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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

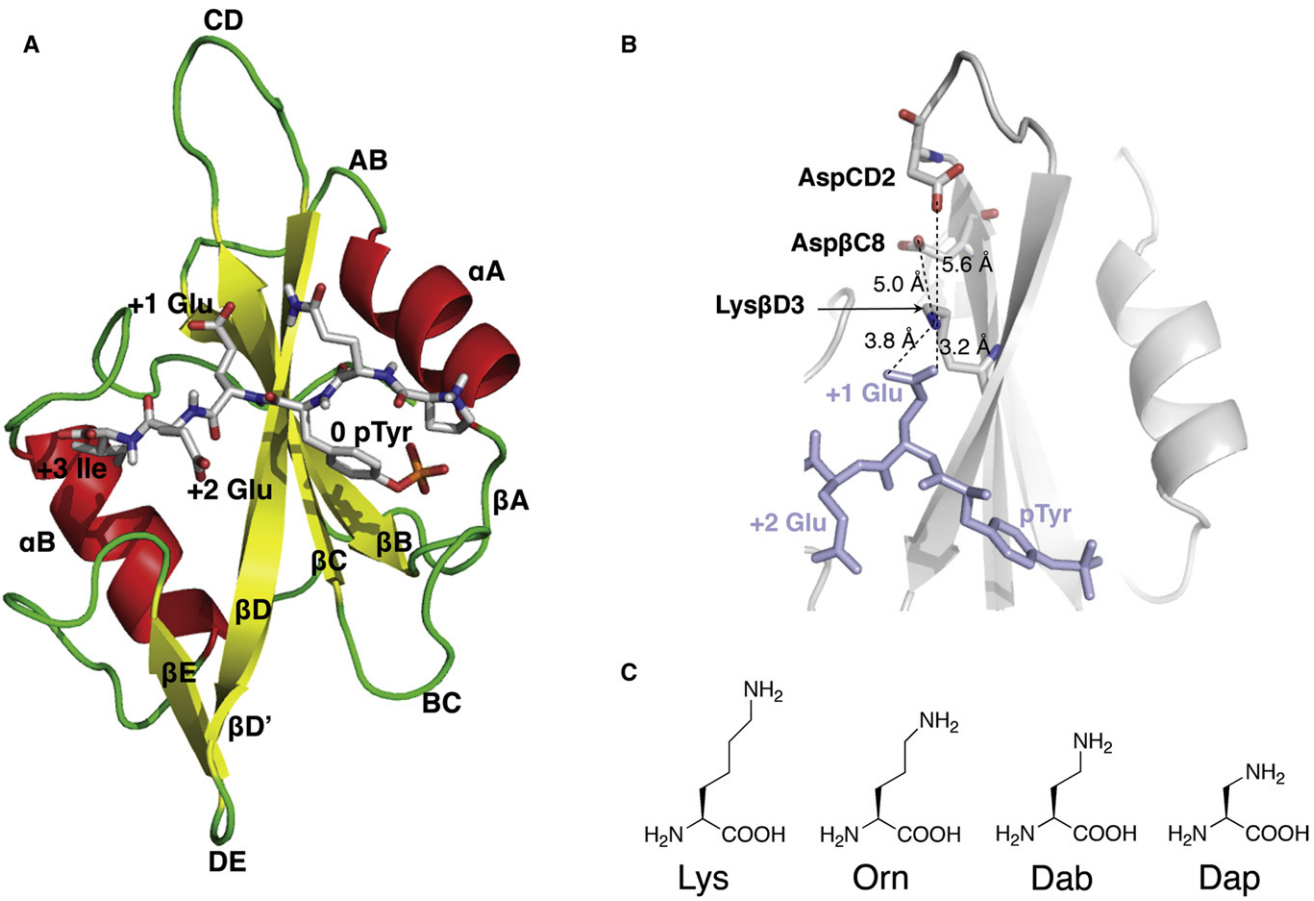

Figure 1. Src SH2 Domain Structure, Phosphopeptide Binding Site, and Incorporated Unnatural Lysine Derivatives

(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