

AD \_\_\_\_\_

Award Number: DAMD17-01-1-0481

TITLE: The Structural Basis for the Role of CHK as a Tumor  
Suppressor Protein in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Jerome E. Groopman, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center  
Boston, Massachusetts 02215-5399

REPORT DATE: May 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> May 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Final (1 May 01-30 Apr 03)	
<b>4. TITLE AND SUBTITLE</b> The Structural Basis for the Role of CHK as a Tumor Suppressor Protein in Human Breast Cancer			<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0481	
<b>6. AUTHOR(S)</b> Jerome E. Groopman, M.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Beth Israel Deaconess Medical Center Boston, Massachusetts 02215-5399  E-Mail: jgroopma@bidmc.harvard.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>		20040116 011		
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  <p><b>Purpose:</b> To obtain structural information on CHK, an important anti-oncogene in breast cancer.</p> <p><b>Scope:</b> A major clinical determinant of breast cancer is the presence in the cancer tumor cells of a protein called the Csk homologous kinase (CHK). CHK recognizes the tail of the breast cancer oncogene ErbB-2/neu receptor via its own SH2 domain and deactivates the bound Src kinases by phosphorylation. This deactivates the proliferative signaling pathways switched on by ErbB-2/neu. To determine how CHK functions at the molecular level, crystals of the cloned and purified, recombinant CHK SH2 domain will be obtained for x-ray diffraction studies from which we can determine its precise, three-dimensional molecular structure. We will also use NMR spectroscopy should we not obtain adequate crystals.</p> <p><b>Major Findings:</b> We obtained structural information from partial crystals of CHK and used NMR spectroscopy to identify binding interactions between CHK and the tail of the ErbB-2/neu receptor.</p> <p><b>Up-To-Date:</b> By identifying the determinants in the SH2 domain of CHK, structural information is now available to help in the design of novel therapeutics for breast cancer.</p>				
<b>14. SUBJECT TERMS</b> CHK, Breast Cancer			<b>15. NUMBER OF PAGES</b> 16	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

## Table of Contents

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>5</b>
<b>Key Research Accomplishments.....</b>	<b>5</b>
<b>Reportable Outcomes.....</b>	<b>6</b>
<b>Conclusions.....</b>	<b>6</b>
<b>References.....</b>	<b>7</b>
<b>Appendices.....</b>	<b>7</b>

PI: Jerome Groopman, M.D.

Award Period: May 1, 2001 – April 30, 2003

Award Number: DAMD17-01-1-0481

Title: The Structural Basis for the Role of CHK as a Tumor Suppressor Protein in Human Breast Cancer

**Abstract:**

**Purpose:** To obtain structural information on CHK, an important anti-oncogene in breast cancer.

**Scope:** A major clinical determinant of breast cancer is the presence in the cancer tumor cells of a protein called the Csk homologous kinase (CHK). CHK recognizes the tail of the breast cancer oncogene ErbB-2/neu receptor via its own SH2 domain and deactivates the bound Src kinases by phosphorylation. This deactivates the proliferative signaling pathways switched on by ErbB-2/neu. To determine how CHK functions at the molecular level, crystals of the cloned and purified, recombinant CHK SH2 domain will be obtained for x-ray diffraction studies from which we can determine its precise, three-dimensional molecular structure. We will also use NMR spectroscopy should we not obtain adequate crystals.

**Major Findings:** We obtained structural information from partial crystals of CHK and used NMR spectroscopy to identify binding interactions between CHK and the tail of the ErbB-2/neu receptor.

**Up-To-Date:** By identifying the determinants in the SH2 domain of CHK, structural information is now available to help in the design of novel therapeutics for breast cancer.

**Introduction:**

The Csk homologous kinase (CHK) acts as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src kinase activity. In human breast cancer tumors, CHK is over-expressed. Importantly, the CHK tyrosine kinase is not over-expressed in normal breast tissue. This provides a potential target for breast cancer therapy, in that, treatments which would augment CHK activity and thereby inhibit ErbB-2/neu-mediated signaling pathways could result in reduced growth and spread of the tumor. The interaction between the

CHK SH2 domain and Tyr(P)<sup>1248</sup> of the ErbB-2/neu receptor is specific and critical for CHK function.

The purpose of the project was to determine the structure of CHK and its interaction with the ErbB-2/neu receptor so as to provide information that could lead to novel therapeutics for breast cancer.

### **Body:**

A series of experiments were conducted during the period of support to obtain high quality crystals of CHK and study interactions of CHK with the ErbB-2/neu receptor. Two different expression systems were utilized. The first was a bacterial system, *E. Coli.* with a pGEX vector. The second was a novel organism, *Pichia pastoris.*

The proteins expressed from each of these two systems were then subjected to various in vitro conditions of temperature and salt incubation in order to obtain crystals. In addition, when our experiments did not succeed in co-crystallizing the SH2 domain of CHK with several synthetic peptide analogs of the ErbB-2/neu receptor, we constructed three CHK SH2 domain binding mutants by cloning them into the pGEX2T vector using the restriction endonuclease sites BamHI and EcoRI. Point mutations were generated by PCR using the QuikChange site-directed mutagenesis system, and mutants were verified by sequencing. The GST-fused CHK SH2 domains were expressed in bacteria, purified on Sepharose 6B columns and then studied with regard to interactions with synthetic peptides containing Tyr (P)<sup>1248</sup> of ErbB-2, ENPEpYLGDV, synthesized using solid phase Fmoc based peptide synthesis with an acetylated N-terminus and amidated C-terminus. All peptides were purified by C<sub>18</sub> reverse-phase high performance liquid chromatography and identities were confirmed using matrix-assisted laser desorption ionization mass spectroscopy. Labeled SH2 mutants with <sup>15</sup>N, <sup>13</sup>C were obtained by growing transformed bacteria in minimal medium containing <sup>15</sup>NH<sub>4</sub>Cl and <sup>13</sup>C-labeled glucose. Purified proteins were then exchanged into the final NMR sample buffer containing 50 mM phosphate (pH 7.5) 50 mM NaCl, 1 mM EDTA and 2 mM perdeuterated dithiothreitol. NMR experiments were performed on Bruker AMX 500-MHz and Avance 600-MHz spectrometers. In addition, the effects of mutants on ErbB-2/neu signaling were studied by transfection into several breast cancer cell lines including MCF-7 (normal level of ErbB-2/neu protein expression), T47D (moderate level over-expression), and BT474 (high level over-expression). All three cell lines were obtained from American Type Culture Collection.

### **Key Research Accomplishments**

- Expressed different truncated variants of the SH2 domain of human CSK homologous kinase (CHK) from a pGEX vector, in *E. Coli.*
- Each of the variants was purified by affinity chromatography and subjected to preliminary crystallization trials using Hampton screens.

- One of the variants yielded preliminary crystals which diffracted to a resolution of 2.5 Å.
- The crystal structure of the SH2 domain of CHK was solved at a resolution of 2.5 Å by molecular replacement method and deposited in the data bank (PDB ID: 1JWO).
- Attempts to co-crystallize the SH2 domain with several synthetic peptide analogs of ErbB-2/neu receptor did **not** yield diffractable crystals.
- The kinase domain of CHK was expressed and affinity purified to ~1mg per liter culture from *Pichia pastoris*.
- Attempts to express full length and some truncated variants of CHK from *Pichia pastoris* were unsuccessful.
- We successfully constructed three CHK SH2 domain binding mutants using the methods described above: G129R (enhanced binding); R147K (inhibited binding), and R147A (disrupted binding).
- NMR spectra for the domains of each mutant construct were used to evaluate their interaction with the Tyr (P)<sup>1248</sup> containing ErbB-2/neu peptide. NMR analysis revealed that the enhanced binding mutant had the same chemical shift changes at the same residues as the wild type CHK.
- Studies in breast cancer cell systems of the mutant that had enhanced binding demonstrated inhibition of Heregulin-stimulated Src kinase activity.
- Heregulin-stimulated Src kinase activity was markedly increased by the R147K mutant that inhibited binding and by the R147A mutant that disrupted binding.

#### **Reportable Outcomes:**

- The preliminary crystal structure of CHK at a resolution of 2.5 Å was deposited in the data bank (PDB ID: 1JWO). Other researchers in the field of breast cancer have access to these data.
- NMR studies with the SH2 domain mutants of CHK demonstrated that the specific interaction of this CHK domain and the receptor accounts for the growth inhibitory properties of CHK. Identification of the binding domain on the ErbB-2/neu receptor with CHK will allow future research to focus on this area for drug design in breast cancer.

#### **Conclusions:**

Considerable work was conducted under this support with both successes and failures. The successes included obtaining initial structural information on the CHK SH2 domain using x-

ray diffraction techniques and on the binding interaction of the CHK SH2 domain with the tail of the ErbB-2/neu receptor using NMR techniques. The failures included the inability to get robust expression of full-length CHK and to obtain crystals of sufficient quality that interacted with the tail of the receptor. Because of this latter obstacle, the alternative strategy was utilized, specifically NMR spectroscopy, that allowed conclusive mapping of the interaction between the CHK SH2 and the receptor. Both successes and failures provide a foundation upon which future research can be conducted against this promising target for the development of therapeutics that inhibit breast cancer growth and spread.

References: None.

Appendix: Addendum to report

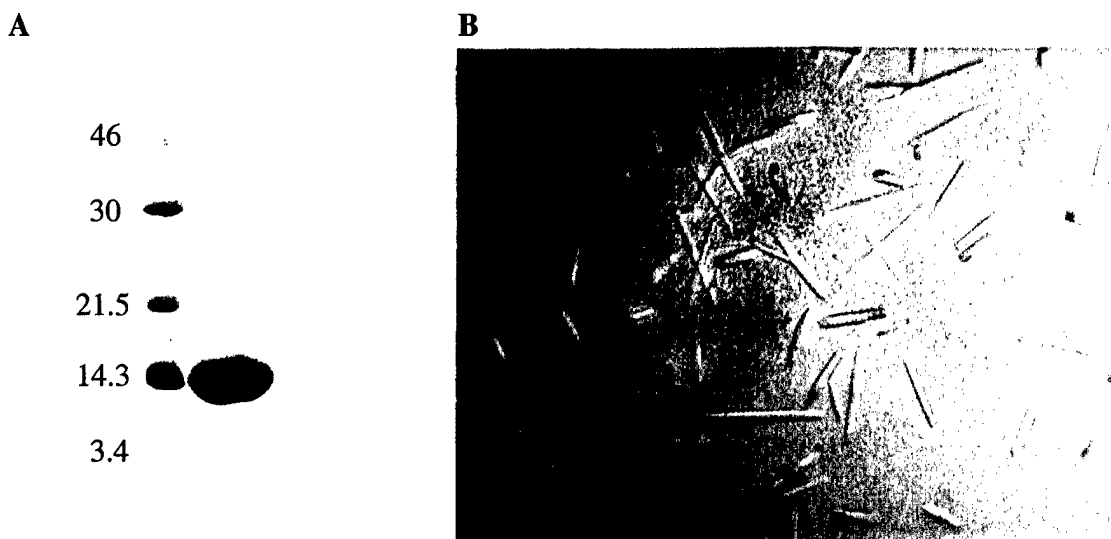
List of Personnel:

Anuradha Balasubramanian, Ph.D.  
Heather Lane, B.A.  
Gordon Webster, Ph.D.

*The Structural Basis for the Role of CHK as a Tumor Suppressor Protein in Human Breast Cancer*

**ADDENDUM: Detailed methods and data**

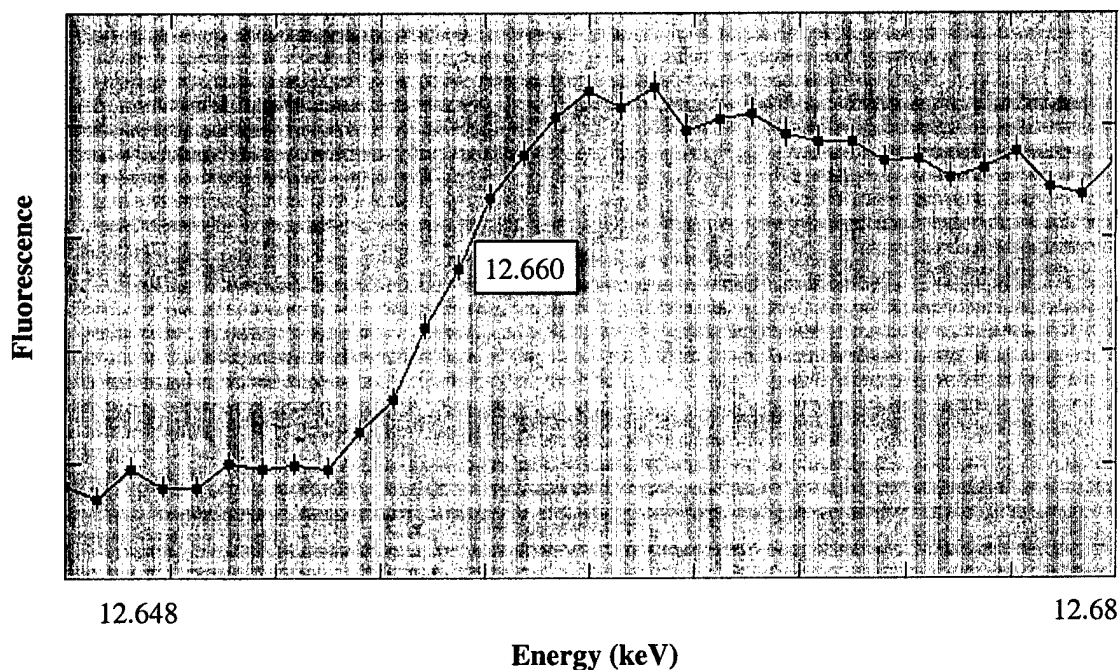
**Production of recombinant CHK-SH2 domain:** We produced large amounts of recombinant CHK-SH2 domain as fusion with Glutathione S-transferase (GST) in *E. coli* BL21 (DE3) cells. Four-liter bacterial cultures were grown at 37° C, until an OD<sub>600</sub> of 0.7 was reached. Subsequently, IPTG was added to a final concentration of 1mM in order to induce the expression of GST-CHK-SH2 protein, then cells were incubated for a further 2 hours. The cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C. The cell pellets were resuspended in 10ml of ice-cold 1X PBS after which the cells were lysed by use of Stansted cell disrupter. Triton X-100 was added to the cell lysate to give a final volume of 1% which was mixed for 30 minutes at room temperature. Following incubation, the cell debris was pelleted by centrifugation at 10,000 rpm for 10 minutes at 40°C. The supernatant was incubated with Glutathione Sepharose 4B beads (Pharmacia) for one hour at room temperature with constant mixing. Following incubation, the beads were washed 3 times with 1X PBS to remove unbound protein, resuspended in 2 ml 1X PBS, and then used to cleave the recombinant CHK-SH2 from the bound GST at a thrombin site in the pGEX-2T vector (Pharmacia). Thrombin cleavage reactions were performed for 18 hours at room temperature using restriction grade thrombin (Novagen), as described by the manufacturer. Reactions were terminated by addition of 50 ul of 10mM PMSF. The tubes were then centrifuged for 5 minutes at 4,000 rpm and the purity of the cleaved protein in the supernatant was monitored by SDS-PAGE. Purified CHK-SH2 protein was dialyzed against 20mM MES pH 6.0, 5 mM DTT for 4 hours at 4°C, followed by concentration to 15 mg/ml using a Centriprep-10 concentrator (Amicon). Using this procedure, 6 mg of SH2 protein per liter of culture could be purified to more than 95% homogeneity, as judged from SDS-PAGE gels (Fig. 1A).



**Figure 1A:** SDS-PAGE analysis of the CHK-SH2 domain produced in *E. coli* BL21 cells as a fusion protein with GST, and cleaved from GST with thrombin (see text for details). The gel was stained with Coomassie Blue. Lane 1, protein markers in kDa. Lane 2, purified recombinant CHK-SH2.

**Figure 1B:** Crystals of the CHK-SH2 domain grown by the hanging drop vapor diffusion method.

**Crystallization of recombinant CHK-SH2 domain:** We used the hanging drop vapor diffusion method and the expanded sparse matrix crystallization method (*Ducruix A, Giegé R: Crystallization of nucleic acids and proteins, a practical approach, IRP press at Oxford University Press, 1992*) at 18°C and 4°C, respectively. Native crystals of the SH2 domain of CHK were grown with dimensions up to 1.0 mm x 0.05 mm x 0.05 mm (Fig. 1B). Diffraction data were collected from a native crystal of the CHK-SH2 domain and a good quality data set obtained, 100% complete to 2.5 Å with a merging R-factor of 8.9% for all reflections. The native protein crystallized in space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> and had unit cell dimensions a=49.5, b=66.9, and c=80.7.



**Figure 2:** The X-ray fluorescence spectrum from a single crystal of SeMet-substituted CHK-SH2 recorded at CHESS. The point of inflexion on the curve at 12.660 keV is characteristic of the presence of selenium, and corresponds to the combined anomalous scattering of the substituted SeMet residues within the protein.

## NMR Studies

*Peptide synthesis and purification.* A peptide containing phosphotyrosine<sup>1248</sup> of ErbB-2, ENPEpYLGLDV, was synthesized using solid phase FMOC-based peptide synthesis with an acetylated N-terminus and amidated C-terminus (Tufts Core Facility, Boston, MA). All peptides were purified by C<sub>18</sub> reverse-phase high performance liquid chromatography (HPLC), and identities were confirmed using matrix-assisted laser desorption ionization (MALDI) mass spectroscopy.

*Construction and Purification of the CHK-SH2 domain.* The CHK-SH2 domain constructs (residues 116-217: G129R, R147A, R147K and wild type) were subcloned into a pGEX2T vector using the restriction endonuclease sites BamHI and EcoRI. Point mutations were generated by PCR using the QuickChange site-directed mutagenesis system (Stratagene) according to the manufacturer's instructions. Mutants were verified by sequencing. At least three independently generated mutants were tested for each construct. The GST-fused CHK-SH2 domains were expressed in bacteria, BL21 DE3 cells, and purified following published procedures. (Zrihan-Licht, S., Lim, J., Keydar, I., Sliwowski, M. X., Groopman, J. E., and Avraham, H. (1997) *J Biol Chem* 272, 1856-63., Zrihan-Licht, S., Deng, B., Yarden, Y., McShan, G., Keydar, I., and Avraham, H. (1998) *J Biol Chem* 273, 4065-72.)

Isolated SH2 domains were generated by thrombin cleavage, followed by purification on a Benzamidine Sepharose 6B column (Pharmacia). Approximately 10 mg of protein from all constructs were purified from 1 L growths in rich media (LB) and 5 mg from growth in minimal media.

*NMR spectroscopy.* [<sup>15</sup>N, <sup>13</sup>C] double-labeled protein samples or [<sup>15</sup>N] labeled samples were obtained by growing the transformed bacteria in minimal medium containing <sup>15</sup>NH<sub>4</sub>Cl and <sup>13</sup>C-labeled glucose or <sup>15</sup>NH<sub>4</sub>Cl and unlabeled glucose as the sole sources of nitrogen and carbon, respectively. Protein was purified following the same procedures as described above, except that the purified proteins were concentrated using Centricon (Millipore) centrifugation filtration units with a 3000 MW cut off. The purified proteins were then exchanged into the final NMR sample buffer containing 50 mM phosphate (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 2 mM perdeuterated dithiothreitol (Cambridge Isotope Laboratories, Cambridge, MA). Optimal conditions were predetermined using microdialysis against a variety of buffers. NMR experiments were performed on Bruker AMX 500-MHz and Avance 600-MHz spectrometers. Titration of the protein with the dissolved peptide, in the same buffer, was monitored by changes in <sup>15</sup>N-<sup>1</sup>H heteronuclear single quantum correlation (HSQC) spectra collected at peptide protein molar ratios of 0, 0.25, 0.5, 0.75, 0.85, 1.0, 1.25, 1.5, and 2.0. Dissociation constants (K<sub>d</sub>) of peptide binding were determined by analyzing the titration data assuming fast exchange, and by using CRVFIT (a nonlinear least-squares fitting program obtained from R. Boyko and B. D. Sykes). For backbone assignment of the protein, triple resonance experiments, including HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB, and <sup>15</sup>N separated 3D NOESY and TOCSY spectra were recorded. The data were processed and analyzed using FELIX 98 (Accelrys, Inc.).

## RESULTS

*Generation of the binding mutants:* Two arginine residues are conserved in the pTyr binding pocket. Alignment of CHK-SH2 with other SH2 domain sequences shows that while the arginine in βB5 is present in these SH2 sequences, the αA2CHK-SH2 has a glycine. This

arginine in the binding pocket of other SH2 domains provides a positive charge to coordinate the phosphate of pTyr, implying either a different mode of binding or weak binding for CHK. R<sup>147</sup> in  $\beta$ B is a critical residue for pTyr binding and is strictly conserved in SH2 domains. Based on these observations, we constructed three CHK-SH2 mutants, G129R, R147A, and R147K. The G129R mutant encodes a CHK-SH2 protein in which the  $\alpha$ A2 glycine at the phosphotyrosine-binding pocket has been replaced by an arginine. For the R147A and R147K mutants, the  $\beta$ B5 arginine has been replaced by an alanine and lysine, respectively. Our hypothesis is that G129R will have enhanced ErbB-2 phosphopeptide binding, R147A will have disrupted binding, and R147K will have reduced binding.

*Binding studies: GST pull-down experiment.* We conducted binding studies with the three CHK-SH2 domain mutants as well as with wild type CHK (Fig. 3). Two different breast cancer cell lines, T47D (moderate-level of ErbB-2/neu expression) and BT474 (high-level expression), were tested. The conserved tyrosine residues of the overexpressed ErbB-2/neu in BT474 have been found to be autophosphorylated in the absence of ligand stimulation. The cells were serum-starved, and then activated with heregulin (20 nM) for 8 min. Unstimulated and stimulated cells were lysed and precipitated with CHK-SH2 fusion proteins as well as with GST protein alone. The precipitates were analyzed on SDS-PAGE and immunoblotted with phospho-HER2/ErbB2 (Tyr<sup>1248</sup>) antibody. In comparison to the wild type, the substitution of G<sup>129</sup> with R<sup>129</sup> resulted in dramatically increased binding in both cell lines. In T47D cells (Fig. 3A), only heregulin stimulation induced the association of ErbB-2 with purified wild type SH2 and also G129R SH2, indicating that G129R SH2-binding is ligand-stimulated to a similar level as the wild type binding. The substitution of R<sup>147</sup> with K<sup>147</sup> slightly decreased binding, whereas substitution with A<sup>147</sup> completely disrupted binding. These results indicate that the positive charge of R<sup>147</sup> is critical for phosphopeptide binding and that the length of the side chain has a moderate effect on binding. Since the R147K mutant showed only a moderate effect on binding in comparison to the wild type, the R127K mutant was not analyzed in further experiments. In BT474 cells, no ligand stimulation was necessary for the CHK-SH2 interaction with the constitutively phosphorylated ErbB-2/neu protein (Fig. 3B). Again, almost no association of R147A SH2 with ErbB-2/neu was seen, while G129R SH2 pulled down markedly more ErbB-2 protein than the wild type CHK-SH2.

*Binding studies: NMR experiments.* The backbone atoms of the CHK-SH2 domain were assigned using triple resonance experiments (Fig. 4A). The <sup>15</sup>N-<sup>1</sup>H heteronuclear single quantum correlation (HSQC) experiment yields a well-resolved spectrum, with single peaks for the backbone amides of most residues in the protein. Changes in the positions of these peaks upon titration of the ligand can identify the residues in the binding site and analysis of the titration data can be used to determine the kinetics of binding.

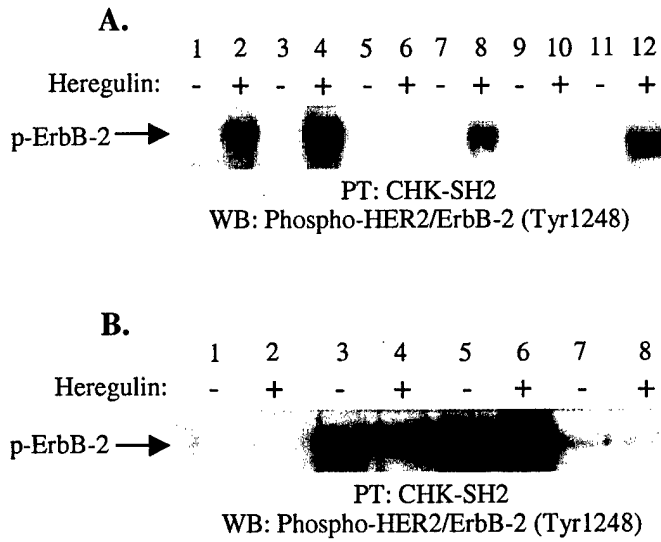
To analyze the interaction between CHK-SH2 constructs and the phosphopeptide, we titrated the peptide into 0.6 mM <sup>15</sup>N-labeled SH2 domains. Progressive changes in <sup>1</sup>H and <sup>15</sup>N chemical shifts were monitored with a series of <sup>15</sup>N-HSQC spectra. Significant chemical shift changes in wild type SH2 were observed for several residues in the  $\alpha$ A,  $\beta$ B, and  $\beta$ D secondary structures as well as in the  $\beta$ D- $\alpha$ B and  $\alpha$ B- $\beta$ E loops, which have been implicated in the binding of other SH2 domains to phosphopeptides (Fig. 4A). In particular, several positive residues in  $\beta$ D (H<sup>168</sup>, R<sup>170</sup>) undergo large chemical shift changes upon complex formation, consistent with the positive charged residues in these secondary structural elements forming contacts with the pTyr of the ligand. In the  $\alpha$ A helix, several residues changed chemical shifts upon complex formation (I<sup>127</sup>, G<sup>129</sup>, Q<sup>134</sup>, and Q<sup>135</sup>). The  $\beta$ B- $\beta$ C loop has also been implicated in the binding of

the peptide, and significant changes in chemical shift were observed for residues in this loop ( $S^{149}$ ,  $R^{151}$ , and  $G^{154}$ ). Interestingly,  $R^{147}$  showed little change in chemical shift despite its presumed role in contacting the phosphate of the ligand. Significant chemical shift changes were also observed in residues in  $\beta D$  ( $Y^{169}$ ,  $V^{171}$ ,  $L^{172}$ ), the  $\beta D$ - $\alpha B$  loop ( $I^{180}$ ,  $D^{181}$ ), and the  $\alpha B$ - $\beta E$  loop ( $I^{203}$ ), which presumably form the hydrophobic environment for the hydrophobic residues of the C-terminal to pTyr region of the peptide (Fig. 5).

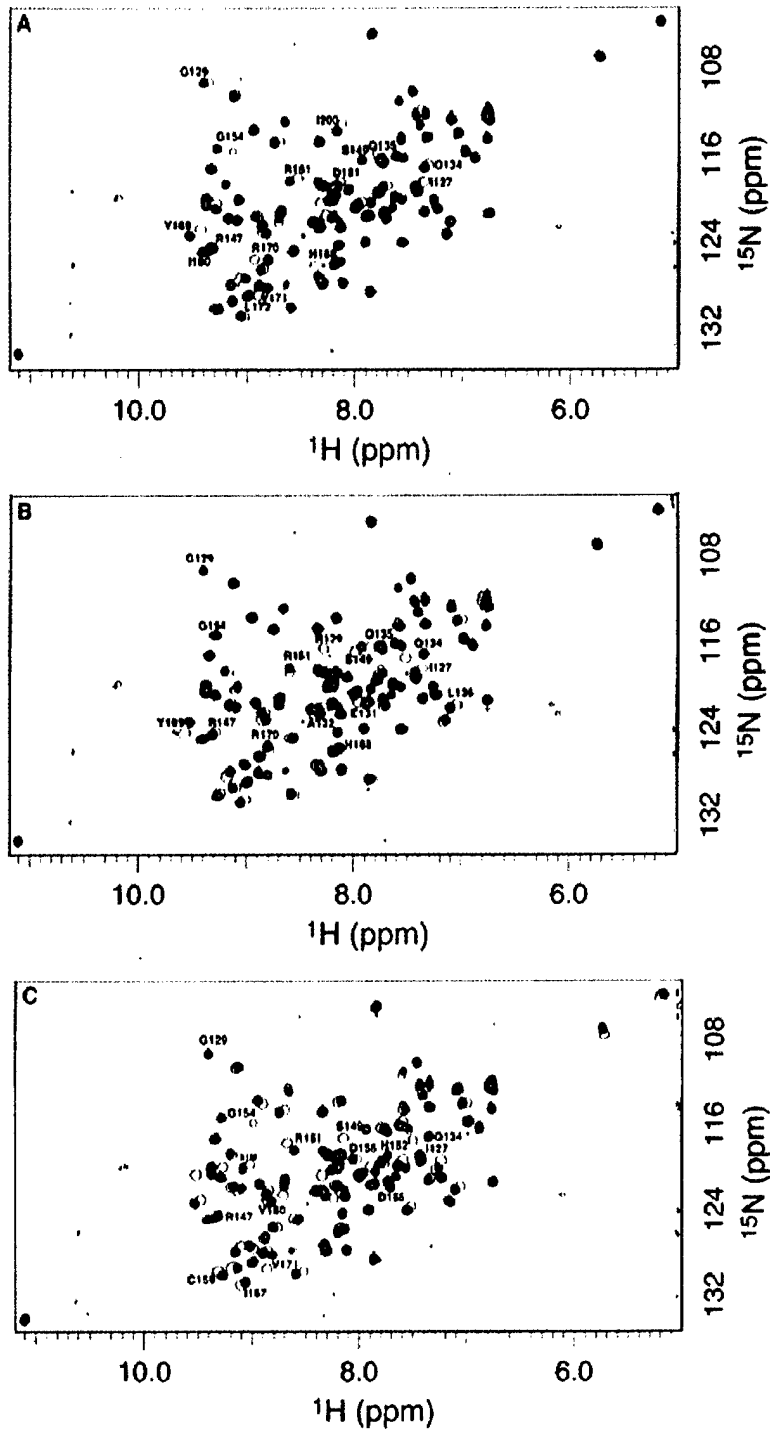
$G^{129R}$ , which showed enhanced binding, was also titrated with the phosphopeptide (Fig. 4B). The  $G^{129}$  resonance was replaced with a new resonance ( $R^{129}$ ), consistent with the substitution of glycine with arginine and the expected changes in chemical shift. Several residues showed chemical shift changes upon mutation. In particular, residues in  $\alpha A$  ( $I^{127}$ ,  $E^{131}$ ,  $A^{132}$ , and  $Q^{134}$ ) experienced chemical shift changes ( $0.3 \pm 0.1$  ppm) presumably due to introduction of the long charged side chain of R. Several residues in  $\beta B$  and  $\beta D$  also showed chemical shift changes, suggesting that they are in proximity to the  $G^{129R}$  mutation site. Upon titration with peptide, residues similar to the wild type underwent significant chemical shift changes, except for residues near the mutation site in  $\alpha A$  and  $\beta B$  (Table 1).

$R^{147A}$  did not show chemical shift changes with any residue upon the peptide titration. With this mutation, the peak for  $R^{147}$  disappeared, but a new  $A^{147}$  peak was difficult to identify, presumably due to resonance overlap (Fig. 4C). Compared with  $G^{129R}$ , the  $R^{147A}$  substitution showed different chemical shifts for residues in regions such as  $\alpha A$  ( $I^{127}$ ,  $S^{128}$ ,  $G^{129}$ ),  $\beta B$  ( $S^{149}$ ,  $H^{152}$ ), the  $\beta B$ - $\beta C$  loop ( $G^{154}$ ,  $D^{155}$ ),  $\beta C$  ( $C^{159}$ ,  $V^{160}$ ), and  $\beta D$  ( $D^{165}$ ,  $I^{167}$ ,  $V^{171}$ ). In addition,  $G^{129}$  disappeared with the mutation, implying line-broadening consistent with the introduction of a motion on the millisecond to microsecond time scale. Although both mutants showed perturbation on several residues, the overall structure seems to be unaltered as the majority of the residues showed little change (Fig. 4).

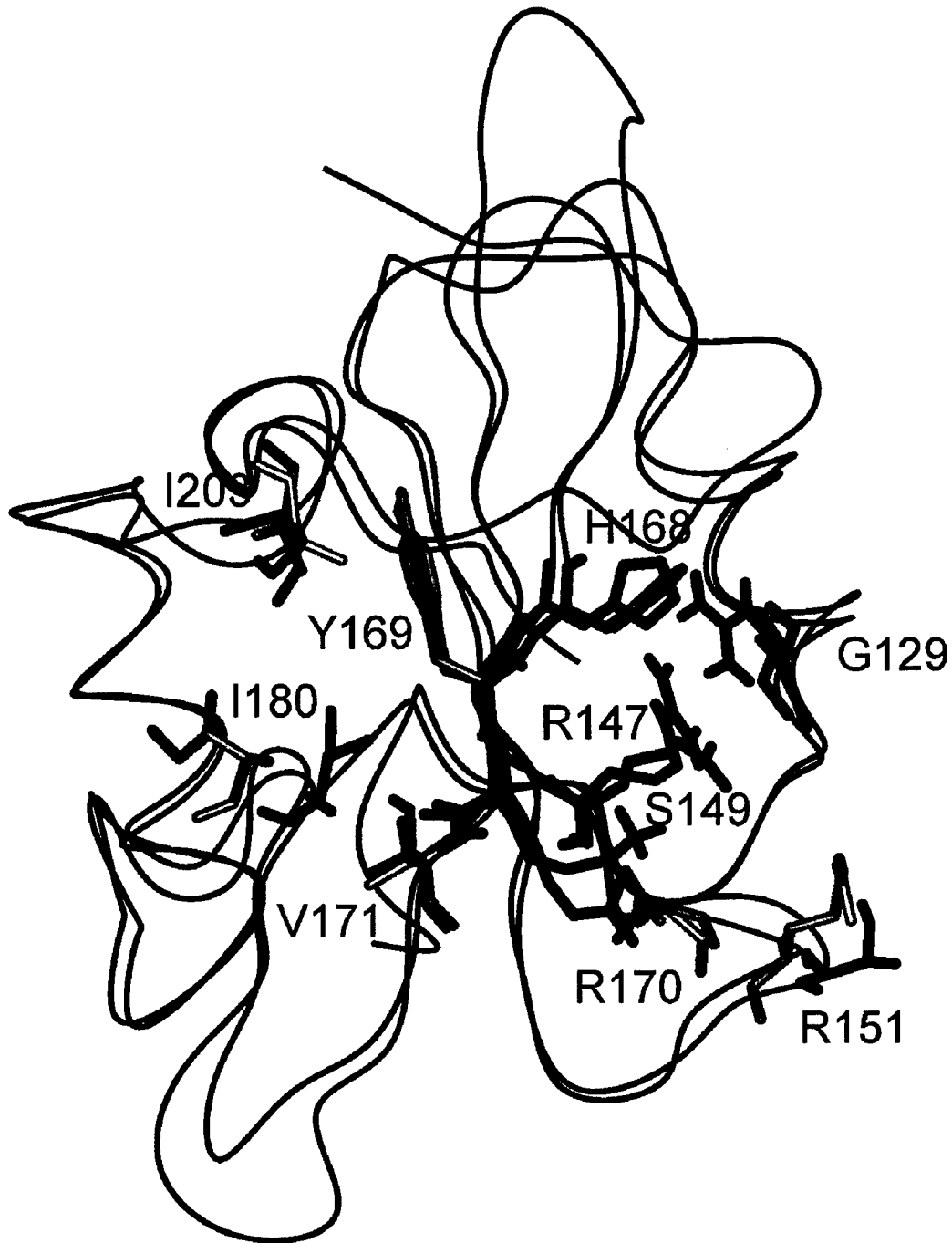
Equilibrium dissociation constants ( $K_d$ ) were determined from a plot of chemical shift changes versus the ratio of peptide to protein. Chemical shifts of the residues  $I^{127}$  ( $\beta A$ - $\alpha A$  loop),  $G^{129}$  ( $\alpha A$ ),  $R^{147}$  ( $\beta B$ ),  $G^{154}$  ( $\beta B$ - $\beta C$  loop),  $Y^{156}$  ( $\beta C$ ),  $H^{168}$ ,  $Y^{169}$ ,  $R^{170}$  ( $\beta D$ ) and  $I^{203}$  ( $\alpha B$ - $\beta E$  loop) were monitored, since these residues were involved in the phosphopeptide interaction and remained well-resolved throughout the titration. In the wild type CHK, residues in  $\beta D$  ( $H^{168}$ ,  $Y^{169}$ ,  $R^{170}$ ) showed tighter binding than  $G^{154}$ ,  $Y^{156}$ , and  $I^{203}$ , which are predicted to be the C-terminal residues of the bound ErbB-2 phosphopeptide. In  $G^{129R}$ , the affinity of  $G^{154}$ ,  $Y^{156}$ , and  $I^{203}$  increased more than 10 times that seen in the wild type, while the residues in  $\beta D$  ( $H^{168}$ ,  $Y^{169}$ ,  $R^{170}$ ) remained the same as that observed in the wild type.



**Figure 3.** GST pull-down experiment. Serum-starved T47D (A) or BT474 (B) cells were stimulated with 20 nM heregulin for 8 min at room temperature and then lysed in protein lysis buffer. Lysates were incubated with GST-fusion proteins coupled to glutathione-Sepharose beads. The precipitated proteins were developed on 7% polyacrylamide SDS-PAGE, then transferred onto Immobilon-PM (Millipore) membranes. Bound proteins were immunoblotted with phospho-HER2/ErbB-2 (Tyr<sup>1248</sup>) antibody. (A) Lanes: 1, 2- wild type CHK-SH2; 3, 4 - G129R; 5, 6 - R147A; 7, 8 - R147K; 9, 10 - empty GST-beads; 11, 12 - total cell lysate. (B) Lanes: 1, 2 - empty GST-beads; 3, 4 - wild type CHK-SH2; 5, 6 - G129R; 7, 8 - R147A.



**Figure 4.** (A) Superimposed  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectra of the CHK-SH2 wild type at 0 (black) and with 1 equivalent (red) of the pTyr peptide (Ac-ENPEpYLGLDV-NH<sub>2</sub>). (B) Superimposed  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectra of the CHK-SH2 wild type (black) and G129R mutant (red). (C) Superimposed  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectra of the CHK-SH2 wild type (black) and R147A mutant (red). The 500 MHz spectra were collected at 25 °C from samples containing approximately 0.6 mM SH2 domain. Peaks that significantly change chemical shift on binding the peptide (A) or on mutation (B, C) are labeled in black by residue number. Residues targeted for mutation are labeled in red.



**Figure 5:** The superposition of the phosphopeptide binding site of the CHK-SH2 domain (red, PDB id#:1JWO) and v-Src SH2 domain (blue, PDB id#:1BKL). Residues involved in binding pTyr ( $G^{129}$ ,  $R^{147}$ ,  $H^{168}$ ,  $R^{170}$ ) are indicated in red for CHK and in blue for the corresponding residues of Src. Residues comprising the hydrophobic site that interact with residues comprising the C-terminal to pTyr region of the phosphopeptide are indicated in pink (CHK) and in green (Src). The nonconserved  $R^{151}$  of CHK is indicated in pink.

**Table 1.** Equilibrium dissociation constants and chemical shift changes of wild type and G129R SH2 domains.

Residue <sup>1</sup>	Wild type		G129R	
	K <sub>d</sub> (mM)	Shift <sup>2</sup> (ppm)	K <sub>d</sub> (mM)	Shift <sup>2</sup> (ppm)
I <sup>127</sup>	n.d.	n.d.	0.41 ± 0.27	0.19 ± 0.04
G <sup>129</sup>	1.1 ± 0.7	0.18 ± 0.06	n.d.	n.d.
R <sup>147</sup>	n.d.	n.d.	0.68 ± 0.50	0.09 ± 0.03
G <sup>154</sup>	0.74 ± 0.28	0.52 ± 0.09	0.09 ± 0.03	0.68 ± 0.50
Y <sup>156</sup>	0.63 ± 0.14	0.33 ± 0.03	0.05 ± 0.05	0.20 ± 0.03
H <sup>168</sup>	0.29 ± 0.04	0.62 ± 0.03	n.d.	n.d.
Y <sup>169</sup>	0.27 ± 0.07	0.42 ± 0.03	0.27 ± 0.07	0.42 ± 0.03
R <sup>170</sup>	0.23 ± 0.05	0.25 ± 0.01	0.26 ± 0.10	0.20 ± 0.02
I <sup>203</sup>	1.89 ± 0.96	0.68 ± 0.22	0.15 ± 0.17	0.20 ± 0.03
Average	0.48 ± 0.05	n.d.	0.13 ± 0.08	n.d.

<sup>1</sup> These residues were selected since they were well resolved during peptide titration. n.d., not determined because chemical shift differences were too small (I<sup>127</sup>, G<sup>129</sup>, R<sup>147</sup>), or because of spectral overlap (H<sup>168</sup>).

<sup>2</sup> The chemical

shift difference was calculated from the absolute value of the change in <sup>1</sup>H chemical shift plus 0.2 times the absolute value of the change in <sup>15</sup>N chemical shift.