Molecular Cell
Article

A Conserved Salt Bridge in the G Loop of Multiple Protein Kinases Is Important for Catalysis and for In Vivo Lyn Function

Rina Barouch-Bentov,[1] Jianwei Che,[1,4] Christian C. Lee,[1,4] Yating Yang,[2,4] Ann Herman,[1,4] Yong Jia,[1] Anastasia Velentza,[1] James Watson,[1] Luise Sternberg,[2] Sunjun Kim,[1] Niusha Ziaee,[1] Andrew Miller,[1] Carie Jackson,[1] Manabu Fujimoto,[3] Mike Young,[1] Serge Batalov,[1] Yi Liu,[1] Markus Warmuth,[1] Tim Wiltshire,[1] Michael P. Cooke,[1] and Karsten Sauer[2,*]
[1]Genomics Institute of the Novartis Research Foundation, 10675 John J. Hopkins Drive, San Diego, CA 92121, USA
[2]Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA
[3]Department of Dermatology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan
[4]These authors contributed equally to this work
*Correspondence: ksauer@scripps.edu
DOI 10.1016/j.molcel.2008.12.024

SUMMARY

The glycine-rich G loop controls ATP binding and phosphate transfer in protein kinases. Here we show that the functions of Src family and Abl protein tyrosine kinases require an electrostatic interaction between oppositely charged amino acids within their G loops that is conserved in multiple other phylogenetically distinct protein kinases, from plants to humans. By limiting G loop flexibility, it controls ATP binding, catalysis, and inhibition by ATP-competitive compounds such as Imatinib. In *WeeB* mice, mutational disruption of the interaction results in expression of a Lyn protein with reduced catalytic activity, and in perturbed B cell receptor signaling. Like *Lyn*$^{-/-}$ mice, *WeeB* mice show profound defects in B cell development and function and succumb to autoimmune glomerulonephritis. This demonstrates the physiological importance of the conserved G loop salt bridge and at the same time distinguishes the in vivo requirement for the Lyn kinase activity from other potential functions of the protein.

INTRODUCTION

Src family protein tyrosine kinases (SFKs) regulate signaling downstream of multiple different membrane receptors in many different cell types. SFK hyperactivity is observed in many cancers (Lowell, 2004; Yeatman, 2004). In immune cells, SFKs mediate signaling from antigen and cytokine receptors, GPCRs, and integrins. They control development and function of T, B, and myeloid cells. Certain SFKs, including Lyn, have both positive and negative functions in the same cell. Thus, SFKs may act as signal-modulating rheostats rather than as binary on-off switches (Lowell, 2004; Saijo et al., 2003; Xu et al., 2005). In particular, Lyn is thought to establish a balance of positive versus negative signals that determines the physiological outcome of B cell receptor (BCR) engagement (Lowell, 2004; Xu et al., 2005). Deregulation of this balance likely underlies the paradoxical observation of B cell hyperactivity and lethal autoimmune disease in both *Lyn*$^{-/-}$ mice (Chan et al., 1997; Hibbs et al., 1995; Nishizumi et al., 1995) and knockin mice expressing hyperactive *Lyn* (Harder et al., 2001; Hibbs et al., 2002).

SFK crystal structures reveal a typical kinase domain fold composed of an N-terminal N lobe and a C-terminal C lobe forming an ATP and substrate-binding active site at the interlobe cleft (Schindler et al., 1999; Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997, 1999; Yamaguchi and Hendrickson, 1996; Zhu et al., 1999). The N lobe is mainly composed of β sheets and involved in anchoring and orienting the ATP. The C lobe is primarily α-helical and mainly responsible for substrate binding and initiating phosphotransfer. The two most N-terminal β strands in the N lobe form a β-strand-turn-β-strand structure. In all canonical protein kinases, the turn comprises a glycine-rich G loop with the conserved amino acid (AA) consensus motif GxGxxG. This flexible clamp covers and anchors the nontransferable ATP α/β-phosphates, leaving the γ-phosphate solvent exposed. By binding and positioning the ATP appropriately for γ-phosphate transfer to the substrate, the G loop (also termed ATP phosphate-binding P loop) controls nucleotide affinity/specificity and γ-phosphoryl transfer rate (Aimes et al., 2000; Grant et al., 1998; Hanks and Hunter, 1995; Hirai et al., 2000; Taylor and Radzio-Andzelm, 1994; Wong et al., 2004).

Here, we show that SFK G loops contain an extended consensus motif $[K/R]_{-4}LGxG_0xF[G/A][E/D]_{+4}V$. The oppositely charged side chains of the conserved N-terminal basic $[K/R]_{-4}$ and C-terminal acidic $[E/D]_{+4}$ interact electrostatically. By constraining G loop flexibility, this salt bridge is required for high-affinity ATP binding and catalysis. In *WeeB* mice, its mutational disruption results in expression of a Lyn protein with little catalytic activity, and in perturbed BCR signaling. Like *Lyn*$^{-/-}$ mice, *WeeB* mice show profound defects in B cell development and function and succumb to autoimmune glomerulonephritis. The *WeeB* mutation thus reveals a functionally important structural feature of SFK G loops and in addition distinguishes the in vivo requirement for the Lyn kinase activity from other potential functions of the Lyn protein.

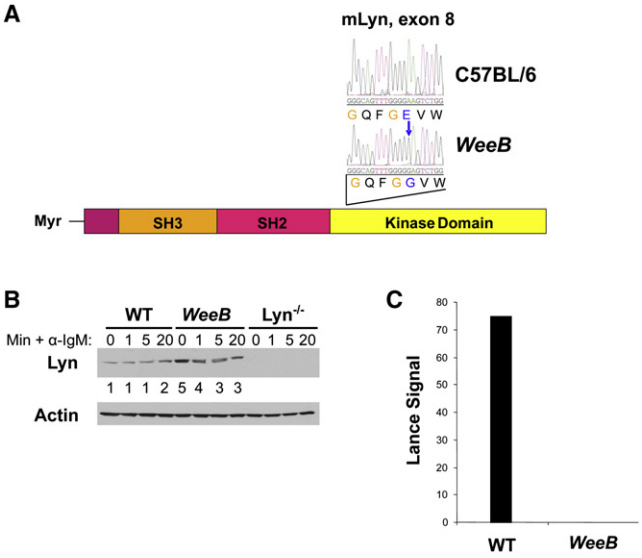

Figure 1. Isolation of *WeeB*, a Mouse Mutant Expressing Catalytically Inactive Lyn

(A) In *WeeB* mice, an $A_{864}G$ transition in *Lyn* exon 8 converts E_{260} in the murine Lyn A kinase domain into a G (blue). E_{260} is immediately C-terminal of the GxGxxG G loop signature motif (last two Gs in orange). Nucleotide numbering is based on the mouse Lyn A cDNA sequence (GenBank accession number M57696), amino acid numbering on AAA39471.

(B) Immunoblot analysis of Lyn (upper) or Actin (lower panel) protein expression in splenic B cell extracts from C57BL/6 controls (WT), *WeeB*, or $Lyn^{-/-}$ mice at the indicated times after αIgM stimulation. LynA/B (Stanley et al., 1991; Yi et al., 1991) were not resolved and are represented by one band here and in Figure 5. Quantified band intensities are indicated.

(C) Lyn was immunoprecipitated from splenic B cell extracts 1 min after αIgM stimulation, followed by Lance in vitro kinase assay. Representative of two independent experiments.

Finally, we found that the G loop salt-bridge-forming motif is conserved among 58 protein kinases and observed the corresponding electrostatic interaction in published structures of six phylogenetically diverse protein kinases, including Abl. Similar to Lyn, its disruption also reduced Lck and Hck catalytic activity. Implication of Bcr-Abl mutants lacking this interaction in Imatinib resistance in human CML patients, molecular dynamics simulations and published biochemical analyses suggest that it controls catalysis and binding of ATP-competitive inhibitors in Abl. These data unveil the conserved G loop salt bridge as a physiologically and pharmacologically important structural feature shared by many protein kinases.

RESULTS

Isolation of *WeeB*, a Mouse Mutant Expressing Catalytically Inactive Lyn

In an ENU mutagenesis screen, we identified several lymphopenic mouse mutants (Huang et al., 2006, 2007). Among these, *WeeB* had ~50% reduced peripheral blood B cell numbers. The phenotype was similar in males and females with >25% frequency, and no overt physical or behavioral abnormalities. Mapping of the underlying mutation (Figure S1), gene sequencing, and genotyping of multiple animals showed a maximal genotype-phenotype correlation for an $A_{864}G$ transition in *Lyn* exon 8 of all *WeeB* affected but no unaffected animals, converting E_{260} in the murine Lyn A kinase domain (E_{239} in Lyn B [Stanley et al., 1991; Yi et al., 1991]) into a G (Figure 1A). Consistent with a causative mutation in *Lyn*, $Lyn^{-/-}$ mice also show peripheral B lymphopenia (Allman et al., 2001; Chan et al., 1997; Hibbs et al., 1995; Meade et al., 2002; Nishizumi et al., 1995; Xu et al., 2005).

Compared to controls, full-length Lyn protein is moderately overexpressed in *WeeB* splenic B cells (Figures 1B and 5B). However, Lyn immunoprecipitates from αIgM-stimulated *WeeB* B cells contained no detectable kinase activity (Figure 1C). Thus, *WeeB* is a loss-of-function *Lyn* allele, resulting in expression of Lyn protein with reduced activity. Its potential overexpression could reflect impaired negative feedback regulation, as constitutive Lyn activation induces its degradation (Harder et al., 2001; Hibbs et al., 2002).

A Conserved Salt Bridge in the G Loop Is Essential for Lyn Activity

E_{260} immediately follows the GxGxxG G loop signature motif in the Lyn A kinase domain. A multiple sequence alignment revealed that all SFKs share the modified G loop AA consensus sequence $[K/R]_{-4}LGxG_0xF[G/A][E/D]_{+4}V$ (Figure 2A). A basic residue at position −4 and an acidic residue at position +4 (E_{260} in Lyn A) are strictly conserved. To explore how E_{260} mutation abrogates Lyn kinase activity, we examined the cognate E_{+4} location in the resolved kinase domain crystal structures of Hck (Sicheri and Kuriyan, 1997), Lck (Yamaguchi and Hendrickson, 1996; Zhu et al., 1999), Fyn (Kinoshita et al., 2006), and c-Src (Sicheri and Kuriyan, 1997). We found that the E_{+4} (Hck NP_002101 E_{275}, Lck E_{258}, Src E_{283}) side chain extends upward from the G loop away from the bound ATP and is juxtaposed to the basic side chain at position −4 (Hck K_{267}, Lck R_{250}, Src K_{275}). In the crystal structures of Quercetin or AMP-PNP-bound Hck, active Lck, or active or inactive c-Src (PDB accession numbers 1QCF, 1AD5; 1QPJ, 3LCK; 1Y57, 1KSW, 1FMK, 2H8H), both side chains are juxtaposed at distances of ≤4 Å and form a salt bridge across the G loop (Figure 2B). This suggests that the salt bridge is a common structural feature of all SFK G loops.

A survey of the human and murine kinomes (Caenepeel et al., 2004; Manning et al., 2002) revealed juxtaposition of oppositely charged basic and acidic amino acids at positions −4 and +4 relative to the invariant G loop G_0 in 58 phylogenetically diverse protein kinases. While most of them share the SFK motif polarity, a subgroup shows reversed charges at −4/+4 (Figure S2). Interestingly, corresponding electrostatic interactions exist in several published crystal structures of Abl (1IEP, 1M52, 2G2F, 2E2B, 1OPJ, 2OIQ), *Zea mays* CK2A1 (1LP4), and with reversed polarity, human SLK (2JFL, Figure 2B). Thus, the conserved sequence motif and corresponding G loop salt bridge exist in multiple phylogenetically diverse protein kinases in mammals and plants (Figure 2C), and their polarity can be reversed.

Based on its position within sheets β1 and β2 in the G loop stem, the salt bridge likely stabilizes this loop and contributes to proper ATP binding and orientation for catalysis. The $E_{260}G$ mutation in Lyn^{WeeB} could impair catalysis through disruption of the salt bridge, resulting in G loop destabilization and perturbed ATP binding. To test this hypothesis, we independently