

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

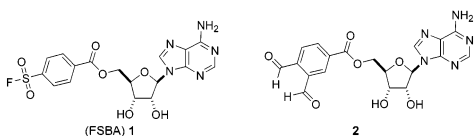
Dustin J. Maly, Jasmina A. Allen, and Kevan M. Shokat*

Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94107, and Department of Chemistry, University of California, Berkeley, California 94720

Received March 9, 2004; E-mail: shokat@cmp.ucsf.edu

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*-phthalaldehyde



(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

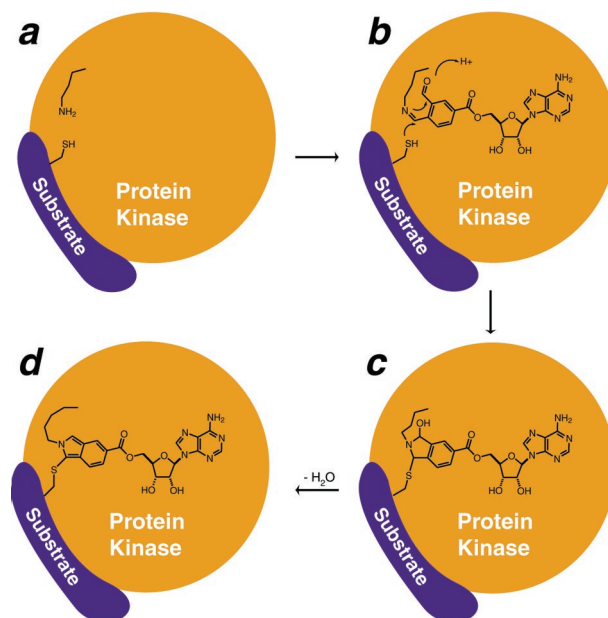


Figure 1. Schematic representation of the substrate–kinase cross-linking reaction.

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,

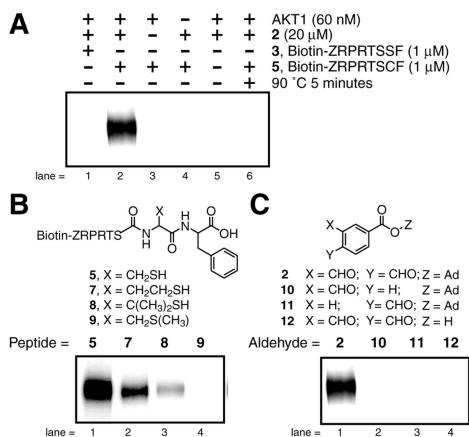


Figure 2. SDS-PAGE analysis of the cross-linking reaction. All gels were transferred to nitrocellulose and probed for a streptavidin–HRP conjugate. (A) Initial characterization of the proposed cross-linking reaction. All reactions were run for 20 min at room temperature. (B) Cross-linking with peptide derivatives (7–9). Each peptide (5, 7–9 [1 μ M]) was incubated with Akt1 (60 nM) and dialdehyde **2** (20 μ M) for 20 min at room temperature. (C) Cross-linking with aldehyde derivatives (10–12). Each aldehyde (**2**, 10–12, [20 μ M]) was incubated with peptide **5** (1 μ M) 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_CDDDD-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-ZIPTXPITTTYF–OH. In addition, a peptide–kinase complex of the serine/threonine kinase PKA and a cysteine-containing (X = Cys) peptide, biotin-ZLRRAXLG-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_CDDDD-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.

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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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