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Mechanism of Lck Activation in Driving Leukemia Cell Proliferation

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Introduction

Figure 1. Lck

In the leukemia cancer cell line CTV-1, researchers have identified four characteristic mutations in the Lck gene. As a member of the Src family kinases, Lck plays an integral role in regulating growth and division in immune system cells. Leukemia develops when a mutational event alters a white blood cell's differentiation pattern and causes the cell to divide without control. As white blood cells begin accumulating in the blood, they affect healthy cell function. With Lck's role in signaling for cell division, we hypothesized that the identified mutations result in Lck's constitutive activation causing leukemia progression. In order to determine how these mutations affect Lck activation, we used molecular techniques to introduce each mutation into a normally functioning Lck enzyme, engineered a substrate specific for Lck, and demonstrated that one of the mutations activates Lck function. This research lays a foundation for understanding the oncogenic basis of the CTV-1 cancer cell.

Methods

DH5α E. coli cell stock stored at -80°C, containing a pGEX vector with the wild type Lck gene was subcultured in 4 mL of LB liquid medium and allowed to grow overnight while shaking at 250 rpm in 37°C. From these cells, the plasmid containing Lck was isolated using QIAprep Spin Miniprep Kit (Qiagen). Subsequent DNA sequencing confirmed the presence of the wild type Lck gene correctly cloned in the reading frame of the N-terminal GST affinity tag.

To purify wild type Lck enzyme, the plasmid was transformed into DH5α cells and streaked on LB agar ampicillin plates and incubated overnight. Transformed colonies were subcultured in 500 mL of LB liquid medium containing 50 µg/mL of ampicillin and allowed to grow overnight while shaking at 250 rpm in 37°C. After 12 hours, the culture was brought to room temperature and IPTG 50 µg/mL was added to induce Lck expression for 6 hours. The cells were spun down at 5000 g for 30 minutes and the pellet saved at -20°C. Cell pellets were thawed and resuspended on ice in PBS lysis buffer. To lyse the cells, the cell mixture was passed through a French Pressure cell press 3 times. The lysate was then subjected to centrifugation at 20,000 rpm at 4°C for 30 minutes. Supernatant was then incubated with 0.06 g of glutathione-agarose beads (Sigma) while shaking at 4°C for 2 hours. This lysate supernatant was passed through an empty chromatography column, washed 3 times with protocols specific for the GST-affinity tag, and finally Lck was eluted from the column. Enzyme concentration was quantified using the Bradford assay according to a standard BSA curve.

We used the QuikChange protocol to generate the Lck mutants from the wild type Lck plasmid and also engineered a substrate specific for Lck. Each QuikChange reaction was transformed into fresh competent DH5α E. coli cells. The plasmids for the mutants were isolated and DNA sequence was confirmed. The same purification protocol was used for each mutant and substrate as described above.

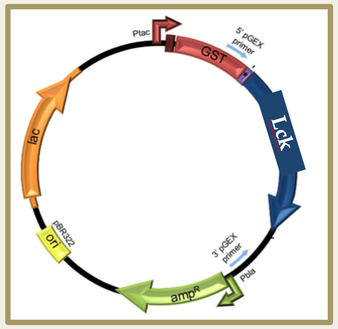

Figure 2. Illustration of pGEX plasmid.

A kinase assay was performed with Lck to test for wild type activity, while a western blot was used to test enzymatic phosphorylation of the Lck substrate by the wildtype and mutant enzyme.

Results

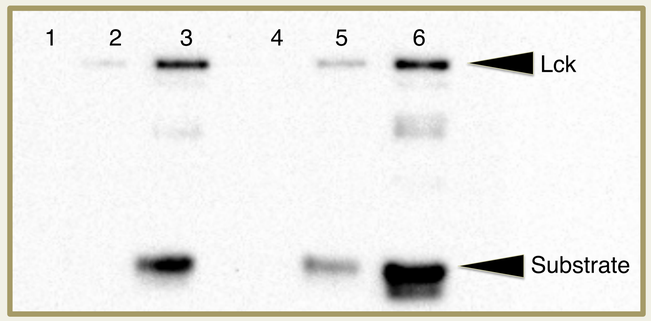

Figure 5. Western blot of wild type Lck (lanes 1-3) and Pinsert Lck (lanes 4-6) enzymatic activity. Enzyme concentrations: lanes one and two 0.02 mg/mL, lanes two and five 0.05 mg/mL , and lanes three and six 0.2 mg/mL. The same concentration of Lck substrate was used for each lane.

Figure 5 shows the relative ability of each enzyme to phosphorylate the Lck substrate and also undergo autophosphorylation. The phosphorylation on Lck and the Lck mutant was determined by Western Blot using an antibody against phosphotyrosine. Lane 3 and lane 6 show the phosphorylation of wt Lck and the Lck insertion mutant and their ability to phosphorylate the Lck substrate. At an enzyme concentration of 0.2 mg/, the Lck band staining levels are comparable between the two lanes, indicating that the two enzymes have a similar ability to autophosphorylate. The significantly darker band at the substrate level in lane six demonstrates that the Pinsert Lck mutant was more active in phosphorylating the substrate than the wild type Lck at the same enzyme concentration. This result indicates that the Pinsert Lck mutant is more active than the wild type enzyme.

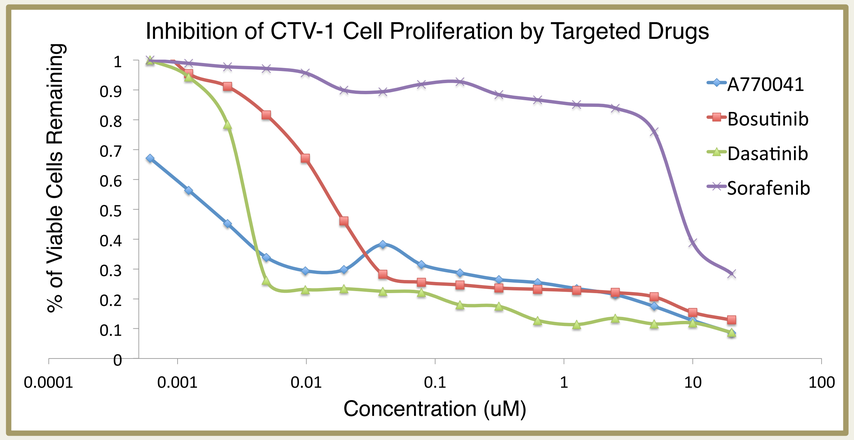

Figure 6. Inhibition of CTV-1 cells by targeted drug therapies. Beginning at a concentration of 0.0006 uM, each drug was added at concentrations increasing by a factor of 10 until the max concentration of 20 uM. Cells were incubated with each drug for 72 hours before data collection. Data is courtesy of Dr. Li Li M.D. Ph.D. and Dr. Gongquin Sun Ph.D.

To determine if the activated Lck is responsible for the proliferation of CTV-1 cells, we determined the sensitivity of CTV-1 cells to various kinase inhibitors that have different levels of inhibition for Lck. The results are shown in figure 6.. Bosutinib, Dasatinib and A770041 are Src family inhibitors with A770041 being the most selective inhibitor for Lck. Sorafenib targets FLT3, an unrelated kinase, and was used as a control. A770041 displayed an IC50 (concentration killing 50% of the cells) of.8 nM. Bosutinib, dasatinib, displayed IC50 values of 18 and 3.6 nM. In contrast sorafenib has an IC50 of 7000 nM toward CTV-1. These results demonstrate that Lck is activated in CTV-1 cells and the activated Lck is responsible for CTV-1 proliferation.

Discussion

Lck contains a linker region located between its SH2 and kinase domains. Previous studies have shown that the linker plays an important inhibitory role in regulating Lck activation through its interaction with the SH3 and kinase domains. With this regulatory mechanism, we hypothesized that the Pinsert mutation in the linker region would disrupt Lck's ability to adopt the inhibitory conformation resulting in increased enzymatic activity. Our preliminary results in figure 5demonstrate that the Pinsert Lck mutation does indeed activate Lck.

Data in figure 6 show that A770041 was most effective in blocking CTV-1 cell proliferation. Although Bosutinib, Dasatinib and A770041 all target Src family kinases, A770041 shows the strongest inhibition. Considering that CTV-1 cells contain mutations only in Lck, and not in other Src family kinases like Src and Fyn, we believe A770041 is suppressing mutant Lck activity. Together these data suggest that the Pinsert mutation in Lck found in CTV-1 cells causes the enzyme's constitutive activation, uncontrolled cell proliferation, and therefore leukemia progression.

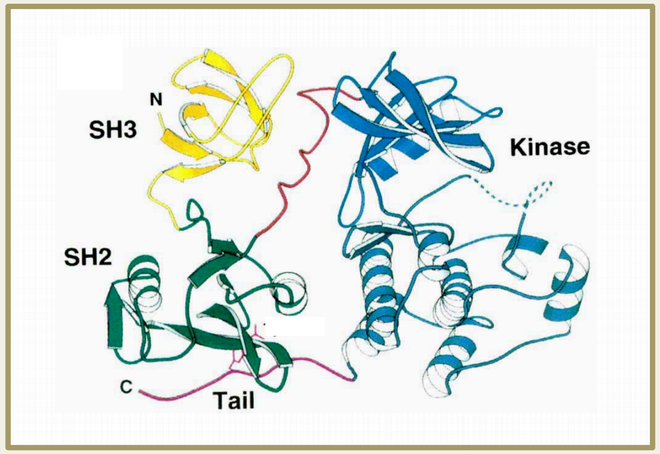

Figure 7. Ribbon structure of wild type Lck with domains labeled. The red region connecting the SH2 domain to the kinase domain is linker region.

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