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Defining the Substrate Specificity Determinants Recognized by the Active Site of C-Terminal Src Kinase-Homologous Kinase (CHK) and Identification of β-Synuclein as a Potential CHK Physiological Substrate

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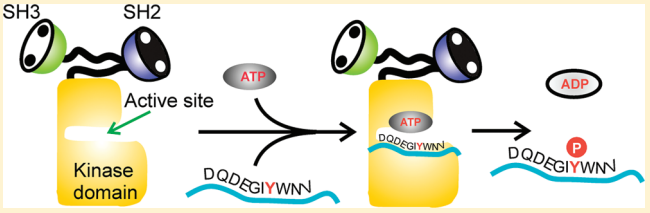
Supporting Information

ABSTRACT: C-Terminal Src kinase-homologous kinase (CHK) exerts its tumor suppressor function by phosphorylating the C-terminal regulatory tyrosine of the Src-family kinases (SFKs). The phosphorylation suppresses their activity and oncogenic action. In addition to phosphorylating SFKs, CHK also performs non-SFK-related functions by phosphorylating other cellular protein substrates. To define these non-SFK-related functions of CHK, we used the "kinase substrate tracking and elucidation" method to search for its potential physiological substrates in rat brain cytosol. Our search revealed β-synuclein as a potential CHK substrate, and Y127 in β-synuclein as the preferential phosphorylation site. Using peptides derived from β-synuclein and positional scanning combinatorial peptide library screening, we defined the optimal substrate phosphorylation sequence recognized by the CHK active site to be E-x-[Φ/E/D]-Y-Φ-x-Φ, where Φ and x represent hydrophobic residues and any residue, respectively. Besides β-synuclein, cellular proteins containing motifs resembling this sequence are potential CHK substrates. Intriguingly, the CHK-optimal substrate phosphorylation sequence bears little resemblance to the C-terminal tail sequence of SFKs, indicating that interactions between the CHK active site and the local determinants near the C-terminal regulatory tyrosine of SFKs play only a minor role in governing specific phosphorylation of SFKs by CHK. Our results imply that recognition of SFKs by CHK is mainly governed by interactions between motifs located distally from the active site of CHK and determinants spatially separate from the C-terminal regulatory tyrosine in SFKs. Thus, besides assisting in the identification of potential CHK physiological substrates, our findings shed new light on how CHK recognizes SFKs and other protein substrates.



Protein kinases relay transmission of specific cellular signaling pathways mainly by selectively phosphorylating unique subsets of cellular proteins. Although a protein substrate contains many serine, threonine, and tyrosine residues, its regulatory protein kinase selects only one or a few of these residues as the phosphorylation sites. A kinase recognizes its protein substrates by two mechanisms: (i) colocalization of the kinase with its protein substrates and (ii) binding of the active site of the kinase to specific structural determinants near the site of phosphorylation in the protein substrates (reviewed in ref 1). Thus, there are two types of structural determinants in a protein substrate that govern its selective phosphorylation by the protein kinase. The first group, termed distal docking determinants, consists of motifs that bind to the interaction domains in the kinase that are distally separated from the active site. For example, the PxxP motifs (where x stands for any amino acid) in both the N- and C-terminal portions of the scaffolding protein $p130^{CAS}$ act as the distal docking determinants that bind to the SH3 domain of its regulatory kinase c-Src.[2,3] The binding facilitates c-Src phosphorylation of $p130^{CAS}$ by colocalizing the two proteins in cells. The

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second type of determinant, the consensus phosphorylation sequence, contains the amino acid residues located near the phosphorylation site that interact with unique structural features within and nearby the active site of the kinase. For example, the c-Src active site recognizes the E/D-E/D-I-Y-E/G-x-F motif as its optimal phosphorylation sequence.[4] The "DIYDVP" motif containing Y253 of $p130^{CAS}$, which conforms to the c-Src optimal phosphorylation sequence, directs the active site of c-Src to selectively phosphorylate Y253.[2,3] Thus, defining the distal docking determinant and the optimal phosphorylation sequence that are recognized by a protein kinase can facilitate the pursuit and identification of its potential protein substrates. In this work, we present our findings using two biochemical approaches to define the optimal substrate phosphorylation sequence of C-terminal Src kinase-homologous kinase (CHK).

Both C-terminal Src kinase (Csk) and its homologous kinase (CHK) are major endogenous inhibitors of Src-family kinases (SFKs) (reviewed in refs 5−8). Overexpression and aberrant activation of SFKs contribute to the formation and disease progression of many forms of cancer. In normal cells, both Csk and CHK function as tumor suppressors by constraining the SFKs in the inactive state. They suppress the activity of SFKs by selectively phosphorylating their conserved C-terminal regulatory tyrosine. Upon phosphorylation, SFKs adopt the inactive conformation stabilized in part by the binding of the phosphorylated C-terminal tail tyrosine to the SH2 domain. Although much is known about the structural basis of Csk inhibition of SFKs (ref 9 and reviewed in ref 10), how CHK specifically recognizes the C-terminal regulatory tyrosine of SFKs as the target phosphorylation site remains to be elucidated.

To date, SFKs are the only known physiological substrates of CHK. However, there is a growing body of evidence suggesting that CHK is capable of phosphorylating other cellular proteins to perform non-SFK-related cellular functions. For example, overexpression of CHK enhanced activation of the MAP kinase signaling pathway and induced neurite outgrowth of the rat pheochromocytoma 12 (PC12) cells upon nerve growth factor stimulation.[11,12] Furthermore, CHK expression enhanced activation of the MAP kinase signaling pathway even in $SFY^{-/-}$ cells lacking SFKs,[12] suggesting that the activation was governed by an SFK-independent mechanism. Relevant to these findings, CHK overexpression could induce tyrosine phosphorylation of the tyrosine-protein phosphatase nonreceptor substrate 1 (SHPS-1) in PC12 cells.[13] The authors provided data suggesting that CHK binds to SHPS-1 and enhances its phosphorylation at specific tyrosine residues. In addition, CHK was known to localize and phosphorylate some unknown cellular proteins in the nucleus of HeLa cells.[14] Identifying the physiological substrates of CHK in cytosol and nucleus is an avenue for deciphering the non-SFK-related functions of CHK.

CHK is expressed at a high level in brain cells and is present predominantly in the cytosolic fraction of rat brain lysate (ref 15 and reviewed in ref 5). In this study, we adopted the "kinase substrate tracking and elucidation" (KESTREL) method to search for and identify potential physiological substrates of CHK in rat brain cytosol extract.[16,17] This method has so far been successfully used to identify new substrates for at least eight different protein kinases.[17−21] Using this method, we identified β-synuclein as a potential CHK substrate. Further analyses revealed that CHK preferentially phosphorylates Y127 in the C-terminal region of β-synuclein in vitro and in transfected HEK293T cells. Although α-synuclein and β-synuclein exhibit a high degree of sequence homology, α-synuclein is a much poorer substrate of CHK. Further studies with peptide analogues derived from β-synuclein allowed us to define several residues in its sequence as the substrate specificity determinants recognized by CHK. Using the combinatorial library approach, we define more completely the substrate specificity determinants in peptide substrates recognized by the CHK active site.

Results from both KESTREL and peptide library studies allowed us to define the consensus target phosphorylation sequence of CHK. The CHK-optimal peptide, which contains this sequence, was found to be an efficient CHK substrate, confirming that the determinants identified by the two approaches govern efficient phosphorylation of the protein and peptide substrates by CHK. Thus, cellular proteins containing motifs conforming to this consensus target phosphorylation sequence are potential physiological substrates of CHK.

MATERIALS AND METHODS

Materials. Recombinant α-synuclein was a gift from C. Pham and R. Cappai (Department of Pathology, University of Melbourne). The expression and purification of α-synuclein were described by Pham et al.[22] The recombinant baculovirus for the expression of wild-type CHK and CHK-His_6 were generated by the BacPAK baculovirus expression system (Clontech) as described previously.[15,23] The CHK-His_6 mutant contains a "GSGS" linker and a polyhistidine tag with a GSGSHHHHHH sequence attached to the C-terminus of the rat CHK sequence. The recombinant kinase-dead mutant of Lyn kinase ([K275M]Lyn), containing the K275M mutation at the ATP-binding site, was generated as described in our previous report.[24] The β-synuclein plasmid was from OriGene Technology, Inc. (catalog number TC300202). Polyclonal anti-CHK antibodies were raised against a GST fusion protein containing a C-terminal segment of rat CHK.[15] The monoclonal anti-Src antibody (MAb327) specifically recognizing an epitope in the SH3 domain of human c-Src was a gift from D. J. Fujita (University of Calgary, Calgary, AB). Rabbit polyclonal anti-pY416 phosphospecific antibody (also known as anti-Src[pY-418]) was from Biosource International. The mouse monoclonal anti-phosphotyrosine antibody (Clone PY69) was from BD Biosciences. The anti-α-synuclein antibody was raised to the C-terminal region of human α-synuclein (residues 116−131); the anti-β-synuclein antibody was generated against the C-terminal domain of human β-synuclein (residues 99−113).[25] The combinatorial peptide library was synthesized by Anaspec, Inc. All other synthetic peptides were synthesized using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based chemistry with the model CEM automated microwave peptide synthesizer. The stock solution of pervanadate used for treatment of cells was freshly prepared prior to each experiment by incubation of the sodium vanadate solution (10 mM) with H_2O_2 (10 mM) for 15 min at 23 °C. The recombinant wild-type CHK and [D311A]CHK expressed in the infected *Sf*9 cells were purified by sequential column chromatography as described previously.[15] The recombinant CHK-His_6 was purified to >90% purity as described previously.[23] Recombinant Csk, expressed in *Sf*9 cells, was purified by sequential column chromatography as described in our previous report.[24] Recombinant 14-3-3β was a gift from A. Dhillon of the Department of Biochemistry and Molecular Biology of the University of Melbourne. Native human hsp90 purified from HeLa cells was from Enzo Life Science. Generation

of the pGEX-6p-3-β-synuclein plasmid and the pGEX-6p-3-β-synuclein mutant plasmids and expression of recombinant GST-β-synuclein and its mutants are described in the Supporting Information.

Comparison of the Rates of CHK Phosphorylation of [K275M]Lyn and β-Synuclein. The reaction mixture (25 μL) contained (i) assay buffer [20 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and Na_3VO_4], (ii) 100 μM [γ-^{32}P]ATP, (iii) 0.11 μM CHK, and (iv) 0−2.2 μM [K275M]Lyn or β-synuclein. The phosphorylation reaction was allowed to proceed at 30 °C for 30 min. The reaction was terminated by the addition of 10 μL of 5× SDS sample buffer. The samples were separated via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the gel was subjected to autoradiography. Protein bands corresponding to [K275M]Lyn and β-synuclein were excised. The amount of ^{32}P incorporated in the two proteins was determined by scintillation counting. The catalytic activity of CHK was expressed as picomoles of phosphate incorporated into the substrate protein per minute.

Phosphorylation of α- and β-Synucleins by CHK. The reaction mixture (25 μL) contained (i) assay buffer, (ii) 100 μM [γ-^{32}P]ATP, (iii) 0.11 μM CHK, and (iv) 0−2.2 μM α- or β-synucleins. Phosphorylation of the synucleins by CHK was conducted at 30 °C for 30 min; 10 μL of 5× SDS sample buffer was added to each reaction mixture to terminate the reaction. The samples were separated by SDS−PAGE, and the gel was subjected to autoradiography. Protein bands corresponding to α- or β-synucleins were excised. The amount of ^{32}P incorporated into the protein substrate was assessed by scintillation counting. The enzymatic activity of CHK was expressed as picomoles of phosphate incorporated into the protein substrate per minute.

Phosphorylation of Peptides Derived from α- and β-Synucleins by CHK. To compare the efficiencies of CHK phosphorylation of peptides derived from α- and β-synucleins, CHK (0.11 μM) was incubated with 0−320 μM synuclein peptides in the presence of assay buffer and 100 μM [γ-^{32}P]ATP. The phosphorylation reaction was performed at 30 °C for 30 min and terminated via addition of 20 μL of acetic acid. Aliquots of the reaction mixture were spotted onto p81 filter paper squares. The paper squares were washed extensively with 4 × 400 mL of 5% (v/v) phosphoric acid. The amount of phosphate incorporated into the peptide was quantitated by scintillation counting.

Search for CHK Protein Substrates in Rat Brain Cytosolic Extract by KESTREL. Cytosolic extract prepared from 20 rat brains (Supporting Information) was used to search for CHK substrates. To minimize background signals due to phosphorylation of proteins by the endogenous kinases in the extract, we partially separated proteins in the extract by DEAE anion exchange chromatography. Bound proteins were eluted using a 0 to 1 M NaCl gradient in ion exchange column buffer and collected in 8 mL fractions. A 10 μL aliquot from each fraction was incubated with KESTREL assay buffer [20 mM Tris-HCl (pH 7.0), 10 mM $MnCl_2$, and Na_3VO_4], and 5 μM [γ- ^{32}P]ATP (specific radioactivity of ≥10000 cpm/pmol) at 30 °C for 5 min in the absence or presence of 1 μg of active recombinant CHK in a final volume of 25 μL. The reactions were terminated by addition of 5× SDS sample buffer prior to SDS−PAGE. ^{32}P-labeled proteins in the stained SDS gel were detected by autoradiography. Fractions containing the CHK-phosphorylated proteins were pooled, dialyzed against the ion exchange column buffer, and subjected to Mono-Q anion exchange column chromatography. To eliminate the interference of the background signal resulting from protein phosphorylation by the endogenous kinases, an aliquot (50 μL out of 1 mL) of each column fraction was subjected to heat treatment at 60 °C for 15 min to inactivate the endogenous kinases. Ten microliters of the heat-treated sample from each fraction was used for screening for CHK-phosphorylated protein as described in the previous paragraph. A number of CHK-phosphorylated proteins were detected in several Mono-Q fractions (Figure 2C). Hence, proteins in these fractions were further separated on the basis of their molecular sizes by SDS−PAGE in gels with different percentages of acrylamide (Figure 2D,E); 15% SDS−PAGE was used to separate low-molecular weight proteins, whereas the high-molecular weight proteins were separated by 7.5% SDS−PAGE. Bands containing the CHK-phosphorylated proteins were excised, digested with trypsin, and analyzed by nano-liquid chromatography−tandem mass spectrometry (nano-LC−MS/MS) as detailed in the next section.

Preparation of CHK-Phosphorylated Proteins for Mass Spectrometry Analysis. The protein bands containing the CHK-phosphorylated proteins in the "+CHK" lanes and those at the corresponding locations in the "−CHK" lanes of the Coomassie-blue stained gel were excised and macerated. To destain the proteins, the gel pieces from each excised band were incubated with 600 μL of a 1:1 (v/v) 100 mM NH_4HCO_3/acetonitrile mixture with agitation for 30 min. The gel pieces were then washed twice for 15 min with 200 μL of a 1:1 (v/v) 100 mM NH_4HCO_3/acetonitrile mixture. The gel pieces were then dehydrated with 200 μL of 100% acetonitrile and air-dried for 5 min. Proteins in the gel pieces were reduced with 200 μL of freshly prepared 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 4 °C. Reduced proteins were subsequently alkylated by incubation of the gel pieces with 20 mM iodoacetamide in 50 mM NH_4HCO_3 for 1 h at room temperature in the dark. After reduction and alkylation, gel pieces from each protein band were washed twice with 200 μL of a 1:1 (v/v) 100 mM NH_4HCO_3/acetonitrile mixture for 15 min, followed by dehydration with 200 μL of 100% acetonitrile, and air-dried for 5 min. Dry gel pieces were rehydrated with 40 μL of 50 mM NH_4HCO_3 containing 250 ng of trypsin (Sigma) and then incubated at 37 °C overnight. Tryptic peptides were extracted from the gel pieces, separated, and analyzed by LC−MS/MS using an Agilent 1100 dual-pump, nano-LC system linked to an Agilent 1100 ion trap XCT Plus mass spectrometer fitted with an Agilent HPLC-Chip CUBE source. Peptides were injected onto a 40 nL Zorbax 300SB-C18 trapping column at a rate of 4 μL/min and then separated by switching the trap column in-line with the separation column (Zorbax 300SB-C18, 43 mm × 0.075 mm) and running an 8 min gradient of 5 to 50% acetonitrile with 0.1% formic acid at a rate of 400 nL/min. MS/MS spectra were analyzed using the Mascot search engine.[26]

Investigation of the CHK Phosphorylation of Flag-Tagged β-Synuclein in HEK293T Cells. The synthetic *Flag-β-synuclein* and *Flag-[Y127F]β-synuclein* genes in the pMA-T vector were ordered from GENEART. The genes encode Flag-β-synuclein and Flag-[Y127F]β-synuclein proteins with the Flag tag attached at the N-terminus. These genes were then subcloned into the pcDNA3 mammalian cell expression vector via the *Eco*RI and *Bam*HI restriction sites. HEK293T cells were transfected with the *pcDNA3-CHK*, *pcDNA3-Flag-β-synuclein*, or *pcDNA3-Flag-[Y127F]β-synuclein* plasmid individually. To determine if CHK can induce tyrosine phosphorylation of Flag-β-synuclein or its mutant, HEK293T cells were cotransfected with *pcDNA3-CHK* and *pcDNA3-Flag-β-synuclein* plasmids or *pcDNA3-CHK*

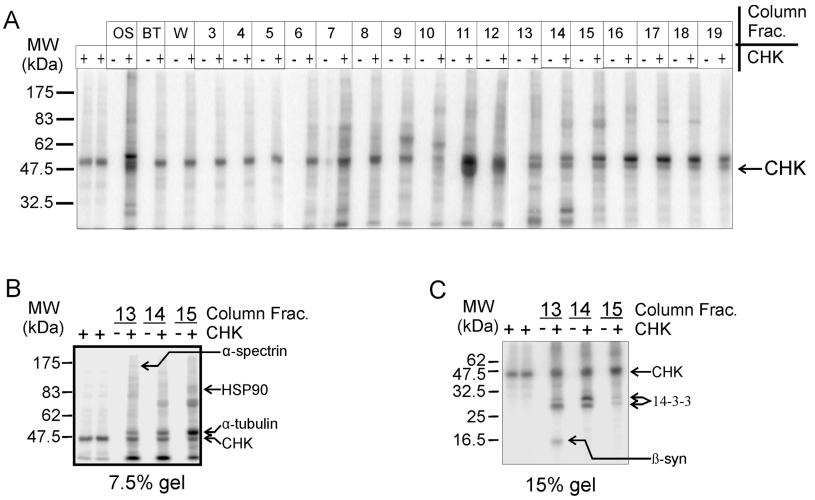

Figure 1. Identification of β-synuclein, α-tubulin, α-spectrin, 14-3-3, and Hsp90 as potential protein substrates of CHK. (A) Autoradiogram showing the profile of CHK-phosphorylated proteins in the Mono-Q anion exchange column fractions. Ten microliters from each fraction was heat-treated (60 °C for 15 min) prior to incubation with [γ-^{32}P]ATP at 30 °C for 5 min in the presence (+) and absence (−) of CHK (0.8 μM) in a total volume of 25 μL. Fractions 13−15 contained several proteins that were preferentially phosphorylated by CHK. (B and C) Autoradiograms showing preferential phosphorylation of proteins of different molecular sizes by CHK in the selected fractions (fractions 13−15) of the Mono-Q column step after SDS−PAGE in 7.5 and 15% polyacrylamide gels. The gel slices containing the CHK-phosphorylated proteins in the +CHK lane and the those in the corresponding locations in the −CHK lane were excised (SFigure 2 of the Supporting Information), digested with trypsin, and then analyzed by mass spectrometry. The proteins identified include β-synuclein, α-spectrin, α-tubulin, 14-3-3, Hsp90, and CHK (SFigure 2 and STable 1 of the Supporting Information). It is noteworthy that recombinant CHK can undergo autophosphorylation in vitro.[23] The radioactive band of ~50 kDa in the +CHK lanes corresponds to autophosphorylated CHK.

and *pcDNA3- Flag-[Y127F]β-synuclein* plasmids. Prior to addition to HEK293T cells, the plasmids were mixed with the FuGENE HD transfection kit (Roche) at a 1:4 ratio (1 μg of DNA to 6 μL of FuGENE HD, 0.5 μg per DNA construct per well) for 20 min at room temperature. The DNA−FuGENE HD complex solution was added dropwise to cells (~3 × 10^5 cells/well). The transfected HEK293T cells were maintained in DME medium containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 μg/mL penicillin and incubated in a humidified atmosphere of 10% CO_2 at 37 °C. The transfected cells were lysed 48 h after transfection with 120 μL of lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 25 μg/mL leupeptin, and 25 μg/mL aprotinin) per well at 4 °C. To prevent dephosphorylation of the recombinant β-synuclein by the endogenous protein tyrosine phosphatases, the transfected cells were treated with 30 μM freshly prepared pervanadate for 20 min prior to cell lysis. After clarification by centrifugation (13000*g* for 5 min at 4 °C), 33 μL of each lysate sample was then diluted with 17 μL of dilution buffer (lysis buffer and 5× SDS sample buffer at a 1:4 ratio). Samples were then heat-denatured at 95 °C for 5 min. Expression of recombinant CHK, Flag-β-synuclein, and Flag-[Y127F]β-synuclein was confirmed by Western blotting.

For immunoprecipitation, 7 μL of the anti-Flag (M_2) antibody-conjugated agarose beads (Sigma) was added to the cell lysate. After incubation for 3 h at 4 °C, the recombinant Flag-β-synuclein and its mutant were isolated from the crude cell lysate by immunoprecipitation. The immune complexes were washed three times in ice-cold wash buffer (0.5% Tween 20 in PBS) and centrifuged (3000*g* for 5 min at 4 °C). The immune complexes were resuspended in 50 μL of dilution buffer. Samples were then heat-denatured at 95 °C for 5 min prior to analysis by Western blotting.

Defining the Substrate Specificity Determinants of CHK by Positional Combinatorial Peptide Library Screening. The CHK phosphorylation site motif was determined using a miniaturized positional scanning peptide library method essentially as described previously.[27] The peptide library is similar in sequence to the one reported previously for analysis of protein Ser/Thr kinase specificity, except that a tyrosine residue replaces the serine-threonine residues at the phosphoacceptor position. The library consisted of 198 peptides having the general sequence G-A-X-X-X-X-X-Y-X-X-X-X-A-G-K-K(biotin), where K(biotin) was ε-biotinamidohexanoyl lysine. In each peptide, eight of the X positions were an approximately equimolar mixture of 18 proteogenic amino acids (all but tyrosine and cysteine), and the remaining position was fixed as one of the 20 unmodified amino acids, phosphothreonine, or phosphotyrosine. Peptides (50 μM) were incubated for 2 h at 30 °C in 1536-well plates with either 30 μg/mL (run 1) or 7.5 μg/mL (run 2) purified CHK-His_6 with 2 μL/well and 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 0.1% Tween 20, and 50 μM ATP (30 μCi/mL [γ-^{33}P]ATP). After incubation, 200 nL aliquots were spotted onto a streptavidin-coated membrane (Promega), which was washed as described previously,[28] dried, and exposed to a phosphor screen. Spot intensities were quantified using QuantityOne. Data were normalized as done previously so that the average value within a position is 1; values greater than 1 therefore indicate positively selected residues.

Synthesis and Purification of Peptides. All synthetic peptides were synthesized by Fmoc-based solid-phase peptide synthesis chemistry on a CEM Liberty automated microwave peptide synthesizer. At the completion of synthesis, peptides were cleaved from the resin by treatment with a TFA/triisopropylsilane/water mixture (95:2.5:2.5) and isolated by precipitation with cold diethyl ether. Crude peptides were purified on an Agilent Zorbax C_{18} reversed-phase HPLC column in 0.1% TFA/

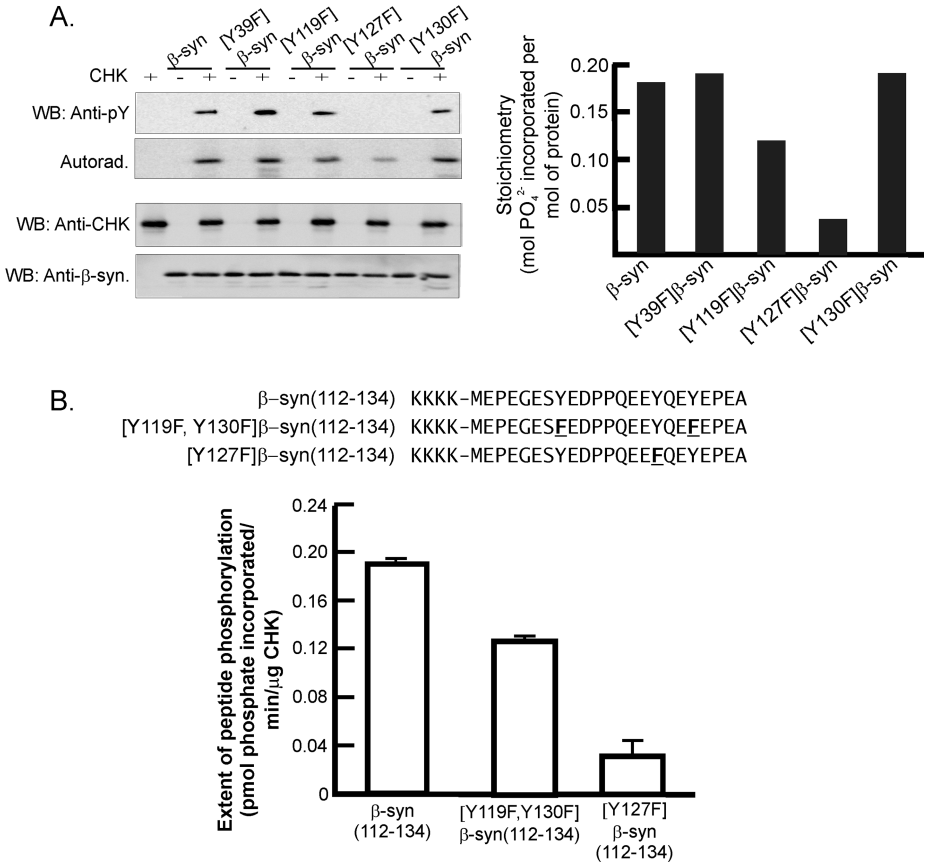

Figure 2. Identification of the tyrosine residues in β-synuclein phosphorylated by CHK. (A) Autoradiogram showing phosphorylation of recombinant β-synuclein (β-syn) and its mutants ([Y39F]β-syn, [Y119F]β-syn, [Y127F]β-syn, and [Y130F]β-syn) by CHK. Briefly, CHK (0.64−0.68 μM) was incubated with β-synuclein or its mutants (0.9−1.1 μM) and [γ-^{32}P]ATP at 30 °C for 30 min. The extent of phosphorylation of β-synuclein and its mutants was monitored by Western blotting with an anti-phosphotyrosine antibody and autoradiography (left). The gel slices corresponding to β-synuclein and its mutants were excised to determine the stoichiometry of phosphorylation (right). The experiment was repeated four times (n = 4), and each gave a similar pattern of difference in the extent of phosphorylation of β-synuclein and its mutants. The data from one of the repeat experiments are shown. (B) CHK phosphorylation of β-syn(112−134) derived from the segment corresponding to residues 112−134 of β-synuclein and its substitution analogues, [Y119F/Y130F]β-syn(112−134) and [Y127F]β-syn(112−134). The four lysines in the N-terminal region were added to facilitate solubility and binding to the P81 phosphocellulose paper squares used in the assay. The tyrosine residues replaced with phenylalanine in the analogues are underlined (top).

water buffer with a linear acetonitrile gradient. The molecular weight of all peptides was confirmed by electrospray mass spectrometry on an Agilent QTOF LC−MS instrument, and peptide purity (>95%) was confirmed by analytical reversed-phase HPLC.

RESULTS

Identification of β-Synuclein as a Potential CHK Substrate. As CHK was reported to reside predominantly in the cytoplasm in cultured cancer cells and brain cells,[15,29] we predicted that some of the physiological substrates of CHK are cytosolic proteins. Thus, we chose rat brain cytosolic extract to search for new CHK substrates. SFigure 1A of the Supporting Information depicts the scheme used to search for potential CHK substrates in rat brain cytosolic extract. Proteins in the extract were first partially separated by DEAE anion exchange column chromatography. Column fractions containing cellular proteins that were preferentially phosphorylated by recombinant CHK (SFigure 1B of the Supporting Information) were combined, dialyzed, and further purified by Mono-Q anion exchange column chromatography. As shown in Figure 1A, several prominent signals reflecting preferential phosphorylation of cellular proteins by CHK were detected in Mono-Q fractions 13−15, of which some were from proteins migrating near the dye front of a 10% polyacrylamide gel. We therefore separated proteins in these fractions with 7.5 and 15% polyacrylamide gels (Figure 1B,C and SFigure 2 of the Supporting Information). The bands corresponding to the CHK-phosphorylated proteins were excised and digested with trypsin. The digests were analyzed by nano-LC−MS/MS followed by MASCOT search analysis. The results showed that these protein bands contained β-synuclein, α-tubulin, α-spectrin, 14-3-3, or Hsp90 (Figure 1B,C and SFigure 2 and STable 1 of the Supporting Information). It is noteworthy that some of these protein bands were not well separated from other protein bands. Thus, it is possible that the proteins identified are just cellular proteins migrating very closely with the CHK-phosphorylated proteins via SDS−PAGE. Among them, the band containing β-synuclein appeared to be better separated from other protein bands (SFigure 2C of the Supporting Information). Furthermore, Western blot analysis using the anti-β-synuclein and anti-α-synuclein antibodies indicated the

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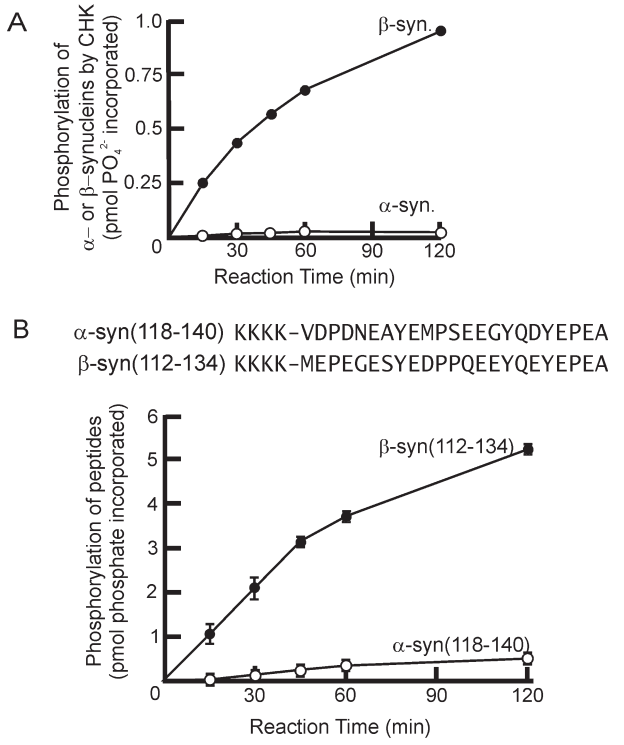

Figure 3. Comparison of the rates of CHK phosphorylation of α- and *β*-synucleins. (A) Time course of phosphorylation of α- and *β*-synucleins (0.56 *μ*M) by CHK (0.04 *μ*M). (B) Sequences (top) of the *β*-syn(112−134) and α-syn(118−140) peptides derived from the C-terminal region of α- and *β*-synucleins. The four lysines at the N-terminus of the peptides were added to improve solubility and to facilitate binding of the peptide to phosphocellulose P81 filter paper used in the kinase activity assay. Time course of phosphorylation (bottom) of *β*-syn(112−136) and α-syn(118−140) peptides by CHK. The reaction mixture contained CHK (0.11 *μ*M), *β*-syn(112−134) or α-syn(118−140) (20 *μ*M), and [*γ*-^{32}P]ATP at 30 °C in a final volume of 25 *μ*L.

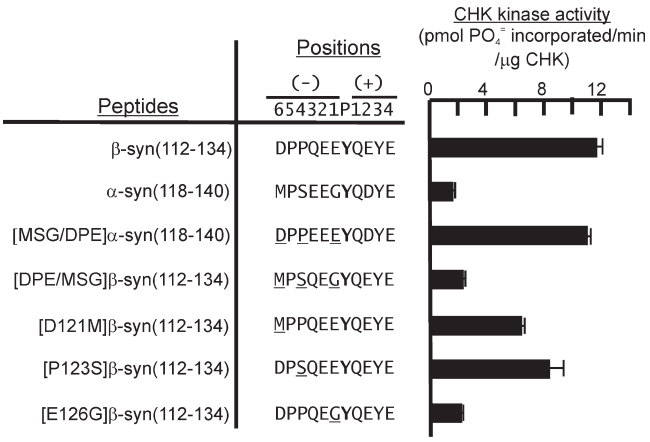

Figure 4. Comparison of the rates of phosphorylation of *β*-syn(112−134), α-syn(118−140), and their substitution analogues by CHK. CHK-His$_6$ (0.4 *μ*M) was incubated with each of the peptides (50 *μ*M), assay buffer, and [*γ*-^{32}P]ATP (50 *μ*M) at 30 °C for 15 min. The following sequences were used: *β*-syn(112−134), KKKK-MEPEGESYEDPPQEEYQEYEPEA; α-syn(118−140), KKKK-VDPDNEAYEMPSEEGYQDYEPEA; [MSG/DPE]α-syn(118−140), KKKK-VDPDNEAYEDPPEEEYQDYEPEA; [DPE/MSG]*β*-syn(112−134), KKKK-MEPEGESYEMPSQEGYQEYEPEA; [D121M]*β*-syn(112−134), KKKK-MEPEGESYEMPPQEEYQEYEPEA; [P123S]*β*-syn(112−134), KKKK-MEPEGESYEDPSQEEYQEYEPEA; [E126G]-*β*-syn(112−134), KKKK-MEPEGESYEDPPQEGYQEYEPEA. Only the residues from the P−6 to P+4 positions of the peptides are presented.

presence of *β*-synuclein but not α-synuclein in the protein band (data not shown). To further separate proteins in the *β*-synuclein-containing Mono-Q column fractions (fractions 13−15), we pooled these fractions and subjected them to hydroxyapatite column chromatography. A 16 kDa protein was found to be preferentially phosphorylated by CHK (data not shown). The band corresponding to this 16 kDa protein was excised, and its identity was confirmed to be *β*-synuclein by mass spectrometry (data not shown).

CHK Preferentially Phosphorylates Recombinant *β*-Synuclein at Tyrosine 127. To further verify *β*-synuclein as a preferential substrate of CHK, we examined if CHK can directly phosphorylate purified recombinant *β*-synuclein, 14-3-3*β*, and Hsp90 in vitro. SFigure 4A of the Supporting Information shows that CHK could directly phosphorylate all three protein substrates in vitro. Among them, CHK phosphorylated 14-3-3*β* at a negligible level; the rate of CHK phosphorylation of *β*-synuclein was ∼23-fold higher than that of hsp90 (SFigure 4B of the Supporting Information). Taken together, the results support *β*-synuclein as a preferential substrate of CHK.

The *β*-synuclein sequence contains four tyrosines, Y39, Y119, Y127, and Y130. To determine which of the four tyrosines are phosphorylated by CHK, we generated *β*-synuclein mutants with each of the tyrosines mutated to phenylalanine and compared the rates of their phosphorylation by CHK. As illustrated in Figure 2A, the Y39F mutation had no effect on *β*-synuclein phosphorylation by CHK, indicating that Y39 was not targeted by CHK. In contrast, the level of *β*-synuclein phosphorylation was reduced by mutation of any of the other three tyrosines, with the greatest reduction induced by the Y127F mutation. These results suggest that CHK can phosphorylate *β*-synuclein at Y119, Y127, and Y130. Among them, Y127 is the preferential target.

Because Y119, Y127, and Y130 reside in the C-terminal region, the results shown in Figure 2A imply that the C-terminal region of *β*-synuclein contains structural features directing CHK to preferentially phosphorylate Y127. To examine this, we synthesized three synthetic peptides derived from this region of *β*-synuclein and compared the efficiencies of their phosphorylation by CHK. These peptides include (i) *β*-syn(112−134), which contains all three tyrosines in this region; (ii) [Y119F/Y130F]*β*-syn(112−134), which contains Y127 as the only tyrosine; and (iii) [Y127F]*β*-syn(112−134), in which Y127 is replaced with phenylalanine (Figure 2B). Substitution of Y119 and Y130 with phenylalanine results in a 30% reduction in the rate of CHK phosphorylation, while the Y127F mutation caused an 84% reduction in the rate of CHK phosphorylation (Figure 2B). From these results, we conclude that the local structure surrounding Y127 in the C-terminal region of *β*-synuclein contains determinants directing its preferential phosphorylation by CHK.

Comparison of CHK Phosphorylation of α- and *β*-Synuclein Reveals α-Synuclein as a Poor CHK Substrate. Among the three members of the synuclein family, α-synuclein and *β*-synuclein show the highest degree of sequence homology

(~60% sequence identity). The amino acid sequences of α- and β-synucleins exhibit 33% sequence homology in their acidic C-terminal regions. For this reason, it is logical to examine whether α-synuclein is also phosphorylated by CHK. Figure 3A shows that the level of CHK phosphorylation of β-synuclein was significantly higher than that of α-synuclein. After a reaction time of 120 min, CHK catalyzed incorporation of ~0.9 pmol of phosphate to β-synuclein but only 0.024 pmol of phosphate to α-synuclein. Thus, despite both synucleins sharing considerable sequence homology, α-synuclein is a much poorer substrate for CHK than β-synuclein. More importantly, the data lend further support to the proposal that β-synuclein contains unique structural features directing CHK to preferentially phosphorylate Y127. Presumably, these structural features reside near Y127 in the β-synuclein sequence. This was confirmed by our demonstration that CHK phosphorylated the β-syn(112−134) peptide more efficiently (~10-fold) than the α-syn(117−140) peptide (Figure 3B).

D121, P123, and E126 in the β-Synuclein Sequence Contain Structural Features Directing Efficient Phosphorylation of Y127 by CHK. Comparison of the sequences of the C-terminal regions of α- and β-synucleins identified D121, P123, and E126 in the β-synuclein sequence and M127, S129, and G132 in the α-synuclein sequence as the dissimilar residues. To evaluate the role of D121, P123, and E126 in directing preferential phosphorylation of β-synuclein by CHK, five more β-synuclein peptide analogues were generated and the rates of their phosphorylation by CHK were compared (Figure 4). When G132, S129, and M127 of α-syn(118−140) were replaced with the corresponding residues in the β-synuclein sequence, the resultant [MSG/DFE]α-syn(118−140) peptide was phosphorylated by CHK with activity as high as that of the β-syn(112−134). E126, P123, and D121 of β-syn(112−134) were replaced with the corresponding residues in α-synuclein to generate the [DFG/MSG]β-syn(112−134) peptide. CHK phosphorylated this peptide with a rate lower (~20-fold) than that of the β-syn(112−143) peptide. These results confirm that D121, P123, and E126 in the β-synuclein sequence govern preferential phosphorylation of β-synuclein by CHK. To further define the role of each of these residues, the rates of CHK phosphorylation of single-residue substitution peptide analogues of β-syn(112−134) were compared. The results shown in Figure 4 indicate that the D121M, P123S, and E126G substitutions induce reductions in the efficiency of CHK phosphorylation of the β-syn(112−134) analogues. Taken together, our results suggest that E126, P123, and D121 participate in directing CHK to preferentially phosphorylate Y127.

To determine whether the secondary structure of these peptides has any input in the preferential phosphorylation of Y127 by CHK, we performed circular dichroism analyses to monitor the secondary structure contents of the SFK C-terminal peptide, α-syn(118−140), β-syn(112−134), and the peptide analogues. The CD spectra reveal the typical profiles of random coil conformation (SFigure 6 of the Supporting Information). Our data indicate that β-syn(112−134) does not need to adopt a specific secondary structure in aqueous solution. In spite of this finding, it is unclear if β-syn(112−134) adopts a specific secondary structure when it is bound to the CHK active site.

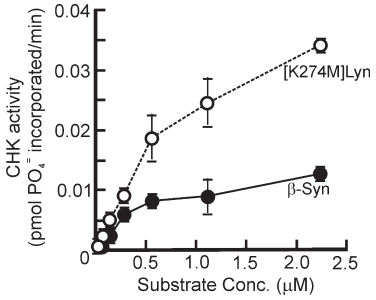

Figure 5. Comparison of the rates of CHK phosphorylation of β-synuclein and the [K274M]Lyn mutant. CHK (0.11 μM) was incubated at 30 °C for 30 min with varying concentrations (35 nM to 2.24 μM) of [K274M]Lyn or GST-β-synuclein. CHK activities at different concentrations of [K274M]Lyn and GST-β-synuclein are presented.

CHK Phosphorylates β-Synuclein and a Src-Family Kinase with Comparable Efficiencies. Because Src-family tyrosine kinases are physiological substrates of CHK, we compared the efficiencies of CHK phosphorylation of β-synuclein and the kinase-dead mutant of the Src-family kinase Lyn, [K274M]-Lyn. As this Lyn mutant lacks catalytic activity, its use as the CHK substrate eliminates the complication of additional tyrosine phosphorylation due to autophosphorylation. As shown in Figure 5, at low substrate concentrations (35−280 nM), CHK phosphorylated both proteins at similar rates. At higher substrate

Table 1. Kinetic Parameters of Phosphorylation of Four Peptide Substrates by CHK-His_6[a]

PEPTIDE SUBSTRATES	SEQUENCES	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
SFK C-terminal Peptide	KKDPEERPTFEYLQSFTATEPQ**Y**QPGENL	255.0 ± 7.8	0.13 ± 0.003	5.1×10^{-4}
β-syn(112-134)	KKKK-MEPEGESYEDPPQEEYE**Y**EPEA	253 ± 18.5	0.17 ± 0.01	6.7×10^{-4}
CHK-optimal peptide	KKK-GESFEDQDEGI**Y**WNVGPEA	103.1 ± 3.8	1.45 ± 0.05	140×10^{-4}
[D9A]CHK-optimal peptide	KKK-GESFE**A**QDEGI**Y**WNVGPEA	114.5 ± 5.6	1.27 ± 0.06	111×10^{-4}

[a] For the phosphorylation reactions, CHK-His_6 (0.2 μM) was used to phosphorylate the substrate peptides at 0−400 μM. The initial velocities of CHK-His_6 were plotted vs the substrate peptide concentration. The data points were transformed to generate Lineweaver−Burk plots (SFigure S6 of the Supporting Information), which yielded the kinetic parameters K_M and k_{cat}.

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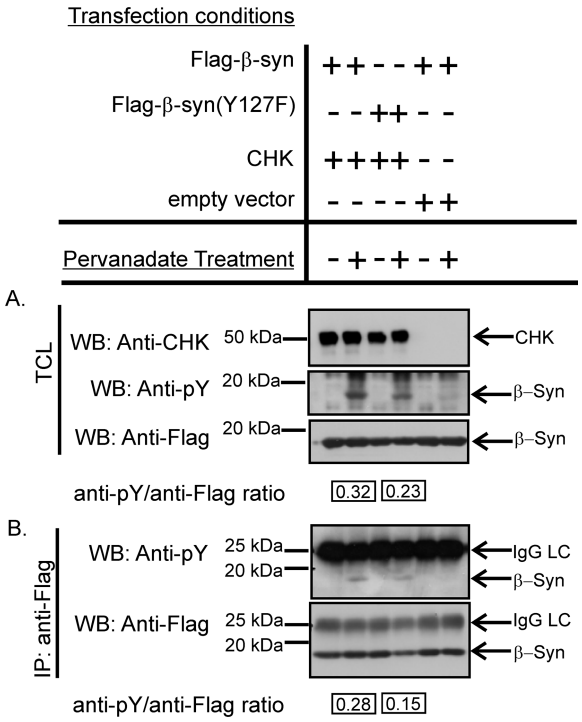


Figure 6. Demonstration of phosphorylation of β-synuclein by CHK in transfected HEK293T cells. Recombinant CHK, Flag-β-synuclein, and its mutant were expressed in HEK293T cells by transfection. The transfection conditions are listed above the panels. The transfected cells with and without treatment with pervanadate were lysed. (A) Western blot analyses of tyrosine phosphorylation of Flag-β-synuclein in total cell lysate (TCL) of the transfected cells using anti-phosphotyrosine (anti-pY), anti-Flag, and anti-CHK antibodies. (B) Western blot analysis of the anti-Flag immunoprecipitates from the TCL using anti-phosphotyrosine (anti-pY) and anti-Flag antibodies. IP denotes immunoprecipitation and WB Western blotting. The anti-pY and anti-Flag signals associated with the recombinant β-synuclein and its mutant on the Western blots were quantitated by densitometry. The ratios of anti-pY and anti-Flag signals in lanes 2 and 4 in the Western blots of panels A and B are shown.

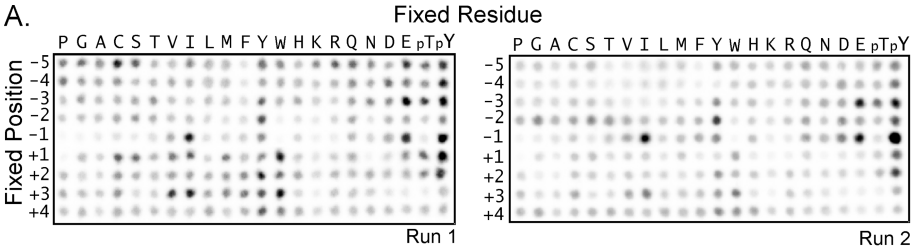
concentrations (560 nM to 2.24 μM), the rate of CHK phosphorylation of [K274M]Lyn was ~2.8-fold higher than that of CHK phosphorylation of β-synuclein. Nevertheless, the similar rates of CHK phosphorylation of both proteins at low substrate concentrations further confirm that β-synuclein contains structural determinants directing its efficient phosphorylation by CHK. To compare the contributions of determinants in the local structures around the phosphorylation sites in SFKs and β-synuclein to the efficiencies of their phosphorylation by CHK, we performed kinetic analyses of CHK phosphorylation of the SFK C-terminal peptide and β-syn(112−134). The kinetic data listed in Table 1 indicate that CHK phosphorylated both peptides with similar rates.

CHK Phosphorylates β-Synuclein in Transfected HEK293T Cells. To examine if CHK can phosphorylate β-synuclein in cells, we expressed recombinant CHK, Flag-β-synuclein, and Flag-[Y127F]β-synuclein in HEK293T cells. As shown in Figure 6, CHK co-expression induced tyrosine phosphorylation of both Flag-β-synuclein and Flag-[Y127F]β-synuclein only in cells pretreated with pervanadate to suppress the endogenous phosphatase activity. Furthermore, replacement of the Y127 in Flag-β-synuclein with phenylalanine reduced the level of CHK-induced phosphorylation. The results are in agreement with results of our in vitro studies shown in Figure 2 that show that CHK phosphorylates Y127 and one or more other tyrosines in β-synuclein. The requirement of pervanadate treatment for the detection of tyrosine phosphorylation of β-synuclein indicates that it is readily dephosphorylated by endogenous phosphatases in cells.

Determination of the Optimal Target Phosphorylation Sequence for CHK by Combinatorial Peptide Library Screening. The data presented in Figure 4 suggest that CHK recognizes the amino acid residues at positions P−6, P−4, and P−1 (positions N-terminal to the phosphorylation site in a protein/peptide substrate are numbered P−1, P−2, P−3, etc., while those C-terminal to the phosphorylation site are numbered P+1, P+2, P+3, etc.) of the β-syn(122−134) peptide as substrate specificity determinants. To more completely identify residues surrounding the site of phosphorylation that influence phosphorylation, we used purified recombinant CHK to screen a positional scanning peptide library previously used to characterize Eph- and Axl-family receptor tyrosine kinases.[30,31] Figure 7 shows the results of the screen. CHK appears to be most selective at position P−1, where it prefers both acidic (primarily E) and hydrophobic (primarily I) residues. CHK also exhibited strong preferences for E at position P−3 and for hydrophobic residues at position P+3. Weaker preferences were also seen at several positions, including hydrophobic residues at P+1 (quantitative preferences at all positions are summarized in Figure 7B). The results from the naïve peptide library screen agree well with the data obtained from the study of the α- and β-synuclein peptide analogues (Figure 4), which support the primary importance of a glutamate residue at position P−1. The β-synuclein sequence also includes a CHK-preferred residue (Y) at position P+3. The presence of CHK-preferred residues at positions P−1 and P+3 of the β-synuclein sequence likely contributes to preferential phosphorylation of rat brain β-synuclein by CHK in vitro. Thus, the data in Figures 4 and 7 together define the CHK-preferred residues in positions P−3 to P+3 as E-X-[Φ/E/D]-Y-Φ-X-Φ, where Y is the phosphorylation site and Φ represents hydrophobic amino acid residues.

A Synthetic Peptide Containing Selected Residues at Positions P−3 to P+3 Is an Efficient Peptide Substrate for CHK. On the basis of the results shown in Figures 4 and 7, the CHK-optimal peptide (KKK-GESFEDQDEGIYWNVGPEA) was designed. This peptide contains part of the β-syn(112−134) sequence and residues that are preferred by CHK at positions P−3 (E), P−1 (I), P+1 (W), and P+3 (V) defined by both the KESTREL and combinatorial peptide library studies. As shown in Table 1 and SFigure 6 of the Supporting Information, the CHK-optimal peptide was phosphorylated with an efficiency higher than those of β-syn(112−134) and the SFK-C-terminal peptide. CHK phosphorylated the CHK-optimal peptide with a slightly lower K_M and a k_{cat} (1.45 s^{-1}) approximately 10-fold higher than those of β-syn(112−134) and the SFK-C-terminal peptide. The data indicate that the CHK-optimal peptide contains determinants that facilitate binding to the active site of CHK and catalysis of the phosphorylation reaction.

Results of analyses of CHK phosphorylation of α-syn(118−140), β-syn(112−134), and their analogues reveal that D121 at position P−6 of β-syn(112−134) is recognized by CHK as a substrate specificity determinant. Because the combinatorial peptide library screen covers only residues P−5 to P+4, further



B.

Position	-5	-4	-3	-2	-1	P	+1	+2	+3	+4
Preferred Residues	Q(1.5) E(1.5)	D(2.1) E(1.6) Q(1.5)	E(3.5)	S(1.6)	I(3.8) E(3.7) D(1.8)		W(2.7) C(1.7) S(1.8) V(1.5) M(1.5)	W(1.5)	I(2.6) W(2.5) V(1.9) Y(1.8)	W(1.7)
optimal phosphorylation sequence	X	X	E	X	ϕ/E/D	Y	ϕ	X	ϕ	X
β-synuclein residues	P	P	Q	E	E	Y	Q	E	Y	E

Figure 7. Defining the CHK-optimal phosphorylation sequence via a positional scanning combinatorial peptide library. (A) CHK was used to phosphorylate a 198-component peptide library in which each peptide had the general sequence G-A-X-X-X-X-X-Y-X-X-X-X-A-G-K-K(biotin). Spot intensities reflect the extent of phosphorylation of peptides having the indicated residue fixed at the indicated position relative to the central tyrosine residue. The results from two runs performed on separate days and with different CHK-His_6 concentrations (30 μg/mL for run 1 and 7.5 μg/mL for run 2) are shown. (B) Summary of residues positively selected at each position. Values in parentheses show normalized quantified spot intensities. Only residues with selectivity values of ≥ 1.5 are shown. Note that the strong signal for peptides with fixed tyrosine residues independent of position is likely to be an artifact due to the presence of two phosphorylatable residues in those peptides.

analysis is needed to establish P−6 aspartate as a specificity determinant recognized by CHK. To this end, we generated the [D9A]CHK-optimal peptide to examine if and how substitution of the aspartate at the P−6 position with alanine affects CHK phosphorylation of the CHK-optimal peptide. Our analysis shows that the substitution resulted in a small increase in K_M and a slight reduction in k_{cat}. Thus, the aspartate at position P−6 plays only a minor role or no role in directing efficient phosphorylation of the CHK-optimal peptide by CHK. For this reason, we do not include the P−6 aspartate in the optimal substrate phosphorylation sequence (E-x-[Φ/E/D]-Y-Φ-x-Φ) of CHK.

DISCUSSION

Clinical Implications of β-Synuclein as a Potential Substrate of CHK. β-Synuclein is a cytoplasmic protein located predominantly in the presynaptic nerve terminals. Its exact physiological function remains unclear. Recently, two mutations of β-synuclein have been identified as risk factors for development of a Parkinson's disease-related neurodegenerative disease, dementia with Lewy bodies (DLB).[32] A valine to methionine substitution at position 70 (V70M) of β-synuclein was found in one sporadic case of DLB in Japan, while a proline to histidine substitution at position 123 (P123H) was identified in a DLB case in Seattle. Furthermore, transgenic mice overexpressing the [P123H]β-synuclein mutant develop progressive neurodegeneration.[33] The P123H mutation occurred at the conserved Pro123 in the highly conserved C-terminal region of β-synuclein. Relevant to this, our result showed that CHK specifically phosphorylated β-synuclein at Y127, which is only four residues from Pro123 in the C-terminal region. Thus, it will be worthwhile in future studies to establish if β-synuclein is a physiological substrate of CHK. If it is indeed a physiological substrate, studies to examine if and how tyrosine phosphorylation contributes to and/or modulates the neurotoxicity of [P123H]β-synuclein should be conducted.

How Does CHK Recognize Its Substrates? There are more than 20 tyrosine residues in a Src-family kinase such as c-Src, yet only the C-terminal regulatory tyrosine is selected by CHK as the phosphorylation site.[15] How might the C-terminal tail sequences of SFKs contribute to their selective phosphorylation by CHK? It is noteworthy that the CHK-optimal phosphorylation sequence (E-x-Φ/E/D-Y-Φ-x-Φ) shares little similarity with the conserved sequences EPQYQPGENL and EGQYQQQP found in the C-terminal tail of most Src-family kinases. We predict that the local structure around the C-terminal regulatory tyrosine of SFKs plays a minor role in directing CHK to recognize SFKs as the substrates. This implies that CHK employs predominantly the docking-based mechanism to recognize SFKs as its substrates. The mechanism involves binding of interaction domain(s) located distally from the active site of CHK to one or more distal docking motifs in SFKs. A full understanding of the structural basis underpinning CHK recognition of SFKs requires identification of the interaction domain(s) in CHK and the distal docking motifs in SFKs.

The crystal structure of CSK/c-Src determined by Levinson et al. provides insights into how CHK recognizes SFKs.[9] In this structure, CSK and c-Src make extensive ionic and hydrophobic interactions at an interface formed by five basic residues residing in helix αD and the αF−αG loop of the CSK kinase domain and

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a motif comprising residues 504−525 near the C-terminal regulatory region of the c-Src kinase domain (reviewed in ref 10). Using surface plasmon resonance, Levinson et al. demonstrated direct binding of CSK to c-Src with the dissociation constant (K_D) for the CSK−c-Src complex ranging between 4 and 64 μM.[9] The crystal structure suggests that the binding of CSK to c-Src is mainly attributed to the aforementioned interactions at the CSK−c-Src interface. Because the sequences of helix αD and the αF−αG loop of CSK and CHK are highly conserved, it is logical to predict that the homologous motifs in helix αD and the αF−αG loop of CHK are involved in binding to the motif containing residues 504−525 of c-Src.

We have previously demonstrated that CHK but not CSK can tightly bind to the SFK kinase domain to form a stable CHK−SFK complex that could be isolated by co-immunoprecipitation.[15,24] We recently used surface plasmon resonance to quantitatively compare the kinetics of CHK and CSK binding to SFKs. Our results showed that CHK binds to a SFK with a dissociation constant 2−3 orders of magnitude lower than that of CSK binding to c-Src (unpublished observations), confirming our previous observation that CHK binds to SFKs with a much higher affinity than CSK. On the basis of these observations, we hypothesized that an unknown motif as well as helix αD and the αF−αG loop are involved in mediating CHK to tightly bind to SFKs and directing the CHK active site to selectively phosphorylate the C-terminal regulatory tyrosine of SFKs. Defining this unknown motif in CHK and the distal docking determinant in SFKs targeted by this CHK motif is the major focus of our current investigation.

For protein substrates with phosphorylation sites exhibiting conformity to the CHK-optimal phosphorylation sequence, their recognition by CHK is governed by interactions between the active site and the phosphorylation sites as well as those between the CHK interaction domains and the distal docking motifs of the protein substrates. We predict that CHK first binds to the distal docking motifs of a protein substrate. Once bound, determinants near the phosphorylation site direct the CHK active site to phosphorylate the protein.

Nevertheless, the discovery of the optimal phosphorylation sequence of CHK reported here is the first step toward elucidation of the structural basis of substrate recognition by CHK. The next step should be definition of the distal docking motifs recognized by CHK (termed the CHK-docking motifs) in SFKs and other protein substrates. Once they have been defined, knowledge of the CHK optimal phosphorylation sequence and the CHK-docking motifs will improve the reliability of the search for potential physiological substrates of CHK by informatics approaches.

We recently reported the procedures for high-yield expression and purification of recombinant CHK-His_6. Using size exclusion chromatography and sedimentation velocity studies, we found that CHK-His_6 is monomeric in aqueous solution.[23] Thus, our recombinant CHK-His_6 is suitable for structural analysis by X-ray crystallography. We have initiated crystallization trials of CHK-His_6 with and without the CHK-optimal peptide. Elucidation of the crystal structure of CHK-His_6 in a complex with the peptide will reveal the structural basis of recognition of the CHK-optimal phosphorylation sequence by the CHK active site.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary procedures, STable 1, and SFigures 1−6. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Ubersax, J. A., and Ferrell, J. E., Jr. (2007) Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol. 8*, 530–541.

(2) Goldberg, G. S., Alexander, D. B., Pellicena, P., Zhang, Z. Y., Tsuda, H., and Miller, W. T. (2003) Src phosphorylates Cas on tyrosine 253 to promote migration of transformed cells. *J. Biol. Chem. 278*, 46533–46540.

(3) Pellicena, P., and Miller, W. T. (2001) Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions. *J. Biol. Chem. 276*, 28190–28196.

(4) Songyang, Z., and Cantley, L. C. (1995) Recognition and specificity in protein tyrosine kinase-mediated signalling. *Trends Biochem. Sci. 20*, 470–475.

(5) Cheng, H.-C., Chong, Y. P., Ia, K. K., Tan, O., and Mulhern, T. D. (2006) CSK-homologous kinase. *UCSD-Nature Molecule Pages*, doi: 10.1038/mp.a000705.01.

(6) Chong, Y. P., Ia, K. K., Mulhern, T. D., and Cheng, H. C. (2005) Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. *Biochim. Biophys. Acta 1754*, 210–220.

(7) Chong, Y. P., Mulhern, T. D., and Cheng, H. C. (2005) C-Terminal Src kinase (CSK) and CSK-homologous kinase (CHK): Endogenous negative regulators of Src-family protein kinases. *Growth Factors 23*, 233–244.

(8) Ingley, E. (2008) Src-family kinases: regulation of their activity level and identification of new pathways. *Biochim. Biophys. Act. 1784*, 56–65.

(9) Levinson, N. M., Seeliger, M. A., Cole, P. A., and Kuriyan, J. (2008) Structural basis for the recognition of c-Src by its inactivator Csk. *Cell 134*, 124–134.

(10) Ia, K. K., Mills, R. D., Hossain, M. I., Chan, K.-C., Jarasrassamee, B., Jorissen, R. N., and Cheng, H.-C. (2010) Structural elements and allosteric mechanisms governing regulation and catalysis of Csk-family kinases and their inhibition of Src-family kinases. *Growth Factors 28*, 329–350.

(11) Kuo, S. S., Armanini, M. P., Phillips, H. S., and Caras, I. W. (1997) Csk and BatK show opposite temporal expression in the rat CNS: Consistent with its late expression in development, BatK induces differentiation of PC12 cells. *Eur. J. Neurosci. 9*, 2383–2393.

(12) Zagozdzon, R., Kaminski, R., Fu, Y., Fu, W., Bougeret, C., and Avraham, H. K. (2006) Csk homologous kinase (CHK), unlike Csk, enhances MAPK activation via Ras-mediated signaling in a Src-independent manner. *Cell. Signalling 18*, 871–881.

(13) Mitsuhashi, H., Futai, E., Sasagawa, N., Hayashi, Y., Nishino, I., and Ishiura, S. (2008) Csk-homologous kinase interacts with SHPS-1 and enhances neurite outgrowth of PC12 cells. *J. Neurochem. 105*, 101–112.

(14) Hamaguchi, I., Yamaguchi, N., Suda, J., Iwama, A., Hirao, A., Hashiyama, M., Aizawa, S., and Suda, T. (1996) Analysis of CSK homologous kinase (CHK/HYL) in hematopoiesis by utilizing gene knockout mice. *Biochem. Biophys. Res. Commun. 224*, 172–179.

(15) Chong, Y. P., Mulhern, T. D., Zhu, H. J., Fujita, D. J., Bjorge, J. D., Tantiongco, J. P., Sotirellis, N., Lio, D. S., Scholz, G., and Cheng, H. C. (2004) A novel non-catalytic mechanism employed by the C-terminal Src-homologous kinase to inhibit Src-family kinase activity. *J. Biol. Chem. 279*, 20752–20766.

(16) Cohen, P., and Knebel, A. (2006) KESTREL: A powerful method for identifying the physiological substrates of protein kinases. *Biochem. J. 393*, 1–6.

(17) Knebel, A., Morrice, N., and Cohen, P. (2001) A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38δ. *EMBO J. 20*, 4360–4369.

(18) Jaleel, M., Nichols, R. J., Deak, M., Campbell, D. G., Gillardon, F., Knebel, A., and Alessi, D. R. (2007) LRRK2 phosphorylates moesin at threonine-558: Characterization of how Parkinson's disease mutants affect kinase activity. *Biochem. J. 405*, 307–317.

(19) Murray, J. T., Campbell, D. G., Morrice, N., Auld, G. C., Shpiro, N., Marquez, R., Peggie, M., Bain, J., Bloomberg, G. B., Grahammer, F., Lang, F., Wulff, P., Kuhl, D., and Cohen, P. (2004) Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem. J. 384*, 477–488.

(20) Murray, J. T., Campbell, D. G., Peggie, M., Mora, A., and Cohen, P. (2004) Identification of filamin C as a new physiological substrate of PKBα using KESTREL. *Biochem. J. 384*, 489–494.

(21) Peng, C., Knebel, A., Morrice, N. A., Li, X., Barringer, K., Li, J., Jakes, S., Werneburg, B., and Wang, L. (2007) Pim kinase substrate identification and specificity. *J. Biochem. 141*, 353–362.

(22) Pham, C. L., Leong, S. L., Ali, F. E., Kenche, V. B., Hill, A. F., Gras, S. L., Barnham, K. J., and Cappai, R. (2009) Dopamine and the dopamine oxidation product 5,6-dihydroxylindole promote distinct on-pathway and off-pathway aggregation of α-synuclein in a pH-dependent manner. *J. Mol. Biol. 387*, 771–785.

(23) Chan, K. C., Lio, D. S., Dobson, R. C. J., Jarasrassamee, B., Hossain, M. I., Roslee, A. K., Ia, K. K., Perugini, M. A., and Cheng, H.-C. (2010) Development of the Procedures for High Yield Expression and Rapid Purification of Active Recombinant Csk-Homologous Kinase (CHK): Comparison of the catalytic activities of CHK and CSK. *Protein Expression Purif. 74*, 139–147.

(24) Chong, Y. P., Chan, A. S., Chan, K. C., Williamson, N. A., Lerner, E. C., Smithgall, T. E., Bjorge, J. D., Fujita, D. J., Purcell, A. W., Scholz, G., Mulhern, T. D., and Cheng, H. C. (2006) C-Terminal Src kinase-homologous kinase (CHK), a unique inhibitor inactivating multiple active conformations of Src family tyrosine kinases. *J. Biol. Chem. 281*, 32988–32999.

(25) Culvenor, J. G., McLean, C. A., Cutt, S., Campbell, B. C., Maher, F., Jakala, P., Hartmann, T., Beyreuther, K., Masters, C. L., and Li, Q. X. (1999) Non-Aβ component of Alzheimer's disease amyloid (NAC) revisited. NAC and α-synuclein are not associated with Aβ amyloid. *Am. J. Pathol. 155*, 1173–1181.

(26) Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis 20*, 3551–3567.

(27) Mok, J., Kim, P. M., Lam, H. Y., Piccirillo, S., Zhou, X., Jeschke, G. R., Sheridan, D. L., Parker, S. A., Desai, V., Jwa, M., Cameroni, E., Niu, H., Good, M., Remenyi, A., Ma, J. L., Sheu, Y. J., Sassi, H. E., Sopko, R., Chan, C. S., De Virgilio, C., Hollingsworth, N. M., Lim, W. A., Stern, D. F., Stillman, B., Andrews, B. J., Gerstein, M. B., Snyder, M., and Turk, B. E. (2010) Deciphering protein kinase specificity through large-scale analysis of yeast phosphorylation site motifs. *Sci. Signaling 3*, ra 12.

(28) Hutti, J. E., Jarrell, E. T., Chang, J. D., Abbott, D. W., Storz, P., Toker, A., Cantley, L. C., and Turk, B. E. (2004) A rapid method for determining protein kinase phosphorylation specificity. *Nat. Methods 1*, 27–29.

(29) Nakayama, Y., Kawana, A., Igarashi, A., and Yamaguchi, N. (2006) Involvement of the N-terminal unique domain of Chk tyrosine kinase in Chk-induced tyrosine phosphorylation in the nucleus. *Exp. Cell Res. 312*, 2252–2263.

(30) Davis, T. L., Walker, J. R., Allali-Hassani, A., Parker, S. A., Turk, B. E., and Dhe-Paganon, S. (2009) Structural recognition of an optimized substrate for the ephrin family of receptor tyrosine kinases. *FEBS J. 276*, 4395–4404.

(31) Huang, X., Finerty, P., Jr., Walker, J. R., Butler-Cole, C., Vedadi, M., Schapira, M., Parker, S. A., Turk, B. E., Thompson, D. A., and Dhe-Paganon, S. (2009) Structural insights into the inhibited states of the Mer receptor tyrosine kinase. *J. Struct. Biol. 165*, 88–96.

(32) Ohtake, H., Limprasert, P., Fan, Y., Onodera, O., Kakita, A., Takahashi, H., Bonner, L. T., Tsuang, D. W., Murray, I. V., Lee, V. M., Trojanowski, J. Q., Ishikawa, A., Idezuka, J., Murata, M., Toda, T., Bird, T. D., Leverenz, J. B., Tsuji, S., and La Spada, A. R. (2004) β-Synuclein gene alterations in dementia with Lewy bodies. *Neurology 63*, 805–811.

(33) Fujita, M., Sugama, S., Sekiyama, K., Sekigawa, A., Tsukui, T., Nakai, M., Waragai, M., Takenouchi, T., Takamatsu, Y., Wei, J., Rockenstein, E., Laspada, A. R., Masliah, E., Inoue, S., and Hashimoto, M. (2010) A beta-synuclein mutation linked to dementia produces neurodegeneration when expressed in mouse brain. *Nat. Commun. 1*, 110.