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Encodable Activators of Src Family Kinases

Crystal D. Zellefrow,[‡] Jennifer S. Griffiths,[§] Sarmistha Saha,[‡] Abby M. Hodges,[§] Jessica L. Goodman,[‡] Joshiawa Paulk,[§] Joshua A. Kritzer,[§] and Alanna Schepartz*[,§,#]

Departments of Chemistry, Molecular Biophysics and Biochemistry, and Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8107

Received October 11, 2006; E-mail: alanna.schepartz@yale.edu

There is considerable interest in encodable molecules that regulate intracellular protein circuitry and/or activity, ideally with high levels of specificity. One class of tightly regulated signaling proteins, the Src family kinases, contains a catalytic kinase domain and regulatory Src homology 2 (SH2) and Src homology 3 (SH3) domains.[1] Src kinases are maintained in an inactive state by virtue of intramolecular interactions between the SH2 domain and a phosphotyrosine sequence in the C-terminal tail and between the SH3 domain and a proline-rich sequence in the SH2-kinase linker.[2] Src kinases can be activated by ligands that disrupt either[3,4] or both[5] of these interactions, and different activation modes may lead to different downstream signaling events.[4,6] Thus, encodable molecules that activate select Src family kinases in well-defined ways could complement selective Src kinase inhibitors[7] to unravel the roles of specific family members in cell signaling events. Here we describe a set of encodable miniature proteins that recognize SH3 domains from distinct Src family kinases with high affinity; two of them activate Hck kinase with potencies that rival HIV Nef, which activates Hck kinase *in vivo*.

The NMR structure of the c-Src SH3 domain in complex with peptide **1**[8] guided design of the molecules studied here (Figure 1A). This structure shows **1** bound as a PPII helix with the side chains of P_1, L_3, and P_4 nestled into grooves of the SH3 domain surface. The side chain of R_6 anchors the peptide in a class II orientation and that of N_7 provides additional affinity.[8] Substitution of these five residues for analogous residues within aPP, the PP-fold protein used previously for miniature protein design,[12,13] led to PP1 (Figure 1B). Inclusion of one (R_8) or three (R_8−R_{10}) additional residues from **1** at the C-terminus of the motif led to PP2 and PP3. To complement the aPP-based designs, we also prepared a pair of miniature proteins (YY1 and YY2) based on the aPP ortholog PYY. PYY also displays a characteristic PP-fold, but its variants are more soluble and less prone to dimerization than aPP variants.[10,14]

First we compared the secondary structures and thermal stabilities of the aPP and PYY-based designs using circular dichroism (CD) spectroscopy (Figure 1C). The CD spectra of the aPP-based molecules (PP1, PP2, and PP3) showed little ellipticity at 208 and 222 nm, indicating little α-helical secondary structure under these conditions. By contrast, the CD spectra of PYY-based YY1 and YY2 showed significant signals at these wavelengths, with mean residue ellipticities at 222 nm (MRE_{222}) of -1.4×10^4 and -1.0×10^4 deg·cm^2·dmol^{-1}, respectively. Temperature-dependent CD studies indicated that YY1 and YY2 undergo cooperative melting transitions with midpoints (T_M) of ∼25 and ∼50 °C, respectively.[11] The spectral signature of YY1 is virtually identical to that of wild-type PYY (MRE_{222} of -1.6×10^4 deg·cm^2·mol^{-1}, $T_m \sim 50$ °C; unpublished data), which suggests that it retains the characteristic PP-fold structure. YY1, like PYY, is a monomer at low micromolar concentrations (dimer $K_d = 180$ μM).[11]

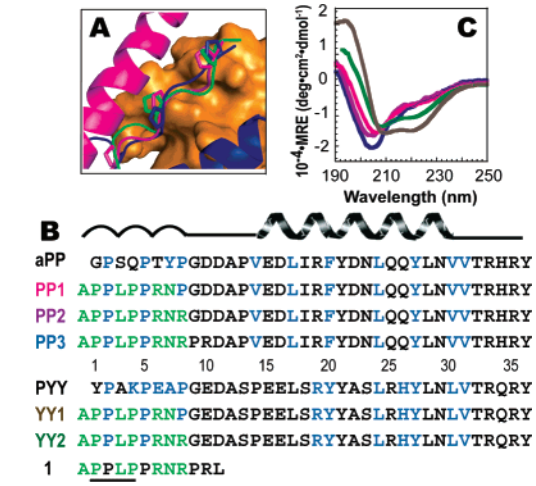

Figure 1. (A) Structure of **1** (APP12 in ref 8, green) in complex with c-Src SH3 (orange, PDB 1QWE), superimposed with aPP (pink, PDB 1PPT), and PYY (blue, PDB 1RU5). (B) Sequences of aPP, PYY, **1**, and miniature proteins studied herein. Residues that contribute directly to c-Src SH3 recognition[8] are in green; those that contribute to aPP/PYY folding[9,10] are in blue. The PxxP core epitope is underlined. (C) CD spectra of miniature proteins color-coded as in (B).[11]

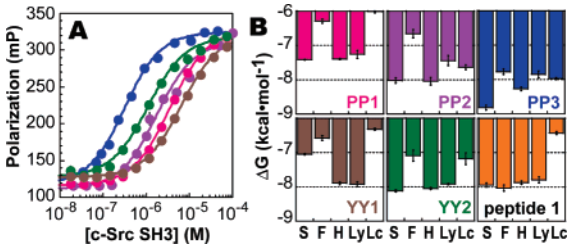

Figure 2. (A) Fluorescence polarization analysis of the binding of c-Src SH3 by miniature proteins (50 nM), color-coded as in (B). (B) Comparison of binding free energies for complexes between aPP- and PYY-based miniature proteins and the SH3 domains of c-Src (S), Fyn (F), Hck (H), Lyn (Ly), and Lck (Lc). Values shown represent the average of at least three trials ± standard error.[11]

The affinity of each miniature protein for the c-Src SH3 domain was determined using fluorescently labeled miniature proteins and a direct polarization assay (Figure 2A).[11] Peptide **1**, whose affinity for c-Src SH3 was optimized by phage display,[15] bound c-Src SH3 well under these conditions, with an equilibrium affinity ($K_d = 1.5 \pm 0.1$ μM) comparable to that reported ($K_d = 1.2$ μM).[8] Although PP1 and PP2 bound c-Src SH3 with affinities close to that of **1** ($K_d = 3.68 \pm 0.06$ and 1.3 ± 0.2 μM, respectively), PP3 bound significantly better ($K_d = 350 \pm 40$ nM). It is notable that the only difference between PP3 and PP1/PP2 is the presence of P_9−R_{10}.

§ Department of Chemistry.
‡ Department of Molecular Biophysics and Biochemistry.
# Department of Molecular, Cellular and Developmental Biology.

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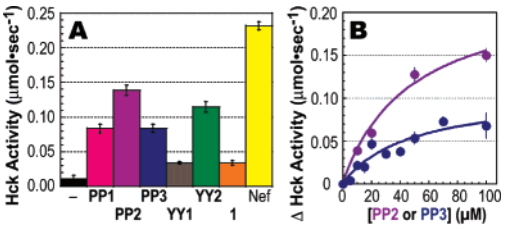

Figure 3. Activity of Hck kinase in the presence or absence of (A) 50 μM miniature protein, **1**, or Nef or (B) PP2 and PP3 at the concentrations indicated. Kinase activities were determined as described.[11,20] Values shown represent the average of three determinations ± standard error.

Neither **1** nor any miniature protein studied here bound detectably (K_d > 20 μM) to c-Src SH3^{P133L}, a variant containing a mutation in the core binding groove that disrupts c-Src activity in *S. pombe*.[16] The affinities of YY1 and YY2 for c-Src SH3 were virtually identical to those of the corresponding aPP-based designs despite differences in intrinsic secondary structure and virtually identical contact surfaces. Taken together, these results indicate that both classes of miniature protein ligands have potential as encodable ligands for SH3 domains. Although miniature protein fold often contributes significantly to binding affinity,[13] in this case, it offers no measurable advantage.

To evaluate whether miniature protein structure contributed to binding specificity, we determined the relative affinity of each miniature protein for SH3 domains within (Fyn, Hck, Lyn, Lck) and outside (Abl, Nck1, Grb2, Abp1) the Src kinase family. None of the miniature proteins, nor peptide **1**, bound well to any non-Src family SH3 domain tested (K_d > 20 μM; data not shown). Peptide **1** showed little specificity within the Src family, binding well to the domains from Fyn, Hck, and Lyn (0.87 μM < K_d < 2.0 μM) and poorly (K_d > 20 μM) to Lck SH3 (Figure 2B). By contrast, all of the miniature proteins, but especially PP3 and YY1, showed significantly greater, and different, specificity. PP3 preferred the Src SH3 domain to all others tested ($\Delta\Delta G$ = 0.6−1.0 kcal·mol^{-1}), whereas YY1 preferred the SH3 domains of Hck and Lyn over Src ($\Delta\Delta G$ = 0.8 and 0.9 kcal·mol^{-1}, respectively). Notably, YY1 and PP1 display different preferences despite the presence of identical sequence over nine N-terminal residues; the same is true for YY2 and PP2. This pattern suggests that SH3 domain specificity, even among close family members, can be fine-tuned by miniature protein sequence and architecture.

SH3 domains regulate the activity of Src family kinases through interaction with an internal proline-rich region that locks the kinase into a catalytically repressed state.[17] Ligands such as HIV Nef[3] and *H. saimiri* Tip,[18] which block this interaction, up-regulate kinase activity. To evaluate whether the miniature proteins studied here could function as encodable activators of a Src family kinase, we monitored their effect on Hck activity using an assay that couples ATP hydrolysis to NADH oxidation.[19] Hck was chosen rather than Src because of the availability of Nef as a potent positive control.[3] As expected, Nef was a potent Hck activator, increasing kinase activity 21-fold at 50 μM concentration (~200K_d). By contrast, at the same concentration, peptide **1** was a modest Hck activator, increasing Hck activity 3-fold. All miniature proteins except YY1 were significantly more potent activators than **1** (Figure 3A). Values of K_{act} determined for the most active molecules PP2 and PP3 were 48 ± 13 and 48 ± 22 μM, respectively (Figure 3B). These values are modestly higher than the published value for Nef (K_{act} = 18.0 μM)[20] and approach the values reported for potent but non-encodable peptoids.[20] Surprisingly, Hck activation correlated with neither SH3 domain affinity nor Hck specificity—PP2, PP3, YY1, YY2, and **1** all bind Hck SH3 with comparable affinities *in vitro*, yet YY1 does not activate; PP1 and PP3 are equipotent activators, yet PP1 binds poorly. These differences could result from differences in affinity for full-length kinases or from differences in binding mode that correspond to alternative activation levels. The evaluation of these molecules as activators of Src family kinases in live cells is currently in progress.[21]

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Supporting Information Available: Materials and experimental methods; K_d values; CD, AU, and activation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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