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Rewiring Kinase Specificity with a Synthetic Adaptor Protein

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Supporting Information

ABSTRACT: Signaling cascades are managed in time and space by interactions between and among proteins. These interactions are often aided by adaptor proteins, which guide enzyme−substrate pairs into proximity. Miniature proteins are a class of small, well-folded protein domains possessing engineered binding properties. Here we made use of two miniature proteins with complementary binding properties to create a synthetic adaptor protein that effectively redirects a ubiquitous signaling event: tyrosine phosphorylation. We report that miniature-protein-based adaptor **3** uses templated catalysis to redirect the Src family kinase Hck to phosphorylate hDM2, a negative regulator of the p53 tumor suppressor and a poor Hck substrate. Phosphorylation occurs with multiple turnover and at a single site targeted by c-Abl kinase in the cell.

Signaling cascades are managed in time and space by interactions between and among proteins.[1] In many cases, the management of these interactions is consigned to adaptor proteins that guide enzyme−substrate pairs into proximity and favor selective reaction.[2−5] Here we describe a strategy that exploits miniature proteins—small, well-folded protein domains possessing engineered binding properties—to create a synthetic adaptor protein that effectively redirects a ubiquitous signaling event: tyrosine phosphorylation. This miniature-protein-based adaptor effectively redirects the Src family kinase Hck to phosphorylate hDM2, a negative regulator of the p53 tumor suppressor and a poor Hck substrate. Phosphorylation occurs with multiple substrate turnover and at a single site targeted by c-Abl kinase in the cell.

Our design strategy began with two previously reported miniature proteins, **YY2**[7] and **3.3**.[8] Miniature protein **YY2** uses residues within its polyproline II (PPII) helix to interact with the SH3 domains of certain Src family kinases, such as Hck (Figure 1A).[7] This interaction disrupts the intramolecular SH3 domain interaction that downregulates kinase activity and results in kinase activation. **YY2** rivals the HIV-1 protein Nef as an activator of Hck.[9,10] Miniature protein **3.3** uses residues within its α-helix to bind to hDM2. This interaction inhibits the association of hDM2 with p53[11] and frees p53 to promote the transcription of p53-dependent genes.[12] We envisioned two ways that these functional domains could be combined in a synthetic adaptor; we chose the arrangement that placed **YY2** on the N-terminus and **3.3** on the C-terminus to allow optimal access to the helices involved in protein binding (Figure 1A). The two miniature proteins were conjoined by 4, 8, and 12 amino acid linker to generate adaptors **1**, **2**, and **3**, respectively

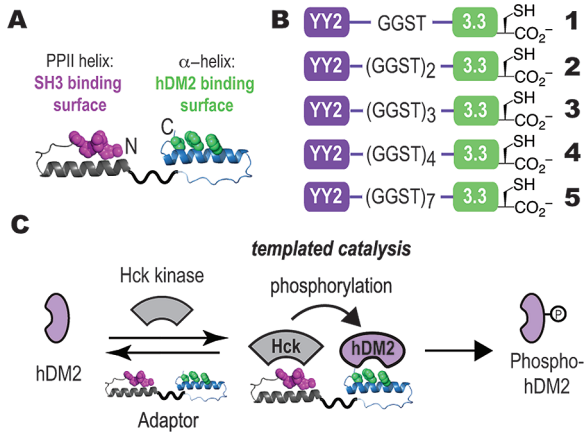

Figure 1. (A) Cartoon illustrating the design of a miniature-protein-based adaptor protein. (B) Structures of adaptors **1**−**5**, illustrating differences in the interdomain linkages. (C) Proposed mechanism for templated catalysis of hDM2 phosphorylation by Hck via ternary complex formation.[6]

(Figure 1B). We hypothesized that these adaptor proteins would be able to form a ternary complex with hDM2 and Hck to promote the phosphorylation of hDM2. As hDM2 would otherwise be a poor Hck substrate, we refer to this process as "templated catalysis" (Figure 1C).

We first performed equilibrium fluorescence polarization experiments to characterize the affinity of adaptors **1**−**3** for hDM2 and Hck. Each adaptor was labeled on a C-terminal cysteine thiol with 5-iodoacetamidofluorescein and titrated with $hDM2_{1-188}$ (hDM2) or GST-Hck-SH3 (Hck-SH3) (Figure 2A,B). We confirmed that none of the miniature protein variants bound appreciably to GST ($K_D \geq 18.1$ μM). Adaptors $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, and $\mathbf{3}^{Flu}$ displayed affinities for hDM2 (K_D = 278 ± 83.5, 76.4 ± 5.9, and 133 ± 8.5 nM, respectively) that are comparable to the affinity of miniature protein $\mathbf{3.3}^{Flu}$ (35 ± 3.3 nM)[8] (Figure 2A). Similarly, adaptors $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, and $\mathbf{3}^{Flu}$ displayed affinities for Hck-SH3 (K_D = 3.1 ± 0.5, 7.6 ± 3.0, and 5.0 ± 0.3 μM, respectively) that are comparable to the affinity of $\mathbf{YY2}^{Flu}$ (K_d = 1.4 ± 0.2 μM)[7] (Figure 2B). In contrast, $\mathbf{YY2}^{Flu}$ bound hDM2 with low affinity (K_d = 70 ± 5.8 μM) and $\mathbf{3.3}^{Flu}$ bound Hck-SH3 with low affinity (K_d = 85.3 ± 19.4 μM). These comparisons indicate that hDM2 discriminates well between the two miniature protein components ($\Delta\Delta G$ = 4.5 kcal mol^{-1}), whereas the discrimination by Hck-SH3 is more modest ($\Delta\Delta G$ = 1.0 kcal mol^{-1}); this difference in

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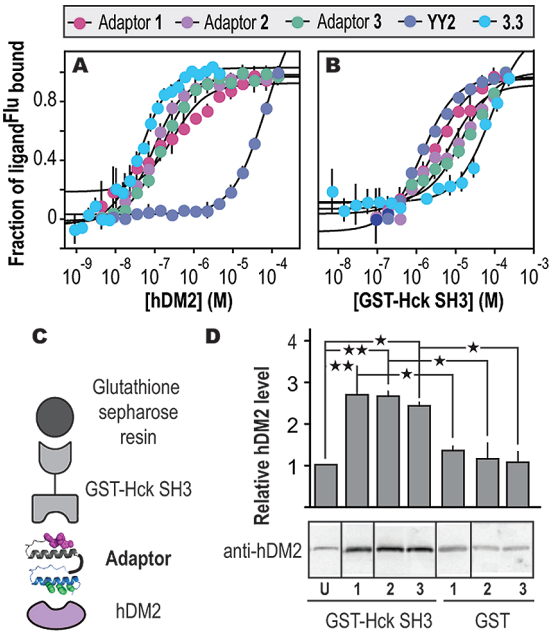

Figure 2. (A,B) Analysis of the equilibrium affinities of adaptors **1**−**3** and miniature proteins **YY2** and **3.3** for (A) hDM2 and (B) Hck-SH3. (C) Schematic and (D) visualization and quantification of the pulldown assay used to analyze ternary complex formation.[6] Adaptors **1**, **2**, and **3** are so indicated (15 μM each); U refers to **YY2** and **3.3** (15 μM each). Symbols in (D): ★, $p \leq 0.05$; ★★, $p \leq 0.01$. ANOVA with the Bonferroni post-test was used.

specificity influenced our choice of reaction conditions (see below). Nevertheless, these in vitro data indicate that incorporation of the miniature proteins **YY2** and **3.3** into adaptors **1**−**3** occurred with little or no loss in equilibrium binding affinity or selectivity.

Next we performed a pulldown assay to verify the formation of a ternary complex between each adaptor and both Hck-SH3 and hDM2 (Figure 2C). Ternary complex formation was performed under conditions of limiting adaptor, with 70 μM Hck-SH3, 157 μM hDM2, and 15 μM adaptor $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, or $\mathbf{3}^{Flu}$. Bound proteins were eluted with glutathione, analyzed by Western blot, and probed with an α-hDM2 antibody. Incubation of immobilized Hck-SH3 with **YY2** and **3.3** (15 μM each) led to little or no retention of hDM2 relative to that observed when GST itself (as opposed to GST-Hck-SH3) was immobilized. However, incubation with adaptors **1**−**3** increased the amount of retained hDM2 by 3-fold (Figure 2D). These results indicate that each of the three adaptors can form a ternary complex with Hck-SH3 and hDM2. Although adaptors **1**−**3** contain different linkers and therefore differ in the relative arrangement of the two component miniature proteins, these differences had no observable effect on the fraction of hDM2 retained. We estimate that under these conditions, only ∼8% of the hDM2 present was assembled into ternary complexes with Hck-SH3 and an adaptor;[6] incubations at higher hDM2 concentrations were not possible because of limited solubility.

With evidence for a ternary complex in hand, we explored the extent to which adaptors **1**−**3** would promote the phosphorylation of full-length $hDM2_{1-491}$ by full-length Hck (Hck). In preliminary experiments, we systematically varied the concentrations of $hDM2_{1-491}$, downregulated[10] Hck, and adaptor $\mathbf{2}^{Flu}$; the relative amounts of phosphorylated $hDM2_{1-491}$ (pY-hDM2) were assessed by Western blot using both α-pY and α-hDM2 antibodies. We observed a bell-shaped dependence on the concentration of adaptor **2** (Figure 3A),

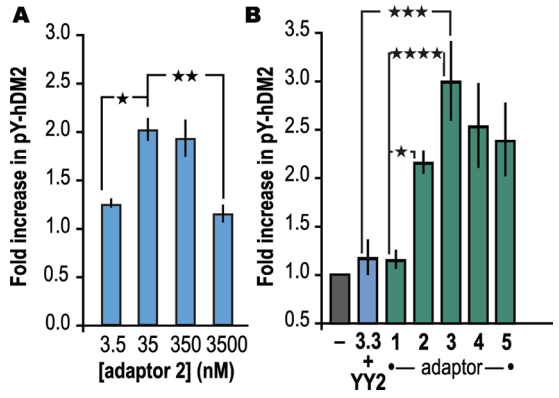

Figure 3. (A) Bell-shaped dependence of the fold increase in phosphorylated hDM2 (pY-hDM2) as a function of [$\mathbf{2}^{Flu}$]. (B) Fold increase in phosphorylated hDM2 (pY-hDM2) produced in vitro in the presence of adaptor **1**−**5** (35 nM) or miniature proteins **YY2** and **3.3** (35 nM each). Symbols: ★, $p \leq 0.05$; ★★, $p \leq 0.01$; ★★★, $p \leq 0.001$; ★★★★, $p \leq 0.0001$. ANOVA with the Bonferroni post-test was used.

whereas no such effect was observed when [Hck] was varied. These experiments revealed that the greatest increase in adaptor **2**-promoted hDM2 phosphorylation occurred in reactions containing 22 nM Hck, 5 μM $hDM2_{1-491}$, and 35 nM $\mathbf{2}^{Flu}$. Under these conditions, the steady-state concentration of the ternary complex was ∼140 pM.[6] The presence of adaptor **2** resulted in a 2-fold increase in pY-hDM2; the increase observed in the presence of adaptor **3** was nearly 3-fold. In contrast, the increase in pY-hDM2 in the presence of adaptor **1** or miniature protein components **3.3** and **YY2** was minimal (Figure 3B). The increase in pY-hDM2 due to adaptors **2** and **3** is not attributable simply to activation of Hck, as incubation of $hDM2_{1-491}$ and Hck with adaptor **1** had no effect on the level of pY-hDM2 (Figure 3B). Moreover, adaptors conjoined by longer linkers (**4** and **5**) showed no improvement in the pY-hDM2 yield. Similar trends in reactivity were observed when the reactions were performed in the presence of bacterial cell lysate.[6] These results indicate that adaptors **2** and **3** act as templates to bring Hck and hDM2 into proximity to favor an otherwise unfavorable phosphorylation reaction.

The phosphorylation state of hDM2 regulates the activity of the tumor suppressor protein p53.[13] In response to DNA damage, hDM2 is phosphorylated at Y276, Y394, and Y405 by c-Abl kinase (Figure 4A).[14,15] These post-translational modifications inhibit the association and ubiquitination of p53 by hDM2 and upregulate the transcription of p53-dependent genes. hDM2 contains 14 tyrosine residues, 13 of which are accessible or located in regions of unknown structure. We used PeptideCutter[16] to identify trypsin and chymotrypsin as a pair of proteolytic enzymes that would fragment hDM2 to report on the phosphorylation of 12 of these 14 positions (Y68 and Y104 could not be observed). Treatment of $hDM2_{1-491}$ (5 μM) with adaptor **3** (35 nM) and Hck (22 nM) was followed by proteolytic digest with chymotrypsin or trypsin.[6] Peptide fragments were resolved and detected using LC/MS/MS and analyzed using BioPharmaLynx software. This analysis revealed phosphorylation at positions Y276 and Y405 but not Y394 or any other observable tyrosine side chain.[6] Thus, adaptor **3**

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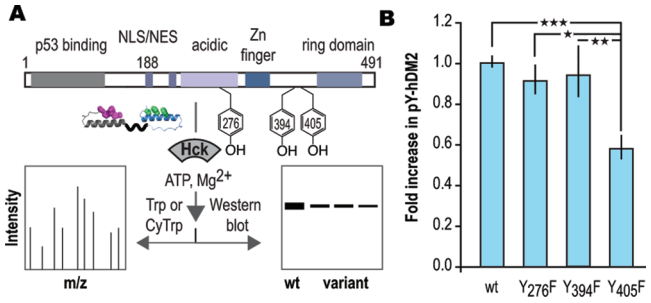

Figure 4. (A) Domain architecture of hDM2 showing the location of tyrosine residues phosphorylated by c-Abl and a reaction schematic demonstrating experimental details. (B) Fold increase in pY-hDM2 and variants thereof produced in vitro in the presence of adaptor **3** and Hck. Symbols in (B): ★, $p \leq 0.05$; ★★, $p \leq 0.01$; ★★★, $p \leq 0.001$. ANOVA with the Bonferroni post-test was used.

promotes phosphorylation of $hDM2_{1-491}$ by Hck at two of the three sites targeted by c-Abl kinase, despite the fact that hDM2 is a poor Hck substrate.[17,18] Subsequent analysis of the phosphorylation of Y276F, Y394F, and Y405F hDM2 using Western blots identified Y405 as the predominant phosphorylation site (Figure 4B). Notably, the redirected specificity of Hck in the presence of adaptor **3** would not be predicted by the known in vitro preferences of either c-Abl or Src/Hck. These results suggest that other factors contribute to the unique reactivity of these two positions.

Our adaptor proteins were designed to interact simultaneously with both an enzyme (Hck) and a latent substrate (hDM2). As a result, these adaptors have the potential to affect multiple substrate turnovers. To calculate a turnover number,[19] we quantified the incorporation of ^{32}P into pY-hDM2 in the presence and absence of adaptor **3** using scintillation counting and storage phosphor autoradiography.[6] Incubation of 5 μM hDM2 with 22 nM Hck, 50 μM ATP (spiked with 14−35 pmol of γ-^{32}P-ATP), and 35 nM adaptor $\mathbf{3}^{\mathbf{Flu}}$ yielded 6.7 ± 0.8 pmol of pY-hDM2 (11% yield), whereas 1.9 ± 0.4 pmol (3% yield) was produced when adaptor **3** was replaced by **YY2** and **3.3** (35 nM each). When calculated on the basis of $[Hck]_T$, the turnover number in the presence of adaptor **3** was 26 (6.7 pmol/0.26 pmol), which is larger than the value of 7 (1.9 pmol/0.26 pmol) calculated in the presence of **3.3** and **YY2**. We note, however, that the true catalytic species of interest in this reaction is not Hck but rather its complex with adaptor **3**. When calculated on the basis of [**3**·Hck] (estimated as 149 pM),[6] the turnover number was 3700 (6.7 pmol/0.0018 pmol). This value is 10 times larger than the value of 302 (1.9 pmol/0.0063 pmol) calculated on the basis of [**YY2**·Hck] (estimated as 524 pM).[6]

In this work, we have applied classic principles of proximity-induced reaction[20−22] to redirect a kinase to an otherwise poor substrate, effectively rewiring a cellular signaling event. In this case, phosphorylation of hDM2 should reactivate p53 and upregulate p53-dependent genes; these studies are in progress. The synthetic adaptor concept could be applied generally as part of an expanding synthetic biology toolkit.[23] Miniature proteins are encodable and evolvable;[24,25] moreover, the strategy does not require the replacement of endogenous cellular proteins[26] and exploits orthogonal domains with less potential for unintended cross-talk.[27]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and experimental methods, K_d values, and pulldown data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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