A Novel Non-catalytic Mechanism Employed by the C-terminal Src-homologous Kinase to Inhibit Src-family Kinase Activity*\[S\]

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Although C-terminal Src kinase (CSK)-homologous kinase (CHK) is generally believed to inactivate Src-family tyrosine kinases (SFKs) by phosphorylating their consensus C-terminal regulatory tyrosine (Tyr\(_T\)) exactly how CHK inactivates SFKs is not fully understood. Herein, we report that in addition to phosphorylating Tyr\(_T\), CHK can inhibit SFKs by a novel non-catalytic mechanism. First, CHK directly binds to the SFK members Hck, Lyn, and Src to form stable protein complexes. The complex formation is mediated by a non-catalytic Tyr\(_T\)-independent mechanism because it occurs even in the absence of ATP or when Tyr\(_T\) of Hck is replaced by phenylalanine. Second, the non-catalytic CHK-SFK interaction alone is sufficient to inactivate SFKs by inhibiting the catalytic activity of autophosphorylated SFKs. Third, CHK and Src co-localize to specific plasma membrane microdomains of rat brain cells, suggesting that CHK is in close proximity to Src such that it can effectively inactivate Src in vivo. Fourth, native CHK-Src complex exists in rat brain, and recombinant CHK-Hck complex exists in transfected HEK293T cells, implying that CHK forms stable complexes with SFKs in vivo. Taken together, our findings suggest that CHK inactivates SFKs (i) by phosphorylating their Tyr\(_T\), and (ii) by this novel Tyr\(_T\)-independent mechanism involving direct binding of CHK to SFKs. It has been documented that autophosphorylated SFKs can still be active, in some cases even when their Tyr\(_T\) is phosphorylated. Thus, the ability of the Tyr\(_T\)-independent mechanism to suppress the activity of both non-phosphorylated and autophosphorylated SFKs represents a fail-safe measure employed by CHK to down-regulate SFK signaling under all circumstances.

Src-family kinases (SFKs)\(^1\) are non-receptor protein-tyrosine kinases that participate in many cellular functions ranging from cell growth and proliferation to memory and learning (1). The kinase activity of SFKs is regulated by phosphorylation, as well as by their interaction with other cellular proteins. Among the various regulatory mechanisms, the most important are autophosphorylation of a consensus tyrosine (Tyr\(_A\)) in the kinase domain and phosphorylation of a consensus regulatory tyrosine near the C terminus (Tyr\(_T\)) (2, 3). Autophosphorylation of Tyr\(_A\) leads to activation of SFKs (1, 4, 5). The crystal structure of the autophosphorylated kinase domain of the Src-family kinase Lck reveals that the phosphorylated Tyr\(_A\) (Tyr\(_P\)) stabilizes the active kinase domain configuration by forming ionic interactions with the conserved Arg in the catalytic loop (6). We previously reported that the Src-family member Hck could undergo autophosphorylation at a novel site (Tyr-29)\(^2\) in the Unique domain and that autophosphorylation of Hck at Tyr-29 contributed to Hck activation (4). However, the structural basis of activation by Tyr-29 autophosphorylation is not yet known. In contrast to the activating effect of Tyr\(_A\) and Hck Tyr-29 autophosphorylation, Tyr\(_T\) phosphorylation results in inactivation (7). Crystal structures of Tyr\(_T\)-phosphorylated c-Src (8) and Hck (9) reveal that the inactive configuration of the kinase domain is stabilized by intramolecular interactions involving binding of (i) the Tyr\(_P\) to the SH2 domain and (ii) the SH2 kinase linker to the SH3 domain.

The consensus Tyr\(_T\) of SFKs is thought to be phosphorylated exclusively by two upstream regulatory tyrosine kinases, C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK) (2, 10–12). CSK is ubiquitously expressed in all mammalian tissues, whereas the expression of CHK is much more restricted; it is expressed predominantly in neurons and hematopoietic cells. Extensive biochemical evidence indicates that CSK inactivates SFKs primarily by phosphorylating their Tyr\(_T\). Although there are several pieces of preliminary evidence suggesting that CHK can also phosphorylate Tyr\(_T\) of several SFK members, including c-Src, Lyn, Lck, and Fyn (10, 11),

\(^1\) The abbreviations used are: SFK, Src-family tyrosine kinase; CSK, C-terminal Src kinase; CHK, CHK-homologous kinase; M5S, 4-morpholineethanesulfonic acid; HEK cells, human embryonic kidney cells.

\(^2\) CHK exists in three isoforms distinguishable by their molecular weight, two variants of the 56-kDa isofom (p56\(^{CSK}\)) and one 52-kDa isoform (p52\(^{CSK}\)). This manuscript all characterizations were made with p52\(^{CSK}\). The residues in Hck, Lyn, and Src are numbered in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

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CHK-mediated SFK inactivation remains largely uncharacterized. Using Hck, Lyn, and Src as model targets, we investigated the mechanism by which CHK inactivates SFKs. We observed that in addition to inactivation of SFKs by specifically phosphorylating their Tyrγ, CHK could inactivate SFKs by a novel inhibitory mechanism. In this mechanism CHK directly binds to SFKs to form stable complexes, and this binding alone is sufficient to inactivate SFKs. To further confirm the validity of our in vitro observations, we attempted to determine if CHK could bind and inactivate Hck in transfected HEK293T cells. Results of our experiments using the transfected HEK293T cells reveal that CHK-Hck complex formation and inactivation of Hck occurred even when Tyrγ of Hck was replaced by phenylalanine. The results indicate that CHK-Hck complex formation and Hck inactivation by this novel inhibitory mechanism are not mediated by binding of Tyrγ of Hck to the active site of CHK. Hence, this novel inhibition occurs by a non-catalytic mechanism. To support the physiological relevance of our findings, we reveal that CHK and Src co-localize to specific microdomains of rat brain plasma membrane and that CHK and Src form stable protein complexes in rat brain. Furthermore, we also demonstrate that the interaction can suppress SFK activity regardless of the level of autophosphorylation of TyrA. We also demonstrate that the interaction can suppress SFK activity by a non-catalytic mechanism. Hence, this novel inhibition occurs by a non-catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant wild type Hck was expressed and purified as described previously (5, 13). The “kinase dead” Hck(I267M) mutant was generated by site-directed mutagenesis and purified by procedures as described previously (4). The anti-CHK antibody was raised against a glutathione S-transferase fusion protein containing the C-terminal 89-aminoc acid segment corresponding to residues 379–467 of CHK. The polyclonal anti-CSK, anti-Hck, and anti-TyrP(29)Hck antibodies were generated and purified as described previously (3, 4). The rabbit polyclonal anti-TyrP(29) phosphoprotein antibody (also called anti-SrcPεP-418) was from BIOSOURCE International. pRK7-BatK-flagC plasmid encoding the 52-kDa isoform of rat CHK (p52CHK) was kindly provided by Drs. I. Caras and S. Ku (12, 14). Poly(Glu,Tyr), a random copolymer of glutamate and tyrosine (4:1) that is a nonspecific peptide substrate for tyrosine kinases, was purchased from Sigma. The two anti-Src monoclonal antibodies mAbe27 and mAbe9 (2–17) were purified and characterized as previously described (15).

**Chemical Cross-linking of Antibodies to Protein A-Sepharose**—Data generated by the use of these reagents are shown in Fig. 1A. The crude membrane was subjected to sucrose gradient centrifugation (25 mM Hepes, pH 7.0, 10 mM MgCl₂, 0.2 mg/ml benzamidine, 0.1 mg/ml phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 100,000 × g for 30 min to separate the plasma membrane and cytoplasm. The crude membrane was further separated into different membrane microdomains by sucrose density gradient chromatography. As shown in the figure, the final CHK preparation was >90% pure. The recombinant CHK(K221M) mutant was also purified using an identical procedure.

**Preparation of Crude Rat Brain Lysate for Detection of CHK-Src Protein Complexes**—All procedures described were performed at 4 °C. Brain tissues from 10 adult rats were homogenized in 140 ml of lysis buffer. The homogenate was centrifuged at 40,000 × g for 40 min. The supernatant was separated into aliquots and frozen at −70 °C before immunoprecipitation analysis to generate the data shown in Fig. 10C.
An SFK C-terminal peptide, modeled after the C terminus of the Src-family kinase Lyn (KEKAERPTFDYLQSVLDDFYTATEGQYQ-QQP, Y₆ is Tyr₆), was synthesized (3). Because the sequence of this peptide exhibits >90% sequence identity with the sequence of Hck C-terminal regulatory domain, we compared the kinetics of CHK phosphorylation on the peptide and Hck(K267M) under identical conditions. The K₅₀ and V₉₅ₐₓ values were determined by double-reciprocal transformation of the Michaelis-Menten curve (Fig. 2B).

Demonstration of Stable Enzyme-Substrate Complex Formed by CHK and Hck(K267M)—This section describes Fig. 2D. Two identical sets of reaction mixtures containing CHK (0.33 μM) and varying amounts of Hck(K267M) (0–5.8 μM) were prepared. The reaction mixtures were incubated at 30 °C for 30 min in the presence of either (i) 100 μM ATP and kinase assay buffer or (ii) Buffer A. After 30 min the reaction mixtures were diluted 4-fold with an EDTA stock solution that gave a final concentration of 5 mM EDTA; this amount of EDTA was sufficient to chelate Mg²⁺ and Mn²⁺ supplied to the reaction mixtures. Immunoprecipitation was performed by adding 4.3 μg of the anti-CHK antibody to each preincubated mixture. CHK was allowed to react with the anti-CHK antibody at 4 °C for an hour before being transferred onto Protein A-Sepharose beads (10-μl bed volume). The antibody-containing mixtures were further incubated at 4 °C for another hour. The immunoprecipitates were then washed extensively with 6 × 1.0 ml of 0.5 M NaCl in Buffer A, and the bound proteins were eluted with SDS-PAGE sample buffer. The proteins were resolved by SDS-PAGE and then blotted onto PVDF membranes. The resultant blots were probed with the anti-Hck antibody followed by horseradish peroxidase-conjugated protein A (protein A-HRP conjugate). Because the IgG heavy chain and CHK (molecular mass, ~50 kDa each) show similar mobility on SDS-PAGE, the use of Protein A-HRP conjugate helped to maximize the detection of CHK immunosignals due to its insensitivity to cross-react with the denatured IgG.

Demonstration of Stable CHK-SFK Complex Formation by Co-immunoprecipitation—This procedure describes Figs. 3 and 5–10. For immunoprecipitation with the anti-CHK antibody, CHK (0–10 μM) was incubated at 30 °C for 30 min with Hck, Lyn, or Src (88 nM to 0.48 μM). As a control, SFKs were omitted from the incubation mixture. The binding assay mixture was applied to a tube containing anti-CHK antibody (1.7 μg) immobilized to Protein A-Sepharose (bed volume, 10–15 μl). The mixture was further incubated for 2 h at 4 °C, and the immunoprecipitates were washed and processed for subsequent Western blot analysis. For anti-Hck immunoprecipitation, the binding assay mixture was transferred to protein G-Sepharose (10 μl bed volume) precoated with a rat monoclonal anti-Hck antibody. As for the in vivo experiment, given that CHK, CSK, and Src are less abundant in the crude brain lysate, immunoprecipitation was performed with antibodies covalently cross-linked to Protein A-Sepharose in an effort to minimize interference from the IgG heavy chain (Fig. 10C).

Assay for the Ability of CHK to Inactivate SFKs (Inactivation Assay)—This section describes Figs. 1E, 4A, 6B, 8A, and 9A. The two-step assay method described previously (3) was used to monitor the ability of CHK to inactivate Hck, Lyn, and Src. In Step 1, each SFK (0.11–0.16 μM Hck, 0.23 μM Lyn, and 0.48 μM Src) was preincubated at 30 °C for 30 min in a reaction volume of 25 μl containing varying amounts of CHK, kinase assay buffer, and 100 μM [γ-³²P]ATP. In this step, SFKs were capable of undergoing autophosphorylation at Tyr₆, whereas Tyr₅ was capable of phosphorylation by CHK. As a control, SFKs were preincubated in the absence of under activated CHK. In Step 2, an activity assay was conducted to specifically monitor the kinase activity of the preincubated SFK. Because cdc2(6–20)K19 peptide was previously reported as a specific and efficient substrate for many SFKs (19, 20) but was a poor substrate for CSK (3) and CHK (data not shown), it was used as the substrate for this assay. The preincubation mixtures (10 μl per sample) were separated into aliquots, and phosphorylation was initiated upon the addition of an assay mix containing 5 μl each 100 μM [γ-³²P]ATP, kinase assay buffer, and 1.5 μM cdc2(6–20)K19 peptide. The reaction was allowed to proceed for 20–30 min. SFK kinase activity was determined from the radioactivity associated with the peptide substrate as previously described (4). The ratio of SFK kinase activity in the preincubation mixtures containing CHK (inactivation reaction) versus that without CHK (control reaction) was determined. From the ratio, the SFK activity in the preincubation mixtures containingCHK and CSK (29), and CHK (23, 24) provide an overview of their mechanisms of action.

Determination of the Stoichiometry of SFK Phosphorylation—This procedure was used to generate Figs. 4B, 8B, and 9B. This method examines the total phosphorylation at all phosphorylation sites (i.e. autophosphorylation sites and Tyr₅). SFK (0.16 μM Hck, 0.23 μM Lyn, or 0.48 μM Src) was phosphorylated in a mixture containing kinase assay buffer, 100 μM [γ-³²P]ATP, and CHK (0–10 μM) in 25 μl. The mixture
was incubated at 30 °C for 30 min. After termination of the reaction, the phosphoproteins were separated by SDS-PAGE. The bands corresponding to the phosphorylated SFKs were excised, and the associated radioactivity was determined.

Phosphopeptide Mapping—This procedure was used to generate Figs. 2A, 4D, 8D, and 9D. Hck, Lyn, and Src were incubated with [γ-32P]ATP at varying CHK concentrations under conditions detailed in the previous section. The phosphoproteins were separated by SDS-PAGE and transferred to a nitrocellulose filter, and the bands corresponding to the radioactively phosphorylated SFKs were excised and processed for tryptic digestion. The tryptic fragments were separated and identified by the two-dimensional thin layer electrophoresis/TLC procedures described previously (13). The spots corresponding to Tyr(P)T, Tyr(P)A (Src and Hck), and Tyr(P)-29 (Hck only) on the phosphopeptide maps were identified as described in our previous reports (4, 13). Similar conditions and procedures were used for phosphopeptide mapping of Hck(K267M) phosphorylated by CHK.

For Lyn, autophosphorylation occurs at both TyrA (Tyr-397) (5) and a novel site YN. Phosphorylated Lyn was analyzed by tryptic (Supplemental Fig. 1) and tryptic/chymotryptic phosphopeptide mappings (Fig. 8D). It is noteworthy that the tryptic digestion of phosphorylated Lyn resulted in seven partially digested Tyr(P)T-containing fragments, whereas the sequential tryptic/chymotryptic digestion successfully reduced these fragments to only one major Tyr(P)T-containing fragment (Supplemental Fig. 1).

Transient Expression of CHK, CHK(K221M), Hck, and Hck(Y499F) Mutant in HEK293T Cells—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For the expression of Hck, Hck(Y499F), CHK, and CHK(K291M), cDNAs encoding these proteins were subcloned into the mammalian expression vectors pEF-BOS or pcDNA3 (4). The plasmids generated were pEF-BOS-Hck, pEF-BOS-Hck(Y499F), pcDNA3-CHK, and pcDNA3-CHK(K221M). The plasmids were used to transiently transfect HER293T...
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RESULTS

Purified Recombinant CHK Displays Both Protein-tyrosine Kinase Activity and the Activity to Inhibit the Src-family Kinase Hck in Vitro—Recombinant CHK was expressed in Sf9 cells and purified by sequential chromatography on ion-exchange, gel-filtration, and phosphotyrosine-agarose affinity columns. Throughout the course of purification, recombinant CHK co-eluted with protein-tyrosine kinase activity as well as the “Hck inhibition” activity (measured by the inactivation assay). Fig. 1, A and B, illustrate co-elution of CHK with these activities in the gel-filtration chromatography. The co-elution indicates that the recombinant CHK was catalytically active and confirms that Hck was inactivated by CHK and not by any low molecular mass contaminants in the CHK preparation. Furthermore, no protein-tyrosine phosphatase activity and negligible ATPase activity (less than 1% of the total ATP present in the Hck inactivation assay was hydrolyzed in 30 min) were detected in the CHK-containing column fractions, indicating that the inhibition of Hck activity was not a result of dephosphorylation of Hck by contaminating phosphatases nor was it due to depletion of ATP. The purity and authenticity of the purified CHK are depicted in Fig. 1C.

CHK Can Specifically Phosphorylate TyrT of Hck by Following a Non-Michaelis-Menten Kinetics Mechanism—To confirm that CHK could specifically phosphorylate the consensus TyrT near the C termini of SFKs, we generated the kinase-dead Hck(K267M) mutant as an in vitro substrate of CHK. Because Hck(K267M) lacks kinase activity, its use as the CHK substrate eliminates the complication of additional tyrosine phosphorylation due to Hck autophosphorylation. As shown in Fig. 2A, despite the existence of multiple tyrosine residues in Hck(K267M), CHK phosphorylates this Hck mutant exclusively at TyrT (Tyr-499), suggesting that CHK can inactivate Hck and possibly other SFKs by phosphorylating the consensus TyrT. To characterize the kinetics of Hck phosphorylation by CHK, we compared the initial velocities of CHK phosphorylation of Hck(K267M) and those of CHK phosphorylation of the SFK C-terminal peptide (Fig. 2, B and C). As shown in Fig. 2B, phosphorylation of the SFK C-terminal peptide conforms to Michaelis-Menten kinetics. A double reciprocal transformation of the data (1/V versus 1/[S]) yields the $V_{\text{max}}$ and $K_m$ values of 16.5 ± 1.4 pmol/min/µg of CHK and 1.1 ± 0.1 mM, respectively (inset). In contrast to the phosphorylation of the SFK C-terminal peptide, increasing the Hck(K267M) concentration from 2.9 to 5.8 µM resulted in an ~2.5-fold increase in initial velocity (inset, Fig. 2C). A double-reciprocal transformation of the data yields a non-linear plot (data not shown), indicating that CHK phosphorylation of Hck$_{TyrT}$ does not follow the Michaelis-Menten kinetic model.

CHK and Hck Form a Tight Complex—The non-Michaelis-Menten kinetics of TyrT phosphorylation by CHK indicates that, in addition to the conventional substrate-enzyme interactions (i.e. binding of TyrT of Hck(K267M) to the CHK active site), TyrT phosphorylation by CHK is also governed by non-catalytic interactions between the two proteins. To examine the validity of this hypothesis, we used co-immunoprecipitation to assess whether the two proteins could bind together to form a stable complex. Fig. 2D shows that CHK bound Hck(K267M) in the presence and absence of ATP and that the complex was stable to extensive washing with a buffer containing 0.5 M NaCl. As the concentration of Hck(K267M) increased in the incubation mixture, the amount of Hck bound to CHK increased. The ability of CHK to bind Hck(K267M) even in the absence of Mg$^{2+}$-ATP further suggests that the CHK:Hck(K267M) complex formation was mediated by non-catalytic interactions between the two proteins, i.e., it was not mediated by the binding of TyrT of Hck(K267M) to the active site of CHK. Conceivably, the interactions increased the effective concentrations of CHK and...
CHK Can Inactivate Hck in Vitro, and the Inactivation Correlates with the Suppression of Autophosphorylation at TyrA and Tyr-29 but Not with Extensive Phosphorylation at TyrT—Autophosphorylation of Hck (Tyr-388) and Tyr-29 is the major mechanism of stimulating Hck activity (4). Therefore, we examined how CHK affected the degree of Hck phosphorylation at these sites. As shown in Fig. 4A, the presence of increasing concentrations of CHK correlates with the extent of Hck inactivation. At the CHK concentration of 7.2 μM, the catalytic activity of Hck was suppressed by more than 95%. Accompanying the Hck inactivation, the increase in CHK concentration resulted in a significant drop in the total phosphorylation level of Hck (Fig. 4B). Because the total phosphorylation level of Hck is contributed by TyrA and Tyr-29 autophosphorylation as well as TyrT phosphorylation by CHK, we used phospho-specific antibodies to examine the extents of autophosphorylation at the two sites at varying CHK concentrations. We also employed phosphopeptide mapping to monitor phosphorylation of Hck at all three sites (i.e. TyrA, Tyr-29, and TyrT) in the presence of 0 and 7.2 μM CHK.

Western blotting of anti-Tyr(P)A and anti-Tyr(P)-29 antibodies shows that the inactivation of Hck correlated with the decrease in the levels of phosphorylation of TyrA and Tyr-29 (Fig. 4C). At the CHK concentration of 7.2 μM, at which the Hck activity was less than 5% of the control Hck activity (i.e. >95% inactivation), autophosphorylation at TyrA and Tyr-29 was suppressed to less than 5% that of the level of Hck autophosphorylation observed in the absence of CHK. To assess the effect of the CHK-mediated TyrT phosphorylation on Hck autophosphorylation levels, we compared the degree of [γ-32P]ATP incorporation at the three phosphorylation sites by two-dimensional phosphopeptide mapping. In the absence of CHK, Hck readily underwent autoactivation by autophosphorylation at TyrA and Tyr-29, reaching a stoichiometry of 0.42 mol of phosphate/mol of Hck in 30 min (Fig. 4B). The presence of increasing concentrations of CHK led to a significant decrease in both the catalytic activity and the total phosphorylation level of Hck (Fig. 4, A and B). Stoichiometry measurement (Fig. 4B) and phosphopeptide mapping (Fig. 4D) of the phosphorylated Hck in the presence of 7.2 μM CHK revealed that the level of phosphorylation of Hck at all sites in total was only 0.03 mol of phosphate per mol of Hck, indicating that the stoichiometry of TyrT phosphorylation must be very low (<0.03 mol of phosphate/mol of Hck). Despite such a low level of TyrT phosphorylation, CHK at this concentration induced 96% inactivation of Hck (Fig. 4A). This clearly demonstrates that TyrT phosphorylation by CHK contributes very little to the Hck inactivation we observed.

CHK Forms a Stable Complex with Hck and Inactivates Hck in HEK293T Cells Even When TyrT of Hck Is Replaced by Phe—The in vitro data presented so far unequivocally demonstrate that Hck was inactivated by CHK by a novel non-catalytic mechanism involving the formation of a stable CHK/Hck complex that occurred without significant TyrT phosphorylation. To examine if Hck and CHK could form a stable complex in mammalian cells, the two proteins were co-expressed in HEK293T cells by transient transfection. Hck was immunoprecipitated from the whole cell lysates of the transfected cells, and the presence of CHK in the anti-Hck immunoprecipitate

![Fig. 4](http://www.jbc.org/...)

**Fig. 4.** CHK inactivation of Hck is associated with suppression of Hck autophosphorylation at TyrA and Tyr-29 and a very low level of TyrT phosphorylation. Hck (0.16 μM) was incubated with varying concentrations of CHK in the presence of assay buffer and [γ-32P]ATP. A, the residual kinase activity of Hck in the presence of CHK. Hck kinase activity was determined by the inactivation assay procedure. B, stoichiometry of Hck phosphorylation at all three sites (i.e. Tyr-29, TyrA, and TyrT) with varying CHK concentrations. C, the effect of CHK on the degree of Hck autophosphorylation at TyrA (pY_A) and Tyr-29. The relative amount of phosphate incorporated at TyrA and Tyr-29 was visualized by Western blotting using the phosphospecific antibodies. Anti-Hck immunoblotting reveals that the same amount of Hck was present in each sample analyzed. D, phosphopeptide maps of Hck phosphorylation in the absence (0 μM) and presence (7.2 μM) of CHK. The key denotes the migration patterns of tryptic phosphopeptides derived from Tyr(P)-29, Tyr(P)-A, and Tyr(P)-T, as determined previously (4). In the presence of 7.2 μM CHK, Hck was phosphorylated at all three sites. A faint spot corresponding to the Tyr(P)-A tryptic phosphopeptide is noticeable in the original autoradiogram.
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was monitored by Western blotting. As shown in Fig. 5A, CHK specifically co-immunoprecipitated with Hck, demonstrating that CHK and Hck formed a stable protein complex in mammalian cells.

Because CHK could specifically phosphorylate TyrT of Hck, it is logical to postulate that binding of the TyrT of Hck to the active site of CHK contributes to the CHK-Hck complex formation. To examine the validity of this hypothesis, we co-expressed CHK and the constitutively active Hck(Y499F) mutant (of which TyrT Tyr-499 was replaced by phenylalanine) in HEK293T cells and determined if CHK and the Hck mutant could form a stable complex. As shown in Fig. 5A, despite the replacement of TyrT by phenylalanine, the Hck mutant still retained the ability to form a stable complex with CHK in HEK293T cells, indicating that the CHK-Hck complex formation did not require a tyrosine at this site. The result further confirms that the CHK-Hck complex formation was mediated by TyrT-independent non-catalytic interactions between the two proteins.

Overexpression of CHK Suppresses Autophosphorylation and Autoactivation of Wild Type Hck and Hck(Y499F) in HEK293T Cells—As shown in Fig. 5B, overexpression of CHK induced a small decrease (17%) in Hck activity. We previously demonstrated that most of the recombinant Hck expressed in HEK293T cells was already in the inactive configuration as it was phosphorylated at TyrT by the endogenous CSK (4). Consequently, the degree of reduction in total Hck activity caused by CHK overexpression is small because the available pool of active Hck was low. Nevertheless, Fig. 5B shows that CHK co-expression suppressed Hck autophosphorylation at TyrA and Tyr-29, indicating that CHK could suppress Hck activation and prevent it from undergoing autophosphorylation. Because Hck(Y499F) is not subject to inhibition by TyrT phosphorylation by the endogenous CSK, it was significantly more active than the wild type Hck (Fig. 5, B–D). Co-expression of CHK, however, resulted in a dramatic reduction in Hck(Y499F) autophosphorylation at TyrA and Tyr-29 and a decrease (>87%) in the catalytic activity of the Hck mutant. These results confirm the hypothesis that CHK can inactivate Hck by a mechanism other than TyrT phosphorylation. Because Hck(Y499F) cannot be phosphorylated by CHK but could form a stable complex with CHK in HEK293T cells (Fig. 5A), we postulate that the inactivation of Hck(Y499F) is solely a consequence of the binding of CHK to the Hck mutant.

The K221M Mutation of CHK Abolishes Its Ability to Bind and Inactivate Hck—Because the binding and inactivation of Hck by CHK is independent of TyrT phosphorylation, it is logical to expect that the same effect can be observed when a CHK mutant lacking tyrosine kinase activity is used in the experiment. To examine the validity of this notion, the kinase-dead CHK(K221M) mutant was generated. Lys-221 in CHK is homologous to the conserved lysine residue critical for ATP binding as well as maintenance of the active configuration of many protein kinases. As expected, the replacement of Lys-221 by methionine rendered the recombinant CHK mutant inactive (data not shown).

In contrast to wild type CHK, CHK(K221M) is unable to form a stable complex with the activated Hck(Y499F) mutant in HEK293T cells (Fig. 5A). Furthermore, overexpression of CHK(K221M) has no significant negative impact on the protein kinase activity and the TyrA and Tyr-29 phosphorylation levels of Hck(Y499F) co-expressed in HEK293T cells (Fig. 5D). The results strongly suggest that the K221M mutation completely abolishes CHK ability to bind and inactivate SFKs. However, it is possible that the mutation causes gross unfolding of the CHK mutant, and as such the recombinant mutant fails to inactivate Hck(Y499F) in HEK293T cells. To address this possibility, we expressed CHK(K221M) in Sf9 cells and purified the recombinant CHK mutant for biochemical and biophysical studies. To ascertain if CHK(K221M) folds correctly, we examined the secondary structure of CHK and CHK(K221M) by circular dichroism. The circular dichroism spectra of CHK and CHK(K221M) indicate similar secondary structure content (data not shown). Furthermore, the CHK mutant remained soluble throughout the course of its purification, and it displayed chromatographic properties very similar to those of wild type CHK. All these results indicate that the K221M mutation does not cause gross unfolding of CHK. Similar to what was observed in HEK293T cells co-expressing Hck(Y499F) and the CHK mutant, the K221M mutation abolishes the ability of CHK to both bind and inactivate Hck in vitro (Fig. 5). Taken together, the results shown in Figs. 5 and 6 suggest that the K221M mutation structurally perturbs or reduces the accessibility of the determinants mediating CHK interaction with and inactivation of Hck.

CHK Can Bind and Inactivate Autophosphorylated Hck—Although Fig. 4 shows that CHK can inactivate Hck by suppressing its autophosphorylation, it remains unclear whether CHK can inactivate the fully active autophosphorylated form of Hck. This issue is relevant to many physiological and pathological situations in which stimulation of growth factor receptors leads to autophosphorylation and activation of the Src family kinases. The fully active autophosphorylated form of the Src kinases mediates the cell growth signals originating from the stimulated receptors. Fig. 7 shows the result of our investigation into this question. In this experiment, Hck was allowed to undergo autophosphorylation by incubation with [γ-32P]ATP and assay buffer for 1 h. The autophosphorylation was stopped by diluting the reaction mixture with the stopping buffer containing EDTA to chelate the Mg2+ and Mn2+ essential for the autophosphorylation. Aliquots of the diluted autophosphorylated Hck preparation were then assayed for peptide kinase activity in the absence and presence of increasing concentrations of CHK (Fig. 7A). In addition, the assay mixture was used for the co-immunoprecipitation experiment to ascertain if CHK could bind the autophosphorylated Hck. As shown in Fig. 7B, autophosphorylated Hck co-immunoprecipitated with CHK, indicating that CHK can form a tight complex with the autophosphorylated Hck. More importantly, the binding effectively inactivates the autophosphorylated Hck, with near complete inactivation of the autophosphorylated Hck occurring when incubated with 10 μM CHK (Fig. 7A). The results indicate that CHK can bind and inactivate autophosphorylated Hck.

CHK Inactivation of Lyn and Src Is Associated with TyrT Phosphorylation and the Formation of Stable CHK-Lyn and CHK-Src Complexes—To ascertain if the mechanism employed by CHK to inactivate Hck is also applicable to CHK inactivation of other SFKs, we studied in vitro whether CHK can specifically phosphorylate TyrT of Lyn and Src and form stable complexes with them. As shown in Fig. 8A, CHK effectively inactivates Lyn protein kinase activity. At CHK concentrations (≥0.37 μM) that are higher than that of Lyn (0.23 μM), CHK is capable of suppressing more than 90% of Lyn kinase activity. In contrast to the effect of CHK on Hck (Fig. 4B), CHK causes a significant increase in the stoichiometry of Lyn phosphorylation (Fig. 8B). Because CHK induces a reduction in Lyn TyrT phosphorylation level (Fig. 8C), the increase in stoichiometry of Lyn phosphorylation in the presence of CHK is very likely attributed to a significant increase in phosphorylation at TyrT. Phosphopeptide mapping of the phosphorylated Lyn digested sequentially by trypsin and chymotrypsin (Fig. 8D) was performed to fur-
FIG. 5. Formation of CHK-Hck complex suppresses autophosphorylation and kinase activity of Hck and Hck(Y499F) in HEK293T cells. Wild type Hck, Hck(Y499F), CHK(K221M), and CHK were overexpressed alone or together in HEK293T cells. As a control, HEK293T cells were transfected with the plasmid that directs the overexpression of green fluorescent protein (GFP). A, demonstration of the formation of stable CHK:Hck and CHK-Hck(Y499F) complexes in HEK293T cells by co-immunoprecipitation (IP). Recombinant Hck and its mutant were immunoprecipitated from the whole cell lysates using the monoclonal anti-Hck antibody. The presence of CHK and the amount of Hck in the immunoprecipitates were monitored by Western blotting (WB). B–D, the effect of overexpression of CHK and CHK(K221M) on the kinase activity as well as the autophosphorylation levels of TyrA and Tyr-29 in Hck and Hck(Y499F). Lysates of the transfected cells were analyzed for Hck and Hck(Y499F) kinase activity using Src-optimal peptide. The specific activities of Hck and its mutant were expressed as pmol of phosphate incorporated/min/densitometry unit of anti-Hck immunoreactivity. The autophosphorylation status of Hck and its mutant was monitored by anti-Tyr(P)A (Anti-pY_A) and anti-Tyr(P)-29 immunoblotting. Anti-Hck immunoblotting was also performed to confirm that equal amounts of Hck and its mutant were present. The multiple immunoreactive bands are consistent with the different degrees of mobility, attributed to various extents of Hck phosphorylation at Tyr-29, TyrA, and TyrT (4). Densitometric analysis of the immunoreactive bands reveals that co-expression of CHK with wild type Hck led to a 26 and 61% reduction in its Tyr(P)A and Tyr(P)-29 levels, respectively, whereas co-expression of Hck(Y499F) with CHK resulted in 67 and 55% reduction in its Tyr(P)A and Tyr(P)-29 levels, respectively. Wt, wild type.
ther examine the effect of CHK on Lyn autophosphorylation and phosphorylation at TyrT. Phosphopeptide maps of the phosphorylated Lyn show that CHK induces a decrease in Lyn autophosphorylation at TyrA, whereas it induces a significant increase in TyrT (Tyr-508) phosphorylation (Fig. 8D).

We recently discovered that Lyn can undergo autophosphorylation in vitro at TyrA Tyr-397 in the kinase domain as well as at a novel site YN (Fig. 8). In addition to suppressing autophosphorylation at TyrA, CHK also induces a reduction in Lyn autophosphorylation at YN (Fig. 8).

Our data, therefore, indicate that both phosphorylation at TyrT and the suppression of autophosphorylation are mechanisms employed by CHK to inactivate Lyn. It is noteworthy that at 0.37 μM, CHK suppresses more than 90% of Lyn kinase activity (Fig. 8A), and densitometric analysis of the corresponding phosphopeptide map (Fig. 8D) reveals that at this CHK concentration only 19% of total Lyn phosphorylation (0.68 mol of phosphates incorporated/mol of Lyn (Fig. 8B)) is contributed by TyrT phosphorylation. From these values, the stoichiometry of Lyn phosphorylation at TyrT is estimated to be 0.13 mol of phosphate incorporated/mol of Lyn. It is obvious from this low level of TyrT phosphorylation by 0.37 μM CHK that TyrT phosphorylation alone cannot fully account for the CHK ability to almost completely suppress Lyn activity. Furthermore, the requirement of CHK at concentrations higher than that of Lyn for effective suppression of Lyn activity implies that the inactivation process involves binding of CHK to Lyn to form the CHK-Lyn complex.

Similar to Hck and Lyn, Src is efficiently inactivated by CHK (Fig. 9A). Intriguingly, incubation of Src with increasing concentrations of CHK leads to a significant increase in the level of autophosphorylation (Fig. 9, B and C). As shown in the phos-
phopeptide maps (Fig. 9D), Src undergoes autophosphorylation mainly at Tyr A. Reminiscent of a previous observation by MacAuley et al. (22), the phosphopeptide map also reveals Src autophosphorylation at Tyr T, albeit only to a very low level. However, the presence of CHK only causes a small increase in TyrT phosphorylation (Fig. 9D). Thus, despite the increased level of TyrA autophosphorylation, CHK effectively inactivates Src without significant Tyr T phosphorylation. The result strongly suggests that CHK also employs the non-catalytic mechanism to inactivate Src. Indeed, results of the co-immu-
FIG. 9. CHK inactivates Src by phosphorylating its TyrA and forming a stable CHK-Src complex. Src (0.48 μM) was incubated at 30 °C for 30 min with varying concentrations of CHK (0–10 μM) in a final volume of 25 μl. A, the residual Src kinase activity was monitored by the inactivation assay procedure. B, the stoichiometry of Src phosphorylation at all sites (TyrA and TyrT) was determined. C, the relative level of Src autophosphorylation at TyrA was monitored by anti-Tyr(P)A (Anti-pYA) Western blotting. D, tryptic phosphopeptide maps of Src phosphorylated in the absence and presence of varying CHK concentrations. The key shows the migration patterns of Tyr(P)A- and Tyr(P)T-derived tryptic fragments, which have been previously identified (13). The origin of each map is marked by an asterisk. TLE, thin layer electrophoresis; TLC, thin layer chromatography. E, demonstration of stable complex formation between CHK and Src. The reaction mixture containing Src phosphorylated in the absence and presence of CHK was subjected to anti-CHK immunoprecipitation. The presence of Src in the immune complex together with its degree of autophosphorylation were monitored by Western blotting (WB). Likewise, the amounts of CHK in the immunoprecipitates (IP) were also monitored.
noprecipitation experiment (Fig. 9E) reveal that CHK forms a stable complex with Src. Western blot analysis of the CHK/Src complex with the anti-Tyr(P)_{A} antibody reveals that CHK is capable of binding to Src even when Src is autophosphorylated. Thus, similar to CHK inactivation of Hck and Lyn, CHK is capable of inactivating Src both by phosphorylating its Tyr_{T} and by the non-catalytic mechanism that involves binding of CHK to Src to form the stable CHK/Src complex.

**CHK and Src Co-localize to Specific Microdomains of the Plasma Membrane, and They Form Tight CHK/Src Complexes in Rat Brain Cells**—An important issue associated with inactivation of SFKs by CHK in vivo relates to their subcellular localizations; SFKs, containing fatty acid moieties at the N terminus, are bound to the plasma membrane, whereas CHK, lacking obvious structural features for its targeting to the plasma membrane, is expected to reside in the cytosol. Furthermore, micromolar concentrations of CHK, Hck, Lyn, and Src are needed for CHK to bind and inactivate the SFKs (Figs. 4, 6, 7, 8, and 9) in vitro. This suggests that efficient inactivation of SFKs by CHK entails their co-localization such that their effective concentrations are significantly increased. Thus, it is important to ask the question, do CHK and SFKs co-localize to specific microdomains of the plasma membrane? Because CHK and Src are co-expressed in brain cells (14), we therefore studied their subcellular localization and whether the CHK/Src protein complexes exist in rat brain. As shown in Fig. 10A, both Src and CHK are present in the plasma membrane fraction of rat brain cells. Further purification by sucrose density gradient ultracentrifugation separates the membrane preparation into the low density and high density membrane microdomains, respectively. All gradient-fractions were again centrifuged. The pellets of the individual fractions were treated with Triton X-100 before an additional round of centrifugation, which gave rise to the Triton X-100-soluble and insoluble portions. The distribution patterns of Src and CHK in the specific membrane microdomains were analyzed by immunoblotting. C, demonstration of the existence of a stable CHK/Src complex by co-immunoprecipitation. 1% of the total crude brain lysate was applied to protein A-Sepharose beads cross-linked to the anti-CHK and anti-CSK antibodies. The beads were also cross-linked to IgG from preimmune serum and were used as a control for the immunoprecipitation analysis. The mixtures were rocked gently at 4 °C for 2 h, and the immunoprecipitates (IP) were processed for Western blotting. MAb, monoclonal antibody.
mains (Fig. 10B). Conceivably, co-localization of CHK and Src in these membrane microdomains significantly increases their effective concentrations and in turn facilitates CHK inactivation of Src. Our finding is in agreement with the observation made by Chow et al. that CHK localizes to the detergent-resistant membrane microdomain (23).

The results presented in Fig. 5 show that CHK can bind and inactivate Hck when they are co-expressed at high levels in HEK293T cells. However, it is still possible that the CHK-Hck complex formation is an artifact resulting from their overexpression. To address this possibility, we need to demonstrate that native stable CHK-SFK complexes exist in cells or tissues where these kinases are co-expressed. As shown in Fig. 10C, Src is present in the anti-CHK immunoprecipitate but not in the preimmune serum immunoprecipitate, suggesting that endogenous CHK and Src form stable protein complexes in brain cells. Because CSK is also expressed in rat brain cells and recently Lee et al. (24) demonstrated that CSK could bind Src in vitro, we therefore examined if CSK could bind Src or to the CHK-Src complexes in vivo. As shown in Fig. 10C, Src is not present in the anti-CSK immunoprecipitate. Furthermore, we failed to detect CSK in the anti-CHK immunoprecipitate of brain lysate (data not shown).

**DISCUSSION**

It is well documented that CHK can down-regulate the activity of SFKs in vitro (25–29). For example, CHK inactivation of Src and Lyn was associated with suppression of breast carcinoma cell growth (29). Because of the significant sequence similarity displayed by CHK and CSK, CHK was postulated to follow the same mechanism as CSK in inactivating SFKs, i.e. CHK inactivates SFKs exclusively by phosphorylating their consensus C-terminal regulatory tyrosine Tyr

In this manuscript we report that CHK can specifically phosphorylate Tyr

three SFK members, Hck, Lyn, and Src, and that CHK can suppress their catalytic activity. Intriguingly, we demonstrated both in vitro and in transfected HEK293T cells that CHK can suppress SFK activity by a novel mechanism that does not require Tyr

phosphorylation. This novel mechanism involves direct binding of CHK to SFKs and suppression of their kinase activity toward exogenous peptide substrates. Further analysis reveals that binding and suppression of SFK activity are independent of Tyr

as CHK could still bind and effectively inactivate the HckY499F mutant, where the Tyr

was substituted with phenylalanine. Moreover, we found that CHK and Src co-localize to specific microdomains in rat brain plasma membrane and that native CHK-Src protein complexes exist in rat brain cells. These findings, therefore, support the physiological relevance of our in vitro observations as co-localization of CHK and Src would increase their effective concentrations and in turn facilitate CHK phosphorylation of Src and binding to Src by the Tyr

-independent mechanism.

What is the physiological significance of CHK ability to inactivate SFKs by this novel non-catalytic mechanism? SFKs are known to be constitutively active and autophosphorylated in cancer cells (for review, see Ref. 30). Furthermore, autophosphorylation permits SFKs to remain active even when their Tyr

is phosphorylated (31). Recently, Lerner and Smithgall (32) reported that human immunodeficiency virus Nef binding or mutations of the SH2 kinase linker caused Hck activation by autophosphorylation even when Tyr

remains phosphorylated and bound to the SH2 domain. All these data indicate that Tyr

phosphorylation and the intramolecular Tyr(P)–SH2 interaction may not be sufficient to completely inhibit SFK activity in vivo. Thus, this novel non-catalytic mechanism, which both inactivates the autophosphorylated Hck and prevents Hck from undergoing autophosphorylation, represents a fail-safe cellular mechanism to suppress SFK signaling under all circumstances.

Despite the high degree of specificity displayed by CHK in phosphorylating Tyr

of SFKs (Figs. 2, 4, 8, and 9), its efficiency in phosphorylating SFKs in vitro is very low. For example, CHK phosphorylated Hck to a stoichiometry of less than 0.03 mol of phosphate/mol of Hck even when CHK was present in a 3–4-fold excess. Similarly, Src is a poor in vitro substrate of CHK (Fig. 9D). Lyn appears to be a better substrate (Fig. 8D), but even so, Lyn Tyr

was phosphorylated only to a stoichiometry of 0.13 mol of phosphate/mol of Lyn when CHK was present in a 1.6-fold excess. Our results suggest that CHK either needs to be activated or requires the assistance of other cellular proteins to enhance its efficiency in phosphorylating Tyr

of SFKs.

It is intriguing that CHK binds Src and suppresses its phosphorylation of exogenous peptide substrates and yet it enhances Src autophosphorylation (Fig. 9C). Nevertheless, despite the elevated Src autophosphorylation, CHK can still bind and suppress Src phosphorylation of exogenous peptide substrates (Fig. 9, A and E). How might CHK binding enhance Src autophosphorylation? Relevant to this question, Wang et al. (33) report that Src can undergo dimerization. We postulate that CHK binding also facilitates Src dimerization and this in turn enhances Src trans-autophosphorylation. But how CHK binding suppresses Src kinase activity toward the exogenous peptide substrates while it permits Src trans-autophosphorylation, is unclear.

Although stable CHK-Src protein complexes exist in rat brain, we failed to detect stable CSK-Src complexes, suggesting either that the CSK-Src complexes are much less abundant than the CHK-Src complexes in rat brain or that the CSK-Src complexes are of transient existence in brain cells. Recently, Lee et al. (24) were able to demonstrate the formation of a stable CSK-Src complex in vitro when both kinases were at high concentrations. Given the high degree of sequence identity of CHK and CSK (>50% in their overall sequences and >70% in their kinase domains), it is possible that both CHK and CSK use similar motifs to bind SFKs.

In an attempt to elucidate the structural basis of the stable association between CHK and SFKs, we revealed that direct binding of CHK to Hck was abolished when Lys-221 in the active site of CHK was mutated to methionine. Based upon the crystal structures of other protein kinases (6, 8, 9), Lys-221 in β-strand 3 of the kinase domain is predicted to fulfill two structural requirements; (i) it binds to the α and β phosphates of ATP, and (ii) it forms a salt bridge with the conserved Glu-235 on α-helix C in the minor lobe of the kinase domain. Our results show that CHK-SFK complexes were maintained even in the absence of ATP (Fig. 2D), indicating that interactions of Lys-221 with ATP play no role in directing CHK to bind SFKs. This implies that formation or accessibility of the motifs critical for SFK binding and inhibition are dependent upon interactions of Lys-221 with other residues of the kinase domain, e.g. formation of the Lys-221–Glu-235 salt bridge.

Because CHK and CSK display significant sequence identity, it is logical to postulate that the three-dimensional structures of the two enzymes also share a high degree of similarity. For this reason we turned to the recently published x-ray crystal structure of CSK for clues to explain how the R221M mutation completely abolishes the CHK ability to bind and inactivate Hck. The unit cell of the CSK crystal structure contains six copies of CSK; four adopt a configuration in which the corresponding Lys-Glu salt bridge is formed, and two adopt configurations in which the salt bridge is broken (Fig. 11). There are significant conformational differences between the two forms.
In the minor lobe of the kinase domain, \(\beta_1\), \(\beta_1-\beta_2\) loop, \(\beta_2\), \(\beta_3\), \(\beta_3-\alpha C\) loop, \(\alpha C\) in the minor lobe and \(\alpha D\) in the major lobe, are displaced. Also, the two lobes of the kinase domain change their relative disposition, and the SH2 domain and its flanking linkers move with respect to the kinase domain (Fig. 11). We suggest that such conformational differences could also exist for CHK. Thus, the disruption of the Lys-221–Glu-235 salt bridge by the K221M mutation could have far-reaching effects on the conformation of CHK that limit the accessibility or prevent the formation of the motifs critical for SFK binding and inhibition. Recently, Lee \textit{et al.} (24) identified a number of residues in and around \(\alpha\)-helix D in the major lobe of the kinase domain of CSK.

**Fig. 11. Conformational changes in CSK.** \(A\), schematic representation of the conformation adopted by CSK (PDB 1K9A chain A) when the salt bridge is formed between the residues corresponding to Lys-221 and Glu-235 in CHK. The side chains involved are shown in stick mode and are labeled Lys and Glu, respectively. The relative position of selected secondary structure elements in the kinase domain (\(\beta_1\), \(\beta_1-\beta_2\) loop, \(\beta_2\), \(\beta_3\), \(\beta_3-\alpha C\) loop, \(\alpha C\) in the minor lobe and \(\alpha D\) in the major lobe) are shown within an outline of the whole molecule. \(B\), conformation adopted by CSK when the salt bridge is broken (PDB 1K9A chain C). The two images were generated with the major lobe of the kinase domain in the same orientation.

**Fig. 12. A model depicting the course of inactivation of SFKs by CHK.** The phosphorylation and activity states of SFKs are mainly determined by autophosphorylation of the consensus tyrosine (Tyr\(_A\)) in the kinase domain and phosphorylation of the C-terminal regulatory tyrosine (Tyr\(_T\)). SFKs can potentially exist in three phosphorylation states, (i) the fully active autophosphorylated state represented as [pY\(_A\)][pY\(_T\)], (ii) the latent non-phosphorylated state represented as [Y\(_A\)][pY\(_T\)], and (iii) the inactive state represented as [Y\(_A\)][pY\(_T\)]. With Tyr\(_T\) phosphorylated by CSK or CHK, in the absence of CHK, the latent non-phosphorylated SFK, \(i.e.\) the [Y\(_A\)][pY\(_T\)] form, readily undergoes autophosphorylation to form the fully active [pY\(_A\)][pY\(_T\)] form. CHK inhibits SFK activity by binding to the latent form and in turn preventing it from undergoing autophosphorylation. Moreover, CHK can bind to the fully active autophosphorylated state Tyr\(_P\_A\)[pY\(_T\)], and the binding alone is sufficient to inhibit SFK activity. In addition to inhibiting SFK activity upon direct binding, CHK can also inhibit its activity by phosphorylating its Tyr\(_T\). The ability of CHK to bind and inhibit both autophosphorylated and latent forms of SFKs suggests that CHK is capable of providing a fail-safe mechanism to shut down SFK signaling regardless of the phosphorylation status of SFKs. The question mark indicates that the mechanisms of efficient Tyr\(_T\) phosphorylation and dissociation of the CHK-Hck complex remain unknown.

In the minor lobe of the kinase domain, \(\beta\)-strand 1, \(\beta\)-strand 2, \(\beta\)-strand 3, the \(\beta_3-\alpha C\) loop, and \(\alpha\)-helix C are displaced. Also, the two lobes of the kinase domain change their relative disposition, and the SH2 domain and its flanking linkers move with respect to the kinase domain (Fig. 11). We suggest that such conformational differences could also exist for CHK. Thus, the disruption of the Lys-221–Glu-235 salt bridge by the K221M mutation could have far-reaching effects on the conformation of CHK that limit the accessibility or prevent the formation of the motifs critical for SFK binding and inhibition. Recently, Lee \textit{et al.} (24) identified a number of residues in and around \(\alpha\)-helix D in the major lobe of the kinase domain of CSK.
Inactive configuration of the kinase domain (the C-terminal tail with the SH2 domain and the interaction phosphorylation at Tyr A to convert the kinase to its fully active, in addition to inactivation of SFKs by the intramolecular mechanism that involves the formation of a tight CHK complex is particularly important because compounds that reconstitute the intramolecular SH2-binding site, and this would allow the SH2 and SH3 domains of the SFK potential therapeutics for the treatment of breast cancer. Further investigations to define the structural determinants in both CHK and SFKs that mediate their stable interactions will facilitate the discovery of such compounds.

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A Novel Non-catalytic Mechanism Employed by the C-terminal Src-homologous Kinase to Inhibit Src-family Kinase Activity

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