C-terminal Src Kinase-homologous Kinase (CHK), a Unique Inhibitor Inactivating Multiple Active Conformations of Src Family Tyrosine Kinases*"[S]

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The Src family of protein kinases (SFKs) mediates mitogenic signal transduction, and constitutive SFK activation is associated with tumorigenesis. To prevent constitutive SFK activation, the catalytic activity of SFKs in normal mammalian cells is suppressed mainly by two inhibitors called C-terminal Src kinase (CSK) and CHK-homologous kinase (CHK), which inactivate SFKs by phosphorylating a consensus tyrosine near the C terminus of SFKs (YT). The phosphorylated YT intramolecularly binds to the SH2 domain of SFKs. This interaction, known as pYT/SH2 interaction, together with binding between the SH2 kinase linker and the SH3 domain of SFKs (linker/SH3 interaction) stabilizes SFKs in a “closed” inactive conformation. We previously discovered an alternative mechanism CHK employs to inhibit SFKs. This mechanism, referred to as the non-catalytic inhibitory mechanism, involves tight binding of CHK to SFKs; the binding alone is sufficient to inhibit SFKs. Herein, we constructed multiple active conformations of an SFK member, Hck, by systematically disrupting the two inhibitory interactions. We found that CHK employs the non-catalytic mechanism to inactivate these active conformations of Hck. However, CHK does not bind Hck when it adopts the inactive conformation in which both inhibitory interactions are intact. These data indicate that binding of CHK to SFKs via the non-catalytic mechanism is governed by the conformations of SFKs. Although CSK is also an inhibitor of SFKs, it does not inhibit SFKs by a similar non-catalytic mechanism. Thus, the non-catalytic inhibitory mechanism is a unique property of CHK that allows it to down-regulate multiple active conformations of SFKs.

The Src family of protein-tyrosine kinases (SFKs) plays crucial roles in controlling a wide array of cellular functions, including growth, proliferation, and differentiation (1, 2). Aberrant regulation of the SFK activity is associated with malignant transformation of healthy cells (see Refs. 3 and 4, for review). In light of the oncogenic potential of SFKs, it is vital that their basal activity is kept low. The major inhibitory mechanism that suppresses SFK activity is contingent upon phosphorylation of a consensus tyrosine in the C-terminal tail of SFKs (YT). Upon YT phosphorylation, SFKs tend to adopt a “closed” inactive conformation in which the catalytically critical residues are not properly aligned for productive substrate phosphorylation. Although YT phosphorylation is generally associated with SFK inhibition, there exist several active conformations of SFKs in which their YT is phosphorylated (5). In this article, we demonstrate that the CSK-homologous kinase (CHK) is a unique inhibitor capable of inhibiting these and other active conformations of SFKs regardless of their YT phosphorylation status.

C-terminal Src kinase (CSK) and CHK are two principal endogenous negative regulators of SFKs that inactivate SFKs by phosphorylating their YT (see Refs. 5 and 6 for review). As revealed by crystal structures of the YT-phosphorylated forms of two SFK members c-Src and Hck, the inactive SFKs adopt a closed conformation stabilized by two major intramolecular inhibitory interactions (7, 8): (i) binding of the phosphorylated YT to the SH2 domain (referred to as the pYT/SH2 interaction), and (ii) binding of a polyproline type II helical motif in the SH2-kinase linker to the SH3 domain (referred to as the linker/SH3 interaction).

In mammalian cells, activation of SFKs is accomplished by multiple mechanisms that disrupt one or both inhibitory interactions (see Refs. 9 and 10 for review). For instance, Hck is activated by the accessory protein Nef of human immunodeficiency virus-1 (HIV-1), which binds to the SH3 domain, and in turn disrupts the linker/SH3 interaction of Hck (11). There is evidence documenting that disruption of the linker/SH3 inter-
action alone is sufficient to induce Hck activation, whereas the pY-T/SH2 interaction remains intact (12, 13). Other examples of ligands capable of activating SFKs by perturbing the linker/SH3 interaction in vivo are p130Cas (14) and the progerin receptor (15). In addition, disruption of the pY-T/SH2 interaction alone can also activate SFKs (12). The pYEEI phosphopeptide from the polyoma virus middle T antigen is a high affinity SH2 ligand shown to activate Hck by directly displacing the engagement of pY-T from the SH2 domain (13, 16, 17). Focal adhesion kinase and a p130Cas-related protein Sin are ligands with dual functions; they activate c-Src by simultaneously disrupting the linker/SH3 and pY-T/SH2 interactions (18, 19).

Phosphorylation of Y-T appears to be a prerequisite for SFKs to adopt the closed inactive conformation. However, there exist multiple endogenous activators that simply activate SFKs by perturbing the linker/SH3 and/or pY-T/SH2 interactions without affecting the Y-T phosphorylation status. This suggests that phosphorylation of Y-T alone is insufficient to restrain SFK activity at the basal level (see Ref. 5 for review). Thus, in addition to phosphorylation of Y-T by CSK and CHK, inhibitors capable of down-regulating SFK activity by Y-T-independent mechanisms are required (see Ref. 5 for review). Previously, we reported that CHK can inactivate SFKs in vitro and in cells by a non-catalytic mechanism that does not involve Y-T phosphorylation. Instead, this mechanism is mediated through direct binding of CHK to SFKs to form stable protein complexes (20). The binding is independent of Y-T phosphorylation by CHK and therefore is referred to as non-catalytic binding. This CHK-SFK non-catalytic binding alone is sufficient to suppress the activity and concomitant autophosphorylation of SFKs. To establish the physiological relevance of our findings, we provided evidence in our previous report that in rat brain cells, (i) CHK and c-Src co-localize in specific membrane microdomains and (ii) CHK and c-Src form a stable protein complex. These two pieces of evidence support the notion that co-localization of CHK and c-Src can significantly increase their effective concentrations in specific membrane microdomains and in turn facilitates their binding to form the stable CHK-c-Src complex. Presumably, CHK binding suppresses the activity of c-Src in the complex.

There are two important outstanding questions arising from our previous findings. (i) Can CHK employ this non-catalytic mechanism to inhibit all active conformations of SFKs? (ii) Can CHK bind to the inactive conformation of SFKs?

In this study, we employed the SFK member Hck as a model and constructed multiple active conformations of Hck by systematically disrupting the linker/SH3 and pY-T/SH2 inhibitory interactions. We observed that CHK binds to and inhibits these active conformations of Hck. However, it does not bind to Hck in the inactive conformation of which both inhibitory interactions are intact. These data suggest that the conformation of SFKs plays a crucial role in governing the non-catalytic CHK-SFK binding and inhibition. Because CSK is another endogenous inhibitor of SFKs (see Ref. 21 for review), we also examined whether it possesses the ability to inactivate SFKs by a similar non-catalytic mechanism. Our data indicate that CSK is incapable of inhibiting SFKs by such a mechanism. Therefore, we conclude that the non-catalytic inhibitory mechanism is a unique ability of CHK enabling it to potentially inhibit multiple active conformations of SFKs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild type CHK expressed in Spodoptera frugiperda 9 (S9) cells was purified as described previously (20). CSK baculovirus was from Dr. David Morgan (22), and CSK was purified by phosphorytir sine-agarose affinity column chromatography (23). Synthesis of the SFK-specific peptide substrate, cdc26(20–20) peptide (KVEKIGEQTVVYK), SFK C-terminal peptide (FFTATEGQYQQQP), pYEEI peptide (THQEEEQPTPY-EEIPYL), and coupling of pYEEI peptide to Affi-Gel 10 (Bio-Rad) were described previously (24, 25). Production of the anti-CHK and anti-Hck antibodies was described elsewhere (20, 24). The phospho-Src(Tyr-416) antibody directed against the consensus phosphorylation site of SFKs (referred to as the anti-pYA antibody) and phospho-Lyn (Tyr-507) antibody recognizing the consensus C-terminal regulatory tyrosine of some SFK members (referred to as the anti-pY-T antibody) were from BIOSOURCE and Cell Signaling, respectively. The general anti-phosphotyrosine antibody p-Tyr-100 (anti-Tyr(P) antibody) was from Cell Signaling. Anti-Lyn antibody was provided by Dr. Margaret Hibbs (26). Protein A-Sepharose with anti-CHK antibody covalently linked to it were generated as described previously with the chemical cross-linker dimethyl pimelimidate (Sigma) (20). Recombinant baculovirus encoding Hck(2PA-YEEI) was generated as previously described (12). Recombinant GST-CD45, a GST fusion protein of the cytoplasmic portion (residues 587–1291) of mouse CD45 was expressed in Escherichia coli (BL21(DE3) strain). The pGEX-KTX-CD45 plasmid (kindly provided by Dr. Irene Stanley) was used to direct its overexpression in E. coli. It was purified by glutathione-agarose affinity column chromatography following the procedures suggested by the manufacturer (GE Healthcare).

**Generation of the pcDNA3-Hck(2PA-YEEI) Plasmid**—The Hck(2PA-YEEI) coding sequence, in which both linker prolines are replaced with alanines and the C-terminal tail is modified to the high-affinity SH2 binding sequence Tyr-Glu-Glu-Ile, has been described elsewhere (12). This clone was subcloned into the plasmid pcDNA3 vector (Invitrogen) for expression in mammalian cells. The plasmid directs the expression of full-length Hck(2PA-YEEI).

**Generation of Recombinant Baculovirus of SFK Mutants**—The Hck-(222–503) construct was generated by PCR. The Lyn(K274M) construct was generated in a two-step PCR procedure as previously described (27). The PCR products were ligated to the pBacPAK9 vector (Clontech) to generate the corresponding pBacPAK9-SFK mutant plasmids. Authenticity of the DNA inserts in these plasmids was confirmed by sequencing. The resultant plasmids were co-transfected with the BacPAK6 baculoviral DNA (Clontech) into S9 cells to produce the recombinant baculoviruses.

5 The Hck(2PA-YEEI) construct was derived from the human Hck sequence.
6 Numbering of amino acid residues is based upon the sequence of the 56-kDa isoform of Hck derived from the mouse Hck gene (GenBank accession number NM_010407).
Expression and Purification of the Recombinant Hck-(222–503) and Lyn(K274M) Proteins—Recombinant FLAG-tagged Hck-(222–503) and Lyn(K274M) proteins in the crude lysate of infected S9 cells were purified by sequential chromatography on dimethylaminoethyl and hydroxylapatite columns. The proteins were further purified by Mono S cation-exchange column chromatography, followed by Mono Q anion exchange column chromatography. Hck-(222–503) in the column fractions was monitored by the protein kinase activity assay as well as anti-FLAG Western blotting. Lyn(K274M) in the column fractions was monitored by anti-Lyn Western blotting.

Generation and Purification of YT-phosphorylated Hck and Hck(K267M) (pYT-Hck and pYT-Hck(K267M))—pYT-Hck and pYT-Hck(K267M) were produced by co-expressing S9 cells with baculoviruses directing the expression of Hck or Hck(K267M) and CSK. The co-expression allows phosphorylation of YT of Hck and its kinase-defective mutant by CSK. It is noteworthy that not all Hck molecules were phosphorylated by CSK. Thus, in addition to the pYT form of Hck proteins, the crude lysates also contained other forms of Hck proteins, including unphosphorylated Hck and pYA-Hck.

Purification of pYT-Hck and pYT-Hck(K267M) involved two steps. First, all forms of Hck and Hck(K267M) were purified by sequential chromatography on ion exchange and hydroxylapatite columns as described previously (27). Second, the enzyme preparations were subjected to chromatography onto a column containing the pYEEI gel, which selectively bound unphosphorylated Hck proteins and pYT-Hck, but not pYT-Hck and pYT-Hck(K267M). Briefly, the enzyme preparations were applied to a pYEEI gel column (1 ml packed volume, loading density = 0.85 μmol of pYEEI peptide/ml of packed gel) pre-equilibrated with Buffer A (25 mM Hepes, pH 7.0, 0.1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 0.2 mg/ml benzamidine, 10 mM β-glycerophosphate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 mM Na3VO4, and 50 mM NaF). The unbound fractions, containing solely or predominantly pYT-Hck and pYT-Hck(K267M), were analyzed by anti-pYT and anti-pYA Western blotting and mass spectrometry to assess phosphorylation states of these proteins.

Preparation of YT-phosphorylated Lyn(K274M) (pYT-Lyn(K274M))—pYT-Lyn(K274M) was generated by phosphorylating Lyn(K274M) with CSK in vitro. Briefly, recombinant Lyn(K274M) and CSK proteins (5 μg each) were incubated in the presence of Assay Buffer (20 mM Tris-HCl, pH 7.0, 10 mM MgCl2, 1 mM MnCl2, 50 μM Na3VO4, and 50 μM ATP) at 30 °C for 1 h. After incubation, the residual unphosphorylated Lyn(K274M) was removed by pYEEI gel affinity chromatography as detailed in the previous section. The final pYT-Lyn(K274M) preparation was analyzed by anti-pYT Western blotting and mass spectrometry.

Generation of Constructs Encoding the Individual Domains of CHK—The GST-SH3 domain construct was generated by amplifying residues 1–80 of CHK by PCR, followed by subcloning into the pGEX6P3 vector. Production of the GST-SH2 domain construct was described elsewhere (28). For the construction of the CHK kinase domain, residues 170–467 of full-length CHK were amplified from pBac-PAK-CHK by PCR. The PCR product was subcloned into the pBacPAK9 vector. The resultant plasmid pBac-PAK-CHK kinase domain was used to generate recombinant baculovirus directing the expression of CHK kinase domain.

Expression and Purification of GST-SH3 and GST-SH2 Domains, and CHK Kinase Domain—The GST fusion proteins were expressed in E. coli (BL21(DE3) strain). Purification of the GST-SH2 and GST-SH3 domains of CHK was conducted as described previously (28). For the CHK kinase domain, its expression was directed by the recombinant CHK kinase domain baculovirus in S9 cells as described previously (20). Briefly, the recombinant protein was purified by sequential chromatography on dimethylaminoethyl and carboxymethyl columns. The protein was further purified by Sephacryl 200 gel filtration column chromatography, followed by Mono S cation-exchange column chromatography. CHK kinase domain in the column fractions was monitored by Western blotting and kinase activity assay as described previously (20).

SH2 Domain Accessibility Assay—This assay monitors accessibility of the SH2 domain of recombinant SFKs. The concentrations of enzymes and amount of pYEEI gel used in the assay were adjusted to allow selective binding of the immobilized pYEEI peptide to unphosphorylated pYA-SFKs, but not pYT-SFKs. Briefly, each protein (3.4–4.2 pmol) was incubated with 5 μl of pYEEI gel (equivalent to 4.3 nmol of the immobilized pYEEI peptide) in a final volume of 30 μl in Buffer A at 4 °C for 30 min. The mixture was centrifuged at 10,000 × g for 5 min, and the supernatant containing the unbound enzyme was removed and analyzed by Western blotting. The pYEEI gel was washed with 2 × 1.3 ml of Buffer A, 2 × 1.3 ml of Buffer A in 0.5 mM NaCl, and 1 × 1.3 ml of Buffer A in 1 mM NaCl. Proteins bound to the pYEEI gel were analyzed by Western blotting.

Co-immunoprecipitation and Ni-NTA Pulldown Assays—CHK was incubated with SFKs in different conformations at 30 °C for 30 min. To perform the anti-CHK immunoprecipitation (anti-CHK IP), the reaction mixtures were transferred to Protein A-Sepharose beads pre-coated with 4.4 μg of anti-CHK antibody (15 μl bed volume). For the anti-FLAG IP, the reaction mixtures were applied to the anti-FLAG antibody covalently coupled to agarose (Kodak), followed by agitation of the samples at 4 °C for 2 h. After separation from the unbound proteins, the immunoprecipitates were washed with Buffer A in NaCl (0 to 1 M). Proteins in the immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting. To study the effect of disrupting the pYT/SH2 interaction on CHK binding, pYT-Hck, pYT-Hck(K267M), and pYT-Lyn(K274M) were preincubated with 500 μM pYEEI peptide for 1 h at 4 °C prior to co-immunoprecipitation. The pulldown assays were performed under similar conditions using Ni-NTA resins (5 μl bed volume).

7 The consensus ATP-binding lysine, autophosphorylation site, and the C-terminal regulatory phosphorylation site are referred to as Lys-267, Tyr-388, and Tyr-499, respectively. For the 59-kDa isofrom, these residues correspond to Lys-287, Tyr-408, and Tyr-519.

8 The constructs of wild type Hck and all Hck mutants, except Hck(2PA-YEEI), were derived from the mouse Hck sequence.
Demonstration of Stable Complex Formation between CHK and Hck(2PA-YEEI) in Transfected HEK293T Cells—HEK293T cells were transfected with pcDNA3-Hck(2PA-YEEI) or pcDNA3-CHK plasmids, or co-transfected with both plasmids. The crude lysates were prepared as described previously (20). Aliquots of the cell lysate (1 mg of total proteins per sample) were incubated with anti-CHK antibody covalently coupled to Protein A-Sepharose beads (with 2.8 μg of antibody covalently coupled to 8 μl of packed gel) at 4 °C for 2 h with gentle agitation. The immunoprecipitates were washed as described previously (20). Hck(2PA-YEEI) bound to CHK was separated on SDS-PAGE and visualized by Western blotting.

Hck Kinase Activity Assays—Hck, Hck(2PA-YEEI), and Hck(222–503), ranging from 20 to 57 nm, were incubated with the designated amount of CHK or CSK at 4 °C for 20 min. An assay mixture containing 50 μM [γ-32P]ATP, Assay Buffer (20 mM Tris-HCl, pH 7.0, 10 mM MgCl2, 1 mM MnCl2, and 50 μM Na3VO4), and 300 μM cdc2(6–20) peptide was introduced to the reaction mixtures. These were incubated at 30 °C for 10 min to permit incorporation of 32P into the cdc2(6–20) peptide catalyzed by Hck and its active mutants. Whereas the cdc2(6–20) peptide is efficiently phosphorylated by Hck proteins, it is a poor substrate of CHK and its active mutants. The cdc2(6–20) peptide was monitored as previously described (29). To study the ability of CHK to inhibit the pYEEI-activated pYT-Hck, CSK-phosphorylated Hck preparation (1 μl) was activated with pYEEI peptide (500 μM), under identical conditions described for the co-immunoprecipitation assay. The catalytic activity of the pYEEI-activated Hck in the presence of varying CHK concentrations was examined by kinase assay.

Assay for the Hck(2PA-YEEI) Activity in Lysate of the Transfected HEK293T Cells—HEK293T cells were transiently transfected with the following plasmids: (i) pcDNA3-Hck(2PA-YEEI) only, (ii) pcDNA3-CHK and pcDNA3-Hck(2PA-YEEI), (iii) pcDNA3-CHK, and (iv) pcDNA3 only, as previously described (20). Hck(2PA-YEEI) activity in the crude lysates (0.1–0.2 μg of total proteins) was measured by the kinase assay method as detailed elsewhere (20). The Src optimal peptide (AEEEIYGEFEAKKK), which is a specific and efficient peptide substrate for Hck, was used as substrate of this assay. The peptide phosphorylation reaction was performed at 30 °C for 20 min and the phosphorylated peptide was separated from free [γ-32P]ATP by the phosphocellulose filter paper assay procedure (24).

Determination of Phosphorylation Sites in the SFK Preparations by Mass Spectrometry—32P radiolabeled SFKs were generated as above, and ~5 μg of protein was precipitated by acetone (acetone:protein solution = 10:1 (v/v)) and dried. The labeled SFKs were then digested with 10 ng/ml of chymotrypsin (Sigma) in 25 mM NH4HCO3 at 37 °C for 24 h. Peptides were separated on a Waters XBridge C18 column (3.5 μm, 2.1 × 10 mm guard column), using a linear gradient of Buffer B (0.1% TFA) to 60% Buffer C (acetonitrile with 0.09% (v/v) trifluoroacetic acid) in 70 min, at a flow rate of 200 μl/min. One-minute fractions were collected and screened for radioactivity (Cherenkov counting). Fractions containing radioactivity were analyzed by direct infusion into an Agilent 1100 MSD SL ion trap mass spectrometer fitted with a microionspray source.

RESULTS

Characterization of the Different Conformations of SFKs and Their Mutants—Fig. 1 depicts the various conformations of Hck and Lyn and their mutants used in this study. Fig. S1 shows the purity of these recombinant proteins. The FLAG-tagged Hck(222–503) mutant consists of the SH2 kinase linker (residues 222–238), kinase domain (residues 239–492), and C-terminal regulatory domain (493–503). No significant anti-pY_A and anti-pY_T immunoreactivities were detected in Hck(222–503), indicating that neither Y_A (Tyr-388) nor Y_T (Tyr-499) are phosphorylated.

Hck(2PA-YEEI) contains the 2PA mutation, whereby Pro-223 and Pro-226 in the SH2 kinase linker capable of forming a type II helical structure that interacts with the SH3 domain, are mutated to alanine. This mutant is constitutively active (12) and is expected to undergo autophosphorylation at Y_A because of the inability of the mutated SH2 kinase linker to engage in the linker/SH3 interaction (Fig. 1). Phosphorylation of Y_A of the enzyme in the purified preparation was confirmed by Western blotting (Fig. S2) and mass spectrometry (data not shown). This mutant also has an altered C-terminal tail sequence whereby the wild type sequence QY_TQQQ was changed to QYEIEIP. The QYEIEIP motif of the modified C-terminal tail is an efficient substrate of SFKs (25).

There is evidence documenting that Hck(2PA-YEEI) can readily undergo autophosphorylation at the QYEIEIP motif in cells (12, 13). We analyzed the phosphorylation status of pY_T of Hck(2PA-YEEI) using the anti-pY_T antibody, directing toward the phosphorylated C-terminal tail of Lyn and Hck (Fig. S2). Our results show that, in addition to recognizing the phosphorylated C-terminal tail sequence (YTAEEQ-pY_T-EEIP), their lack of phosphorylation at YA and YT was confirmed in Fig. S2C, treatment of the purified Hck(2PA-YEEI) with CD45 readily dephosphorylated YA, whereas it had no effect on anti-pY_T immunoreactivity. The lack of CD45 effect on anti-pY_T immunoreactivity was likely due to its inability to dephosphorylate pY_T, which was tightly bound to the SH2 domain. Further evidence substantiating that Y_T of this mutant is constitutively phosphorylated and engaged in intramolecular pY_T/SH2 interaction is presented in Fig. 3A (to be discussed in a later section).

For the enzyme preparations with wild type Hck, Hck(K267M) and Lyn(K274M) phosphorylated by CSK, Western blotting (Fig. S2) confirms that they are phosphorylated at Y_T but not Y_A. Mass spectrometry analysis verified Y_T phosphorylation in the pY_T-Lyn(K274M) preparation. For the unphosphorylated Hck(K267M) and Lyn(K274M) preparations, their lack of phosphorylation at Y_A and Y_T was confirmed by Western blotting (Fig. S2).
CHK Binds and Inhibits Hck-(222–503) That Lacks the SH3 and SH2 Domains—The published crystal structure of inactive Hck shows that the linker/SH3 and pYT/SH2 interactions are essential to stabilize SFKs in a closed inactive conformation. In this study, we investigate how disruption of both intramolecular inhibitory interactions affects the ability of CHK to inhibit SFKs. Hck-(222–503) is a truncated Hck mutant that lacks the SH3 and SH2 domains and therefore is unable to establish the inhibitory interactions. As shown in Fig. 2A, CHK effectively inhibits the catalytic activity and suppresses pYT autophosphorylation of this mutant. Fig. 2B shows that CHK binds to this mutant to form a stable protein complex; and the complex formation occurs even in the absence of Mg^{2+}/ATP. Given that Hck-(222–503) lacks the SH3 and SH2 domains and therefore is not subject to inhibition by pYT phosphorylation, CHK must have employed exclusively the pYT-independent mechanism to inhibit this mutant. Because this mechanism allows CHK to inhibit SFKs simply by binding to them, we previously referred it as the non-catalytic inhibitory mechanism (20).

Although inhibition of Hck-(222–503) is mediated by its non-catalytic binding with CHK, we were uncertain if the binding involves neighboring residues surrounding Y_T near the C terminus of Hck. For this reason, we examined the impact of introducing a synthetic peptide encompassing the last 11 amino acids of SFK (SFK C-terminal peptide) on the CHK-SFK binding and inhibition. We observed that this peptide did not significantly reduce the ability of CHK to bind to and inhibit Hck-(222–503) (Fig. 2, C and D). These data imply that the non-catalytic CHK/SFK interaction is not mediated by the local structure surrounding Y_T of SFKs. In addition, the data indicate that a high affinity CHK-binding motif resides in the segment encompassing residues 222–503 of Hck.
FIGURE 2. CHK binds and inhibits Hck lacking the SH3 and SH2 domains. A, CHK suppresses the catalytic activity and autophosphorylation of Hck-(222–503). The kinase assay was conducted to assess the efficiency of Hck-(222–503) to phosphorylate an SFK-specific substrate, cdc2(6–20) peptide in the presence of varying concentrations of CHK. The concentration (69 nM) of the Hck mutant remained constant throughout the assay, whereas the concentration of CHK varied from 0 to 5 μM. Autophosphorylation of the Hck mutant was monitored by anti-pY immunoblotting (inset). The anti-FLAG blot confirms that equal amounts of Hck-(222–503) were loaded for each sample analyzed. B, CHK directly binds to Hck-(222–503) to form a tight physical complex. The complex formation between CHK and Hck-(222–503) was verified by both anti-FLAG and anti-CHK IP. For the anti-FLAG IP, Hck-(222–503) (2.6 μM) in the reaction mixture was isolated using the anti-FLAG antibody immobilized on agarose. Non-catalytic binding of CHK to Hck-(222–503) was detected by Western blotting (WB). For the anti-CHK IP, CHK (2.1 μM) in the samples was isolated by its corresponding antibody immobilized on protein A-Sepharose. The presence of Hck-(222–503) in the immunoprecipitates was monitored by anti-FLAG Western blotting. C, SFK C-terminal peptide does not interfere with CHK binding to Hck-(222–503). Hck-(222–503) (57 nM) was incubated with CHK (2.9 μM) in the presence of 0–0.8 mM SFK C-terminal peptide at 4 °C for 30 min. CHK was immunoprecipitated and the presence of CHK and Hck-(222–503) was detected by Western blotting. D, SFK C-terminal peptide does not interfere with CHK inhibition of Hck-(222–503). Hck-(222–503) (69 nM) was incubated with CHK of varying concentrations (0–6 μM) in the absence (−) and presence (+) of 0.8 μM SFK C-terminal peptide. The residual kinase activity of Hck-(222–503) was monitored.
CHK Inhibits the Active Conformation of Hck Generated by Disruption of the Linker/SH3 Interaction—SFK activation in vivo is usually associated with pY_T dephosphorylation. Thus, it has been a general belief that dephosphorylation of pY_T is a prerequisite for SFK activation. However, there are exceptions to this belief, we and others observed that HIV-1 accessory protein Nef binds to the SH3 domain of Hck and in turn displaces the linker/SH3 interactions (12, 17, 30). This structural perturbation is sufficient to activate Hck without pY_T dephosphorylation (12, 17). Based on these findings, we generated the Hck(2PA-YEEI) mutant that resembles the Nef-activated conformation of Hck, the linker/SH3 interaction of this mutant is abolished by substitution of the conserved prolines in the PXXP motif with alanine. However, the pY_T/SH2 interaction is strengthened by replacing the C-terminal QYQQQP sequence with the QYEEIP sequence. As the QYEEIP sequence is an optimal SFK phosphorylation sequence, this mutant readily undergoes autophosphorylation at the modified C-terminal tail (12). The study by Bradshaw et al. (31) demonstrated that affinity of the SFK-SH2 domain toward the pYEEI motif is ~150-fold higher than that toward the native C-terminal tail sequence (pYQQQP) of an SFK member. Such a property is expected to significantly strengthen the pY_T/SH2 interaction in this mutant. Indeed, Hck(2PA-YEEI) did not bind to the pYEEI-agarose (Fig. 3A), confirming that its SH2 domain is engaged in the intramolecular pY_T/SH2 interaction.

Despite displaying elevated kinase activity and transforming potential, Hck(2PA-YEEI) is inhibited by CHK (Fig. 3B). Because pY_T of Hck(2PA-YEEI) is constitutively phosphorylated and firmly bound to the SH2 domain, CHK can only employ the non-catalytic mechanism to inhibit this mutant. This notion is confirmed by our observation that CHK forms a stable complex with this mutant (Fig. 3C).

CHK Down-regulates Hck(2PA-YEEI) in Transfected HEK293T Cells—To ascertain if CHK is capable of inhibiting the active conformation of Hck generated by disruption of the linker/SH3 interaction in cultured cells, we expressed full-length Hck(2PA-YEEI) in HEK293T cells and studied the functional interplay between CHK and this constitutively active Hck mutant. As shown in Fig. 4A, whereas expression of recombinant Hck(2PA-YEEI) in HEK293T cells induced a significant increase in tyrosine phosphorylation of many cellular proteins, CHK expression only induced a slight increase in the tyrosine phosphorylation level of a few proteins. Co-expression of CHK with Hck(2PA-YEEI) in HEK293T cells induced a significant increase in tyrosine phosphorylation of many cellular proteins, suggesting that CHK could inhibit the activity of this Hck mutant in cells. In agreement with this suggestion, Fig. 4B demonstrates that the peptide kinase activity of Hck(2PA-YEEI) was inhibited by co-expression of CHK. Furthermore, CHK co-expression also suppressed Hck(2PA-YEEI) autophosphorylation at Y_A in the kinase domain as well as the novel site Tyr-29 in the unique domain (Fig. 4C) (27).

We previously showed that the modified C-terminal QYEEIP motif of recombinant Hck(2PA-YEEI) could readily undergo phosphorylation in transfected mammalian cells (12). Furthermore, upon phosphorylation, the QpYEEIP motif could establish tight pY_T/SH2 intramolecular interaction. Based on these findings, we postulate that CHK could only down-regulate Hck(2PA-YEEI) by employing the non-catalytic inhibitory mechanism. This notion is substantiated by the results presented in Fig. 4D, which demonstrates complex formation between CHK and Hck(2PA-YEEI) in the transfected HEK293T cells. Taken together, it is clear from Figs. 3 and 4 that CHK employs the non-catalytic inhibitory mechanism to down-regulate the active conformation of Hck generated by disruption of the linker/SH3 interaction in vitro and in cells.

Inactive pY_T-Hck Is Unable to Bind CHK, but Disruption of the pY_T/SH2 Interaction Restores the CHK-Hck Binding—Can CHK inhibit SFKs activated by ligands disrupting the pY_T/SH2 interaction?
Conformation-dependent Inhibition of Src Family Kinases by CHK

A. Transfection conditions

Hck(2PA-YEEI) + + +
CHK - + +
WB: Anti-pY?
WB: Anti-Hck
WB: Anti-CHK

B. Transfection conditions

Hck(2PA-YEEI) + + +
CHK - + +
WB: Anti-pY?
WB: Anti-Hck
WB: Anti-CHK

C. Transfection conditions

Hck(2PA-YEEI) + + +
CHK - + +
WB: Anti-pY?
WB: Anti-pY?
WB: Anti-Hck

D. Transfection conditions

Hck(2PA-YEEI) + - +
CHK + + +
WB: Anti-Hck
WB: Anti-CHK

IP: Anti-CHK

FIGURE 4. CHK employs the non-catalytic inhibitory mechanism to down-regulate both the kinase activity and autophosphorylation of Hck(2PA-YEEI) in transfected HEK293T cells. Hck(2PA-YEEI) and CHK were coexpressed alone or together in HEK293T cells. A, analysis of the tyrosine phosphorylation of endogenous proteins in HEK293T cells. The levels of tyrosine phosphorylation of endogenous proteins in crude cell lysates (~30 μg of total proteins per sample) were examined using a generic anti-phosphotyrosine (anti-pY100) antibody. B and C, the effects of CHK on the kinase activity, as well as autophosphorylation levels of Y? in Hck(2PA-YEEI). Lysates of the transfected samples were analyzed for Hck(2PA-YEEI) kinase activity using Src-optimal peptide as the substrate. The "specific" enzymatic activity of Hck(2PA-YEEI) was expressed as pmol of phosphate incorporated in the peptide substrate per minute per densitometry unit of anti-Hck immunoactivity. The effects of CHK on the levels of pY? and pY? autophosphorylation of Hck(2PA-YEEI) were monitored by Western blotting (WB) using the appropriate antibodies. The anti-Hck Western blotting confirms that equal amounts of Hck were present in the samples analyzed. The multiple immunoreactive bands of different mobilities are attributed to various phosphorylation states of Hck(2PA-YEEI) at Y? and Y? (D). Demonstration of stable CHK-Hck(2PA-YEEI) complex formation in HEK293T cells by co-immunoprecipitation. CHK was immunoprecipitated from the crude cell lysates (~1 mg total proteins per sample) using the anti-CHK antibody cross-linked to Protein A-Sepharose. The samples were incubated at 4 °C for 2 h with gentle agitation, and the IP were washed with 1× IP buffer (1 M NaCl, 1× IP buffer) and 3× IP buffer. The presence of Hck(2PA-YEEI) and CHK in the immunoprecipitates was visualized by Western blotting.

Interaction? To address this question, we generated pY?–Hck adopting the closed inactive conformation and then activated it by displacing its SH2 domain using the exogenous pYEEI peptide. The ability of CHK to bind and inhibit this activated pY?–Hck was examined.

Previously, Porter et al. (13) demonstrated that full activation of pY?–Hck was achieved by incubating the enzyme with 500 μM pYEEI peptide. We therefore used identical phosphopeptide concentrations to activate pY?–Hck in our enzyme preparation. As shown in Fig. 5A, 0.2 μg of this enzyme preparation gave a basal activity of 1.39 ± 0.06 pmol of PO? incorporated/min. Upon preincubation with the phosphopeptide, the basal activity was increased by ~5-fold to 7.52 ± 0.06 pmol PO? incorporated/min, indicating that pY?–Hck was activated upon disruption of the intramolecular pY?/SH2 interaction. CHK in increasing concentrations could completely inhibit both basal and the pYEEI-activated Hck activity. We next examined the ability of CHK to bind pY?–Hck with and without disruption of pY? by CSK to a higher extent than that of wild type Hck (data not shown). Using a combination of pYEEI-agarose affinity column and ion exchange column chromatography, we were successful in obtaining an enzyme preparation containing only pY?–Hck(K267M) with pY? bound to the SH2 domain (Figs. 1 and supplemental S3). As shown in Fig. 5C, CHK does not bind pY?–Hck(K267M) (lanes 1 and 2). However, when the closed conformation of pY?–Hck(K267M) was perturbed by the pYEEI peptide, CHK restores its ability to form a stable protein complex with pY?–Hck(K267M) (Fig. 5C, lanes 3 and 4).

Because both unphosphorylated Hck and phosphopeptide-activated pY?–Hck lack the pY?/SH2 interaction, they are postulated to adopt similar three-dimensional structures. Recently, the crystal structure of unphosphorylated c-Src reveals that the linker/SH3 interaction of the enzyme remains intact even in the absence of the pY?/SH2 interaction. It is logical to predict that unphosphorylated Hck and the phosphopeptide-activated
pYT-Hck also exist in a structure similar to that of unphosphorylated c-Src. Fig. 5
D reveals that unphosphorylated Hck(K267M) forms a stable protein complex with CHK, further confirming that CHK binds and inhibits Hck and its mutants with linker/SH3 interaction intact but lacking pYT/SH2 interaction. In contrast, CHK does not bind pYT-Hck(K267M), again indicating that Hck adopting the closed inactive conformation is not the target of CHK binding.

The Non-catalytic CHK-Lyn Interaction Is Abolished When Lyn Assumes the Closed Inactive Conformation— It is clear from data in Fig. 5 that CHK cannot bind pYT-Hck and pYT-Hck(K267M) in the closed inactive conformation. To study if this observation also applies to other SFKs, we examined if CHK can bind pYT-Lyn(K274M). Consistent with data in Fig. 5, pYT-Lyn(K274M) does not form a stable protein complex with CHK, whereas its unphosphorylated counterpart does (Fig. 6). Collectively, the results presented in Figs. 5 and 6 suggest that the non-catalytic CHK-SFK binding is governed by conformational changes in SFKs, CHK binds to the active, but not the inactive conformation of SFKs.

CSK Lacks the Ability to Inhibit Hck via the Non-catalytic Mechanism—Because of the significant similarity between CSK and CHK, we were interested in examining whether CSK also exhibits the ability to inactivate SFKs by the non-catalytic mechanism. As shown in Fig. 7, CSK fails to inhibit Hck(2PA-YEEI) and Hck-(222–503), whereas CHK effectively suppresses the catalytic activity of both mutants. No complex formation between CSK and the mutants was detected (data not shown), further substantiating the fact that CSK does not employ the non-catalytic mechanism to inhibit SFKs. Therefore, our data confirm that although CSK and CHK are closely related, only CHK possesses the ability to down-regulate SFKs by the non-catalytic inhibitory mechanism.
CHK Kinase Domain Forms Stable Complex with Hck-(222–503), but Is Unable to Inhibit Hck-(222–503) Activity—We also attempted to define the domains of CHK required for binding to Hck-(222–503). We generated the individual domains of CHK, i.e., GST-SH3 domain (residues 1–80), GST-SH2 domain (residues 69–186), and kinase domain (residues 170–467). We then investigated the ability of these CHK domains to bind and inhibit Hck-(222–503). The results demonstrate that only the kinase domain, and not the SH3 and SH2 domains of CHK, form stable complexes with Hck-(222–503). Intriguingly, the complex formation does not inhibit the catalytic activity of Hck (Fig. 8).

It is evident from data presented so far that inhibition of Hck-(222–503) and Hck(2PA-YEEI) by CHK is mediated by the non-catalytic mechanism involving formation of the stable CHK-Hck protein complex. Because the SH3 domain of CHK does not bind to Hck-(222–503), we anticipated that it would not affect Hck activity. As expected, the CHK-SH3 domain does not influence the activity of both Hck mutants (Fig. 4), supporting the claim that CHK-SFK binding is required for effective SFK inactivation.

**DISCUSSION**

**CHK Can Potentially Down-regulate Multiple Active Conformations of SFKs—**SFKs can adopt multiple active conformations. Among them, some still retain the pYT/SH2 interaction and escape regulation by CSK. How all these active conformations of SFK are inhibited remains an important outstanding question. In this article, we present biochemical evidence that CHK employs a non-catalytic inhibitory mechanism to target the various active conformations of SFKs arising from disruption of one or both of the intramolecular inhibitory interactions. However, once SFKs adopt the closed inactive conformation, CHK is unable to bind to them. Despite the high degree of structural similarities between CSK and CHK, CHK lacks the ability to inhibit SFKs via a similar non-catalytic mechanism. Our results therefore reinforce the claim that CHK is a unique inhibitor capable of suppressing active SFKs using two distinct mechanisms: (i) YT phosphorylation and (ii) the non-catalytic inhibitory mechanism.

To establish the physiological significance of the CHK non-catalytic inhibitory mechanism, we previously demonstrated that in rat brain cells, CHK and c-Src exist in the same microdomains of the plasma membrane (20). Presumably, co-localization of CHK and c-Src significantly increases the effective concentrations of both proteins, and in turn facilitates CHK activity.
inhibition of c-Src. We also demonstrated in our previous study that CHK, but not CSK, forms stable protein complexes with c-Src in rat brain cells (20). The results suggest that CHK is capable of employing the non-catalytic mechanism to bind and down-regulate SFKs in vivo.

The ability of SFKs to adopt multiple active conformations allows them to respond specifically to different activating inputs (32). These activating inputs include: (i) upstream regulatory proteins containing motifs that bind to the SH2 and SH3 domains of SFKs, and (ii) protein phosphatases that dephosphorylate pYT of SFKs. Additional active conformations are produced when the activated SFKs undergo autophosphorylation at YA. These various active conformations of SFKs (Fig. 9) modulate the functions of specific downstream target proteins. The active SFKs deliver two types of output signals: (i) phosphorylation of specific protein substrates and in turn modulation of their functions; and (ii) direct binding to specific cellular proteins via their SH2 and SH3 domains. An example for the first type of output is modulation of the focal adhesion kinase activity by c-Src phosphorylation (33). This implies that binding of CHK to SFKs is a reversible process, CHK binds to SFKs when they are in the “open” active conformations, but dissociates from them once they adopt the closed inactive conformation. Based upon our results, we postulate the following stepwise mechanism to describe the process of inactivation of SFKs by CHK. Step 1, CHK binds to the active conformations of SFKs. Step 2, pYA of active SFKs is dephosphorylated by protein tyrosine phosphatases such as proline-enriched tyrosine phosphatase and T-cell protein-tyrosine phosphatase (35, 36); ligands bound to the SH2 and SH3 domains dissociate from SFKs. Step 3, CHK in the CHK-SFK complex phosphorylates YT of SFK. Step 4, CHK dissociates from the pYT-SFK once it adopts the inactive conformation (Fig. 9). Confirmation of this model awaits (i) elucidation of how CHK cooperates with proline-enriched tyrosine phosphatase and T-cell protein-tyrosine phosphatase to down-regulate SFKs, and (ii) demonstration of dissociation of the CHK-SFK complexes upon YT phosphorylation by CHK.

As presented in our previous article (12), the conformation of Hck(2PA-YEEI) mimics the conformation of Hck activated by HIV-1 Nef binding to its SH3 domain. The results shown in Figs. 3 and 4 therefore suggest that CHK is capable of inhibiting Nef-activated pYT-Hck. Investigating if CHK can employ the non-catalytic mechanism to inhibit Nef-activated Hck is a focus of our future investigation. In addition, it is also worthwhile to examine in our future studies if CHK binding can promote dissociation of the NefpYT-Hck complex.

Interplay between CSK and CHK in Regulation of SFKs in Vivo—It is evident from Fig. 7 that CSK inhibits SFKs only by phosphorylating their YT (the catalytic mechanism), whereas...
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CHK can employ both catalytic and non-catalytic inhibitory mechanisms to suppress SFK activity. Nevertheless, we cannot conclude from our findings that CHK is more important than CSK for SFK suppression in vivo because regulation of SFKs by CHK and CSK is governed by many factors including (i) the subcellular localization of CSK, CHK, and SFKs, (ii) catalytic efficiency of CSK and CHK in phosphorylating Y₅ of SFKs, and (iii) interaction of SFKs with activator proteins such as HIV-1 Nef and autophosphorylated FAK (18, 37). Furthermore, contributions of these factors may vary in different cell types and under different physiological states. Nonetheless, in neuronal and hematopoietic cells where CSK and CHK are co-expressed, both proteins can potentially cooperate to down-regulate SFKs. For cells expressing CSK only, other endogenous non-catalytic SFK inhibitors such as RACK1, caveolin, and WASP may cooperate with CSK to down-regulate SFK activity (5, 38 – 40).

Defining Structural Basis of SFK Inhibition by the CHK Non-catalytic Mechanism May Facilitate Development of Specific Synthetic SFK Inhibitors—Constitutive activation of SFKs is a major underlying cause of cancer progression. Because CHK can employ the non-catalytic mechanism to target multiple active conformations of SFKs, small molecule compounds mimicking these non-catalytic inhibitory properties are potential anti-cancer therapeutics. Fig. 8 suggests that the motif direct binding of CHK to SFKs resides in the CHK kinase domain, whereas the motif(s) responsible for inhibition of SFKs resides in other parts of CHK. Ongoing studies in our laboratories to define determinants mediating the binding and inhibition of SFKs by the non-catalytic mechanism of CHK may facilitate development of these small molecule compounds.

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