Tyrosine Phosphorylation of the Lyn Src Homology 2 (SH2) Domain Modulates Its Binding Affinity and Specificity*

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Src homology 2 (SH2) domains are modular protein structures that bind phosphotyrosine (pY)-containing polypeptides and regulate cellular functions through protein-protein interactions. Proteomics analysis showed that the SH2 domains of Src family kinases are themselves tyrosine phosphorylated in blood system cancers, including acute myeloid leukemia, chronic lymphocytic leukemia, and multiple myeloma. Using the Src family kinase Lyn SH2 domain as a model, we found that phosphorylation at the conserved SH2 domain residue Y194 impacts the affinity and specificity of SH2 domain binding to pY-containing peptides and proteins. Analysis of the Lyn SH2 domain crystal structure supports a model wherein phosphorylation of Y194 on the EF loop modulates the binding pocket that engages amino acid side chains at the pY+2/+3 position. These data indicate another level of regulation wherein SH2-mediated protein-protein interactions are modulated by SH2 kinases and phosphatases. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.044404, 695–706, 2015.

Src homology 2 (SH2) domains are modular protein structures that are important for signal transduction due to their ability to bind phosphotyrosine (pY)-containing polypeptides within defined amino acid sequence motifs (1). SH2 domains are found in various signaling enzymes and adaptor proteins. Given the reversibility of protein tyrosine phosphorylation and the affinity of SH2-pY binding, the interactions of SH2 domains are inherently dynamic and diverse. Indeed, selective, transient binding to pY motifs is a key mechanism through which intracellular signaling networks are dynamically assembled, localized, and regulated. In addition to mediating protein interactions in trans, SH2 domains bind intramolecularly (2). For example, in Src family kinases (SFKs), the SH2 domain binds in cis to the phosphorylated C-terminal tail as a mechanism to constrain and thereby auto-inhibit the intervening tyrosine kinase domain (3, 4). As well, SH2 domains of cytoplasmic tyrosine kinases have been shown to affect the kinase activity of adjacent kinase domains through allosteric interactions (5). The SFKs are therefore highly regulated as a function of their SH2 domains, which exist in dynamic equilibrium between intra- and intermolecular interactions (6). Hence, as discussed by Pawson (7), the transient and diverse interactions of an SH2 domain can regulate signaling enzymes and constitutes a major mechanism of signal transduction in response to extracellular signals.

The structure of the SH2 domain has been extensively characterized. At its core is a conserved antiparallel β-sheet sandwiched between two α-helices (8). SH2 domains bind phosphotyrosine-containing peptides in an extended conformation across the central β-sheet, with the pY residue inserted in a deep recognition pocket formed by conserved residues from strands βB, βC, and βD, helix αA, and the phosphate binding loop. Peptide binding specificity is determined by more variable binding surfaces on the SH2 domain, which recognize residues C-terminal to the pY residue. For the SFK SH2 domains, the three residues C-terminal to the pY residue (pY+1,+2,+3) are dominant determinants of specificity (9, 10), with the domain binding most tightly to sequences containing the motif pYEEI (11, 12). The hydrophobic pY+3 residue inserts in a deep hydrophobic specificity pocket defined by residues of the EF and BG loops (8, 13, 14). Indeed, structural analysis of the SH2 domain revealed that...
the configuration of the EF and BG loops is critical in dictating SH2 domain specificity by shaping the ligand-binding surface and controlling accessibility of the pY+3 binding pocket (15). Mutation of a single residue of the EF loop can drastically impact peptide binding specificity by altering the pY+3 pocket (15–17), indicating the importance of the pY+3 pocket in substrate selectivity for the SFK SH2 domains.

In addition to binding pY-containing polypeptides, SH2 domains themselves may be modulated by phosphorylation. For example, phosphorylation of the Src SH2 domain at conserved Y213 resulted in activation of the cognate kinase domain, possibly by impairing SH2 binding to the phosphorylated C-terminal tail (18). Similarly, phosphorylation of Lck at the equivalent SH2 residue (Y192) generally reduced binding to pY-peptides and proteins (19). Phosphorylation at S590 in the SH2 domain of the p85α subunit of PI 3-kinase decreased its affinity for pY-containing proteins and promoted feedback inhibition of PI 3-kinase and Akt in response to cellular starvation (20). Conversely, tyrosine phosphorylation of the tensin-3 SH2 domain stimulated substrate binding and biological activity (21). Therefore, phosphorylation of SH2 domains appears to be a general mechanism for modulating their binding properties.

Here, we report that Y192 in the SH2 domain of the SFK Lyn, a residue conserved in SFK SH2 domains, is frequently phosphorylated in hematopoietic and other cancers. In vitro protein and peptide interactions with the Lyn SH2 domain were affected by this phosphorylation. Our results suggest that tyrosine phosphorylation of the SFK SH2 domain modulates both its binding affinity and specificity and may constitute another layer of regulation in signaling networks.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Lyn SH2 and Phosphorylated Lyn SH2—Residues 124 to 231 of mouse Lyn kinase, corresponding to the Lyn SH2 domain, were cloned in frame into a pGEX expression vector. The Lyn SH2 construct and a GST-EphA4 kinase construct (residues 591–896) were individually transformed into Escherichia coli BL-21 cells and grown in LB media supplemented with 100 μg/ml ampicillin overnight at 18 °C (A600 = 0.6–0.8 and 0.25 ml IPTG induction). Cells were collected by centrifugation, resuspended in lysis buffer (50 mM Hepes (pH 7.5), 0.4 M NaCl, 10% glycerol, with 1.5 mM aprotinin, 20 mM leupeptin, and 0.4 mM AEBSF). Protein concentrations were determined by the BCA protein assay (Thermo Fisher Scientific). Equal amounts of isolated Lyn SH2 or phosphorylated Lyn SH2 were immobilized on streptavidin (SA) resin and mixed with 6 mg of Mv4–11 lysate protein (4 °C, 4 h) in Nonidet P-40 lysis buffer. The resin was washed three times with Nonidet P-40 lysis buffer, followed by two washes in 20 mM Hepes (pH 7.2). Bound proteins were alkylated and digested by the addition of trypsin, eluted from the resin, desalted with a C18 ziptip (Thermo Fisher Scientific), and analyzed by the Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

Affinity Purification of Phosphorylated Proteins—Cells from the acute myeloid leukemia (AML) cell line MV4–11 were treated with 0.06 mM pervanadate for 10 min at 37 °C, pelleted, and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, with 1.5 mM aprotinin, 20 mM leupeptin, and 0.4 mM AEBSF). Protein concentrations were determined by the BCA protein assay. Equal amounts of isolated Lyn SH2 or phosphorylated Lyn SH2 were immobilized on streptavidin (SA) resin and mixed with 6 mg of Mv4–11 lysate protein (4 °C, 4 h) in Nonidet P-40 lysis buffer. The resin was washed three times with Nonidet P-40 lysis buffer, followed by two washes in 20 mM Hepes (pH 7.2). Bound proteins were alkylated and digested by the addition of trypsin, eluted from the resin, desalted with a C18 ziptip (Thermo Fisher Scientific), and analyzed by the Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

Patient Selection and Sample Preparation—Chronic lymphocytic leukemia (CLL) patients were selected as previously described (22). Treatment naive symptomatic CLL patients were given Revlimid at 2.5 mg daily for 21 days during Cycle 1, followed by another 21-day cycle at 5 mg daily. Peripheral blood samples, peripheral blood mononuclear fractions of diagnostic bone marrow aspirates were obtained with Research Ethics Board (REB) approval. In vitro kinase reaction monitoring.

1 The abbreviations used are: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AML, acute myeloid leukemia; AP, affinity purification; CLL, chronic lymphocytic leukemia; IPTG, isopropyl β-D-1-thiogalactopyranoside; MS, mass spectrometry; PTB, phosphotyrosine binding; pY, phosphotyrosine; rt, room temperature; SA, streptavidin; SFK, Src family kinase; SH2, Src homology 2; SRM, selected reaction monitoring.
approval from the Princess Margaret Hospital leukemia repository. Sterile, viable cryopreserved AML cell suspensions were stored at −2 × 10^7 cells per aliquot in liquid nitrogen until usage.

Cell Lysis and Enrichment of pY-containing peptide for mass spectrometry analysis—Peripheral blood mononuclear cells collected from AML or CLL patients were sonicated in urea lysis buffer (20 mM Hepes (pH 8), 9 mM urea, 1 mM Na_2VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate) and the lysates cleared by centrifugation. The supernatants were trypsin-digested and enriched for pY-containing peptides as described previously (23).

Measurement of Phosphorylation Stoichiometry by Selective Reaction Monitoring (SRM)—Mass Spectrometry (MS)—Lyn kinase was enriched from whole cell lysate samples by immunoprecipitation; trypsin digestion of Lyn and analysis by mass spectrometry were carried out as described previously (24). The peak areas of the extracted ion currents corresponding to the Lyn Y194 or pY194-containing peptides were correlated by using a response rate ratio factor, and the absolute stoichiometry of pY194 was calculated based on the adjusted peak area ratios.

Mass Spectrometry Analysis of Tryptic Peptides—For LC-MS/MS analysis, the tryptic peptides were loaded onto a 50-cm Easy-Spray column with a 75-μm inner diameter packed with 2 μm C18 resin (Thermo Scientific) and eluted over 120 min at 250 nl/min in a 0 to 40% acetonitrile gradient (0.1% formic acid) with an EASY nLC 1000 chromatography system (Thermo Fisher Scientific). The chromatography system was connected to a Q Exactive or Elite mass spectrometry analysis with a nano-ESI source (Thermo Fisher Scientific). Mass spectra were obtained with an automatic switch between a full MS scan and up to 10 MS/MS scans. A dynamic exclusion list of ion mass was maintained to avoid repeated sequencing of the same ion within a 20 s time window.

Computational Analysis of Mass Spectrometry Data—The raw files generated by the mass spectrometer were searched in MaxQuant software (25) (version 1.3.0.5; search engine: Andromeda) to generate peak lists and peptide identifications by using the default parameters and minimum peptide length 5, multiplicity 1, maximum charge 5, and incorporating carboxyamidomethylation (C + 57.02146 Da) modification, and variable modifications, including oxidation (M + 15.99491 Da), acetylation (protein N-terminal + 42.01056 Da), and phosphorylation (STY + 79.96633 Da), against the most recent UniProtKB/Swiss-Prot human protein database (2013 September release; 540,958 sequence entries). Protein modification sites were assigned automatically by the software. Peptide/protein quantification was achieved by using the default parameters in MaxQuant as well as the “match between runs” and “label-free quantification” features. For AP of phosphopeptides, three technical replicates were performed and the reported quantification was averaged from three replicates. Three phosphopeptides that consistently bound SA negative control at high levels were manually excluded from further analysis. The reported 66 peptides all bound to Lyn SH2 or pSH2 but not SA. For AP of phosphopeptides, two biological repeats, with three technical replicates for each, were performed. The reported protein levels were averaged from a total of six replicate proteins. Proteins significantly enriched (levels increased by at least 2.5 standard deviations compared with SA negative controls) in the SH2 or pSH2 bound fractions were reported as SH2 or pSH2 binders (p value < 0.0124).

Fluorescence Polarization Binding Assay—Synthetic peptides with sequences CGGGGY[ph]EEIA (pYEEI), THDCGY[ph]EEIA (pYEEI), THDCGY[ph]EELLT (pYEEL; modeled after Lyn pY508 site), and CENTITY[ph]QQQP (pYQQQ; modeled after MS4A6A pY242 site), CATEGQ[ph]QQQP (pYQQQ; modeled after Lyn pY508 site), and CENTITY[ph]SSLM (pYSLL; modeled after FcγRIIB pY392 site), where Y[ph] denotes phosphorylated tyrosine, labeled N-terminally by Alexa Fluor 488, were obtained from the SPARC BioCentre (The Hospital for Sick Children, Toronto, ON). The peptides were dissolved in fluorescence polarization binding buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 0.5 mM DTT) to a concentration of 10 μM and stored protected from light at −80 °C. Fluorescence polarization measurements were obtained on a fluorometer (Analyst HT, Molecular Devices, Sunnyvale, CA) by mixing serial dilutions of SH2 or pSH2 in fluorescence polarization binding buffer with 1 μM labeled peptide. A binding curve was created by plotting fluorescence polarization against protein concentration and the dissociation constant (K_d) calculated using Prism 4 software (GraphPad Software, La Jolla, CA). The binding assay was repeated five times and the average K_d was reported.

Prediction of Direct Binders of Lyn SH2—The prediction algorithm was performed using HHiSuite software and a profile hidden Markov model method (26). The 69 pY sites enriched in binding to Lyn SH2 domains were used to build a matrix model as a predictor, which was subsequently used to search against a custom database containing 1988 potential pY sites from the 398 proteins that bound to Lyn SH2 and phospho-Lyn SH2 differentially. This custom database was generated by combining our observed pY sites and the catalogued pY sites from PhosphoSitePlus (27). Algorithms were run with default parameters.

Cryocrystallization, Data Collection, and Structure Determination of Lyn SH2—A Lyn construct containing the SH2 domain (residues 115–229) crystallized at 4 °C by the sitting drop diffusion method by mixing 0.8 μl of protein (20 mM Hepes (pH 7.0), 75 mM NaCl, 1 mM DTT, 11.8 mg/ml) with 0.8 μl of well solution (0.25 M ammonium acetate, 17.5% w/v PEG3350). Crystals were crushed, resuspended in 50 μl of well solution, vortexed for 2 min with a seed bead (Hampton Research) Aliso Viejo, CA, and used as seed stock. A single, large, cubic crystal grew at 4 °C by the sitting drop diffusion method by mixing 0.1 μl seed stock with 0.2 μl well solution (0.1 M Tris (pH 8.5), 20% v/v ethanol) and 0.3 μl of protein (20 mM Hepes (pH 7.0), 150 mM NaCl, 1 mM DTT, 22.5 mg/ml) using an Oryx8 automatic protein crystallization system (Douglas Instruments, Ltd., Berkshire, UK). The crystal was cryoprotected prior to flash freezing in liquid nitrogen by soaking briefly in 0.075 M Tris (pH 8.5), 15% ethanol, and 12.5% glycerol, followed by 0.05 M Tris (pH 8.5), 10% ethanol, and 25% glycerol. Diffraction data were collected at 100K on a home source (λ = 1.5418) with Rigaku Saturn944+ detector. High-resolution data were collected at a detector distance of 45 mm, 20 s exposure time, followed by collection of low resolution data at a detector distance of 75 mm, 2 s exposure time. Data integration and scaling were carried out using iMosfilm and Aimless (28–30). The Lyn SH2 structure was solved by molecular replacement with the program PHASER (31) using the low-resolution data set and a model based on the structure coordinates of the human Hck SH2 domain (PDB: 3HCK), with non-identical residues mutated to Ala (32). The initial model was improved to R/Free 26.0/33.8% through cycles of refinement and manual building with programs Phenix REFMAC and Coot (31, 33). High- and low-resolution data sets were scaled and merged with Scala (34) and subsequent cycles of refinement and manual building performed against this merged data set. Relevant data collection and refinement statistics for the merged data set are listed in Table S1. Superpositions of the Lyn SH2 coordinates with other structures were performed in Coot (33).

RESULTS

The SH2 Domain of SFKs Is Tyrosine Phosphorylated in Cancer Samples and Derived Cell Lines—To identify sites of protein tyrosine phosphorylation in primary hematopoietic cancer samples or their derived cell lines, we combined anti-phosphotyrosine immunoprecipitation with MS. Phosphory-
equivalent Hck Y209 or Lck Y192, was detected in 11 out of 12 white blood cell samples obtained from newly diagnosed AML patients (Fig. 1A). Similarly, phosphorylation of Lyn Y194, Lck Y192, or their equivalents Fgr Y209 or Blk Y188, was detected in five white blood cell samples of Revlimid-treated CLL patients (Fig. 1B). Consistent with these results, phosphoproteomic studies have similarly identified Y194 or its equivalent as a site of phosphorylation in lung cancer and in cell lines containing Bcr-Abl fusion protein, which drives oncogenesis in chronic myeloid leukemia. Specifically, Lyn Y194, or its equivalent residue in Fyn, Hck, and Lck, is frequently phosphorylated in Bcr-Abl containing pre-B cell or myeloid cell lines (35). As well, Y194 or its equivalent tyrosine was identified as a site of phosphorylation in Fyn, Yes, Hck, Lck, and Lyn in nonsmall cell lung cancer cell lines and tumors (36), and human epidermal growth factor receptor 2 (HER2)-overexpressing breast tumor specimens showed increased levels of Src pY213 (37). Together, these results indicate that Y194 in the SH2 domain of Lyn, or its equivalent in SFK family members, is frequently phosphorylated in cancer samples and
cancer-derived cell lines, although these data do not indicate which kinases or protein-tyrosine phosphatases regulate this modification.

Stoichiometry of Lyn SH2 Domain Tyrosine Phosphorylation in Multiple Myeloma Cells—To determine if SFK SH2 domain tyrosine phosphorylation at Y194-equivalent sites is susceptible to protein-tyrosine phosphatase inhibition in cells, we tested the stoichiometry of Lyn Y194 phosphorylation in four multiple myeloma cell lines under normal conditions and after treatment with pervanadate. We measured the absolute phosphorylation stoichiometry of Lyn pY194 by SRM-MS. This showed that Lyn Y194 was phosphorylated 0.7% and 0.4% in untreated KMS18 and LP1 cell lines, respectively. In response to pervanadate treatment, these respective values increased to 4.7% and 0.9% (Fig. 1C). Similarly, Lyn pY194 was not detected in untreated KMS11 and RPMI 8226 cell lines but was phosphorylated 4.3% and 2.0%, respectively, following pervanadate treatment. The increase in Lyn Y194 phosphorylation in pervanadate-treated cells suggests that Lyn pY194 is subject to regulation by tyrosine kinase and pY phosphatase activities in vivo.

Tyrosine Phosphorylation of the Lyn SH2 Domain Modulates Its Binding to pY Peptides—Next, we sought to further investigate the role of SH2 domain tyrosine phosphorylation on substrate binding and specificity. Since phosphorylation at Lyn Y194 has been repeatedly observed in cancer tissues, Lyn SH2 was chosen as a model for further experimentation. To this end, unphosphorylated and tyrosine phosphorylated Lyn SH2 were purified as shown in Fig. 2A and 2B. Purified phosphorylated SH2 domain samples were analyzed by SRM-MS and estimated to have a stoichiometry of phosphorylation at Y194 exceeding 95% (Fig. 2C).

To understand if tyrosine phosphorylation of the Lyn SH2 domain influences its binding to phosphorylated ligands, the
specificity of the Lyn SH2 domain for pY peptides was compared with that of phosphorylated Lyn SH2 domain. Immobilized phospho- or unphosphorylated Lyn SH2 domain (hereafter referred to as pSH2 and SH2, respectively) was mixed with a pool of highly phosphorylated peptides generated from pervanadate-treated AML Mv4–11 cells. Bound peptides were eluted into acid solution and analyzed by MS. As a basis for comparison, a total cellular pY-peptide profile for the AML cell line was generated using an anti-pY immunoprecipitation method combined with MS analysis. Repetition of this experiment three times identified a collection of 504 pY sites.

A total of 66 pY-containing peptides, on which 69 pY sites were identified, bound to pSH2 and/or SH2. Among these, 18 singly phosphorylated peptides bound to both SH2 and pSH2, while the remaining 48 peptides, which contain 51 pY sites, bound to SH2 only (Fig. 3A; Table S2). Interestingly, 27 pY sites captured by one or both SH2 ligands were not identified by the anti-pY immunoprecipitation approach. Sequence logos were generated using the WebLogo tool (38) (http://weblogo.berkeley.edu/logo.cgi) to assess the 69 captured pY sites, the 18 pY sites able to bind pSH2, and the collection of total cellular 531 pY sites identified in Mv4–11 cells (Fig. 3B). The sequence logos based on the SH2 or pSH2 bound pY sites showed enrichment of the canonical pYEE[V/I/L] motif known to bind the SFK SH2 domains (1, 11, 39), while the sequence logo based on the total pY did not show enrichment of any distinct motif. Statistical analysis by motif-x (40) indicated that the SH2-bound motifs pYExI and pYExV were enriched 28-fold and 14-fold, respectively, compared with the total pY background, while the pSH2-bound motif...
pYE was enriched fourfold compared with the background (p value < 0.001). These data show that both SH2 and pSH2 were able to selectively enrich for defined binding motifs. Moreover, the preference of pSH2 for pYE, but not pYEEx or pYExV, suggests that the selectivity of the SH2 domain toward the residue at +3 position of the pY site is reduced by SH2 domain tyrosine phosphorylation at Y194. Conversely, these results suggest that SH2 phosphorylation at Y194 does not appear to impact specificity at the pY pocket or pY+1 site.

To gauge the impact of Lyn SH2 Y194 phosphorylation on peptide binding selectivity, the relative quantities of the 18 pY peptides that bound to both SH2 and pSH2 were determined using MaxQuant software (25). Quantifications across three replicates were averaged and used to generate a binding heat map (Fig. 3C). Notably, for all 18 pY peptides, the relative degree of binding was higher for SH2 than pSH2 (3 to 779-fold increase), indicating that the pY peptides have a preference for unphosphorylated Lyn SH2. These data suggest that tyrosine phosphorylation of the SH2 domain at Y194 decreases its affinity for pY-containing peptides compared with unphosphorylated SH2.

To further assess if tyrosine phosphorylation of the SH2 domain modulates its binding affinity, the dissociation constants (K_d) of isolated Lyn SH2 and pSH2 domains for a set of four pY-containing peptides were determined by an in vitro fluorescence polarization binding assay. Two of the peptides, pYEEL and pYEEL, contain the preferred pYEEV/I/L SH2-binding motif, while the other two peptides were derived from biomolecules that are known to bind Lyn SH2 and induce functional consequences, namely the Lyn autoinhibitory C-terminal tail (pYQQQ) (3) and the FcγRIIb immunoreceptor tyrosine-based inhibition motif (pYSLL) (41). K_d values for binding of the isolated Lyn SH2 domain to the pYEEL and pYEEL peptides were determined to be 0.75 ± 0.2 μM and 0.94 ± 0.2 μM, respectively, consistent with the 0.58 μM K_d value reported for Lyn SH2 and a peptide containing the pYEEL motif (42) and the 0.3–0.6 μM K_d values reported for other SFK SH2 domains (43). The affinity of the Lyn SH2 domain for pYQQQ and pYSLL was found to be significantly lower, with K_d values measuring 4.9 ± 0.6 μM and 3 ± 0.9 μM, respectively (Fig. S1), consistent with the 4 μM K_d value reported for Lck SH2 and a low affinity peptide (pYQPG) (43). Notably, all four peptides were found to have lower affinity for pSH2 than SH2 (Fig. 3D), regardless of whether the peptide bound SH2 with relatively high or low affinity. Specifically, the affinity of pYEEL and pYEEL for pSH2 was measured as 1.9 ± 0.2 and 2.8 ± 0.5 fold less than for SH2, respectively. Similarly, the affinity of pYQQQ and pYSLL for pSH2 decreased 1.5 ± 0.1 and 2.1 ± 0.3 fold, respectively, as compared with SH2. These data indicate that phosphorylation of the Lyn SH2 domain at Y194 decreases its binding affinity for pY-containing peptide ligands. The fact that the binding affinity for SH2 versus pSH2 differs by only 1.5–2.8 fold may reflect that the majority of the binding energy is contributed by pY binding while the pY-proximal residues contribute significantly less to binding affinity and suggests that the majority of the binding energy from pY binding is not significantly altered by SH2 domain tyrosine phosphorylation (9, 44).

Tyrosine Phosphorylation of the Lyn SH2 Domain Modulates Its Binding to pY-Containing Proteins—To investigate whether phosphorylation at Lyn Y194 affects binding to pY-containing proteins, the binding of cellular proteins to Lyn SH2 and pSH2 was compared. SA immobilized Lyn SH2 and pSH2 were used to affinity-purify pY-containing proteins from pervanadate-treated Mv4–11 whole cell lysates. Bound proteins were assessed by immunoblotting with a pY-specific antibody or, alternatively, by MS following trypsin-digestion of captured, immobilized proteins. Notably, immunoblot analysis showed dramatically reduced binding of pY-containing proteins by pSH2 compared with SH2 (Fig. 4A; compare Lanes 4 and 5). For MS analysis, quantitative profiles of the proteins were generated based on MS ion current intensities. Compared with the SA-only negative control, a total of 539 proteins were found to be significantly enriched in the SH2- or pSH2-bound fractions, including 36 known Lyn-interacting proteins, based on the GeneCards data archive (45) (www.genecards.org).

A total of 166 pY sites were identified on these proteins. The fact that the number of identified pY sites was lower than the number of detected proteins indicates direct SH2-binding, and indirect SH2-independent protein complexes were recovered by the AP method. Consistent with the peptide binding experiments described above, fewer pY-containing proteins (containing fewer pY sites) bound pSH2 compared with SH2 (Fig. 4A and 4B), suggesting that phosphorylation of Lyn SH2 at Y194 decreases its ability to interact with pY motifs in the context of native proteins. This is consistent with findings for Lck, whereby phosphorylation of the equivalent SH2 tyrosine residue reduced its ability to bind cellular proteins from pervanadate-treated Jurkat T cells (19). A total of 352 proteins bound twofold or better to SH2, and 42 proteins bound twofold or better to pSH2, suggesting that pSH2 has an altered binding specificity for phosphoproteins (see also Table S3).

A hidden Markov model method (26) was used to predict which proteins, if tyrosine-phosphorylated, based on their potential pY motifs, would bind directly to Lyn SH2. This analysis predicted that of the 394 proteins that showed a preferred binding to SH2 or pSH2 as described above (i.e. 352 + 42), 66, including 16 known interaction partners of Lyn, would bind directly (Fig. 4C). Among these 66 proteins, 62 were predicted to bind directly SH2 and four to bind directly pSH2. Comparison of captured proteins and peptides revealed 27 instances of overlap, wherein a native protein and a pY-peptide containing the same pY site were captured by SH2 or pSH2 (Table S4).

In the above protein binding experiment, it is conceivable that a protein containing an SH2 or phosphotyrosine binding
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A

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B



C

Prefer SH2

> 100 50-100 20-50 10-20 2-10 lyn SH2 > 100 Fold change

Prefer pSH2

> 100 50-100 20-50 10-20 2-10 lyn SH2 > 100 Fold change

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(PTB) domain might bind the pY194 moiety of the immobilized pSH2 domain. In this scenario, such a protein would be expected to bind preferentially pSH2. However, no SH2/PTB domain-containing proteins captured in the AP experiments bound preferentially to pSH2, suggesting the data are not contaminated by "reverse" binding to the pY194 moiety itself (Table S5).

Crystal Structure of Lyn SH2—To further understand the impact of Y194 phosphorylation on Lyn SH2 binding to pY-peptides, the crystal structure of the Lyn SH2 domain was determined. Attempts to obtain high-quality crystals of Lyn pSH2 or peptide-bound Lyn SH2 were unsuccessful, and thus, we used the Lyn SH2 structure to model Y194 phosphorylation and peptide binding. The structure contains two molecules in the asymmetric unit, which are very similar in conformation (0.6 Å root mean square deviations) and generally well ordered, except for the N-terminal four residues and the last C-terminal residue. A structure-based protein sequence alignment of Lyn SH2 and other SFK SH2 domains is shown in Fig. 1D.

The domain adopts the conserved SH2 fold, containing a central, seven-stranded antiparallel β-sheet (βA, βB, βC, βD, βE, βF, and βG) flanked by two α-helices (αA and αB) (8, 13) (Fig. 5A) and superimposes well with other SFK SH2 domains (root mean square deviations of 1.0 Å with v-Src, PDB: 1SPS) (Fig. 5B). Despite the absence of a phosphopeptide in the Lyn SH2 structure, the phosphotyrosine pocket is intact, formed by the side chains of conserved residues from strands βD

Fig. 5. Crystal structure of Lyn SH2. A Ribbon representation of the Cα atoms of the Lyn SH2 crystal structure, with the EF and BG loops highlighted in green as in Fig. 1D. B Superposition of Lyn SH2 with v-Src SH2 bound to peptide PQpYEEIP (PDB: 1SPS), aligned using main chain atoms. Lyn SH2 is colored as in A; v-Src and the bound peptide are colored cyan and yellow, respectively. Key residues are shown in stick, with carbon atoms colored according to their respective backbones and oxygen, nitrogen, and phosphate atoms colored red, blue, and orange, respectively. C As in B, with the addition of surface representation for Lyn SH2, colored according to electrostatic potential as calculated by MacPyMol, with negative, positive, and neutral potential shaded red, blue, and white, respectively. This figure was prepared with MacPyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.).

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Fig. 4. Tyrosine phosphorylation at Y194 modulates the binding affinities of Lyn SH2 domain and pY-containing proteins. A Mv4–11 cells untreated (Lane 1) or treated with pervanadate (Lane 2) were lysed and incubated with SA beads alone (Lanes 3, 6), SH2 (Lanes 4, 7), or pSH2 (Lanes 5, 8) to allow binding. The bound proteins (Lanes 3–5) and the flow-through (Lanes 6–8) were analyzed by anti-pY Western blotting. The two lanes in the lower panel were blotted with streptavidin–horseradish peroxidase showing equivalent loading of SA–immobilized biotin-labeled SH2 (left) and pSH2 (right) preparations. B A volcano plot showing Student’s t test p value (y axis), calculated from six replicates versus protein quantification fold changes (x axis). A statistical significance p value cut-off is indicated. C Schematic representation of proteins bound differentially (twofold or higher) to Lyn SH2 (shades of blue/purple) or pSH2 (shades of green). Proteins predicted to bind directly are connected to Lyn SH2 by a line. Known Lyn interacting proteins are highlighted with red boarders. Asterisks denote Student’s t test p values: *p < .05; **p < .01; ***p < .001.
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(H^{182} and K^{184}) and βC (S^{165}), and two conserved arginines (R^{136} and R^{156}). As observed for other peptide-free SH2 domain structures, the phosphate binding loop is in a more open conformation in Lyn SH2 than in peptide-bound SFK SH2 domains (14) (Fig. 5B). The pY+3 specificity pocket is similarly intact in the Lyn SH2 structure, formed by the side chains of residues from loops EF and BG, as well as conserved residues Y^{183} and Y^{211} (Fig. 5B and 5C).

Structural Impact of Phosphorylation at Lyn SH2 Y^{194}—The side chain hydroxyl group of Y^{194} is positioned on the surface of the protein, accessible for phosphorylation in trans (Fig. 5C). The side chain adopts the same conformation in both peptide-free and peptide-bound SFK SH2 domains, suggesting it is accessible for phosphorylation both before and after substrate binding (Fig. 5B). The negative charge of phosphorylated Y^{194} could potentially form a stable electrostatic interaction with the side chain of conserved R^{186}, as predicted for the tyrosine phosphorylated Src SH2 domain (18). Y^{194} is located sufficiently distant from the pY recognition pocket that phosphorylation at this position is not expected to significantly impact pY affinity or specificity. Given that SH2 peptide binding is energetically dominated by pY binding, this likely explains our observation that phosphorylation of Lyn SH2 at Y^{194} results in only a modest reduction in phosphate-binding affinity (Fig. 3D) (9, 44).

In contrast, Y^{194} is located near the pY+2 and pY+3 recognition sites, such that phosphorylation could significantly impact the specificity of peptide binding at these positions. In the unphosphorylated SH2 domain, the side chain of R^{186} contributes to the pY+2 binding surface. However, if R^{186} alters its conformation to interact with the phosphate group on pY^{194}, then binding at the pY+2 site may be affected. Furthermore, Y^{194} is located at the N-terminal end of the EF loop, which dictates SH2 domain specificity by shaping the ligand-binding surface and controlling accessibility of the pY+3 binding pocket (15). Since minor alterations in the EF loop and pY+3 pocket significantly impact kinase function as well as peptide binding affinity and specificity (15–17, 46, 47), it is feasible that alteration of the EF loop by the addition of a bulky, negatively charged phosphate group at Y^{194} could alter the configuration of the EF loop and thus impact peptide binding at the pY+3 pocket. This would explain why we observe modulation of Lyn SH2 binding of both pY-peptides and pY-containing phosphoprotein complexes upon phosphorylation at Y^{194}.

DISCUSSION

Through proteomics analysis, we discovered that a conserved tyrosine in the SH2 domain of Src family kinases is phosphorylated in acute myeloid and chronic lymphocytic leukemia and in multiple myeloma cell lines. Review of the literature revealed phosphorylation of this tyrosine in other cancer samples and cancer-derived cell lines, including neuroblastoma cell lines and tumors (36) and HER2-overexpressing breast tumor specimens (37). Using Lyn SH2 domain as a model, we investigated the impact of tyrosine phosphorylation on SH2 binding of pY-containing peptides and pY-containing phosphoprotein complexes. We found that phosphorylation at Y^{194} generally decreases the affinity of Lyn SH2 for tyrosine-phosphorylated peptides, and modulates interactions with pY-containing protein complexes.

Our results indicate that tyrosine phosphorylation of Lyn SH2 increases in multiple myeloma cells following broad inhibition of protein-tyrosine phosphatases, but our analysis did not implicate specific tyrosine kinases and protein-tyrosine phosphatases in the modulation of Lyn SH2 phosphorylation. Regulation of SFK SH2 phosphorylation at the equivalent tyrosine residue has been reported for other Src family members. Both platelet-derived growth factor (PDGF) and HER2/heregulin signaling induced phosphorylation of Src Y^{213} in vitro (18, 37), while Lck Y^{192} was phosphorylated in response to treatment of Jurkat T cells with anti-CD3 antibodies (48). Notably, direct phosphorylation of Src Y^{213} by the PDGF receptor in vitro was found to diminish SH2 binding to the C-terminal inhibitory phosphotyrosine sequence, resulting in Src activation (18). HER2/heregulin stimulated Src Y^{213} phosphorylation also increased Src kinase activity, and selectively increased tyrosine phosphorylation of focal adhesion kinase (FAK) at Y^{61} (37). As well, pY^{193} was found to have a distinct effect on localization of Src, suggesting that phosphorylation of Src at Y^{213} activates a new signaling pathway that influences metastasis of breast cancer cells (37). Together these results suggest that SFK SH2 domain tyrosine phosphorylation may impact SFK activity by influencing localization, substrate specificity of the SH2 domain and possibly allosteric interactions with the kinase domain (18, 37). Consistent with this, the affinity of Y^{194}—phosphorylated Lyn SH2 for pY-containing proteins decreased in general, while a small subset of proteins exhibited increased binding to pSH2. Thus, SH2 tyrosine phosphorylation may serve as a means for SFKs to regulate their protein-protein interactions and downstream signaling events.

The phosphorylated Lyn SH2 showed reduced affinity for high- and low-affinity synthetic pY-peptides in vitro. In contrast, tyrosine phosphorylation of Src SH2 did not affect binding to a high affinity peptide but significantly reduced binding to a peptide representing the low affinity C-terminal inhibitory tail of Src (18). Phosphorylation of the Y^{194}—equivalent site in Lck SH2 (Y^{192}) reduced binding to a high affinity peptide but had no effect on binding to a lower affinity peptide (19). These conflicting results may be due to differences in the pY-peptides or SFK SH2 domains tested or even the method of assessment. These observations may also reflect the fact that the interaction between a specific SFK SH2 domain and a given pY-peptide may be differentially modulated by SH2 tyrosine phosphorylation, thereby serving as a mechanism to control SFK SH2 substrate binding profiles and finely regulate and differentiate signaling by SFK members.
Structural analysis of the Lyn SH2 domain and other SFK SH2 domains suggests that Y¹⁹⁴ is equally accessible for phosphorylation before and after substrate binding and that phosphorylation at this site is unlikely to impact pY³ binding affinity. However, the proximity of Y¹⁹⁴ to the pY³ + 2 and pY³ + 3 recognition sites suggests that phosphorylation at this residue would significantly impact the specificity of peptide binding. Residues of the EF loop modulate the accessibility and shape of the pY³ pocket and thus peptide specificity (15). Notably, Y¹⁹⁴ is part of a conserved EF loop sequence (G/G/W)[Y/F/L]I[V/F/T/S/R] found in SFK SH2 domains but not in other SH2 domains (B. Liu; www.SH2domain.org/alignment) (49) (Fig. 1D). Therefore, the EF loop conservation in SFK SH2 domains likely confers their characteristic modest level of binding specificity, which can be further modified by phosphorylation at Y¹⁹⁴ (44). However, this conserved consensus sequence may also be required for substrate recognition by the tyrosine kinase(s) that phosphorylate SFK SH2 domains at Y¹⁹⁴ sites. Here, we show that EphA4 kinase phosphorylated Lyn SH2 in vitro. Syk kinase has also been shown to phosphorylate Lck at the equivalent SH2 tyrosine (Y¹⁹２) in COS cells (19, 50), and platelet-derived growth factor receptor kinase phosphorylated the Src SH2 domain in vitro (18). Further investigation of the tyrosine kinases that mediate SFK SH2 phosphorylation is required to assess the role of the EF loop consensus sequence in substrate recognition.

In conclusion, the modulation of Lyn SH2-ligand interactions by SH2 domain tyrosine phosphorylation illustrates yet another layer of complexity within signaling networks mediated by phosphorylation-dependent protein-protein interactions. The prevalence of SH2 domain phosphorylations in phosphoproteomics datasets suggests this is a widespread phenomenon subject to regulation by SH2 kinases and pSH2 phosphatases.

**Accession Numbers**—Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 4TZI.

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§§ This article contains supplemental material Fig. S1 and Tables S1–S5.

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