Communication

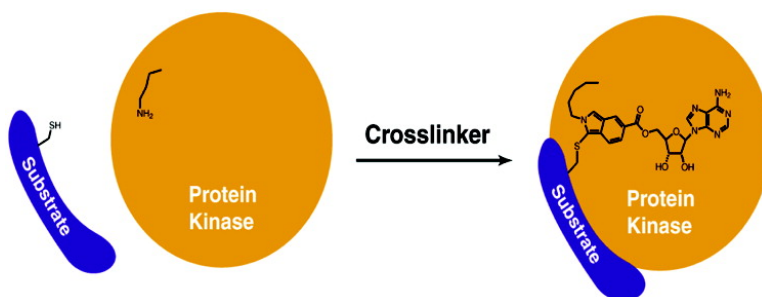# A Mechanism-Based Cross-Linker for the Identification of Kinase–Substrate Pairs

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A Mechanism-Based Cross-Linker for the Identification of Kinase−Substrate Pairs

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Protein phosphorylation is one of the most dominant forms of information transfer in cells, and the dissection of phosphorylation cascades is essential for our understanding of signal transduction.[1] To this end, biochemical and proteomic efforts have been invaluable in identifying numerous phosphoproteins and phosphorylation sites.[2] Unfortunately, the full potential of this information remains unrealized because there is a paucity of techniques that allow a researcher to use a phosphoprotein to identify its upstream kinase.[3] Standard methods for the identification of protein−protein interaction partners such as yeast 2-hybrid screens and pull-down assays are typically not effective, as kinase−substrate interactions are transient and usually of low affinity. To this end, we have developed a chemical method that allows the covalent cross-linking of a substrate of interest to its upstream kinase, thus facilitating purification of the entire substrate−kinase complex (Figure 1).

To convert a transient enzyme−substrate interaction into a covalent complex, we have developed a novel mechanism-based cross-linker. For the identification of bona fide substrate−kinase pairs we designed our cross-linker to be (1) selective in that it only cross-links a defined phosphorylation site of interest with the kinase responsible for phosphorylating it and (2) general for any kinase−substrate pair. To ensure that only the substrate of interest reacts with the cross-linker, the serine/threonine residue that is phosphorylated is replaced with a reactive cysteine residue.[4] This substitution provides the substrate with a reactive moiety that is positioned in close proximity to the kinase's catalytic lysine. To guarantee generality, the cross-linker was designed to interact with the conserved ATP-binding site and target the invariant catalytic lysine residue. We based the scaffold of our cross-linker on 5′-fluorosulfonylbenzoyl adenosine (FSBA) **1**, a general covalent inhibitor of protein kinases that modifies the catalytic lysine.[5] To convert FSBA from a monofunctional electrophile into the bifunctional cross-linker **2**, the aryl sulfonyl fluoride was replaced with an *o*-phthaldialdehyde
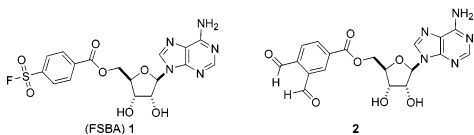
(OPA) moiety. OPA and its derivatives have previously been utilized to selectively cross-link proximal lysines and cysteines in an intramolecular fashion.[6] In contrast, we have designed **2** to intermolecularly cross-link the lysine of a kinase and the cysteine of a substrate.

When cross-linker **2** occupies the ATP-binding pocket of a kinase, the catalytic lysine is expected to form a reversible imine adduct (Figure 1b) that will then be trapped by the cysteine residue of the substrate (Figure 1c). This reaction results in the formation of a stable isoindole linkage between the cognate kinase−substrate pair (Figure 1d). In this context, **2** serves as a mechanism-based cross-linker that is selectively activated by the kinase's conserved
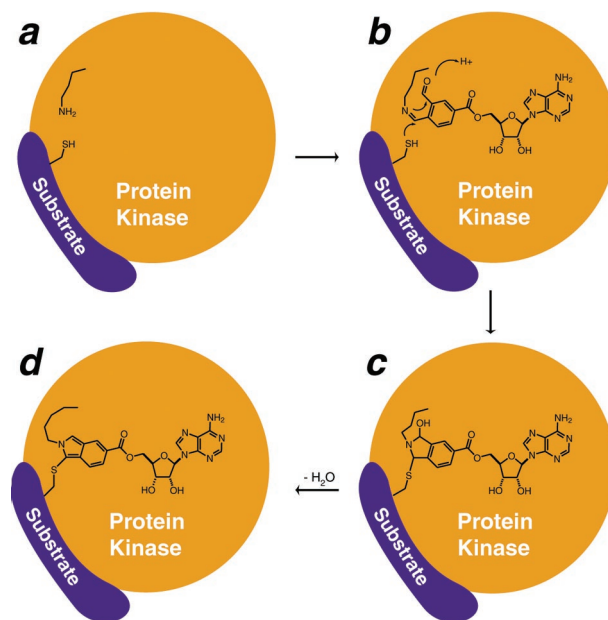

Figure 1. Schematic representation of the substrate−kinase cross-linking reaction.

catalytic machinery. To our knowledge this is the first example of the use of a cofactor analogue as a mechanism-based cross-linker for the identification of enzyme−substrate interactions.

To evaluate dialdehyde **2** as a mechanism-based cross-linker, a set of peptide substrate derivatives with and without a cysteine (biotin-ZRPRTSSF-OH **3**, fluorescein-ZZRPRTSSF-OH **4**, biotin-ZRPRTSCF-OH **5** and fluorescein-ZZRPRTSCF-OH **6**) were incubated with the kinase, Akt1.[7] As shown in Figure 2A, incubation of Akt1 and the serine-containing peptide **3** in the presence of dialdehyde cross-linker **2** led to no detectable cross-linking (lane 1). However, when cysteine-containing peptide **5** and Akt1 were incubated with dialdehyde **2** rapid formation of the peptide−Akt1 complex was observed (lane 2),[8] demonstrating the necessity of a free thiol for cross-linking to occur. As expected, the peptide−Akt1 covalent complex runs at a slightly higher molecular weight than unmodified Akt1 on SDS-PAGE (Supporting Information, Figure 3). All three components were found to be necessary, as labeling is not observed in the absence of dialdehyde **2** (lane 3), Akt1 (lane 4), or peptide **5** (lane 5). In addition, the absence of the peptide−Akt1 complex upon prior heat denaturation of Akt1 demonstrates that the protein must be properly folded for the cross-linking reaction to occur (lane 6). The cross-linking reaction is independent of the reporter group, as identical results were obtained for peptides **5** and **6** (data not shown).

To determine the sensitivity of the cross-linking reaction to the presence of competing thiol nucleophiles, Akt1 was incubated with peptide **6** and dialdehyde **2** in the presence of increasing concentrations of β-mercaptoethanol or Ac-Cys-OH (Supporting Information,
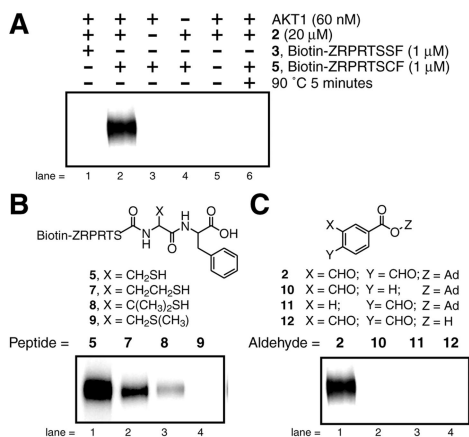
Figure 2. SDS-PAGE analysis of the cross-linking reaction. All gels were transferred to nitrocellulose and probed for biotin with a streptavidin–HRP conjugate. (A) Initial characterization of the proposed cross-linking reaction. All reactions were run for 20 min at room temperature. (B) Cross-linking with peptide derivatives (**7**–**9**). Each peptide (**5**, **7**–**9** [1 μM]) was incubated with Akt1 (60 nM) and dialdehyde **2** (20 μM) for 20 min at room temperature. (C) Cross-linking with aldehyde derivatives (**10**–**12**). Each aldehyde (**2**, **10**–**12**, [20 μM]) was incubated with peptide **5** (1 μM) and Akt1 (60 nM) for 20 min at room temperature. Ad = adenosine.

Figure 4). The cross-linking reaction appears to be shielded from exogenous nucleophiles, as the presence of either thiol in 100-fold excess of peptide **6** did not significantly reduce the amount of cross-linking observed. Furthermore, a peptide substrate of casein kinase II (biotin-ZRRADD**C**DDDD-OH) was not cross-linked to Akt1 when incubated in the presence of dialdehyde **2** (data not shown).

To probe the requirements of the thiol nucleophile, several derivatives of peptide **5** (**7**–**9**) were tested for their ability to undergo the cross-linking reaction (Figure 2B). The orientation of the attacking thiol relative to the formed imine has a dramatic effect on the efficiency of cross-linking, as demonstrated by the reduced amount of peptide–Akt1 complex observed for the homocysteine-substituted peptide **7** (lane 2). In addition, the greatly reduced cross-linking efficiency observed for a peptide containing methyl groups geminal to the attacking thiol **8** demonstrates that increased steric bulk has a detrimental effect on cross-linking (lane 3). Consistent with the mechanism of isoindole formation, the Akt1–peptide complex is not formed with a peptide that contains an alkylated cysteine **9** (lane 4).

Competition experiments were performed to confirm that cross-linking occurs in the ATP-binding site and the protein-binding groove (Supporting Information, Figure 3). Increasing concentrations of biotin-ZRPRTSSF–OH **3** effectively diminished the formation of the peptide–Akt1 complex. In addition, increasing amounts of ATP also reduced the amount of cross-linking observed. In both cases, a 1000-fold excess of competitor was necessary to prevent detectable formation of the peptide–Akt1 complex, demonstrating the speed and efficiency of this reaction. The robustness of the cross-linking reaction should be beneficial in an intracellular environment that contains a high concentration of ATP and competing nucleophiles.

To further determine whether the cross-linking reaction is occurring in the ATP-binding site via isoindole formation, a series of alternative aldehyde derivatives (**10**–**12**) were tested (Figure 2C). The necessity of the dialdehyde moiety was demonstrated by the inability of monoaldehydes **10** or **11** to cross-link peptide **5** with Akt1 (lanes 2 and 3). Without adenosine to guide the dialdehyde to the ATP-binding pocket, 3,4-diformylbenzoic acid **12** displays greatly reduced cross-linking (lane 4).

The generality of our method was demonstrated by the ability

of dialdehyde **2** to facilitate the cross-linking of a diverse set of serine/threonine kinases with their respective cysteine-containing pseudosubstrates (Supporting Information, Figure 5). The MAP kinase p38 was observed to be cross-linked to a cysteine version (X = Cys) of the peptide biotin-ZIPT**X**PITTTYF–OH. In addition, a peptide–kinase complex of the serine/threonine kinase PKA and a cysteine-containing (X = Cys) peptide, biotin-ZLRRA**X**LG-OH, was formed in the presence of **2**. In both cases, cross-linking did not occur with the serine version (X = Ser) of these peptides. Furthermore, the serine/threonine kinase casein kinase II was also found to undergo facile cross-linking with a cysteine version (X = Cys) of a peptide pseudosubstrate, biotin-ZRRADD**X**DDDD-OH. The diversity of the kinases that undergo the cross-linking reaction with cysteine-containing pseudosubstrates demonstrates that **2** should be a general cross-linker for most serine/threonine kinases. Further experiments are underway to determine if this method is useful for protein tyrosine kinases as well.

In conclusion, we have described a general method for the identification of kinase–substrate pairs. This method relies upon the replacement of a phosphorylated residue with an engineered cysteine and a novel, mechanism-based ATP-analogue cross-linker. The cross-linking reaction is robust and appears to be general for a number of serine/threonine kinases. Use of this method for the identification of novel kinase–substrate pairs will be reported in due course.

Supporting Information Available: Complete protocols for cross-linker and peptide synthesis and characterization, as well as full experimental procedures for all cross-linking reactions. This material is provided free of charge via the Internet at http://pubs.acs.org.

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(8) Under the reaction conditions described in Figure 2A, 20−25% of the kinase is cross-linked to the peptide substrate (Supporting Information, Figure 1). For an analysis of the kinetics of the cross-linking reaction see Supporting Information, Figure 2.

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