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A Small Molecule-Regulated Guanine Nucleotide Exchange Factor

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Cell-permeable, small molecules are powerful reagents for studying signal transduction networks because they provide rapid, reversible, and dose-dependent control over protein function. However, the identification of agonists and antagonists with useful selectivity and potency is challenging for many protein targets. For this reason, there has been a great deal of interest in using genetics to generate proteins that are uniquely sensitive to a pharmacological agent of choice.[1] A major advantage of these chemical genetic techniques is that multiple proteins can be engineered to respond to the same small molecule. One strategy that has not been exploited to date is the use of a small molecule-controllable protein−protein interaction to allosterically regulate enzyme catalytic activity.[2] As many signaling enzymes have evolved to be regulated by modular protein−protein interactions, a chemical genetic method based on this principle has the potential to be of broad utility.[3]

To develop a chemical genetic method for controlling signaling enzymes, we envisioned replacing their autoinhibitory regulatory domains with a protein−protein interaction that can be disrupted with a small molecule. The protein binding partners used for regulating catalytic activity must fulfill two important criteria. First, both binding partners must be continuous functional domains that are capable of being transported to a diverse number of signaling proteins. In addition, a potent, selective, and cell-permeable small molecule that is capable of disrupting the interaction between these proteins must exist.[4] The interaction between antiapoptotic protein Bcl-xL and BH3-only proteins is an ideal candidate for generating a protein switch because it fulfills both of these criteria. Bcl-xL can be reduced to a single, functional binding domain of less than 25 KDa that has a high affinity (K_d = <10 nM) for short BH3 peptides (<30 amino acids) derived from BH3-only proteins (Figure 1A).[5] Most importantly, several cell-permeable, small molecules that competitively displace BH3 peptides from Bcl-xL have been identified.[6] Two potent inhibitors of this interaction (K_i = <10 nM), **ABT-737** and **A-385358**, are shown in Figure 1B.

The guanine nucleotide exchange factor (GEF) Intersectin was selected as our initial target for generating a signaling enzyme that can be controlled with a small molecule.[7] This enzyme is a member of the Dbl-family of GEFs, which activate Rho-family GTPases by catalyzing the exchange of GDP for GTP.[8] Intersectin, like all other Dbl-family GEFs, contains a conserved catalytic Dbl homology (DH) domain of ∼200 residues. The catalytic nucleotide exchange activity of the DH domain of Intersectin is repressed by intramolecular regulatory interactions; disruption of these autoinhibitory binding events allows Intersectin to act upon its GTPase substrate, Cdc42. The general strategy for generating small molecule-controllable Intersectin constructs is shown in Figure 1C. Replacement of Intersectin's regulatory and localization domains with Bcl-xL and a BH3 peptide represses the catalytic activity of the DH domain. Disruption of the interaction between these artificial regulatory domains leads to an increase in nucleotide exchange activity.

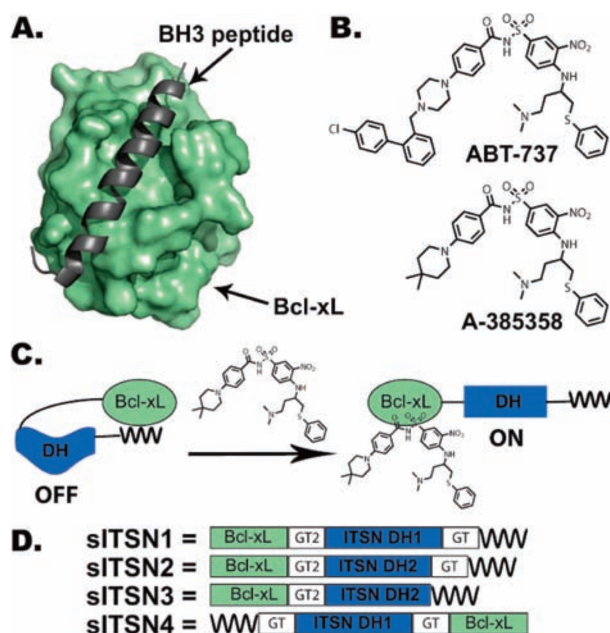

Figure 1. Intersectin constructs that can be regulated with a cell-permeable small molecule. (A) Structure of Bcl-xL bound to the BH3 peptide Bad (PDB: 2BZW). (B) Chemical structures of **ABT-737** and **A-385358**. (C) General strategy for regulating the nucleotide exchange activity of Intersectin with a small molecule. (D) sITSN constructs (**sITSN1**−**sITSN4)** that were generated in this study.

A small panel of synthetic Intersectin (sITSN) fusion proteins was generated to determine the optimal orientation and position for the small molecule-controllable regulatory domains. All of the synthetic GEF constructs (**sITSN1**−**sITSN4**, Figure 1D) contain three core components: (1) the catalytic DH domain of Intersectin, (2) Bcl-xL (residues 2−215), and (3) a BH3 peptide that binds tightly to Bcl-xL (residues 137−163 of the protein Bad). **sITSN1**−**sITSN3** have Bcl-xL at their *N*-termini, followed by a flexible glycine-threonine repeat linker $(GT)_2$ that is fused to the catalytic DH domain of Intersectin. While the *N*-termini of **sITSN1**−**sITSN3** are identical, these constructs contain variable linkers between the DH domain and the BH3 peptide. **sITSN1** contains residues 1229−1445 of the DH domain (DH1) and is connected to the BH3 peptide with a glycine-threonine linker (GT). Both **sITSN2** and **sITSN3** contain 16 fewer *C*-terminal residues from the DH domain (DH2) than **sITSN1**. **sITSN2** contains a (GT) linker between the catalytic domain and the BH3 peptide, while **sITSN3** does not. In contrast to **sITSN1**−**sITSN3**, **sITSN4** contains an *N*-terminal BH3 peptide and Bcl-xL at the *C*-terminus. For **sITSN4**, both Bcl-xL and the BH3 peptide are linked to the DH domain (DH1) with a (GT) linker.

An *in vitro* assay that measures the rate at which Intersectin catalyzes nucleotide exchange in the GTPase Cdc42 loaded with a fluorescently labeled GDP analogue (mant-GDP) was used for

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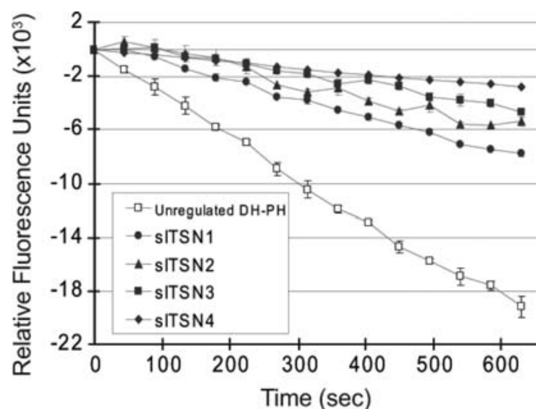


Figure 2. Fusing Bcl-xL and a BH3 peptide to the DH domain of Intersectin represses its catalytic activity. The nucleotide exchange activities of wild type **sITSN1−sITSN4** (50 nM) and the unregulated DH-PH domain of Intersectin (50 nM) were determined in the presence of a control peptide (5 μM Noxa). The rate of nucleotide exchange was determined by measuring the loss of fluorescence (Ex: 355 nm, Em: 460 nm) due to exchange of mant-GDP with free nucleotide in Cdc42 (2 μM). All assays were performed in triplicate.

characterization of the sITSN constructs. The BH3 peptides Bad and Noxa were used as competitors in this assay. The Bad peptide should disrupt the autoinhibitory interaction of the artificial regulatory domains due to its high affinity for Bcl-xL (K_i = 6.0 nM), whereas the Noxa peptide serves as an inactive control due to its lack of affinity for Bcl-xL (K_i > 10 000 nM) (Figure S1). As shown in Figure 2, the catalytic activities of all four synthetic Intersectin constructs were repressed in the nucleotide exchange assay. In the absence of a competitor or in the presence of 5 μM Noxa, **sITSN1−sITSN4** were at least 3-fold less active than the unregulated DH-PH domain of Intersectin. In the absence of a competitive ligand, **sITSN1** had the highest catalytic activity and **sITSN4** had the lowest. Unfortunately, all four constructs were only weakly activated in the presence of a ligand for Bcl-xL (Figure 3).

We hypothesized that it may be difficult to disrupt the intramolecular interaction between the BH3 peptide and Bcl-xL regulatory domains with an intermolecular competitor of similar affinity. Therefore, we designed a panel of BH3 peptide mutants that have lower affinities for Bcl-xL. Based on previous analyses that have been performed with the BH3-only protein Bim, two conserved hydrophobic residues in the BH3 peptide used in our sITSN constructs (residues 137−163 of Bad: APPNLWAAQRYGRE**L**RRMSDE**F**EGSFK) were selected as sites of mutagenesis.[9] A peptide variant with little or no affinity (K_i > 10 000 nM) for Bcl-xL was generated by converting Leu151 into a Glu residue (Figure S1). Substitution of Phe158 with a Ser and Gly resulted in peptides that have 33-fold and 75-fold lower affinities for Bcl-xL than wild type, respectively (Figure S1). Synthetic Intersectin constructs that have a range of affinities between the binding partners in their artificial regulatory domains were obtained by generating F158S, F158G, and L151E mutants of **sITSN1−sITSN4**.

To determine how Bcl-xL and the BH3 peptide affect the catalytic activity of the DH domain in the absence of an autoinhibitory interaction, the nucleotide exchange assay was performed with the L151E mutants of **sITSN1−sITSN4**. Gratifyingly, the L151E mutants of **sITSN1−sITSN3** showed comparable catalytic activities (>90%) as the unregulated DH-PH domain (Figure S2). However, the L151E mutant of **sITSN4** was 33% less active. Next, the ability of each synthetic GEF to be activated with a competitor that disrupts the interaction between Bcl-xL and the BH3 peptide was ascertained by testing their nucleotide exchange activity in the

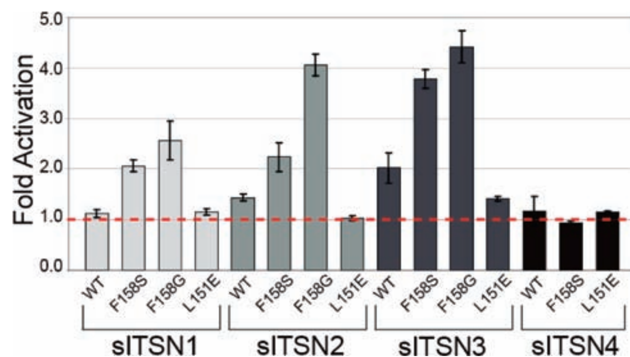


Figure 3. Activation of the sITSN constructs in the presence of a competitive ligand. All sITSN constructs were assayed in the presence of 5 μM Bad (competitor) or 5 μM Noxa (control) peptide using the conditions in Figure 2. Data are presented as the ratio of catalytic activity in the presence of Bad divided by the catalytic activity in the presence of Noxa. Any sITSN's with a ratio >1.0 are activated by a competitor. Values shown are the average of three independent assays ± SEM.

presence of 5 μM Bad. The level of autoinhibition for each construct was also determined by performing assays in the presence of 5 μM Noxa. The fold increase in catalytic activity for each construct is shown in Figure 3. As expected, the L151E mutants of **sITSN1−sITSN4** did not demonstrate a significant increase in nucleotide exchange rate in the presence of a competitive ligand due to their lack of autoinhibition. However, the F158G and F158S mutants of **sITSN1−sITSN3** were activated significantly more than their corresponding wild type variants, verifying that the lack of activation observed for the wild type constructs is due to the high affinity between their artificial regulatory domains. Significantly, in the presence of 5 μM Bad the F158G mutants of **sITSN2** and **sITSN3** show a greater than 4-fold increase in activity, which is a greater dynamic range in catalytic activity than is afforded by the endogenous SH3 regulatory domains of Intersectin.[10]

An advantage of using competitive ligands to control signaling enzyme activity is that dose−response analyses can be performed. To determine the range of activities that can be achieved with our constructs, the nucleotide exchange activities of several sITSN constructs (F158G **sITSN3**, F158S **sITSN3**, and F158G **sITSN2**) were determined in the presence of varying concentrations of Bad competitor peptide (Figures 4 and S3). As expected, all three sITSN's had higher catalytic activities in the presence of increasing concentrations of Bad peptide competitor. Both F158G **sITSN2** and F158G **sITSN3** exhibited ~50% maximum catalytic activity in the presence of 5 μM of competitor, while 25 μM Bad was required for a similar effect with F158S **sITSN3**. In the presence of 25 μM Bad, F158G **sITSN2** and F158G **sITSN3** catalyzed nucleotide exchange at a similar level as the unregulated DH-PH domain of Intersectin, which represents an 8-fold increase in catalytic activity over the autoinhibited state.

Unfortunately, **ABT-737** and **A-385358** are not compatible with the fluorescence-based nucleotide exchange assay due to their UV absorbance properties. Therefore, a GST pull-down assay was used to determine whether these small molecules are able to activate the DH domain of Intersectin. This assay relies on the use of a GST-tagged version of the Cdc42/Rac interactive binding (CRIB) domain of Pak1,[11] which selectively binds to GTP-bound Cdc42 over the GDP-bound form. By incubating Cdc42·GDP in the presence of free GTP and selectively purifying Cdc42·GTP with the GST-CRIB fusion, the efficiency of nucleotide exchange can be determined. Results for pull-down assays performed with F158G **sITSN3** are shown in Figure 5 (top panel). As expected, in the absence of **sITSN3**, GTP, Cdc42·GDP substrate, or a competitive

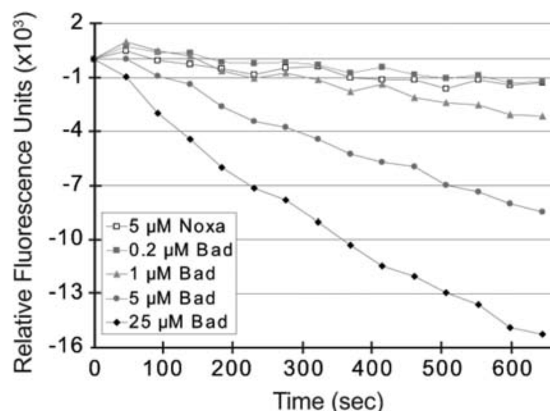
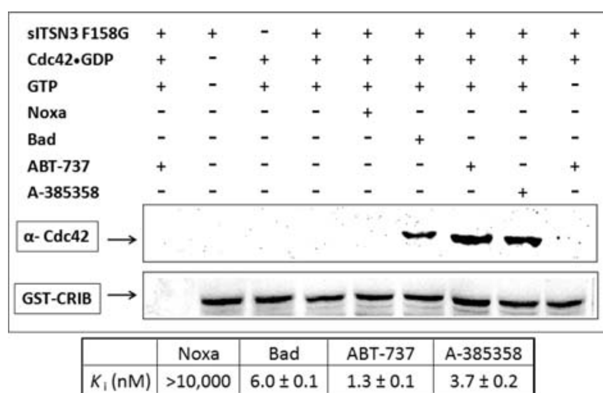


Figure 4. Dose−response analysis of the catalytic activity of F158G **sITSN3**. All assays were performed using the conditions described in Figure 2. Assays were performed in triplicate.



	Noxa	Bad	ABT-737	A-385358
K_i (nM)	>10,000	6.0 ± 0.1	1.3 ± 0.1	3.7 ± 0.2

Figure 5. **ABT-737** and **A-385358** activate F158G **sITSN3**. (*Top panel*) GST pull-down assays performed with the CRIB domain of Pak1 (GST-CRIB). GST-CRIB selectively binds to Cdc42·GTP. F158G **sITSN3** was incubated with Noxa peptide (5 μM), Bad peptide (5 μM), **ABT-737** (10 μM), or **A-385358** (10 μM). All reactions were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with α-Cdc42. (*Bottom panel*) Affinities (K_i's) of Noxa peptide, Bad peptide, **ABT-737**, and **A-385358** for Bcl-xL determined with a fluorescence polarization competition assay. Values shown are the average of three assays ± SEM.

ligand, no nucleotide exchange occurs (lanes 2, 3, 4, and 9, Figure 5). Furthermore, in the presence of the Noxa control peptide (5 μM), very little Cdc42 is detected due to minimal formation of Cdc42·GTP (lane 5, Figure 5). However, 5 μM of the Bad competitor peptide activates the nucleotide exchange activity of F158G **sITSN3**. Gratifyingly, 10 μM **ABT-737** and **A-385358** appear to activate F158G **sITSN3** more effectively than the Bad peptide competitor (lanes 6, 7, and 8, Figure 5). The greater ability of **ABT-737** and **A-385358** to disrupt autoinhibition of F158G **sITSN3** is consistent with the 2-fold higher concentration of these ligands and their higher affinities for Bcl-xL (bottom panel, Figure 5).

While an ideal ligand for controlling synthetic signaling enzymes would not interact with any endogenous cellular proteins, the redundant function of the antiapoptotic Bcl-2 family proteins should allow **ABT-737** and **A-385358** to be used in most mammalian cell lines. **ABT-737** binds tightly to the Bcl-2 family members Bcl-2, Bcl-xL, and Bcl-w but has a much lower affinity for Mcl-1, Bcl-b, and A1.[6] For this reason, the viabilities of cells that are not already primed for cell death genetically or with cytotoxic agents are not affected by high concentrations of this molecule.[6,12] Furthermore, **A-385358**, which is selective for Bcl-xL over other Bcl-2 family members, including Bcl-2, has a minimal effect as a single agent on most cells lines.[6b] To confirm this, we subjected three cell lines that are commonly used for studying GTPase function (Cos-7, HeLa, and HEK-293) to a 10 μM concentration of **ABT-737** or **A-385358**. Continuous exposure for 8 h to these compounds did not affect the viability of these cells (Figure S4). Furthermore, an enhanced apoptotic response over a DMSO control was not detected by Western blot analysis after 4 h (Figure S5). The concentration and exposure times in these assays represent the upper limit of what would be used in cellular studies with our synthetic GEFs.

In summary, we have demonstrated that the small-molecule-controllable interaction between Bcl-xL and a BH3 peptide can be used to control the catalytic activity of the GEF Intersectin. This method should be useful for engineering additional Dbl-family GEFs that can be regulated with a small molecule because many other members of this enzyme family are regulated by modular allostery. Furthermore, this strategy may be applicable to other signaling enzymes including protein kinases and phosphatases. Current efforts are underway to expand the scope of this methodology and to use the constructs generated to study signaling enzyme function in living cells.

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Supporting Information Available: Figures S1−S5, experimental details, and the complete citations for ref 6a−6d. This material is available free of charge via the Internet at http://pubs.acs.org.

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