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Conversion of a Tyrosine Kinase Protein Substrate to a High Affinity Ligand by ATP Linkage

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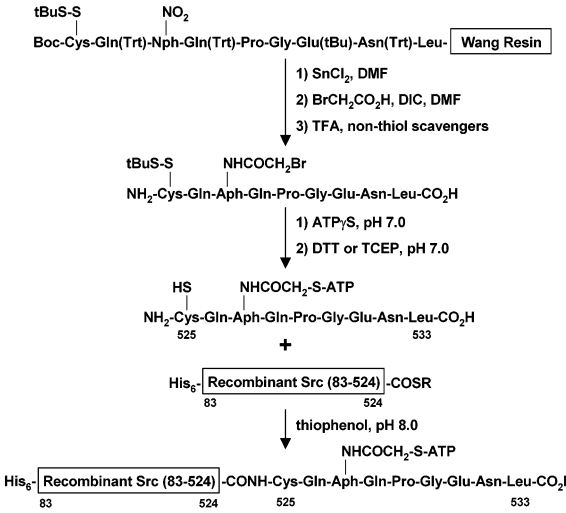
Protein kinases are key enzymes in cell signal transduction and are now viewed as therapeutic targets for a host of disease states.[1] Despite the development of a variety of creative and powerful new technologies to understand their function,[2] for many kinases key cellular protein targets remain elusive, an obstacle to the development of cell-based screens for drug discovery. Unlike many protein−protein interactions such as those underlying SH2 domain-phosphotyrosine and 14-3-3-phosphoserine recognition,[3] protein kinase−protein substrate interactions are often low affinity and not reliably discovered by techniques such as affinity pull-downs or two-hybrid screens. Furthermore, it is not uncommon for protein kinase−protein substrate interactions to be specified by long-range interactions between each protein partner, rather than the local amino acids surrounding the phosphorylation site.[4] Thus, for many phosphoproteins, identifying the protein kinase responsible for targeted phosphorylation can be an extremely difficult task. Here, we show that by merging bisubstrate analogue technology along with the method of expressed protein ligation it is possible to convert a protein tyrosine kinase Csk substrate into a high affinity inhibitor which can be used to isolate Csk from a protein mixture.

Protein tyrosine kinase Csk is responsible for site-specific tail phosphorylation of Src and Src family members, and this phosphorylation negatively regulates Src activity.[5] Although heavily studied, the precise details about this selectivity have not been elucidated, although long-range interactions are thought to be critical. It has been shown recently that it is possible to convert a peptide substrate into a tyrosine kinase inhibitor by linking ATPγS to an aminophenylalanine (Aph), replacing Tyr at the phosphorylation site via an acetyl spacer.[6] We hypothesized that it might be possible to generate a Src-ATP conjugate by first generating the requisite ATP-conjugated peptide and then fusing this to Src by the method of expressed protein ligation[7,8] (Scheme 1). The ATP-conjugated peptide sequence was derived from the Src tail but included a substitution of a Cys at the N-terminus in place of a Pro normally found at this site. Previous studies have shown that this amino acid substitution in Src is well tolerated in phosphorylation by Csk.[8] The peptide was prepared by using nitrophenylalanine (Nph) as a precursor to aminophenylalanine (Aph) with reduction by $SnCl_2$ on the solid phase.[6] It was established that this reduction was compatible with disulfide protection of the Cys which was carried throughout the deblocking and cleavage of the bromoacetylated peptide and removed by DTT or TCEP reduction after the ATPγS ligation.

The recombinant Src (K295M) fragment fused in frame with an intein-chitin binding domain segment (Scheme 1) was prepared as described previously[8] for subsequent ligation with ATP-conjugated peptide. The semisynthetic Src-ATP protein generated in this fashion was isolated after extensive dialysis to remove excess unreacted peptide and concentrated by ultrafiltration to 0.5 mg/mL and was found to be approximately 70−80% pure by SDS-PAGE stained with Coomassie blue (Figure 1). MALDI-TOF MS confirmed that the semisynthetic protein had the correct mass.

Scheme 1. Generation of the Src-ATP Conjugate through Sequential Peptide ATPγS Ligation and Expressed Protein Ligation



The semisynthetic Src-ATP protein so produced was evaluated as a Csk inhibitor using the standard assay of phosphorylation of a synthetic tyrosine peptide substrate.[9] In this fashion, it was found that the Src-ATP conjugate was a fairly potent inhibitor of Csk catalyzed phosphorylation, exhibiting an IC_{50} of 600 nM (Figure 2a). As a control, recombinant Src with a Phe in place of the conjugated ATP (Src-Phe)[10] showed no inhibition up to 10 μM (Figure 2b). While Src-ATP was a fairly strong Csk inhibitor, we suspected that this level of inhibition may have been suppressed by the possibility of inter- or intramolecular interactions between Src-ATP molecules facilitated by nucleotide binding pocket interactions as exemplified in Figure 2c. To evaluate this possibility, the Csk kinase inhibition assays were carried out in the presence of the purine analogue PP1 which is a Src selective inhibitor.[11] Addition of PP1 led to enhanced blockade of Csk by Src-ATP as illustrated in Figure 2d. Assuming a competitive inhibition model, in the presence of PP1, Src-ATP shows a K_i of approximately 100 nM for Csk inhibition, which is approximately 100-fold lower than the K_m of ATP under these assay conditions (see Supporting Information).

It was of interest to examine whether Src-ATP could selectively pull down Csk from a mixture of proteins. Src-ATP was immobilized onto a metal-chelating column via its N-terminal His_6 tag[8,10] and was incubated with mammalian (NIH3T3) cell extracts spiked with Csk (approximately 1:100 Csk:cellular proteins). After aggressive wash and elution, Csk was found to be selectively retained (Figure 3). In a separate experiment in which the incubation

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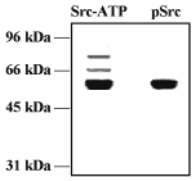

Figure 1. Coomassie blue-stained 10% SDS-PAGE of the Src-ATP semisynthetic protein. Approximately 2 μg of the dialyzed Src-ATP semisynthetic protein and the phosphorylated Src recombinant protein (pSrc, for comparison)[13] are shown.

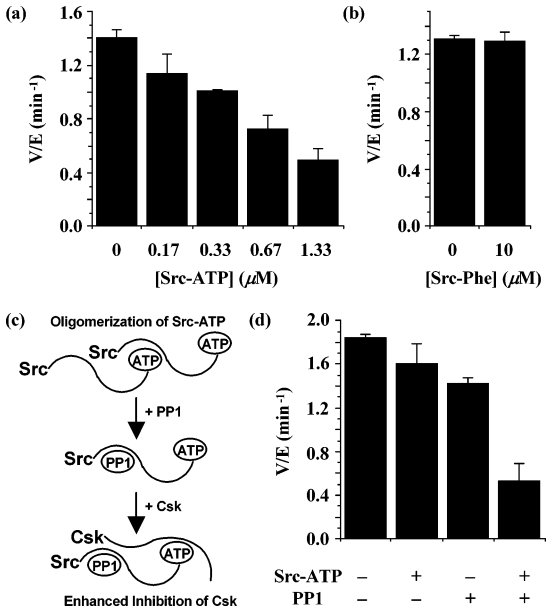

Figure 2. Csk inhibition assayed in the presence of 5 μM of ATP and 200 μM of an optimized substrate peptide $K_4E_2IYF_3$.[9] (a) Csk activity versus [Src-ATP]; (b) Csk activity versus [Src-Phe]; (c) hypothesized PP1 enhancement of Csk inhibition; (d) Csk activity in the presence of Src-ATP (0.33 μM) and/or PP1 (0.25 μM).

was carried out in the presence of 10 mM ADP, SDS-PAGE analysis showed a rather specific disappearance of a band comigrating with Csk (Figure 3). This suggests that ADP is selectively disrupting the Csk/Src-ATP interaction. In an additional control experiment in which Src-Phe was immobilized on the resin instead of Src-ATP, Csk was also minimally retained (Figure 3). Selectivity in binding to Src-ATP by Csk versus other protein kinases (including casein kinase 2, insulin receptor tyrosine kinase, and protein kinase A) not known to phosphorylate the Src tail was also demonstrated using *E. coli* cell extract systems (see Supporting Information).

Thus, these studies outline a strategy that could in principle be used to identify an unknown kinase that might be responsible for the phosphorylation of a protein substrate of interest. In addition, the use of such protein-ATP conjugates could also facilitate high-resolution X-ray crystal structural determination of a protein kinase with its physiologic substrate by enhancing the stability of this complex. Such a strategy is complementary to the elegant covalent trapping approach that has been pioneered in the DNA polymerase/repair enzyme arena.[12]

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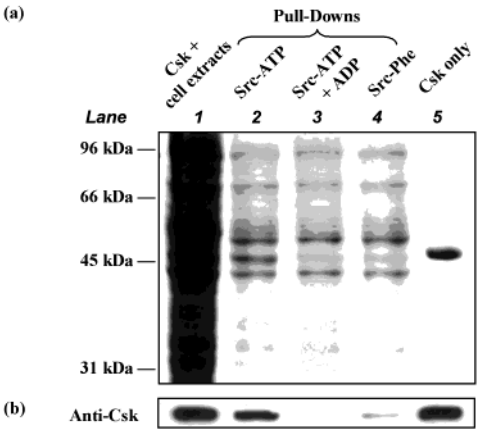

Figure 3. 10% SDS-PAGE of pull-downs from dialyzed mammalian (NIH3T3) cell extracts spiked with Csk detected by (a) Coomassie blue staining, and (b) anti-Csk antibody immunoblotting, respectively [lanes 1 and 5 (each containing about 0.5 μg of Csk): Csk + extracts (2.7 μL, 20 mg/mL), and Csk only; lanes 2−4: pull-downs by Src-ATP and Src-ATP in the presence of 10 mM ADP and Src-Phe, respectively].

Supporting Information Available: Experimental details for ATP-conjugated peptide synthesis, protein semisynthesis, kinase assays, and pull-down assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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