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Structural Characterization of BRCT-Tetrapeptide Binding Interactions

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Abstract

BRCT(BRCA1) plays a major role in DNA repair pathway, and does so by recognizing the conserved sequence pSXXF in its target proteins. Remarkably, tetrapeptides containing pSXXF motif bind with high specificity and micromolar affinity. Here, we have characterized the binding interactions of pSXXF tetrapeptides using NMR spectroscopy and calorimetry. We show that BRCT is dynamic and becomes structured on binding, that pSer and Phe residues dictate overall binding, and that the binding affinities of the tetrapeptides are intimately linked to structural and dynamic changes both in the BRCT(BRCA1) and tetrapeptides. These results provide critical insights for designing high-affinity BRCT(BRCA1) inhibitors.

Keywords

BRCT(BRCA1); pSXXF tetrapeptide; NMR; structure; thermodynamics

Introduction

BRCT(BRCA1) interacts with several cell cycle related proteins, and plays an essential role in the DNA-damage response and repair pathway [1–7]. Mutations in BRCT(BRCA1) lead to breast cancer, and also sensitize cells to DNA damaging agents. Therefore inhibitors targeting this domain offer a novel approach to cancer therapy.

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Structural and biochemical studies show that BRCT(BRCA1) recognizes and binds a conserved sequence pSXXF in these proteins [8–13]. We have recently shown that peptides containing the pSXXF sequence as the minimal structural unit required for inhibitor design [14]. The small size (4-amino acids) and the micromolar (μM) binding affinity of these peptides make them an excellent template for a peptidomimetic approach for designing more potent nanomolar (nM) inhibitors. The knowledge of the structural and thermodynamic basis of the binding interactions is essential for such an approach. Towards this goal, we have now characterized the binding of a series of peptides, SRSTpSPTFNK, pSPTF, pSPAF and Flu- β A-pSPTF to BRCT(BRCA1) using NMR spectroscopy and isothermal titration calorimetry.

Our results indicate that the binding of pSXXF tetrapeptides is accompanied by global dynamic changes, and that the tetrapeptide captures all of the interactions observed for the longer peptides. We further show that the fluorescein moiety in Flu- β A-pSPTF binds to the previously identified hydrophobic site adjacent to the pSXXF binding site, and that its higher binding affinity should be due to favored interactions at this site. Most importantly, correlating the NMR and calorimetry data show that the binding affinities of the tetrapeptides are intimately linked to structural and dynamic changes both in BRCT(BRCA1) and the tetrapeptides.

Materials and Methods

Protein Purification and NMR Sample Preparation

The BRCT(BRCA1) construct was expressed, and purified as discussed previously [14]. ^{15}N labeling was achieved by growing the transformed cells in M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories) as the sole nitrogen source. The peptides were synthesized using standard Fmoc chemistry and HPLC purified either in house or by the Tufts University Core Facility [14]. The NMR sample consisted of $\sim 150\ \mu\text{M}$ BRCT in 50mM KH_2PO_4 , pH 7.0 containing 10% D_2O , 1mM DSS and 1mM sodium azide.

NMR Spectroscopy

NMR experiments were carried out on a Varian Inova 800 MHz spectrometer equipped with gradients and a cryoprobe at 30 °C. For all NMR titration experiments, unlabeled peptides were titrated into ^{15}N -labeled BRCT and binding was followed by changes in 2D ^1H - ^{15}N TROSY spectral characteristics [15]. The fractional bound population of the last titration point was $\sim 99\%$ for all the experiments. The data were processed and analyzed as described previously [16]. The backbone ^1H and ^{15}N chemical shifts of the BRCT(BRCA1) construct are from earlier published data [8]. Apparent dissociation constants (K_d) were determined by fitting the binding-induced chemical shift changes as a function of peptide:BRCT(BRCA1) molar ratios as described previously [16].

Results and Discussion

Previous structural studies of BRCT(BRCA1)-phosphopeptide complexes reveal a well defined binding pocket for pSer and for Phe(P+3) residues (Fig. 1), and mutational studies have also shown that these residues are critical for binding, and so pSXXF sequence has been implicated as the recognition motif for BRCT(BRCA1) interaction and function [8–13]. We have recently shown that the tetrapeptides containing the pSXXF bind only with slightly lower affinities compared to the longer peptides, indicating that these peptides are the minimal structural unit required for inhibitor design [14].

In the present study, we have used NMR chemical shift changes and line broadening characteristics as probes to map the binding of a series of BACH1 derived peptides, SRSTpSPTFNK, pSPTF, pSPAF and Flu- β A-pSPTF to BRCT(BRCA1). The decapeptide and

the tetrapeptides, which were initially designed and characterized by ITC and FP studies, were used as the starting set for the NMR binding studies [14]. The pSPAF peptide was chosen to understand the role of P+2 position in tetrapeptide binding. The fluorescein-tagged tetrapeptide was designed to probe whether the previously identified hydrophobic site could be exploited for designing high affinity inhibitors.

Characterization of BRCT(BRCA1)

The NMR spectrum of the unbound BRCT(BRCA1) is crowded and many of the peaks are weak or fully broadened out (Fig. 2). This is a consequence of its large molecular weight (273 amino acids; 31 kDa) and intrinsic conformational flexibility. A similar observation for BRCT (BRCA1) has been reported previously [8]. The dynamic nature could be an essential feature that allows binding to multiple target proteins. Peaks that showed characteristic chemical shifts could be nevertheless assigned in a straightforward manner based on available data [8], though collected on a slightly different construct and at a different pH (pH 7.0 vs. pH 7.5). A total of ~100 cross peaks were unambiguously identified, with a majority of them in and around the BRCT(BRCA1)-pSXXF binding interface.

Binding of BACH1 derived peptides to BRCT(BRCA1)

For all peptides, we observe binding resulted in majority of the peaks becoming more intense with additional appearance of new bound peaks, suggesting transition to a more structured state (Fig. 2). This is most likely due to reduced motions of the loops that are in the binding interface, which in turn impose conformational restraint on the flexibility of the tertiary scaffold. In addition, the network of interactions involving pSer and Phe (P+3) in the pSXXF binding site could act as a clutch to arrest the dynamic nature of the free BRCT tertiary scaffold. Our data clearly show that binding of the tetrapeptides captures all of the interactions observed for the longer peptides. In contrast to our observations, crystal structures have not provided any role for the dynamics in the binding process. NMR measurements are carried out in solution, and so are quite powerful for giving insights into the dynamic processes and their role in binding and function.

Data from all peptides consistently indicate that the pSXXF motif interact with the BRCT (BRCA1) in a two-point binding mode through the phosphate group of pSer and the phenyl ring of Phe(P+3), while the (P+1) and (P+2) could act as spacers so as to orient the phosphate group and the phenyl ring for optimal interactions.

We first carried out NMR titration experiments of the decapeptide SRSTpSPTFNK. Ideally, NMR mapping of the binding site and measurement of binding constants require that the binding is in the fast exchange regime on the NMR time scale so that only one set of peaks are observed before and after the titrations. However, binding of the decapeptide was in the slow exchange regime resulting in doubling of peaks due to the presence of both the unbound and bound peaks, similar to that observed in a previous study with a pSXXF containing 13-mer peptide [8]. Binding induced chemical shift changes are observed for residues in and around the pSXXF binding site and are similar to those observed previously [8].

Unlike the decapeptide, the binding of pSPTF was in fast exchange regime resulting in only one set of peaks. The BRCT(BRCA1) residues whose chemical shifts are maximally perturbed are either in the binding surface or in close proximity to the binding site (Fig 3). For example, chemical shift perturbations are observed for F₁₇₀₄ and L₁₈₃₉, which are involved in packing interactions with the Phe (P+3) side chain of the pSPTF peptide, and G₁₇₀₆, which is adjacent to F₁₇₀₄ in the hydrophobic core (Fig 3). The localized perturbation in the pSXXF binding site clearly demonstrates that pSPTF binds to the same site as previously reported for the longer

peptides [8]. From NMR titrations, we also measured the binding affinities ($K_d \sim 1 \mu\text{M}$), which are comparable to those obtained from ITC studies (Table 1).

Analysis of the crystal structure of the BRCT(BRCA1)-peptide complex shows that the Thr(P+2) side chain is solvent exposed, and not involved in intermolecular interactions [13]. However, pSPAF shows weaker binding compared to pSPTF, indicating a role for Thr in the binding process [14]. To explore the role of Thr in the P+2 position, we have carried out NMR binding studies of an Ala mutant pSPAF. The overall chemical shift perturbation for pSPAF was similar to that observed for the parent pSPTF peptide indicating that binding is predominantly driven by pSer and Phe(P+3) interactions. However, the extent of chemical shift perturbations for pSPAF was smaller suggesting weaker binding (Fig. 3). The measured binding constant from NMR ($K_d 21 \pm 11 \mu\text{M}$) is also comparable to those obtained from ITC (Table 1). Our NMR data could not identify any discernible changes between pSPAF and pSPTF spectra suggesting that Thr(P+2) is not involved in intermolecular interactions. However, ITC data do show that the binding of pSPAF, compared to the pSPTF, is entropically disfavored (Table 1). Therefore, it is possible that intramolecular interactions between Thr(P+2) and Phe(P+3) minimizes loss of conformational entropy and/or stabilizes Phe(P+3) interactions in the hydrophobic pocket resulting in the increased affinity of the pSPTF peptide.

Our NMR data show that both the tetrapeptides and the decapeptide bind to BRCT in a similar fashion, and residues corresponding to the tetrapeptide in the decapeptide provide the specificity. Therefore, differences in binding affinities are not structural and should be due to dynamics, which should reflect in the thermodynamics of binding. Indeed, ITC data show that the enthalpic component is the same and the tighter binding of the decapeptide is due to more favorable entropic component (Table 1). These observations further emphasize that the pSXXF motif drive the binding interactions, and that binding-induced loss of conformational entropy in the tetrapeptides is the major reason for the differences in binding affinities.

Towards designing high affinity inhibitors

We had proposed previously that the hydrophobic site (V_{1653} , V_{1654} , L_{1657} , T_{1658} , F_{1662} , Y_{1666} , L_{1676} and T_{1677}) identified adjacent to the pSXXF binding site could be exploited to increase the potency of binding. We have now characterized the binding of pSPTF tagged with an N-terminal fluorescein (Flu- β A-pSPTF), and show that it indeed binds BRCT(BRCA1) with higher affinity (Table 1; Figures S1 and S2).

Our NMR studies of the fluorescein-labeled tetrapeptide (Flu- β A-pSPTF) show that the signature of the chemical shift perturbation to be distinctly different from those observed for the tetrapeptides. In addition to perturbation of residues that can be attributed to the pSXXF binding site, differential perturbation of the putative-hydrophobic pocket residues were also observed. In particular, residues M_{1652} , Y_{1666} and T_{1677} show much larger perturbations compared to that observed for pSPTF or pSPAF (Fig. 4). Further, another set of residues in this hydrophobic pocket (V_{1654} , T_{1658} , F_{1662} , L_{1664} , A_{1669} and L_{1676}) fully broaden out in the bound form. As most residues sharpen on binding, the latter observation also indicates that fluorescein binding to this independent binding site actually induces increased dynamics of this site.

Increased dynamics in this hydrophobic pocket suggest that the binding to this site is not only enthalpically favored but also entropically favored. Our observation that local binding is entropically favored is novel, especially in light of the observation that overall binding is entropically disfavored and enthalpically favored (Table 1). Though NMR data indicate that the binding induced change in BRCT is entropically favored, the large unfavorable entropy component of overall binding can be attributed to the conformational flexibility of the linker. In summary, our data indicate that binding affinities are dictated by structural and dynamic

changes both in BRCT and the tetrapeptides, and has shown the importance of maximizing both enthalpy and entropy components to be successful in designing high-affinity inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ITC	isothermal titration calorimetry
NMR	nuclear magnetic resonance
TROSY	Transverse relaxation optimized spectroscopy

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Fig 1. Molecular plot showing interactions that define the pSXXF binding site (PDB id: 1T29). Dotted lines indicate hydrogen bonding and salt bridge interactions involving pSer. The Phe(P+3) side chain is packed against the hydrophobic pocket (shown as a space filled patch). Molecular plot was generated using Pymol.

Fig 2.
Overlay of the 2D ^1H - ^{15}N TROSY spectra of BRCT(BRCA1) (black) and BRCT(BRCA1)-pSPTF complex (red).

Fig 3.
2D ^1H - ^{15}N TROSY spectra of BRCT bound to the pSPTF (green), pSPAF (red) and SRSTpSPTFNK (magenta). Binding-induced chemical shift changes for residues F₁₇₀₄, L₁₈₃₉ and G₁₇₀₆ are shown.

Fig 4.

Characterization of the binding of Flu- β A-pSPTF to BRCT(BRCA1). Panel A shows chemical shift changes and line broadening of selected residues in the 2D ^1H - ^{15}N TROSY spectra of BRCT(BRCA1) bound to pSPTF (green), pSPAF (blue) and Flu- β A-pSPTF (red). Y₁₆₆₆ show differential perturbation, while the residues F₁₆₆₂ and L₁₆₇₆ totally broadened out in the Flu- β A-pSPTF bound BRCT(BRCA1). These residues are in the hydrophobic pocket adjacent to the pSXXF binding site. Panel B is a molecular plot of BRCT(BRCA1) (PDB id: 1T29) showing the binding surface of the fluorescein moiety to the hydrophobic pocket. The residues that are differentially perturbed/broadened compared to that observed for pSPTF binding are shown as red sticks and spheres. Molecular plot was generated using Pymol.

Table 1

Thermodynamic parameters for binding of BACH1 derived peptides to BRCT(BRCA1) domain from isothermal calorimetry.

	Peptides	n	K _d (μM)	ΔG ^a (kcal/mol)	ΔH (kcal/mol)	-TΔS ^a (kcal/mol)
1 [*]	Ac-SRSTpSPTFNK-CONH ₂	0.98	0.43 ± 0.03	-8.7 ± 0.2	-12.4 ± 0.3	3.7 ± 0.9
2	Ac-pSPTF-CONH ₂	0.89	2.31 ± 0.06	-7.7 ± 0.1	-14.0 ± 1.1	6.3 ± 1.1
3	FTTC-βA-pSPTF-CONH ₂	0.98	0.63 ± 0.07	-8.5 ± 0.1	-20.7 ± 0.4	12.4 ± 0.4
4 [*]	Ac-pSPAF-CONH ₂	1.05	47.7 ± 2.3	-5.9 ± 0.2	-14.7 ± 0.8	8.8 ± 1.4

^{*} values from ref. 14