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

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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 hyper-active 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]4L[GxG][F/G][E/D]4V. 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.
phenotype correlation for an A864G transition in Lyn exon 8 of all WeeB affected but no unaffected animals, converting E260 in the murine Lyn A kinase domain (E239 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 αlgM-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
E260 immediately follows the GxGxG 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]−εGxG−xF[G/A][E/D]− emphasized, the salt bridge likely stabilizes this loop and contributes to proper ATP binding and orientation for catalysis. The E260G substitution abrogates Lyn kinase activity, we examined the cognate E4, 4 position 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 E4+4 (Hck NP_002101 K267, Lck E258, Src E283) 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−268, Lck R259, Src R282) in the crystal structures of Quercetin or AMP-PNP-bound Hck, active Lck, or active or inactive c-Src (PDB accession numbers 1QCF, 1AD5, 1PQJ, 3LCK; 1Y57, 1KSW, 1FMK, 2HBH), 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 (Canepeel 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 G0 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 E260G mutation in LynWeeB could impair catalysis through disruption of the salt bridge, resulting in G loop destabilization and perturbed ATP binding. To test this hypothesis, we independently...
disrupted the SFK salt bridge through mutation of the basic K252.

Compared to wild-type (WT) Lyn, recombinant E260G, K252G, charge-reversing K252E, or K252G/E260G double mutant Lyn proteins all had similar, strongly reduced catalytic activities in vitro (Figure 2D). This suggests an essential role for the salt bridge in SFK G loop function and catalysis. Indeed, salt bridge disruption also reduced Lck and Hck catalytic activity (Figure S3). Surprisingly, a Lyn K252E/E260K mutant, which might generate a “reversed” salt bridge of opposite polarity, still had little activity (Figure 2D), suggesting that steric factors prevent salt bridge formation, or that the charge reversal perturbs G loop function and impacts ATP binding through additional factors such as an altered electrostatic environment. The residual <17% activities measured for all mutants in the in vitro assay may contrast the lack of detectable activity in LynWeeB immunoprecipitates from splenic B cells (Figure 1C). Potential explanations are enzyme concentrations, coprecipitated interacting proteins, or posttranslational modifications that could all differ between endogenous and purified recombinant Lyn.

To further explore how the conserved salt bridge contributes to G loop function, we homology modeled the WT and WeeB (E260G) mutant Lyn A protein kinase domains based on the active Hck/AMP-PNP crystal structure (1AD5, Figures 3A and 3D). We then simulated the effects of the WeeB mutation on G loop structure and conformational flexibility using molecular dynamics. An elevated root-mean-square displacement (RMSD, Figure 3B) suggests that the LynWeeB G loop has greater conformational flexibility than that of WT Lyn. Increased atomic fluctuations for residues remote from the mutation suggest that the increased flexibility extends beyond the G loop and includes hinge, P loop, and C helix residues (Figure 3C). For example, the
characteristic electrostatic interaction between E290 in the αC helix and the catalytic K275 in sheet β3 was weakened by 1.5 kcal/mol in LynWeeB. Moreover, the LynWeeB kinase domain had elevated overall conformational flexibility (data not shown). Thus, LynWeeB shows strongly increased protein plasticity compared to WT Lyn, and nearly all functional motifs necessary for catalysis exhibit enhanced conformational flexibility. The dramatic impact of the single E260 point mutation on Lyn plasticity underscores the importance of the conserved K252/E260 salt bridge in restraining and conformationally stabilizing the G loop in active WT Lyn and possibly other SFKs.

Superposition of the average structures suggests a more compact ATP-binding site in WT than WeeB Lyn (Figure 3D). The E260G mutation results in a G loop shift away from the ATP-binding cleft, weakening estimated ATP binding by ~1.7 kcal/mol. Thus, the WeeB mutation should impair ATP binding and profoundly perturb catalysis. To test this prediction biochemically, we determined the K_m values of purified recombinant full-length WT or E260G mutant murine Lyn for ATP or a peptide substrate in vitro. Consistent with strongly reduced ATP binding and impaired catalytic activity, LynWeeB had an ~7-fold higher K_m for ATP than WT Lyn (wt, 1.6 μM, WeeB, 11.4 μM; Figure S4). Moreover, an ~5-fold higher K_m for the substrate (WT, 0.24 μM, WeeB, 1.25 μM) indicates impaired substrate binding and/or phosphorylation. We next assessed the function of WT or WeeB Lyn in a cellular assay. Retroviral expression of WT but not WeeB Lyn/TEL fusion proteins conferred IL-3-independent survival and growth to BaF/3 cells (Figure S5; Table S1). Thus, disruption of the SFK G loop salt bridge impairs B cell development. Since WeeB mice express full-length Lyn protein (Figures 1B, 5B, and 5D), this also shows that the Lyn kinase activity is required for splenic B cell development.

The above defects occur at stages where BCR signals mediate a developmental transition, suggesting that LynWeeB perturbs BCR signaling, which is impaired in Lyn^−/− mice.

Perturbed B Cell Development and BCR Signaling in WeeB Mice
To determine how G loop salt bridge disruption affects Lyn function in vivo, we first compared B cell development in WT, WeeB, and Lyn^−/− mice (Figures 4, S6, and S7). As previously reported (Allman et al., 2001; Chan et al., 1997; Hibbs et al., 1995; Meade et al., 2002; Nishizumi et al., 1995; Xu et al., 2005), Lyn^−/− mice showed normal early B cell development in the bone marrow, but reduced total numbers of all splenic B cell subsets and of mature B220^high IgM^+ recirculating B cells. Consistent with its hypomorphic character, WeeB had a similar, but milder phenotype. Thus, disruption of the Lyn G loop salt bridge impairs B cell development. Since WeeB mice express full-length Lyn protein (Figures 1B, 5B, and 5D), this also shows that the Lyn kinase activity is required for splenic B cell development. The above defects occur at stages where BCR signals mediate a developmental transition, suggesting that Lyn^WeeB perturbs BCR signaling, which is impaired in Lyn^−/− mice.

Figure 3. Disruption of the Conserved Salt Bridge Destabilizes the SFK G Loop and Enhances Its Flexibility
(A) Blowup of the G loop region (dark blue) of mLyn, homology modeled into the active Hck/AMP-PNP kinase domain structure (Figure 2B), showing the side chains of the conserved basic K_4 (K252) and acidic E_4 (E260). Juxtaposition at 2.3 Å suggests a salt bridge interaction.
(B) Molecular Dynamics simulation of the flexibility of the modeled WT (red) or WeeB mutant (E260G, blue) Lyn G loop. Shown is the root-mean-square displacement (RMSD) over time.
(C) Simulated atomic fluctuations for amino acids around the catalytic site in the WT (red) or WeeB mutant (blue) Lyn model.
(D) Superimposed average structures of the WT (red) and WeeB mutant (blue) Lyn models from a 5 ns molecular dynamics simulation. The position of the docked ATP is shown for reference.

Figure 4. Perturbed Splenic B Cell Development in WeeB Mice
Bar graph depicting mean numbers ± standard deviation of total B220^+ , follicular mature (FM, B220^+ IgM^high CD21^+ ), marginal zone (MZ, B220^+ IgM^high CD21^+CD23^- ), transitional T2 (B220^+ IgM^low CD21^+CD23^+ ), and T1 (B220^+ IgM^low CD21^- ) B cells in WT, WeeB, or Lyn^−/− mice. Average live cell numbers in three WT, four WeeB, or two Lyn^−/− spleens were 5.6 ± 0.7 × 10^7, 2.3 ± 0.7 × 10^7, and 1.5 ± 0.1 × 10^7, respectively. Representative FACS data are shown in Figure S7.
To determine the specific functions of the Lyn kinase activity in BCR signaling, we compared Lyn downstream signaling between WeeB and Lyn$^{-/-}$ mice. WeeB splenic B cells showed intermediate defects between WT and Lyn$^{-/-}$ B cells in IgM-induced overall tyrosine phosphorylation, which is severely impaired in Lyn$^{-/-}$ mice (Figure 5A). Lyn deficiency impairs activation of both positive and negative BCR signaling pathways (Campbell, 1999; Chan et al., 1997, 1998; Cornall et al., 1998; DeFranco et al., 1998; Hibbs et al., 1995, 2002; Lowell, 2004; Nishizumi et al., 1995, 1998; Smith et al., 1998; Xu et al., 2005). Positive Lyn signaling involves tyrosine phosphorylation of the PTKs Syk and Btk and the Btk effector PLC$\gamma$2. Inhibitory Lyn signaling involves phosphorylation of negative regulatory proteins, including CD22, which then recruit phosphoprotein phosphatases like SHP-1. Compared to IgM-treated WT controls, induction of all these events was reduced moderately in WeeB and severely in Lyn$^{-/-}$ B cells (Figures 5C–5E and S8A). Disrupted positive and negative proximal BCR signaling results in augmented distal signaling in Lyn$^{-/-}$ B cells (Figures 5C and S8B). Thus, WeeB splenic B cells showed intermediate increased distal signaling, including elevated basal p38 and Jnk phosphorylation, strongly sustained IgM-induced Erk and JNK phosphorylation (Figure 5C), and intermediately augmented IgM-induced store-operated Ca$^{2+}$ influx (Figure S8B). Therefore, WeeB B cells showed intermediately increased distal signaling, including elevated basal p38 and Jnk phosphorylation, strongly sustained IgM-induced Erk and JNK phosphorylation (Figure 5C), and intermediately augmented IgM-induced store-operated Ca$^{2+}$ influx (Figure S8B). Thus, WeeB splenic B cells showed intermediate defects between WT and Lyn$^{-/-}$ B cells in all signaling events investigated. Hence, Lyn G loop salt bridge and kinase activity are essential for both positive and negative BCR signaling.

Perturbed B Cell Function and Immune Pathology in WeeB Mice

Strong perturbation of positive and negative Lyn signaling results in B cell hyperactivity in Lyn$^{-/-}$ mice. To determine how the intermediate perturbation of BCR signaling in WeeB affects B cell activity in vivo, we analyzed cellular parameters of B cell function. B cells from young Lyn$^{-/-}$ mice show augmented BCR but normal LPS-induced proliferation; B cells from old Lyn$^{-/-}$ mice show severe proliferation defects (Chan et al., 1997; Hibbs et al., 1995; Nishizumi et al., 1995). We found that B cells from 7- to 11-week-old WeeB mice hyperproliferated after IgM F(ab')2, but not LPS treatment (Figures 6A and S9A), while Lyn$^{-/-}$ B cells showed intermediately increased distal signaling, including elevated basal p38 and Jnk phosphorylation, strongly sustained IgM-induced Erk and JNK phosphorylation (Figure 5C), and intermediately augmented IgM-induced store-operated Ca$^{2+}$ influx (Figure S8B). Thus, WeeB splenic B cells showed intermediate defects between WT and Lyn$^{-/-}$ B cells in all signaling events investigated. Hence, Lyn G loop salt bridge and kinase activity are essential for both positive and negative BCR signaling.
hyperproliferated after either treatment. Moreover, Lyn–/– mice contained elevated basal serum IgM and IgA levels as reported (Hibbs et al., 1995; Nishizumi et al., 1995). WeeB mice showed elevated basal serum IgM, but normal IgA levels (Figure S9B). Thus, WeeB showed intermediate B cell hyperactivity between WT and Lyn–/– mice.

Lyn–/– mice develop splenomegaly due to myeloid hyperplasia (Harder et al., 2001, 2004; Nishizumi et al., 1995). Aging WeeB mice developed similar, characteristically enlarged spleens with accumulation of unusual lymphoblast-like and plasma cells, reduced B cell numbers, and perturbed B cell zone organization (Figures 6B and 6D). The WeeB phenotypes were milder than those of Lyn–/– mice at 8–9 months, but similar in older mice.

At least in part due to the B cell hyperactivity, Lyn–/– mice develop a Lupus-like glomerulonephritis with accumulation of autoimmune antibodies (AAB) in the kidneys (Hibbs et al., 1995; Nishizumi et al., 1995). Aging WeeB mice showed intermediate increased α-dsDNA AAB serum levels, thought to be most closely correlated with Lupus-like pathology (Figure 6C). Consistent with previous reports, Lyn–/– mice developed severe glomerulonephritis at ~8 months, evidenced by enlarged glomeruli, glomerular hypercellularity, lobularity and sclerosis, mesangial interposition in peripheral capillary loops, and strong IgG deposition in the glomeruli (Figure 6D). A very similar renal pathology was observed in WeeB, but with a later onset of 12–14 months. In addition, we observed IgG deposition in the livers of Lyn–/– and older WeeB mice, but no evidence for significant liver pathology. Thus, WeeB mice develop a pathology that is overall similar to that in Lyn–/– mice but less severe and of delayed onset.

G Loop Salt Bridge Disruption in Imatinib-Resistant Bcr-Abl Mutants

A G loop electrostatic interaction between K247 and E255 analogous to the Lyn salt bridge is observed in several published
crystal structures of the non-Src family PTK Abl (1IEP, 1M52, 2G2F, 2E2B, 1OPJ, 2OIQ) (Shah et al., 2002). Chronic myelogenous leukemia (CML) results from expression of hyperactive Bcr-Abl fusion proteins in hematopoietic stem cells. Intriguingly, Bcr-Abl E255K or E255V mutations have been strongly implicated in clinical resistance to the ATP competitive Abl inhibitor Imatinib, a current standard CML treatment (Cowan-Jacob et al., 2004, 2007; Shah et al., 2002). Both mutations reduce Imatinib binding to Bcr-Abl, and Imatinib inhibition of its catalytic activity and ability to render Ba/F3 cells IL-3 independent. They worsen prognosis of Imatinib-treated CML patients. This suggests that the Abl G loop salt bridge is required for Imatinib binding and efficacy in patients.

The effects of both mutations on Bcr-Abl activity in vitro are controversial. One study reported moderately reduced full-length E255K Bcr-Abl activity, but WT activity of a mutant GST-cacy in patients.

prognosis of Imatinib-treated CML patients. This suggests that binding to Bcr-Abl, and Imatinib inhibition of its catalytic activity and peptide substrate in vitro (Griswold et al., 2006). Another group found elevated activity for an E255K mutant FLAG-tagged incomplete Bcr fragment lacking its dimerization motif, fused to the Abl SH3 domain, immunoprecipitated from COS7 cells and assayed with 2 μM ATP toward GST-CrkL-F protein (Yamamoto et al., 2004). A third study reported augmented Bcr-Abl E255K, but reduced E255V activities toward a peptide substrate after immunoprecipitation from 293T cells (Skggs et al., 2006). Gel-based kinase assays with 200 μM ATP and various substrate proteins did not detect major differences between either mutant and WT Bcr-Abl, likely due to excess ATP and substrate. The conflicting results very likely reflect the different assay conditions and Abl fragments used. In any case, most of the analyses suggest that E255 mutation, which disrupts the Abl G loop salt bridge, alters catalytic activity. Altered transforming potential of E255K/V Abl (Griswold et al., 2006; Skggs et al., 2006) supports this view.

To analyze how G loop salt bridge disruption affects Abl kinase domain function, we simulated the impact of the E255K mutation by molecular dynamics based on Dasatinib-bound human active Abl (Figure S10). The effects were generally lower than in the case of Lyn. However, several residues even outside the G loop showed significantly altered atomic fluctuations. The average kinase domain structure suggests slightly increased G loop flexibility, resulting in its downfolding into the ATP-binding cleft, which might affect ATP and/or Imatinib binding or orientation and catalysis. Thus, G loop salt bridge disruption has significant effects on Abl kinase domain plasticity.

**DISCUSSION**

Like the catalytic domain of most protein kinases, the SFK kinase domain is composed of N- and C-terminal lobes flanking an ATP and substrate-binding 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 G loop is critically involved in catalysis by acting as a flexible clamp that extends from the N lobe to cover and anchor the nontransferable ATP a'/b'-phosphates, orienting the ATP appropriately for optimal γ-phosphoryl transfer (Hanks and Hunter, 1995; Schenk and Snaar-Jagalska, 1999). It controls nucleotide affinity/specificity and γ-phosphoryl transfer rate (Aimes et al., 2000; Grant et al., 1998; Hirai et al., 2000; Taylor and Radzio-Andzelm, 1994; Wong et al., 2004).

Here, we show that SFK G loops contain a modified amino acid sequence consensus motif [K/R]4LGGxG[F/G][A]/E[D]4V (Figure 2A). The conserved basic [K/R]4 and acidic [E/D]4 side chains form a salt bridge in published crystal structures of Hck, Lck, and c-Src (Figure 2B) (Cowan-Jacob et al., 2005; Yamaguchi and Hendrickson, 1996; Zhu et al., 1999). Homology modeling of murine Lyn into the active Hck/AMP-PNP structure suggests that the analogous residues E260/R/K252 also form a salt bridge in Lyn (Figure 3A). Its location in the stem suggests that the salt bridge stabilizes the G loop in active SFKs, limits its flexibility, and thereby contributes to ATP binding and orientation. This view is supported by the proximity of the salt bridge to the residues involved in direct interactions with Staurosporine in Lck and AMP-PNP in Hck (Hubbard, 1997; Zhu et al., 1999).

Our molecular dynamics analyses support this notion, suggesting enhanced conformational flexibility not only for the G loop, but also for hinge, P loop, and C helix residues important for catalysis in E260G mutant LynWheeB. The less ordered LynWheeB G loop engages less strongly with the ATP and is less suited to orient it appropriately for optimal γ-phosphoryl transfer (Figure 3D). Supporting an important role for the G loop salt bridge in ATP binding and catalysis, the E260G mutation increased the Lyn K_m for ATP (Figure S4). Moreover, salt bridge disruption by mutation of either K252 or E260 strongly reduced Lyn catalytic activity to a similar extent as double mutation of both residues or K252 charge reversal to E (Figures 1C and 2D).

Finally, the E260G mutation abrogated the ability of Lyn to confer IL-3-independent growth to Ba/F3 cells (Figure S5; Table S1) and resulted in expression of catalytically inactive, functionally impaired Lyn protein in mice (Figures 1 and 5). Thus, disruption of the G loop salt bridge profoundly impairs Lyn catalytic activity and in vivo function.

Juxtaposition of oppositely charged basic and acidic amino acids at positions –4 and +4 relative to the invariant G loop G_0 is highly conserved in 58 phylogenetically diverse mammalian protein kinases, including the non-Src family PTK Abl and the serine/threonine kinases CK2A1 and SLK (Figures 2 and S2). Similar to Lyn, salt bridge disruption also reduced Lck and Hck catalytic activity (Figure S3). Thus, the potential to form a G loop salt bridge is of specific importance for SFKs but could represent a more general feature. Indeed, we found a corresponding electrostatic interaction in several published crystal structures of Abl, Zea mays CK2A1, and with reversed polarity, SLK (Figure 2B). Hence, the conserved G loop salt bridge exists in multiple phylogenetically diverse protein kinases in mammals and even in plants (Figure 2C), and its polarity can be reversed.

Varying effects of G loop point mutations on ATP/ATP analog binding and catalytic activity have been described for several protein kinases (Chailiot et al., 2000; Hemmer et al., 1997; Hirai et al., 2000; Wan et al., 2004). In Bcr-Abl, E_{255} (E255) mutation to K or V altered catalytic activity and transforming potential in most cases (Griswold et al., 2006; Skaggs et al., 2006; Yamamoto et al., 2004). It reduced inhibition by the ATP competitive inhibitor Imatinib. Both mutations contribute to Imatinib resistance in human CML patients, indicating the critical importance
of the G loop salt bridge for Abl function in vitro and in vivo (Cowan-Jacob et al., 2004, 2007; Griswold et al., 2006; Shah et al., 2002; Skaggs et al., 2006; Yamamoto et al., 2004). Interestingly, conservative K247R mutation, which might preserve the salt bridge, does not significantly affect Abl function or inhibition by ATP analogs (Crossman et al., 2005).

Consistent with a role for the G loop salt bridge in Abl kinase domain plasticity, our molecular dynamics (MD) analysis suggests altered atomic fluctuations for multiple residues in the E255K mutant kinase domain even outside the G loop and significant changes in G loop conformation and electrostatic charge distribution (Figure S10). An independent MD analysis based on the Imatinib-bound inactive Abl kinase domain (1IEP) found overall similar results and proposed that reduced Imatinib binding to E255K/KV Abl reflects primarily G loop contributions to the binding free energy (Lee et al., 2008). Since E255 does not directly contact Imatinib, this suggests that its mutation affects either overall G loop conformation or the electrostatic charge distribution within the G loop, either of which could affect Imatinib interactions indirectly. Detailed analysis of the electrostatic and van der Waals contributions of various G loop residues to the relative binding free energy (RBFE) confirmed this view, suggesting primarily electrostatic effects for the E255K, and a combination of electrostatic and conformational effects for the E255V mutation. Consistent with our findings, both mutations also affected RBFE contributions of non-G loop residues. Altogether, the E255K/KV mutation effects on Imatinib binding, catalytic activity, transforming potential, and kinase domain conformation are consistent with an important role of the G loop salt bridge in Abl function in vitro and in vivo, further supported by the contribution of both mutations to Imatinib resistance in CML patients.

Imatinib binding is associated with G loop downfolding into a distorted conformation that increases surface complementarity with the drug (Schindler et al., 2000). E255K/KV mutations have been suggested to reduce Imatinib binding by destabilizing this conformation (Cowan-Jacob et al., 2004, 2007; Shah et al., 2002). Our observation of the K247-E255 interaction in the Abl/PD173955 structure (1M52), whose conformation differs from that of Abl/Imatinib through an oppositely oriented activation loop (Nagar et al., 2002), suggests a broader role for the G loop salt bridge beyond the Imatinib-stabilized inactive conformation. Consistent with this view, E255K/V mutation mildly reduced Abl inhibition by BMS-354685/Dasatinib, which binds both the active and inactive conformations (Burgess et al., 2005; Shah et al., 2004).

Abl kinase domain function is modulated through autoinhibitory intramolecular interactions with other domains and through phosphorylation of its activation and G loops. Indicating relevance for these interactions, E255 mutation differentially affected full-length versus truncated Bcr-Abl activities (Griswold et al., 2006; Skaggs et al., 2006; Yamamoto et al., 2004), and activation loop phosphorylation activated full-length Bcr-Abl but not kinase domain fragments (Schindler et al., 2000). These findings could explain the differences observed between Abl full-length and kinase domain proteins, and possibly also the quantitatively different effects of E255 versus E260 mutation in Abl versus Lyn.

Lyn is the predominant SFK in B cells and pivotal for positive and negative BCR signaling (Campbell, 1999; Lowell, 2004; Xu et al., 2005; Yamanashi et al., 1992). WeeB B cells express full-length Lyn protein with strongly reduced catalytic activity (Figures 1B, 1C, 2D, 5B, and 5D). They show defects in the BCR-induced phosphorylation and activation of positive and negative signaling mediators with Lyn−/− directionality (Figure 5) (Chan et al., 1998; Hibbs et al., 1995, 2002; Nishizumi et al., 1998), opposing that in B cells overexpressing cataclysmically hyperactive Lyn (Hibbs et al., 2002). Thus, WeeB is a loss-of-function Lyn allele, consistent with inability of LynWeeB to confer IL-3-independent growth to Ba/F3 cells (Figure S5; Table S1). Therefore, the perturbation of overall tyrosine phosphorylation and in particular of Syk, Btk, CD22, SHIP-1, and SHP-1 phosphorylation both in sorted Lyn−/− and WeeB splenic B cells formally establishes that the Lyn kinase activity is required for these events.

However, WeeB B cells showed milder defects than Lyn−/− B cells in all biochemical and functional parameters investigated (Figures 5, 6, and S6–S9). The preservation of significant PTK signaling in WeeB compared to Lyn−/− B cells could reflect the low residual LynWeeB catalytic activity, or the preservation of Lyn protein interactions that might allow other PTKs to partially substitute for Lyn function. Evidence for kinase-independent SFK functions, and for Lyn/Fyn/Blk interactions in B cells, has indeed been reported (Lowell, 2004; Saijo et al., 2003; Xu et al., 2005). A conclusive determination of the kinases that preserve PTK signaling in WeeB will require genetic perturbation of candidates on a WeeB background and a characterization of their molecular interactions with Lyn.

Taken together, our results establish that the Lyn kinase activity is essential for preventing B cell hyperactivity and disease. Moreover, they functionally define a conserved salt bridge as a physiologically important structural feature in the G loops of multiple, phylogenetically diverse protein kinases, including all SFKs and Abl. The salt bridge stabilizes the G loop in an optimal conformation for ATP binding and catalysis and controls binding of ATP-competitive inhibitors. Its further investigation will promote our mechanistic understanding of protein kinase function and aid the development of small-molecule kinase inhibitor therapeutics.

**EXPERIMENTAL PROCEDURES**

**Mice, Cell Preparation, and Analysis**

*Lyn−/−* mice (Chan et al., 1997) were obtained from Dr. Clifford Lowell (UCSF). WeeB mice were backcrossed to C57BL/6 for ≥3 generations. All animal experiments were approved and supervised by the GNF Institutional Animal Care and Use Committee. Cells were prepared and analyzed by FACS as in Miller et al. (2007), using the ABs listed in the Supplemental Experimental Procedures.

**Immunoblotting**

B Cells were stimulated with αIgM F(ab′)2 for the indicated times at 37°C, followed by lysis and analysis via SDS-PAGE and immunoblotting as described in the Supplemental Experimental Procedures, using NIH Image band intensity quantification. Values were normalized to actin controls and are shown as fold change over time 0.

**Histology and Immunohistochemistry**

A detailed description of the procedures used is provided in the Supplemental Experimental Procedures. Briefly, fixed tissues were paraffin embedded,
Molecular Cell
A G Loop Salt Bridge Is Essential for Lyn Function

sectioned, and stained with Mayers H&E or Gomori’s periodic acid-methanamine silver. Immunohistochemistry was performed with heat-induced epitope retrieval and avidin/biotin blocking.

cDNAs
All Lyn mutant cDNAs were generated from WT LynA or LynAThr37Ala cDNA templates using a Quik-Change kit (Stratagene). Identities were confirmed by DNA sequencing.

Molecular Dynamics
Hck/AMP-PNP structure-based WT and WeeB Lyn protein kinase domain homology models were built by Prime (Schrödinger, Inc.). The molecular dynamics simulations were done as described in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA
The Supplemental Data include Supplemental Experimental Procedures, ten figures, and three tables and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(08)00894-0.

ACKNOWLEDGMENTS
We thank Nathanael Gray, Yina H. Huang, and Christian Schmedt for valuable discussions and critical reading of the manuscript, the GNF vivarium for mouse husbandry, and Bill Young and TSRI Research Computing for LINUX cluster support. All authors except M.F., Y.Y., and L.S. were paid employees of the Genomics Institute of the Novartis Research Foundation while most of the studies reported here were conducted and therefore declare a potential financial conflict of interest. K.S. has partial salary support through NIAID grant R01AI070845.

Received: September 7, 2007
Revised: June 30, 2008
Accepted: December 23, 2008
Published: January 15, 2009

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