

## Identification of potential binding sites for the FHA domain of human Chk2 by in vitro binding studies

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### Abstract

Human Chk2 is a newly identified tumor suppressor protein involved in signaling pathways in response to DNA damage. The protein consists of a forkhead-associated (FHA) domain and a kinase domain. Identification of binding partners of the Chk2FHA domain is important in understanding the roles of Chk2 in signaling. We report development of an approach involving the use of combinatorial libraries, pull-down assays, surface plasmon resonance (SPR), and nuclear magnetic resonance (NMR) methods to identify possible candidates for the binding sites of Chk2FHA. The approach has been used to identify Thr329 of p53 and Thr1852 of breast cancer type 1 susceptibility protein (BRCA1) as very likely biological binding sites of Chk2FHA. The results provide useful leads for further biological analyses of cell signaling involving the FHA domain of Chk2 protein.

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FHA domains are found in more than 400 proteins, such as protein kinases, protein phosphatases, transcriptional factors, and other signaling proteins in various organisms, from prokaryotes to eukaryotes <http://smart.embl-heidelberg.de>. FHA domains bind to phosphorylated substrates and have been considered as protein–protein interaction regulatory modules [1,2]. Identification of possible biological binding partners of the FHA domains has been the subject of active research in many laboratories. Such research involves two major approaches: (a) use of in vivo cell biological analyses to identify biological binding partners (which would then be further validated by in vitro biochemical and structural analyses) and (b) use of in vitro biochemical and structural methods to identify potential ligand sequences (which could serve as leads for cell biological studies). Using the latter approach, we have identified the ligand specificity and solved the solution structures of FHA

domains from yeast Rad53 [1,3,4,6,7] and human Ki67 [5], in both the free form and in complexes with phosphopeptides from possible biological binding sites. In a parallel approach, Jackson and co-workers [2] used a different combinatorial library screening method, different binding analysis (isothermal titration calorimetry, ITC instead of SPR), and different structural method (X-ray instead of NMR) to achieve similar results for the FHA1 domain of yeast Rad53.

This paper concerns the human Chk2 protein, which consists of an FHA domain near the N-terminus and a kinase domain at the C-terminus. The DNA damage checkpoint pathway is vital to the maintenance of genomic integrity. Chk2 plays crucial roles on this pathway by phosphorylating several important down-stream proteins, such as tumor suppressor p53 [8]. Chk2 phosphorylates p53 at Ser20 and this phosphorylation enhances the stability of p53 by disrupting the association of Mdm2 [9,10]. It has been demonstrated that naturally occurring mutations in the FHA domain abolish the kinase activities of Chk2, as well as the

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ability of Chk2 to be activated by ataxia-telangiectasia, mutated (ATM) [11,12]. These results suggest that the major function of FHA domain is to regulate the kinase activities in Chk2 by interacting with (possibly many) other proteins.

Both in vivo and in vitro approaches have been actively used to probe the interaction of Chk2FHA with partner proteins. While a number of possible partner proteins and binding sites have been suggested for Chk2FHA based on cell biological studies, only one has been shown with certainty—that Chk2FHA binds the phosphorylated Thr68 from Chk2 itself [13,14]. In the in vitro approach by Jackson and co-workers [15], the results of library screening have been reported and the crystal structure of the Chk2FHA domain in complex with a short artificial phosphopeptide was solved. On the other hand, our study has encountered difficulty since determination of solution structures by NMR requires larger amounts of sample and better stability. However, NMR can be used as a binding probe. Considering the enormous significance of the subject, and the fact that little is known with certainty about the biologically relevant binding site (the phosphopeptide used in the crystal structure is *not* from a known partner protein), our approach provides an independent and convenient way to identify possible candidates of biological binding sites for Chk2FHA, as illustrated by the identification of two possible binding sites, Thr329 of p53 and Thr1852 of BRCA1.

## Materials and methods

**Expression and purification of Chk2FHA domain.** Two versions of Chk2FHA domain were constructed as fragments 40–229 and 64–219; the latter was reported as the minimal fragment [15] and was also the one used in this work. They were cloned into the *Bam*H/*Eco*RI sites of pGEX-4T1 vector (Amersham Bioscience) for expression in *Escherichia coli* BL21(DE3) cells and purification as glutathione *S*-transferase (GST) fusion proteins. The fusion protein was purified using glutathione agarose (Sigma) and GST tag was removed by thrombin (Sigma) digestion. Gel filtration chromatography was applied to further purify Chk2FHA prior to binding and structural studies.

**Synthesis and screening of resin-bound peptide libraries.** The phosphothreonine (pThr or pT) library, acetyl-AX<sub>-3</sub>X<sub>-2</sub>X<sub>-1</sub>(pT)X<sub>+1</sub>X<sub>+2</sub>X<sub>+3</sub>ABBRM, was synthesized as previously described [7]. The relative positions to pT are numbered as shown above. Chk2FHA was first biotinylated by sulfo-NHS-biotin (Pierce) to generate biotinylated protein. Beads containing peptide libraries were transferred to a polypropylene column connected to a vacuum manifold (Bio-Rad) and pre-treated with buffers as reported before [6]. The peptide libraries were then incubated with 1 μM biotinylated Chk2FHA at room temperature for 4–6 h and positive beads containing peptides were identified following published procedures [6,16]. The sequence of the peptide on each positive bead was determined by mass spectrometry (MS) on a Kratos Kompact MALDI-III mass spectrometer as described before [7]. Peptides with known sequences were purchased in large quantity (Genemed Synthesis) with ~90% purity for the binding and structural studies.

**Peptide pull-down assay.** Activated CH Sepharose 4B (Pharmacia) gel was pre-treated following the manufacturer's standard protocol.

For every peptide immobilization, about 65 μL gel solution, equaling roughly 30 mg dry gel, was transferred to a polypropylene column (Bio-Rad) and 25 μL peptide solution in phosphate buffered saline (PBS) (20 mM in 20 mM Na<sub>2</sub>HPO<sub>4</sub> and 150 mM NaCl, pH 8.0) was added to reach ~5 μmol peptide per 1 mL Sepharose gel. Incubated at room temperature for 2 h, the gel was then drained and washed with coupling buffer (0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.0). The peptide-Sepharose gel (ready gel) was blocked with blocking buffer (0.1 M Tris, pH 8.0) and equilibrated with elution buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaCl, and 2 mM DTT, pH 8.0).

For pull-down assay, 25 μL ready gel was incubated with 1.5 μL Chk2FHA protein solution (0.2 mM) in the elution buffer for 1 h at 4 °C. After extensive washing, SDS–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer was applied to the column and the mixture was incubated at 80 °C for 5–10 min before the collection of the eluent. The eluent was then analyzed by SDS–PAGE.

**Binding analysis by SPR.** The dissociation constant ( $K_d$ ) between Chk2FHA (residues 64–219) and the peptides was determined on a BIAcore 3000 instrument [17]. The target peptides were biotinylated by treating with sulfo-NHS-biotin (Pierce). Sensor chip SA, with streptavidin bound on the surface, was conditioned according to the manufacturer and biotinylated peptides were then immobilized on the surface. Chk2FHA solutions of increasing concentration were injected to the sensor chip at a rate of 25 μL/min. The maximal response at equilibrium binding condition was then plotted against the protein concentration to determine the  $K_d$  value. At least eight different protein concentrations were performed.

**NMR experiments.** NMR experiments were carried out on a Bruker DRX-800 spectrometer at 20 °C. The protein concentration was 0.4 mM in elution buffer with 90% H<sub>2</sub>O/10% <sup>2</sup>H<sub>2</sub>O. 2D <sup>15</sup>N–<sup>1</sup>H HSQC experiments were performed with the free protein samples. Peptide binding experiments were performed by recording a series of 2D <sup>15</sup>N–<sup>1</sup>H heteronuclear single quantum coherence (HSQC) spectra [18] with different peptide concentrations.

## Results and discussion

### Screening of a pT peptide library for binding to Chk2FHA

We carried out a pT peptide library screening against Chk2FHA by previously established procedures [1,6]. The sequences of positive beads were sequenced by MS and the amino acid distributions at each position are shown in Fig. 1. The high abundance of the positively charged arginine is likely caused by non-specific interactions similar to what we observed previously with other FHA domains, where high Arg abundance was also observed at all positions but when a peptide containing Arg at all positions, ARRR(pT)RRR, was designed and tested no specific binding could be observed [3]. When Arg is not considered, the selection of Ile at the key +3 position as well as the +2 position agrees with the published results [15]. As to other positions, there are no clear preferences and the distributions between the two methods seem to differ. This is not unusual since for all of the FHA domains studied, only the pT + 3 position shows a clear preference independent of the methods of analysis.

While Fig. 1 shows only the distribution of amino acids at each position, it was obtained from a compila-

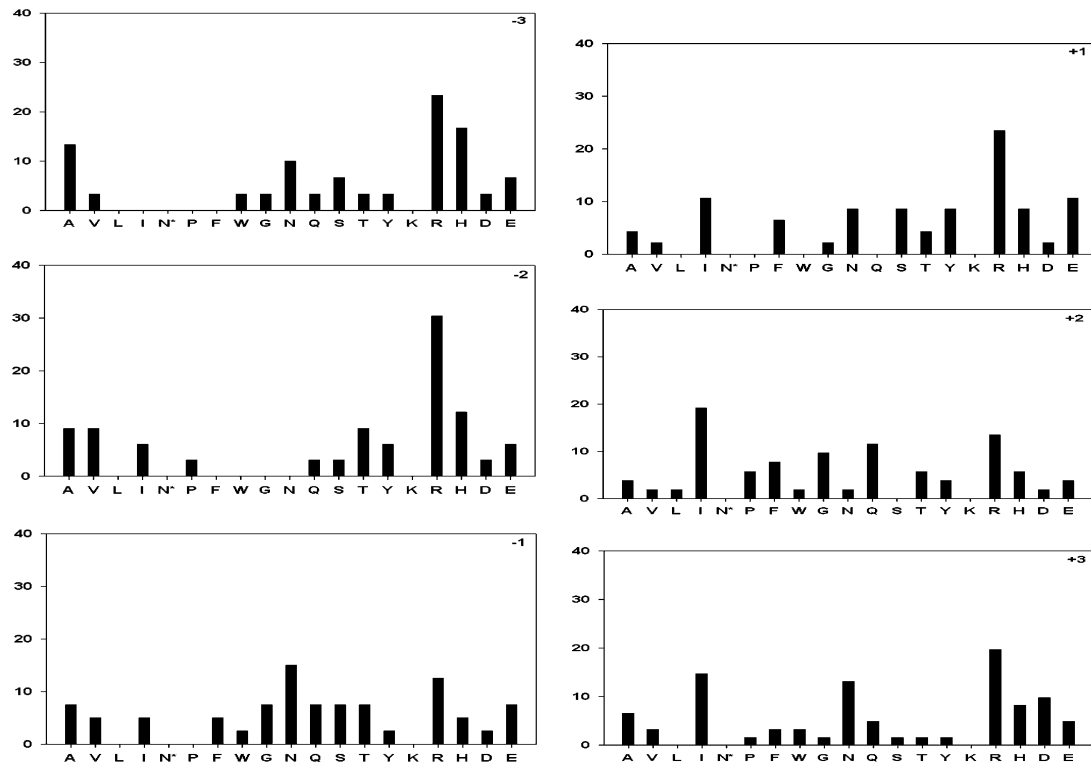


Fig. 1. Result of the pT peptide sequence specificity for binding to Chk2FHA. Displayed are the amino acid residues selected at the  $-3$  to  $+3$  positions relative to the pT residue. The  $x$ -axis indicates the identity of the 19 amino acids in single letters, whereas abundance on the  $y$ -axis represents the percentage of occurrence of an amino acid at a certain position. N\*, norleucine.

tion of a number of individual sequences based on MS analyses. This differs from the published method [15], which only provides the relative mole percentage of each amino acid at a particular sequencing cycle of automated Edman degradation [2]. Our method offers a distinct advantage in that it allows us to consider not only the statistical matches at each position, but also the actual matches of specific sequences, as described in the next section.

#### Possible Chk2FHA binding sites of p53 and BRCA1

Our consideration is based on the library screening results described above, as well as on the biological information available. In 2001, Falck et al. [11] demonstrated the formation of Chk2–p53 complex *in vivo*, which prompted us to examine the possibility that the interaction occurs through binding of Chk2FHA with a specific pThr of p53. Based on the consensus sequence pTXX(I/L), only one positive motif,  $^{329}$ TLQI, was found among the 22 Thr residues in the sequence of p53. In addition, when residues from  $-3$  to  $+3$  positions are all considered, we found that EYF(pT $^{329}$ )LQI matches quite well with the sequence RYF(pT)IRI actually detected in library screening (considering that the two R residues are likely nonspecific). Furthermore, Thr329 of p53 is located in a loop region of the tetramerization domain (PDB Accession No. 1A1E), suggesting that it is

accessible for contacting other proteins. A longer pT peptide based on the actual sequence of p53, DGEYF(pT $^{329}$ )LQIRGRE (designated as pT329), was then synthesized for further binding studies described in later sections.

We then considered another possible binding target of Chk2FHA, BRCA1. Biological evidence suggests that Chk2 can form physical complex with BRCA1 [19], and that Chk2 with mutations in the FHA domain abolishes this interaction *in vivo* [15]. It is unclear if FHA interacts with BRCA1 directly, since BRCA1 is known to form multiprotein complexes *in vivo* [20] and the interaction with Chk2FHA could be through another phosphoprotein in the same cluster [15]. It is thus interesting to examine whether or not BRCA1 harbors some direct binding sites for Chk2 FHA. There are 111 Thr residues in BRCA1 and 13 of them have Ile or Leu at the  $+3$  position. Specifically, there are six TXXI motifs ( $^{617}$ TRHI,  $^{922}$ TVNI,  $^{963}$ TGLI,  $^{1677}$ TNLI,  $^{1720}$ TQSI,  $^{1852}$ TYLI) and seven TXXL ( $^{509}$ TSGL,  $^{715}$ TSEL,  $^{1249}$ TECL,  $^{1550}$ TSYL,  $^{1561}$ TPYL,  $^{1597}$ TSAL, and  $^{1777}$ TDQL) motifs. When all residues from  $-3$  to  $+3$  for all 13 possible sites were compared with all actual sequences obtained from library screening, the best match was found between ELD(pT $^{1852}$ )YLI and a positive peptide from the library, NRD(pT)YII (again considering R as nonspecific). Interestingly, Thr1852 in BRCA1 is located close to the C-terminus of BRCT2

domain and is also an accessible residue in a loop region (PDB Accession No. 1JNX). Therefore, we synthesized the peptide YQCQELD(pT<sup>1852</sup>)YLIPQI (designated as peptide pT1852) from the sequence of BRCA1 for further binding studies.

#### Binding studies with pull-down assays, SPR, and NMR

Binding of peptides pT329 and pT1852 to Chk2FHA was first demonstrated by peptide pull-down assays as shown in Fig. 2, in which the artificial pT peptide used to solve the crystal complex structure (designated as peptide pT+) [15] was used as a positive control and a non-phosphorylated peptide (T control) was used as a negative control. SPR was then used to determine the dissociation constants ( $K_d$ ) of peptides pT329, pT1852, pT+, and one additional positive control peptide pT68, whose sequence is based on the only confirmed biological binding site of Chk2FHA [13] mentioned above. As shown in Table 1, reasonably tight binding with  $K_d$  values in the 1–12  $\mu$ M range was observed for all four phosphopeptides.

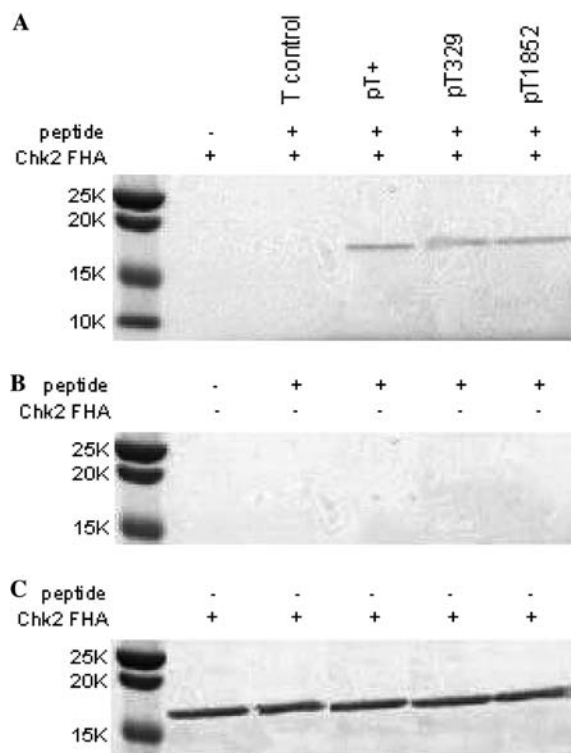


Fig. 2. Peptide pull-down assay. (A) The existence of Chk2FHA and a selected peptide in each reaction is indicated by “+” (added) or “-” (not added). A single, 18 kDa band indicates Chk2FHA, if retained by the corresponding peptide. Peptide pT+ is the positive control. (B) Negative controls. Chk2FHA or any other proteins were not detected, indicating that the peptide-bound Sepharose gel is contamination free. (C) Total amount of Chk2FHA loaded in each reaction. T control is the non-phosphorylated version of pT68 [13]. It has been demonstrated as incapable of binding to Chk2FHA and is thus used as a negative control.

Table 1  
Dissociation constants of Chk2FHA–pT peptide complexes

Peptide	Sequence	$K_d$ ( $\mu$ M)
pT+	RHFD(pT)YLIRR	$3.3 \pm 0.4^a$
pT68	ETVS(pT <sup>68</sup> )QELYS	$12.3 \pm 2.1$
pT329	DGEYF(pT <sup>329</sup> )LQIRGRE	$11.7 \pm 1.8$
pT1852	YQCQELD(pT <sup>1852</sup> )YLIPQI	$1.2 \pm 0.2$

<sup>a</sup>The  $K_d$  value of pT+ was previously reported as 0.9  $\mu$ M [15]. The difference possibly comes from different determination methods and experimental conditions.

Binding of the phosphopeptide to the Chk2FHA domain was further examined by <sup>15</sup>N-HSQC titration experiments. Although no assignment or structure is reported here (the protein was very difficult to be obtained in large quantity and precipitated out in NMR tube in 2–3 days), titration experiments are useful in qualitatively comparing the relative binding affinity of different peptides on the basis of the shifts (fast, slow, or intermediate exchange on the NMR time scale) of various peaks during the titration. Detailed analyses of the titration results (data not shown due to space limitation) confirmed the SPR result that the peptide pT1852 binds Chk2FHA most tightly, followed by pT+, and then the remaining two peptides with comparable affinity. Furthermore, as shown in Fig. 3, binding of pT1852 also induces largest changes in the overall HSQC spectra under fully saturated condition, again followed by pT+ and then the other two comparably. Since binding of pT+ has already been demonstrated by X-ray structure, and Thr68 is an established binding site of Chk2FHA based on biological studies, our results strongly support the fact that Thr329 of p53, and particularly Thr1852 of BRCA1, are likely binding sites of Chk2FHA.

#### Potential biological significance

It is important to point out that both p53 and BRCA1 have also been demonstrated to be substrates of the Chk2 kinase, with phosphorylation occurring at Ser20 of p53 [10] and Ser988 of BRCA1 [19]. It is interesting to note that the proposed FHA binding sites are C-terminal to the kinase phosphorylation sites in both proteins. On the other hand, the kinase domain of Chk2 is C-terminal to the FHA domain. Based on these results, we propose a hypothetical “head-to-tail” model of interaction between Chk2 and its partner proteins: the N-terminal FHA domain of Chk2 binds to a pT site at the C-terminus of its partner protein, which activates the C-terminal kinase domain of Chk2, which then phosphorylates an N-terminal Ser residue of the partner (substrate) protein.

The Chk2FHA binding sites suggested for p53 and BRCA1 are only possible candidates and the purpose of this work was to provide positive leads for further studies. Neither binding site suggested here has ever

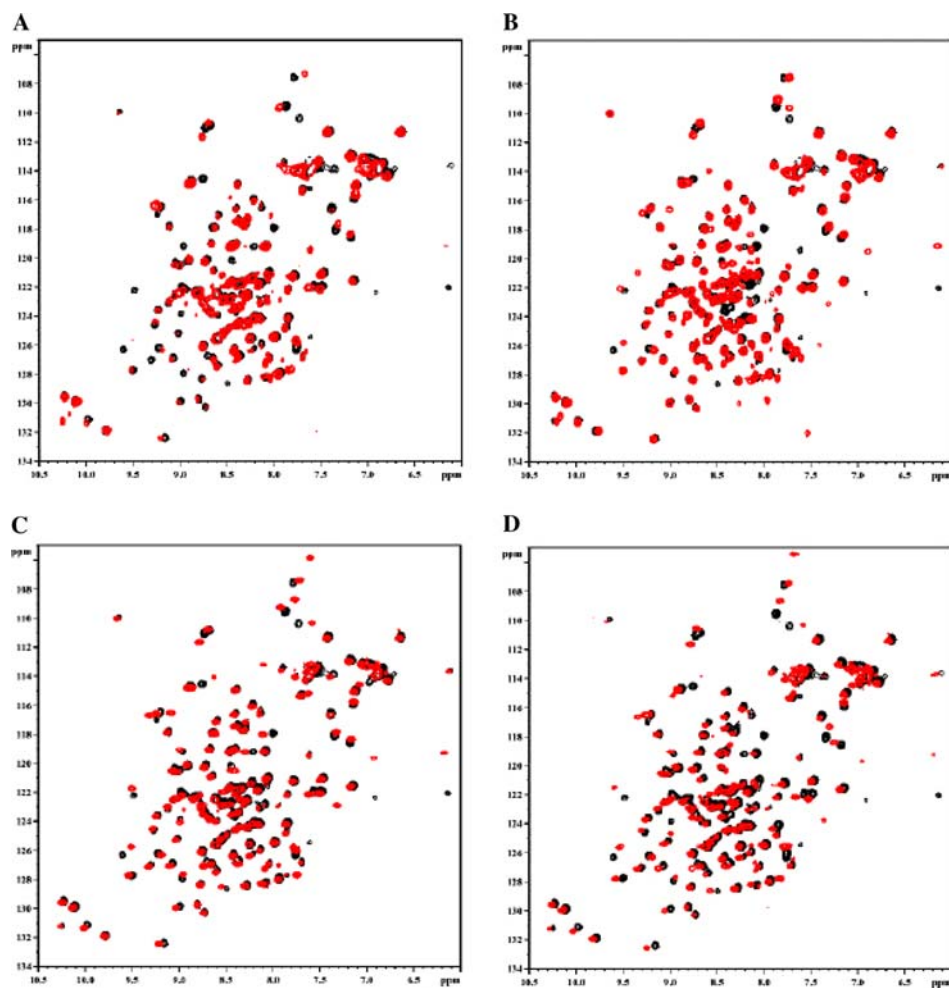


Fig. 3. Superimposed  $^{15}\text{N}$ -HSQC spectra of the free Chk2FHA (black) and Chk2FHA complexed with different pT peptides (red): (A) pT+, (B) pT68, (C) pT329, and (D) pT1852. Due to the limit of space, a crossing peak at 11.7 ppm ( $^1\text{H}$ ) from free Chk2FHA, which disappears upon peptide titration, is not shown here. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

been suggested by cell biological studies. In fact, neither Thr residue has been shown to be phosphorylated by a protein kinase *in vivo*. Thus a great deal of biological studies is necessary before our suggested leads can be verified. Nonetheless, our data suggest that the leads could be very valuable for biological research and the suggested sites have a good possibility to be verified by *in vivo* studies. Furthermore, the approach reported here will allow us to continue to examine other possible binding sites in the same and different binding partners (possibly many) of Chk2FHA. Such an interplay between *in vitro* and *in vivo* studies is very important in providing definitive answers.

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