Functional Diversity of Csk, Chk, and Src SH2 Domains due to a Single Residue Variation*

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The C-terminal Src kinase (Csk) family of protein tyrosine kinases contains two members: Csk and Csk homologous kinase (Chk). Both phosphorylate and inactivate Src family kinases. Recent reports suggest that the Src homology (SH) 2 domains of Csk and Chk may bind to different phosphoproteins, which provides a basis for different cellular functions for Csk and Chk. To verify and characterize such a functional divergence, we compared the binding properties of the Csk, Chk, and Src SH2 domains and investigated the structural basis for the functional divergence. First, the study demonstrated striking functional differences between the Csk and Chk SH2 domains and revealed functional similarities between the Chk and Src SH2 domains. Second, structural analysis and mutagenic studies revealed that the functional differences among the three SH2 domains were largely controlled by one residue, Glu127 in Csk, Ile167 in Chk, and Lys200 in Src. Mutating these residues in the Csk or Chk SH2 domain to the Src counterpart resulted in dramatic gain of function similar to Src SH2 domain, whereas mutating Lys200 in Src SH2 domain to Glu (the Csk counterpart) resulted in loss of Src SH2 function. Third, a single point mutation of E127K rendered Csk responsive to activation by a Src SH2 domain ligand. Finally, the optimal phosphopeptide sequence for the Chk SH2 domain was determined. These results provide a compelling explanation for the functional differences between two homologous protein tyrosine kinases and reveal a new structure-function relationship for the SH2 domains.

Mutations in the SH2 domain of Csk or Chk significantly decrease the ability of Csk or Chk to suppress Src, even when such mutations do not directly affect Csk or Chk kinase activity (3, 4). These findings indicate that the SH2 domains play critical roles in the cellular functions of Csk and Chk.

Consistent with the above conclusion, both the Csk and Chk SH2 domains bind to a number of phosphotyrosine-containing proteins in the cells. Interestingly, there is very little overlap between the two sets of proteins that bind to the Csk and Chk SH2 domains. Csk SH2 domain-binding proteins include the Csk-binding protein (CBP) (5), protein tyrosine phosphatase-HSCF (6), β-dystroglycan (7), an SHP2-interacting transmembrane adaptor protein (8), insulin-like growth factor-1 receptor (9), T-cell receptor ζ and ε (10), and GTPase-activating protein complex (11, 12). Chk SH2 domain-binding proteins include ErbB2 (13, 14), c-KIT receptor tyrosine kinase (15), paxillin (16), TrkA receptor (17), and RAFTK/Pyk2 (18). This comparison raises the possibility that the Csk and Chk SH2 domains may have different binding preferences. Detailed studies of Csk and Chk function in breast cancer cells support this possibility. In normal and cancerous breast cells, Csk is highly expressed and fully active (19). In contrast, Chk is not expressed in normal breast cells, but expression of Chk is induced in breast cancer cells (14). The increased expression level of Chk correlates to suppression of Src, although constitutively expressed Csk fails to suppress Src in these cells (19). The specific ability of Chk to suppress Src correlates to the binding of the Chk SH2 domain to the receptor tyrosine kinase ErbB2 on pTyr1248 (4). Side-by-side experiments demonstrate that the Csk SH2 domain does not bind to ErbB2 on pTyr1248 (19). These studies suggest that the Csk and Chk SH2 domains bind to different sets of pTyr-containing proteins, which may distinguish the cellular function of Csk and Chk.

The SH2 domain is a ~100-residue protein module that binds to pTyr-containing proteins (20, 21). It is present in a large number of signaling proteins, including protein tyrosine kinases, protein tyrosine phosphatases, phospholipid kinases, transcription factors, adaptor proteins, and others (22, 23). The SH2-pTyr interaction, in conjunction with tyrosine phosphorylation, is a fundamental mechanism of mammalian signal transduction (20).

Optimal phosphopeptides for a number of SH2 domains have been determined by screening phosphopeptide libraries (24–26). The structures of several SH2-phosphopeptide complexes have been determined (27–29). The SH2 domains within each family of proteins are generally similar in structure and binding preference. For example, Src family kinases contain nine members, and the SH2 domains of all tested Src family kinases prefer to bind to the sequence of pYEEI (24). Structural, mutagenic, and binding studies on the Src SH2 domain reveal a
“two-socket” binding mechanism, in which pTyr and Ile of the phosphopeptide respectively bind to two cavities of the SH2 domain (30–32). Alignment of amino acid sequence and comparison of available crystal structures of the Src family SH2 domains reveal that such a two-socket binding surface is well conserved within this family.

In the current study, we investigated functional and structural divergence between the Csk and Chk SH2 domains in relation to Src regulation. These studies revealed that the Chk SH2 domain shared a higher functional similarity to the Src SH2 domain than to the Csk SH2 domain. Furthermore, structural and mutational studies demonstrated that the functional comparison among the Csk, Chk, and Src SH2 domains was largely determined by one key residue. These studies shed new light on the functional comparison between Csk and Chk and the general structure-function relationships of the SH2 domain architecture.

MATERIALS AND METHODS

Reagents and Chemicals—All reagents used for bacterial culture and protein expression were purchased from Fisher. Chromatographic reagents were purchased from Sigma. A human fetal brain cDNA library was obtained from Stratagene. DNA primers were synthesized by Integrated DNA Technologies. Phosphopeptides were synthesized by solid phase synthesis and purified by HPLC, and their molecular weights were confirmed by electrospray mass spectrometry.

Plasmid Construction and Protein Purification—Human Csk and Chk coding sequences were amplified by polymerase chain reaction from a fetal brain cDNA library (33, 34), and the chicken Src SH2 domain coding sequence was amplified from chicken Src cDNA (35). The SH2 domains and other fragments were expressed as fusion proteins with glutathione S-transferase in DH5α using pGEX-2th (36). The GST-Csk SH2 and GST-Src SH2 fusion proteins were used as the Csk and Src SH2 domains. The GST-Src SH2 domain resulting in an increase of fluorescence polarization of 485 and 530 nm, respectively recorded using PerkinElmer Life Sciences LS55 luminescence cuvette, for about 1 min, and the fluorescence polarization was determined using fluorescence polarization assay. As previously described (37). This peptide also bound to the Chk SH2 domain was determined by the fluorescence polarization assay using bovine serum albumin as a standard. GST-Csk SH2 domain was extensively digested during recombinant expression and purification, and the GST-Chk SH2 fusion was used as the Chk SH2 domain.

Fluorescence Polarization Assay and Kd Determination—To determine the binding of a phosphopeptide to an SH2 domain, fluorescein was coupled to the phosphopeptide at the N terminus and purified by HPLC. The fluorescence polarization assay was performed as described previously (37). Briefly, fluorescein-labeled phosphopeptide at a fixed concentration (80 nM) and varying concentrations of the SH2 domain were incubated in 50 mM Tris-Cl, pH 8.0 (in a 500-μl reaction volume) for 1 min, and the fluorescence polarization was then recorded using PerkinElmer Life Sciences LS55 luminescence spectrometer at 25 °C. The excitation and emission wavelengths for the fluorescence polarization measurement were 485 and 530 nm, respectively. The fluorescence polarization value with no SH2 domain present was used as a background and subtracted from the FP values in the presence of the SH2 domain. The increase in FP as a function of the SH2 domain concentration was fitted into the following equation: 

\[
FP = FP_{\text{max}} \times \frac{[\text{SH2}] 
\times \frac{1}{K_{d1} + [\text{Pept}]}}{1 + \frac{[\text{SH2}]}{K_{d2} + [\text{Pept}]}}
\]

where \( FP_{\text{max}} \) was the maximum fluorescence polarization value when all the fluoresceinated phosphopeptide was bound to the SH2 domain, and \( K_{d1} \) is the dissociation constant of the phosphopeptide binding to the SH2 domain. Data fitting was performed with a curve-fitting software, LabFit (www.extension.hp.com.br).

To determine the binding of an unlabeled phosphopeptide to an SH2 domain, the fluorescence polarization of 80 nM Flu-GpYEEI in the presence of 700 nM SH2 domain and varying concentrations of the unlabeled phosphopeptide was determined. The fluorescence polarization as a function of the total unlabeled phosphopeptide concentration was curve-fitted using the following equation: 

\[
FP = A \times (\frac{[\text{SH2}] \times [\text{Pept}]}{K_{d1} + [\text{Pept}]}) + \frac{[\text{SH2}] \times [\text{Pept}]}{K_{d2} + [\text{Pept}]}
\]

where \( K_{d1} \) was the dissociation constant of Flu-GpYEEI binding to the SH2 domain, which was pre-determined by the direct binding assay, and \( K_{d2} \) was the dissociation constant of unlabeled peptide binding to the SH2 domain. A is a conversion factor between the concentration of probe SH2 complex and the fluorescence polarization value. \([\text{SH2}]\), and \([\text{Pept}]\) were total concentrations of the SH2 domain and the fluorescent probe, Flu-GpYEEI, respectively. \([\text{Pept}]\) was the total concentration of the unlabeled phosphopeptide. All binding assays were performed in duplicates and repeated at least twice with similar results.

Phosphopeptide Screening to Determine the Optimal Ligand for the Chk SH2 Domain—To determine the optimal Chk SH2 domain phosphopeptide ligand, a phosphopeptide library was synthesized and screened. The library contained a basal phosphopeptide, GpYAAA, and three groups of phosphopeptides. Each group contained 17 individual phosphopeptides, substituting one of the Ala with 17 other standard residues. Cys and Trp were not included in the screening to avoid complications in peptide synthesis. All phosphopeptides were individually synthesized and purified. The binding of each phosphopeptide to the Chk SH2 domain was determined by the fluorescence polarization competition assay. The binding of each phosphopeptide was measured by the decrease in FP of Flu-GpYEEI caused by the presence of 50 μM phosphopeptide. Residue preference for each position was calculated by comparing the binding of a phosphopeptide carrying a particular residue at the given position with that of GpYAAA.

RESULTS

The Chk and Src SH2 Domains Have Similar Binding Preferences—The optimal phosphopeptides for the Src and Csk SH2 domains have been previously reported to be pYEEI (24) and pYTKM (25), respectively. The optimal pTyr peptide for the Chk SH2 domain has not been reported. To compare the binding of the SH2 domains to pTyr peptides, two fluorescein-phosphopeptides, Flu-GpYEEI and Flu-GpYTKM, were synthesized. Binding of these peptides to each of the SH2 domains was determined using fluorescence polarization assay. As shown in Fig. 1A, Flu-GpYEEI bound to the Src SH2 domain tightly, with an apparent \( K_{d} \) of 0.13 ± 0.01 μM. This affinity is similar to a previous report (37). This peptide also bound to the Chk SH2 domain, with a \( K_{d} \) of 3.3 ± 0.2 μM. In contrast, the peptide did not bind to the Csk SH2 domain under identical conditions, with 10 μM Csk SH2 domain resulting in an increase of fluorescence polarization of <0.005.

Binding of these three SH2 domains to Flu-GpYTKM was
Indeed, both the CBP peptide and GpYTKM activated Csk binds to the Csk SH2 domain and activates Csk activity (39). Previously shown to activate Csk. These two phosphopeptides are the tightest-binding ligands for the Csk SH2 domain.

then determined. To our surprise, Flu-GpYTKM did not bind to the Csk SH2 or the other two SH2 domains to any significant extent (data not shown). To ensure that our Csk SH2 domain fusion protein was not defective in some way, we generated an SH3+SH2 domain and full-length Csk, but neither of them bound to Flu-GpYTKM significantly. In an attempt to find a phosphopeptide that binds to Csk SH2 domain as a potential probe, we also synthesized Flu-AMPYSSV, a phosphopeptide that mimicked the pTyr314 phosphorylation site of CBP that was known to bind to the Csk SH2 domain (5). However, this phosphopeptide also failed to bind to the Csk SH2 domain, SH3+SH2 fragment, or full-length Csk significantly in the direct binding assays. One possible explanation for the discrepancy between these observations and previously reported binding is that previous reports did not determine the binding affinity (5, 25). It is possible that these peptides bind to the Csk SH2 domain too weakly to be determined by the direct binding assay in the current study, which is limited by the concentration of the Csk SH2 domain that can be used in the assay.

To test this possibility, we determined the activation of Csk kinase activity by the two phosphopeptides (Fig. 2). It has been previously demonstrated that CBP pTyr114 phosphopeptide binds to the Csk SH2 domain and activates Csk activity (39). Indeed, both the CBP peptide and GpYTKM activated Csk –78% with $K_d$ values of 166 ± 17 μM for CBP peptide and 39 ± 3 μM for GpYTKM. The CBP phosphopeptide activation data are consistent with the previous report (39). Both the $K_d$ values were significantly higher than the highest concentration of the Csk SH2 domain (10 μM) used in the FP assay. These data together indicated that these phosphopeptides did bind to the Csk SH2 domain, but not strongly enough to be determined by the direct FP assay.

The similarity between the Chk and Src SH2 domains in binding to Flu-GpYEEI was intriguing. To determine whether the similarity in the Chk and Src SH2 domains in binding to Flu-GpYEEI would extend to a physiological phosphopeptide, we synthesized and tested a fluoresceinated phosphopeptide mimicking pTyr1248 of ErbB2, Flu-NEPEpYLGLDV (Flu-pY1248) (14, 19). This phosphopeptide site is responsible for ErbB2 binding to the Chk SH2 domain (14). Flu-pY1248 bound to both the Chk and Src SH2 domains (Fig. 1B), with $K_d$ values of 17.9 ± 3.7 μM and 0.8 ± 0.06 μM, respectively. In contrast, the Csk SH2 domain did not bind to Flu-pY1248 to any significant extent. This result is consistent with a previous study that demonstrates that the Chk SH2 domain but not the Csk SH2 domain binds to ErbB2 on pTyr1248 (19). However, tight binding of Flu-pY1248 to Src SH2 domain has not been previously reported and is rather surprising because the peptide bears only minor similarity to the Src SH2 optimal peptide, pYEEI. Although Src binding, through its SH2 domain, to autophosphorylated ErbB2 is well established (40, 41), the phosphorylation site on ErbB2 for this binding has not been determined to our knowledge. The tight binding of Flu-pY1248 to the Src SH2 domain makes pTyr1248 a prime candidate for this function. Taken together, these binding studies suggested a functional similarity between the Chk and Src SH2 domains and a functional divergence between the Csk and Chk SH2 domains.

**Tertiary Structural Comparison Suggests Potential Structural Bases for the Inability of the Csk SH2 Domain to Bind GpYEEI**—The functional similarity between the Chk and Src SH2 domains and the functional divergence between the Csk and Chk SH2 domains are intriguing. The Chk SH2 domain shares 61% sequence identity with the Csk SH2 domain but has only 40% sequence identity with the Src SH2 domain. The lack of correlation between functional similarity and the overall sequence identity raises the possibility that a few critical residues, rather than the overall sequence similarity, dictate binding properties of these SH2 domains. To investigate this possibility, we compared the tertiary structures of the Chk (Protein Data Bank accession code 1JWO) and Csk SH2 domains (Protein Data Bank accession code 1K9A) (42) with that of the Src SH2 domain complexed with PqpYEEIP (Protein Data Bank accession code 1SPS) (27), using K2 structural alignment program (43). This program seeks the best alignment between two protein structures by minimizing the difference of distance matrices using a genetic algorithm. By such structural alignments, we were able to compare the binding surfaces of the Csk and Chk SH2 domains with that of the Src SH2 domain. Although the K2 program does not recognize the phosphate moiety in pTyr, it superimposes the rest of the phosphopeptide (PqpYEEIP) in the conformation bound to the Src SH2 domain onto the Csk or Chk SH2 domain. We were particularly interested in identifying any residues in the Csk SH2 domain that might clash with PqpYEEIP, thus preventing its binding. The alignment revealed that the Csk and Src
SH2 domains shared a similar overall tertiary structure (Fig. 3A), but the Csk SH2 domain contained two residues that would clash with the phosphopeptide. Asn111 would sterically clash with the pTyr residue from the phosphopeptide, with Asn111 side chain essentially occupying the binding pocket for the phosphate of the phosphopeptide (Fig. 3B). Glu127 would sterically and electrostatically clash with Glu at the Y1 position in PQpYEEIP. These structural analyses suggested that Asn111 and/or Glu127 prevented its binding to GpYEEI.

\[ \text{Csk Glu}^{127} / \text{Src Lys}^{200} \text{ Is Critical in Controlling the SH2 Domain Function—To test whether Asn}^{111} \text{ and/or Glu}^{127} \text{ were indeed responsible for the inability of the Csk SH2 domain to bind to Flu-GpYEEI, two Csk SH2 domain mutants were generated, individually mutating Asn}^{111} \text{ to Gly and Glu}^{127} \text{ to Lys, respectively. The N111G mutation removed the interfering side chain of Asn}^{111} \text{, whereas mutation of Glu}^{127} \text{ to Lys replaced the Csk residue with the Src counterpart. Mutation of N111G did not alter the binding, but E127K mutation significantly improved the binding of the Csk SH2 domain to Flu-GpYEEI, with a Kd of 1.8 ± 0.6 M (Fig. 4A). This result indicated that Glu}^{127} \text{ of Csk was indeed a key residue preventing the Csk SH2 domain from binding to Flu-GpYEEI. This residue is located at the third position of } \beta \text{-sheet D (6D3). The fact that Asn}^{111} \text{ was not responsible for preventing the binding suggested that a conformational change at this residue occurred during ligand binding. The Csk residue equivalent to Csk Glu}^{127} \text{ is Ile}^{167} \text{, but this hydrophobic side chain does not protrude from the binding surface. We determined whether mutation of Ile}^{167} \text{ to Lys or Glu would respectively strengthen and weaken the binding of the Csk SH2 domain to Flu-GpYEEI (Fig. 4B). Mutation Ile}^{167} \text{E reduced the binding to Flu-GpYEEI somewhat, changing the}

### Table I

| SH2 domain | Flu-GpYEEI Kd | Flu-pY1248 Kd | Fig. |
|------------|---------------|---------------|-----|
| wt Csk SH2 | 3.3 ± 0.2 M   | 17.9 ± 3.7 M  | A and B |
| Chk SH2-1167K | 0.18 ± 0.01 M | 2.5 ± 0.2 M   | A and B |
| Chk SH2-1167E | 11.3 ± 1.9 M  | 23.4 ± 4.9 M  | 5A and 4B |
| wt Csk SH2 | NB            | NB            | 1, A and B |
| Csk SH2-1167E | NB            | NB            | 4A |
| N111G | 1.8 ± 0.18 M | NB            | 4A |
| Csk SH2-1167E | 0.13 ± 0.01 M | 0.8 ± 0.06 M  | 1, A and B |
| wt Src SH2 | 6.3 ± 2.1 M   | 20.4 ± 2.6 M  | 5B and 4C |
| Src SH2-K200E | 0.01 M         | 0.8 M         | 1, A and B |

`a` wt, wild type.

`b` NB, no binding detected. In these cases, the fluorescence polarization increased <0.005 even at the highest concentrations of the SH2 domain (up to 10 μM).
Kd from 3.3 to 11.3 ± 1.9 μM. However, mutation I167K dramatically increased the binding affinity to a Kd of 0.18 ± 0.01 μM. This affinity is comparable with that of the Src SH2 domain for the same probe.

The above results with the Csk and Chk SH2 domains suggested that Lys and Glu represented two extremes at the D3 position, with Lys conferring the strongest binding to GpYEEI. To further test this relationship, we mutated Lys200 of the Src SH2 domain to Glu. Consistent with the above trend, the mutation decreased the Src SH2 domain binding to GpYEEI dramatically, increasing the Kd from 0.13 to 6.3 ± 2.1 μM (Fig. 4C).

This result confirmed the critical role of Lys200 in the Src SH2 domain function. These results together compellingly demonstrated that the identity of the βD3 residue played a major role in determining the binding affinity of the SH2 domains to Flu-GpYEEI.

We next determined whether the structure-function relationship at the βD3 position could be extended to the ErbB2 peptide, Flu-pY1248. The Chk SH2 I167E mutant displayed a Kd of 23.4 ± 4.9 μM (Fig. 5A), very similar to the Kd for the wild type Chk SH2 domain in binding to this peptide. However, the Chk I167K mutant displayed a Kd of 2.5 ± 0.2 μM, about 7-fold better than the wild type Chk SH2 domain. Consistent with this trend, the Src SH2 K200E mutant displayed a Kd of 0.18 ± 0.01 μM (Fig. 5B), about 25-fold lower than that of the wild type Src SH2 domain. The Src SH2 E127K mutant displayed a slightly better binding to this peptide, but it was still not strong enough to determine a meaningful Kd value. Overall, these results revealed that the βD3 position was a key determinant for the function of the three SH2 domains. All reported Kd values in the above sections are summarized in Table I.

To determine whether the mutational effects of the βD3 residues could be extended to other phosphopeptides, we compared the binding of the SH2 domains and mutants to a group of various phosphopeptides binding to the SH2 domains.

Table I

| Peptide                  | Src SH2 | Chk SH2 | Chk SH2 |
|--------------------------|---------|---------|---------|
|                          | Kd (μM) | Kd (μM) | Kd (μM) |
| GpYEEI                  | 0.7 ± 0.1 | 1.5 ± 0.3 | 1.5 ± 0.3 |
| DSpYAEI                 | 1.9 ± 0.2 | 13 ± 1.6 | 13 ± 1.6 |
| ESpYDYV                 | 1.2 ± 0.9 | 3.3 ± 0.2 | 25 ± 2.2 |
| EFpYEND                 | 0.5 ± 0.3 | 3.2 ± 0.2 | 11 ± 1.3 |
| ESpYENE                 | 1.5 ± 0.2 | >100 | 38 ± 3.1 |
| DSpYAEI                 | 0.5 ± 0.3 | 4.6 ± 0.2 | 14 ± 0.9 |

Fig. 7. Residue preference at the Y1, Y2, and Y3 positions for the Chk SH2 domain. Each of the three positions was optimized by screening 17 phosphopeptides, each containing a standard amino acid residue at the position. Binding of each of the phosphopeptides to the Chk SH2 domain was determined by the fluorescence polarization competition assay using Flu-pYEEI as a probe. Residue preference, which reflects the preference for a given residue at the position, was calculated as described under "Materials and Methods." The reported values were the average of two screenings.

Fig. 8. Comparison of GpYYYL and GpYEEI in binding to the Chk and Src SH2 domains. Binding of the two phosphopeptides to the Chk (A) and Src (B) SH2 domains was measured by their ability to compete against a fluorescent probe, Flu-GpYEEI.
phosphopeptides. The current study was initiated to compare the properties of the Csk, Chk, and Src SH2 domains and to investigate the structural basis for this functional divergence. We confirmed that the Csk and Chk SH2 domains had striking functional differences, whereas the Csk and Src SH2 domains had a certain level of functional similarity. These functional comparisons did not correlate to the comparisons in the primary structures among the three SH2 domains. Surprisingly, the functional comparisons among the SH2 domains were mainly dictated by the differences at the βD3 position. GpYYYL has been identified to bind to the Src SH2 domain, but GpYAAA has no effect on the Src SH2 domain. The Chk SH2 domain prefers pYYYL—

| Peptide position, SH2 position | Y+1 | Y+2 | Y+3 |
|-------------------------------|-----|-----|-----|
|                              | βD3 | βD5 | βD1 | βE5 | βE4 | EF1 | BG4 |
| Src                           | Lys<sup>170</sup> | Tyr<sup>200</sup> | Arg<sup>205</sup> | Tyr<sup>200</sup> | Tyr<sup>129</sup> | Met<sup>132</sup> | Leu<sup>192</sup> |
| Csk                           | Glu<sup>127</sup> | Tyr<sup>129</sup> | Ile<sup>140</sup> | Tyr<sup>129</sup> | Ile<sup>169</sup> | Ile<sup>172</sup> | Leu<sup>192</sup> |
| Chk                           | Ile<sup>167</sup> | Tyr<sup>169</sup> | Thr<sup>179</sup> | Thr<sup>179</sup> | Thr<sup>179</sup> | Ile<sup>160</sup> | Leu<sup>193</sup> |

TABLE III

Residues of the Src, Csk, and Chk SH2 domains proposed to interact with various positions on phosphopeptides

of phosphopeptides that had been identified to bind to the Src SH2 domain (Table II). For convenience, these phosphopeptides were not labeled with fluorescein, and their binding to the SH2 domains was determined by the competition assay using Flu-GpYEEI as the fluorescent probe. The wild type Csk SH2 domain displayed weak affinity toward these phosphopeptides, with K<sub>d</sub> values ranging from 10- to 100-fold higher than those of the wild type Src SH2 domain. Mutation of Ile<sup>167</sup> to Lys decreased the K<sub>d</sub> values significantly, to levels much closer to those of wild type Src SH2 domain. We could not determine the binding of these phosphopeptides to the wild type Csk SH2 domain for lack of a suitable fluorescent probe. However, we could determine the binding of these phosphopeptides to the Csk SH2 E127K mutant using Flu-GpYEEI as the probe. These phosphopeptides bound to the Csk SH2 domain E127K mutant, albeit with somewhat higher K<sub>d</sub> values than those for the Chk SH2 domain mutant or the wild type Src SH2 domain. These data indicated that the βD3 position determined the functional divergence of these SH2 domains toward a large number of phosphopeptides.

**Mutation E127K Renders Csk Responsive to GpYEEI Activation**—As reported previously (5, 39) and demonstrated earlier in this study, the SH2 domain containing protein or phosphopeptide activates Csk kinase activity. Apparently, binding of a phosphopeptide causes a conformational change in the Csk SH2 domain, which can be communicated to the catalytic domain (44). Upon demonstrating that E127K mutation confers GpYEEI binding to the Csk SH2 domain, we determined whether this mutation would render Csk responsive to GpYEEI activation. Fig. 6A shows the responses of the wild type Csk to activation by AMpYSSV and GpYEEI. Whereas Csk was activated by the CBP phosphopeptide—about 60% at 200 μM, GpYEEI activated Csk only ~20% at 200 μM. Fig. 6B shows the opposite responses of Csk E127K to activation by these two phosphopeptides. The mutant Csk was activated by the CBP phosphopeptide only ~30% at 200 μM, but it was activated by GpYEEI ~70% at 200 μM. Curve-fitting of the GpYEEI activation data yielded a K<sub>d</sub> of 100 μM and a maximal activation of 95%. This K<sub>d</sub> value was significantly higher than that determined from the SH2 domain binding to the fluoresceinated probe, likely due to a number of factors, such as the influence of the catalytic domain in the full-length protein or phosphopeptide activating Csk kinase activity. Upon demonstrating that E127K mutation confers GpYEEI binding to the Csk SH2 domain (K<sub>d</sub> = 10 versus 15 μM) and was worse than GpYEEI in binding to the Src SH2 domain (K<sub>d</sub> = 2.1 versus 0.7 μM). These results confirmed that GpYEEI was indeed specifically preferred by the Chk SH2 domain. A comparison among the phosphopeptides preferred by the Csk SH2 domain (pYTKM), Src SH2 domain (pYEIE), and Chk SH2 domain (pYYYL) also confirmed the functional divergence between the Csk and Chk SH2 domains and the similarity between the Chk and Src SH2 domains.

**DISCUSSION**

The Csk family of protein tyrosine kinases contains two members: Csk and Chk. As in many other protein tyrosine kinase families, whether they play redundant or distinct roles in cellular regulation is an important and interesting question. Whereas the major function of Chk is to activate Src family kinases (14, 19). Indeed, the Csk and Chk SH2 domains bind to two different sets of pTyr-containing proteins, potentially placing Csk and Chk under the control of different upstream Tyr phosphorylation events. In this scenario, Csk and Chk would link different signaling pathways to the regulation of Src family kinases. Thus, the function of the SH2 domains may distinguish the cellular function of Csk and Chk.

The current study was initiated to compare the properties of the Csk, Chk, and Src SH2 domains and to investigate the structural basis for this functional divergence. We confirmed that the Csk and Chk SH2 domains had striking functional differences, whereas the Csk and Src SH2 domains had a certain level of functional similarity. These functional comparisons did not correlate to the comparisons in the primary structures among the three SH2 domains. Surprisingly, the functional comparisons among the SH2 domains were mainly dictated by the differences at the βD3 position, GpYAAA in Csk, Chk, and Src, respectively. Swapping this residue among the three SH2 domains resulted in switched binding properties, providing compelling evidence for the crucial role of this residue in determining the functional diversity of these SH2 domains.

Whereas the critical determinant role of the βD3 residue in the function of the three SH2 domains is surprising, it is consistent with several recent studies of the SH2 domains. Mutation of Lys<sup>200</sup> to Ala in the Src SH2 domain resulted in a 7-fold reduction in binding affinity for GpYEEI (32). In a recent computational analysis, the βD3 position is identified to be an energetically important residue in the function of a large num-
number of SH2 domains, such as Hck, Lck, Src, Fyn, SHPTP2 N, Grb2, SAP, p85a C, and so forth (45). An alignment of various families of SH2 domains indicates that the β3D position is variable between families but is often conserved within a family. For example, all the Src family SH2 domains contain a Lys at the β3D position, consistent with the finding that all the Src family SH2 domains prefer GPYEEI as the optimal ligand (24). Additional studies will be needed to determine whether the β3D position is a functional determinant in other SH2 domains.

Based on crystal structures and sequence alignments, the SH2 domains are divided into five groups, 1A, 1B, 2, 3, and 4 (24). The Src family and Csk family SH2 domains belong to groups 1A and 1B, respectively. The key residues from all three SH2 domains that are expected to interact with Y+1 through Y+3 positions are shown in Table III. At the six key residues, the Csk SH2 has three residues in common with the Src SH2 domain (at βD5, βE4, and BG4), whereas the Chk SH2 domain shares only one common residue with the Src SH2 domain at βD4 and one similar residue at BG4. Thus, even such classifications based on crucial binding residues would not have predicted the functional comparisons among the three SH2 domains. The finding of one residue controlling the binding preference of the SH2 domain supports the view of the SH2 domain as a common protein module with tunable specificity. This result also demonstrates how easily SH2 domains with new properties could be evolved. Two cases in which a single point mutation could switch the binding specificity have been reported (46, 47), but two homologous SH2 domains possessing dramatically different binding preferences due to the difference in one residue has not been previously observed.

Another surprising finding in the current study is the strong binding of the Src SH2 domain to Flu-pY1248, representing pTyr1248 of ErbB2. One key mechanism for ErbB2 oncogenicity is through its recruiting and activation of c-Src by autophosphorylated ErbB2 binding to the Src SH2 domain (40, 41). However, the pTyr site on ErbB2 that binds to the Src SH2 domain and activates c-Src has not been previously identified. On the other hand, autophosphorylation of ErbB2 on pY1248 is critical for ErbB2-mediated oncogenicity in breast cancer (38), but the mechanism of pTyr1248-mediated oncogenicity remains unknown. Tight binding of Flu-pY1248 to the Src SH2 domain strongly suggests pTyr1248 as the likely site responsible for activating c-Src. The binding of both the Chk and Src SH2 domains to pY1248 of ErbB2 also suggests a model of how Chk could inactivate c-Src that is activated by ErbB2, whereas Csk could not: the Chk SH2 domain can compete against the Src SH2 domain in binding to ErbB2, thus preventing c-Src activation. These implications await additional structural and cellular studies. Another issue raised by the finding is how the Src SH2 domain binds to pYLGL, which is very different from pYEEL. In fact, two other tight-binding phosphopeptides, EFpYEND and ESpYENE (Table II), also do not conform to the pYEEL mode.

The optimal phosphopeptide ligand for the Chk SH2 domain has not been previously reported. We used a novel library screening approach to determine the phosphopeptide preference of the Chk SH2 domain and identified pYYYL as the optimal phosphopeptide. A comparison among the ligands for the Csk (pYTKM), Src (pYEEL), and Chk (pYYYL) SH2 domains confirmed the functional divergence between the Csk and Chk SH2 domains and the functional similarities between the Chk and Src SH2 domains. The library screening approach may be generally useful for characterizing other SH2 domains. Altogether, this library contains just 52 phosphopeptides, and it can be used to determine the sequence preference of any SH2 domain with an available fluorescence probe. One drawback to this approach is the requirement of a fluorescent probe for the given SH2 domain. Similar to the approach by Songyang et al. (24), it looks at each position independently of the other positions, thus ignoring any potential interactions between positions. One major advantage of this approach is the convenience and simplicity. Once a library of 52 phosphopeptides is synthesized, screening against any SH2 domain takes about 1 day to complete.

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