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Selective photolabeling of Lck kinase in complex proteome

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Abstract—A molecular probe that selectively tags Lck, a Src-family kinase, was developed. This probe was one of many compounds originally designed to target the active site of tyrosine kinases in general. To our surprise, however, the probe almost exclusively labeled Lck even in a lysate of Jurkat cells. This finding led us to further characterize this probe-Lck complex by a series of photolabeling and mass spectrometric analyses. The probe-binding site on Lck was located within the well-conserved region of Src-family kinases, as we originally expected. However, the unexpected selectivity of this probe toward Lck suggests that subtle factors, which are difficult to predict based on static crystal structures, play important roles in probe recognition.

Lck is a Src-family kinase, playing multifarious roles in thymocyte differentiation and T-cell activation.[1] Its roles are also implicated in various diseases, including lymphocyte malignancy[2] and immunodeficiency.[3] Molecules that selectively tag Lck, therefore, can serve as useful tools for both clinical and basic biomedical research.

Our group is developing molecular probes to examine the expression and function of kinases.[4] Such compounds can serve as useful tools to profile kinases in a manner analogous to other chemical proteomics probes.[5] During this effort, we unexpectedly found a compound that tags Lck in a highly selective manner. Here, we present the characterization of this compound. This study revealed subtle nature of probe recognition by Lck, which is difficult to predict from static crystal structure of this kinase.

The study started out with a different and, in a sense, less ambitious goal. We were originally targeting tyrosine kinases (TKs) in general. TKs possess several hydrophobic regions within the substrate binding sites. Therefore, they could be selectively photo-crosslinked by divalent ligands containing adenine and benzophenone, which can, in theory, target the hydrophobic regions of TKs (Fig. 1). Once a compound with modest selectivity toward TKs is identified, the tethering region can be modified to tune the affinity and selectivity. The concept of tethering two weak ligands to obtain a higher affinity ligand, that is, the principle of thermodynamic additivity,[6] has been successfully employed for the development of many enzyme inhibitors.[7] We, therefore, simply tried to apply this principle for the development of tunable photo-probes for TKs.

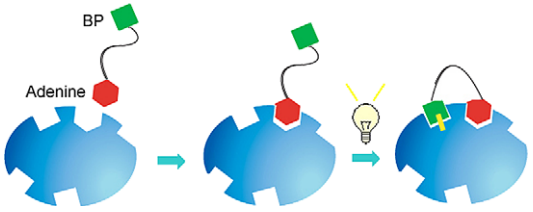

Figure 1. Original tagging strategy for tyrosine kinases (TKs). TKs possess hydrophobic pockets in the substrate-binding sites. Thus, tethering of adenine and benzophenone (BP) with appropriate linkers can lead to bidentate photoligands that can selectively tag TKs.

A series of adenine–benzophenone conjugates were synthesized using a standard Fmoc chemistry on solid phase as reported earlier.[4] Synthesized probes were purified by reversed phase HPLC, and their structures were confirmed with NMR and mass spectrometry (see Supplementary data). In this paper, we focus on two newly synthesized compounds, **1S** and **1R**, in which the letters, **R** and **S**, denote the configurations of the chirality center in the benzophenone moiety.

Keywords: Src family kinases; Molecular recognition; Photo-crosslinking; Benzophenone; Chemical proteomics.
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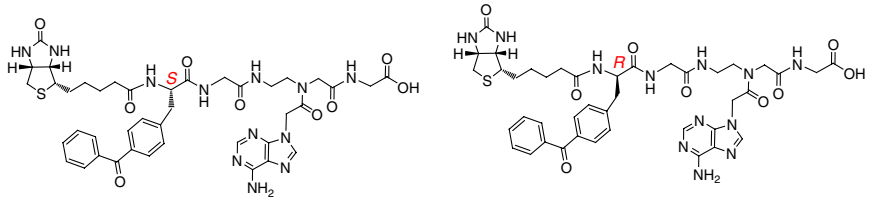

As a preliminary study to characterize the binding selectivity, we screened a panel of six commercially available kinases, PKA, GSK3, CK1, Src, Fyn, and Lck. Kinases were incubated with the probes and irradiated under a UV-A lamp (λ_{max} 350 nm). Samples were then subjected to SDS–PAGE in denaturing condition and blotted onto PVDF membrane. Blotted membrane was treated with an anti-biotin horseradish peroxidase-conjugated antibody, and biotinylated proteins were visualized by chemiluminescence. To our surprise, **1S** exhibited distinct selectivity toward Lck (Fig. 2a). **1R**, on the other hand, did not tag any of the kinases in the panel (Fig. 2b). Thus, a single chirality center at the base of benzophenone makes a dramatic difference in the recognition by Lck. Further study of **1S** with additional kinases, that is, VEGFR2, EGFR, PKC-α, CK2, and MAPK did not identify other targets of **1S** (see Supplementary data).

A series of blocking experiments was subsequently carried out to gain insights into the interaction between Lck and **1S**. Lck was pre-incubated with an excess (10×) amount of benzophenone, adenine, or biotin prior to the photolabeling with **1S**. This study revealed that the labeling can be significantly blocked by benzophenone and adenine (Fig. 2c, lanes 2 and 3), in which adenine exhibited more pronounced blocking effect. The labeling was not affected by biotin at all (Fig. 2c, lane 4). These results indicated that Lck recognized adenine and benzophenone of **1S** but not the biotin moiety. As expected, however benzophenone, an unnatural ligand to Lck, was recognized more weakly than adenine.

The binding site was then determined by MALDI-TOF mass spectrometry and LC/MS/MS. MALDI-TOF mapping of the trypsin digest, which covered 82% of the Lck sequence, identified a peptide fragment (*m/z* 1746.8), corresponding to Ile379-Arg386 (IADFGLAR) tagged with **1S**. This fragment was separated by gradient elution with the Dionex capillary/nano-HPLC system and subjected to MS/MS analyses by Applied Biosystems QSTAR XL mass spectrometer using information-dependent, automated acquisition.

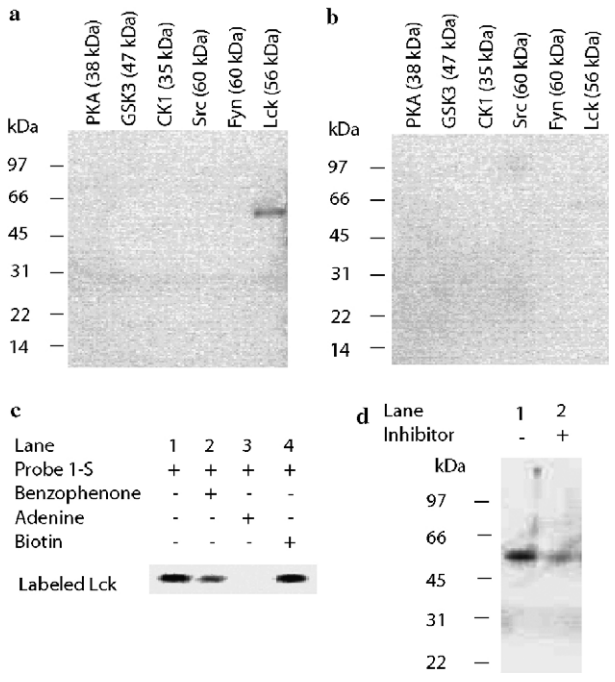

Figure 2. Photolabeling studies of purified kinases and cytosolic extract of Jurkat cells. (a) Selective labeling of Lck with **1S**, (b) None of the six kinases was labeled with **1R**. (c) Blocking experiments. (d) Selective labeling of Lck in cytosolic extract of Jurkat cells.

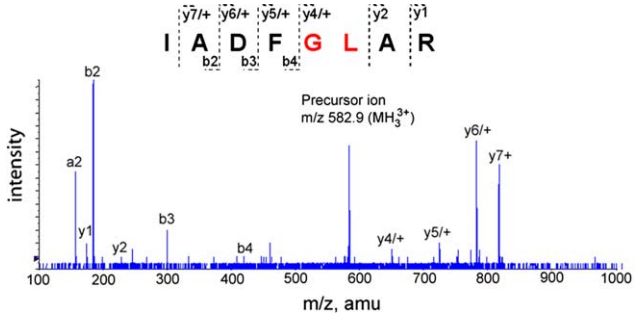

Figure 3. Identification of the benzophenone-binding site using MS/MS fragmentation analysis.

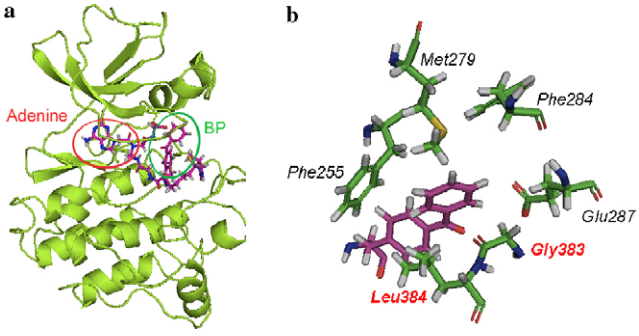

Figure 4. The current model of **1S**-Lck complex. (a) The image of the whole complex: Lck (lime green) and **1S** (backbone in pink). (b) The close-up of amino acid residues surrounding the benzophenone moiety.

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sp|P06239|LCK_HUMAN     TRLSRPCQTQKP----QKPWWEDEWEVPRETLKLVERLGAGQFGEVWMGYYNGHTKVAVK
sp|P06241|FYN_HUMAN     CRLVVPCHKGMPRLTDLSVKTKDVWEIPRESLQLIKRLGNGQFGEVWMGTWNGNTKVAIK
sp|P12931|SRC_HUMAN     HRLTTVCPTSKP---QTQGLAKDAWEIPRESLRLEVKLGQGCFGEVWMGTWNGTTRVAIK

sp|P06239|LCK_HUMAN     SLKQGSMSPDAFLAEANLMKQLQHQRLVRLYAVVTQEPIYIITEYMENGSLVDFLKTPSG
sp|P06241|FYN_HUMAN     TLKPGTMSPESFLEEAQIMKKLKHDKLVQLYAVVSEEPIYIVTEYMNKGSLLDFLKDGEG
sp|P12931|SRC_HUMAN     TLKPGTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSEEPIYIVTEYMSKGSLLDFLKGETG

sp|P06239|LCK_HUMAN     IKLTINKLLDMAAQIAEGMAFIEERNYIHRDLRAANILVSDTLSCKIADFGLARLIEDNE
sp|P06241|FYN_HUMAN     RALKLPNLVDMAAQVAAGMAYIERMNYIHRDLRSANILVGNGLICKIADFGLARLIEDNE
sp|P12931|SRC_HUMAN     KYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFGLARLIEDNE
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Figure 5. Sequence alignment of Lck, Fyn, and Src. The probe-binding region of Lck was aligned with Fyn and Src using CLUSTAL W (1.82) on Expasy. The ATP-binding site is colored in green. The photo-crosslinking site on Lck is highlighted in red.

The MS/MS analyses revealed that **1S** is covalently attached to either Gly383 or Leu384 (Fig. 3); fragment ions resulting from the cleavages between these two residues were not observed despite numerous attempts.

Selectivity of **1S** toward Lck in complex proteome was also examined using cytosolic proteins of Jurkat cells, a lymphocyte cell line known to express Lck. The cytosolic proteins were obtained by lysing Jurkat cells in a hypotonic buffer (see Supplementary data). Photolabeling of the cytosolic proteins with **1S** gave an intense band at ~60 kDa, which corresponded to the molecular weight of Lck (Fig. 2d, lane 1). On the other hand, when the lysate was pre-incubated with an ATP-competitive inhibitor of Lck,[8] the band intensity significantly decreased (Fig. 2d, lane 2). These results confirmed that **1S** selectively tagged Lck even in a complex mixture of proteins.

Our current model of the **1S**–Lck complex, obtained through a conformational search of **1S** within the binding site, indicates that the benzophenone moiety is surrounded by Phe255, Met279, Phe284, Glu287, Gly383, and Leu384 (Fig. 4). This model now provides us with a structural basis to design new Lck ligands with higher affinity and selectivity.

It is noted that the probe binding site is located within the well-conserved region of Src family kinases. As illustrated in Figure 5, the photo-crosslinking site, Gly383/Leu384, is conserved in Src and Fyn. Another binding site of **1S** is the ATP-binding pocket, which is highly conserved among all kinases. Our current model of the **1S**–Lck complex indicates that all hydrogen bonding and hydrophobic interactions are mediated through the amino acid residues that are well conserved in Src family kinases.

This binding site is what we had originally expected at the onset of this study. What was surprising, however, was the fact that **1S** did not tag other kinases with seemingly identical binding pocket. These observations suggest that subtle differences in structure and/or dynamics between Lck and other Src family kinases culminated in the distinct difference in the recognition of **1S**. Therefore, it may be possible to alter the selectivity by making small changes in the probe structure as exemplified by **1R**.

Another important point to note is that the concept of our method is applicable to proteins other than kinases. The adenine moiety of probe can be replaced with other recognition motifs, such as sugars and short peptides, to target many different proteins. Thus, it is possible to develop highly selective photo-probes targeting a variety of proteins using the same principle.

In conclusion, we identified a photoactive Lck ligand, **1S**, which can selectively tag Lck in complex proteomes. Lck recognizes both adenine and benzophenone moieties of **1S**. The chirality center at the base of benzophenone is important for the recognition. The probe-binding site turned out to be located within the well-conserved region of Src family kinases. Thus, the **1S**–Lck complex is an interesting system for further study to understand the subtleties of molecular recognition, which, in turn, can lead to new approaches for the selective interrogation of kinases.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.08.023.

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