCEP). To facilitate dissolution the sample was sonicated. The $SrcSH2^{Thz54\text{-}65}$-Bn thioester peptide (1.69 mg, 1.04 μmol) with the desired lysyl derivative was then added. The reaction was monitored to completion by analytical RP-HPLC using 5 μl samples of the ligation mixture quenched with 3% TFA. A gradient of 15%–45% B was applied over 30 min with an initial 5 min isocratic phase. Mobile phase B = 0.1% TFA in MeCN. Solid $MeONH_2 \cdot HCl$ was then added to a concentration of 200 mM and the pH was adjusted to 4 on ice by careful addition of 8 M HCl. Deprotection was left to commence overnight. The deprotected ligation product was purified by semi-preparative RP-HPLC. A gradient of 23%–48% B was applied, with a 5 min isocratic stage and at a flow rate of 3 ml min^{-1}. Fractions containing $SrcSH2^{Cys54\text{-}106}$ were confirmed by ESI-MS (representative observed mass for LysβD3 peptide = 6093.8; expected mass = 6094.1). The recovered yield of lyophilized peptide was 61% (3.55 mg). This procedure was repeated for the three other lysyl derivatives.

Native Chemical Ligation 2

$SrcSH2^{1\text{-}53}$-MES thioester (3.5 mg, 0.55 μmol) was dissolved in 0.92 ml solvent consisting of 85% buffer (300 mM HEPES [pH 7.5], 7.5 M GdmCl, 60 mM MPAA, 60 mM TCEP) and 15% MeCN. The peptide was left to transthioesterify for 30 min. $SrcSH2^{Cys54\text{-}106}$ (2.25 mg, 0.37 μmol) containing the desired lysyl derivative was then added. The reaction was monitored by analytical RP-HPLC using 10 μl samples quenched with 3% TFA. A gradient of 25%–40% B over 45 min with an initial 5 min isocratic phase was applied. Buffer A = 0.1% TFA in H_2O, Buffer B = 0.1% TFA in MeCN. The ligation product ($SrcSH2^{1\text{-}106}$) was purified by semi-preparative RP-HPLC applying the same gradient at 4 ml min^{-1}. Isopropanol was immediately added to the fractions at a final concentration of 10% and fractions containing the peptide were determined by ESI-MS and lyophilized. Src SH2(LysβD3), expected mass = 12301.9 Da, observed = 12302.4 Da; Src SH2(OrnβD3), expected mass = 12287.9 Da, observed = 12288.0 Da; Src SH2(DabβD3), expected mass = 12273.9 Da; observed = 12274.1 Da; Src SH2(DapβD3), expected mass = 12259.9 Da, observed = 12260.1 Da. The recovered yield of the $SrcSH2^{1\text{-}106}$ lyophilized peptides was ~21% (0.96 mg).

Protein Folding

Lyophilized $SrcSH2^{1\text{-}106}$ was dissolved in degassed buffer (20 mM HEPES [pH 7.5], 7.5 M GdmCl, 20 mM 2-mercaptoethanol, 1 mM EDTA) at

a concentration of 0.5 mg ml^{-1}. The solution was then added drop-wise to 10 volume equivalents of ITC buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA) while stirring. The protein was then dialyzed overnight against ITC buffer (1 l) at 4°C using a 6 – 8 kDa cut-off membrane (Spectrum Labs). The protein was then centrifuged (20,000 × g, 20 min) and the supernatant concentrated to ~25 μM with a 10 kDa cut-off centrifugal spin filter (Millipore). The protein was then dialyzed against fresh ITC buffer (1 l) using a 500 Da cut-off Float-a-lyzer (Spectrum Labs). The pYEEI or pYDEI peptide was dissolved at a concentration of 0.5 mM in ITC buffer and dialyzed in parallel against the same buffer for 48 hr at 4°C. Folding efficiency was 70% yielding ~0.7 mg of protein per synthesis.

Isothermal Titration Calorimetry

ITC experiments were carried out with a VP-ITC microcalorimeter (Microcal) as previously described. Experimental protein concentrations were 16–20 μM and peptide concentrations were 0.2–0.3 mM. Protein and peptide concentrations were determined by electronic spectroscopy using extinction coefficients of 14,700 M^{-1} (280 nm) and 695 M^{-1} (268 nm), respectively. Phosphopeptides consisted of the sequence Ac-PQpYXEIPI-NH_2 (where X = E or D) and were obtained at > 95% purity (Biosynthesis Inc., TX, USA). Titrations were carried out at 25°C using 8–11 μl injections. This resulted in a ~3-fold excess of peptide, which allowed the enthalpy of ligand dilution to be determined from the mean average of the injections after protein saturation. Data were fitted with the VP-ITC routine within the ORIGIN software using the single site model. Stoichiometry, n, for all titrations was 0.8–1.1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures, and can be found with this article online at doi:10.1016/j.chembiol.2010.01.015.

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