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Structure of the BRCT Repeat Domain of MDC1 and Its Specificity for the Free COOH-terminal End of the γ-H2AX Histone Tail*

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Megan S. Lee[1], **Ross A. Edwards, Gina L. Thede, and J. N. Mark Glover**[2]

From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

MDC1 (mediator of DNA damage checkpoint protein 1) regulates the recognition and repair of DNA double strand breaks in mammalian cells through its interactions with nuclear foci containing the COOH-terminally phosphorylated form of the histone variant, H2AX. Here we demonstrate that the tandem BRCT repeats of MDC1 directly bind to the phosphorylated tail of H2AX-Ser(P)-Gln-Glu-Tyr, in a manner that is critically dependent on the free carboxylate group of the COOH-terminal Tyr residue. We have determined the x-ray crystal structure of the MDC1 BRCT repeats at 1.45 Å resolution. By a comparison with the structure of the BRCA1 BRCT bound to a phosphopeptide, we suggest that two arginine residues in MDC1, Arg[1932] and Arg[1933] may recognize the COOH terminus of the peptide as well as the penultimate Glu of H2AX, while Gln[2013] may provide additional specificity for the COOH-terminal Tyr.

Tandem BRCT[3] repeats, initially discovered at the COOH terminus of the breast cancer-associated protein 1, BRCA1, are phosphoprotein recognition modules that play key signaling roles in the cellular response to DNA damage (1). Individual repeats are ~90–100 amino acids in size. While they can fold independently, they often exist in tandem pairs where they pack in a head-tail manner (2–4). The BRCT repeats of BRCA1 have been shown to specifically bind to pSer-*X*-*X*-Phe peptide targets, such as the BACH1 helicase (5, 6), and the transcriptional co-repressor CtIP (7). Structural studies reveal that the NH_2-terminal BRCT repeat is responsible for phosphoserine recognition, while the phenylalanine side chain is recognized by a pocket at the interface between the NH_2- and COOH-terminal repeats (8–10). Mutations that perturb the phenylalanine recognition pocket disrupt phosphopeptide binding and explain the enhanced cancer risks associated with some of these mutations.

MDC1 (mediator of DNA damage checkpoint protein 1) is another BRCT repeat protein that plays a critical role in the DNA damage response. MDC1 has been implicated in the recognition and repair of DNA double strand breaks through its rapid co-localization with γ-H2AX, the COOH-terminally phosphorylated form of the histone variant H2AX, at the sites of double strand breaks in mammalian nuclei (11, 12). MDC1 also facilitates the recruitment of other repair proteins to these foci, including the MRE11 complex and the BRCT proteins 53BP1 and BRCA1, and is required for the efficient repair of ionizing radiation-induced DNA damage. The COOH-terminal BRCT repeats of MDC1 can specifically bind to phosphopeptides with specificity for a tyrosine residue at the +3 position relative to the phosphoserine and some specificity for a glutamic acid at the +2, matching the sequence of the γ-H2AX tail: Ser(P)-Gln-Glu-Tyr (13). Here we demonstrate that the MDC1 BRCT repeats bind to the γ-H2AX tail in a manner that is critically dependent on the free carboxylate group of the COOH-terminal tyrosine residue. The crystal structure of the MDC1 BRCT repeats reveals a phosphoserine binding pocket and adjacent structural features that may explain the novel selectivity of this domain for the free COOH terminus of its phosphopeptide target.

MATERIALS AND METHODS

BRCT Expression and Purification—Human MDC1 BRCT domain (residues 1,891–2,086) was expressed in *Escherichia coli* strain BL21 Gold99 and purified as a GST fusion protein by glutathione affinity chromatography. MDC1 was then cleaved from GST using PreScission protease (Amersham Biosciences), and the COOH-terminal MDC1 polypeptide was purified from GST by ion exchange chromatography.

Crystallization and Structure Determination of MDC1 BRCT—MDC1 BRCT crystals were grown by vapor diffusion in hanging drops at room temperature. Native MDC1 (10 mg ml^{-1}) in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol was mixed with an equal volume of the reservoir solution (22% polyethylene glycol 8000, 0.1 M HEPES, pH 8.0). Crystals of Se-methionine-substituted MDC1 were grown by mixing 2 μl of 10 mg ml^{-1} BRCT domain with a 1.5-fold molar excess of a γ-H2AX peptide in protein solution (150 mM NaCl, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5) with 2 μl of well solution (1.4 M NaH_2PO_4, 0.4 M K_2HPO_4, 0.1 M citrate, pH 4.2). No γ-H2AX peptide was found to be bound to MDC1 in these crystals.

For cryopreservation, single crystals were soaked in the appropriate well solution supplemented with 26% (v/v) glycerol and then flash-frozen in liquid nitrogen. All data were collected at beamline 8.3.1 of the Advanced Light Source, Lawerence Berkeley National Laboratory. Data reduction and scaling were done using the HKL package (14).

Crystallographic phases for MDC1 were determined from a multiple wavelength anomalous dispersion experiment on the Se-methionine-substituted protein derivative to 2.7 Å resolution. Multiple MDC1 molecules were assumed to be present in the asymmetric unit, and eight selenium sites were located with SOLVE (15) using data collected at two wavelengths. Electron density maps calculated from this solution were improved by density modification and NCS averaging in RESOLVE (FOM 0.67) and revealed three molecules in the asymmetric unit. Automatic (RESOLVE) and manual model building (using XFIT (16)) produced a complete MDC1 model, which was partially refined using CNS (17) and REFMAC (18). This model was then used as the search model for molecular replacement using MOLREP (19) against the native 1.5 Å data. Two molecules were placed in the native asymmetric unit and these were rebuilt using ARP/wARP (20). Cycles of minimization and individual isotropic *B*-factor refinement and manual model building produced the completed model having 390 residues and 256 waters with an R_{free} of 0.195 and an R_{factor} of 0.212 (TABLE ONE). The atomic coordinates and structure factors have been submitted to Protein Data Base (accession code 2ADO).

Isothermal Titration Calorimetry—Peptides used for microcalorimetry contained the COOH-terminal sequence of H2AX, with an NH_2-terminal "KKK" sequence to improve peptide solubility (acetyl-KKK**TQApSQEY**, γ-H2AX sequence in bold) and were prepared with or without an amide group blocking the COOH terminus (Alberta Peptide Institute). MDC1 and the phosphopeptides were prepared in 10 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, and 2 mM TCEP. The Micro Calorimetry System (Microcal, Amherst, MA) was used to perform the ITC measurements for the interaction between the BRCT repeats and the peptides. The titration data, collected at 22 °C, were analyzed using the ORIGIN data analysis software (Microcal Software, Northampton, MA). Thermodynamic parameters reported are the average of three independent experiments.

RESULTS AND DISCUSSION

Intriguingly, not only the sequence of the H2AX tail, but also its position at the COOH terminus of the chain, is absolutely conserved in mammals (21). To test if the free carboxyl terminus of the tail is an important determinant of recognition, we used isothermal titration calorimetry to determine the thermodynamics of binding between recombinant MDC1 tandem BRCT repeats

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The atomic coordinates and structure factors (code 2ADO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http/www.rcsb.org/).

[1] Supported by an Alberta Heritage Foundation for Medical Research Studentship.

[2] Awarded a Canada Research Chair. To whom correspondence should be addressed: Dept. of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2136; Fax: 780-492-0886; E-mail: mark.glover@ualberta.ca.

[3] The abbreviations used are: BRCT, BRCA1 COOH-terminal repeat; MDC1, mediator of DNA damage checkpoint protein 1; H2AX, histone 2A, variant X; BRCA1, breast cancer associated protein 1; BACH1, BRCA1-associated COOH-terminal helicase; GST, glutathione *S*-transferase; ITC, isothermal titration calorimetry; 53BP1, p53-binding protein; FOM, figure of merit.

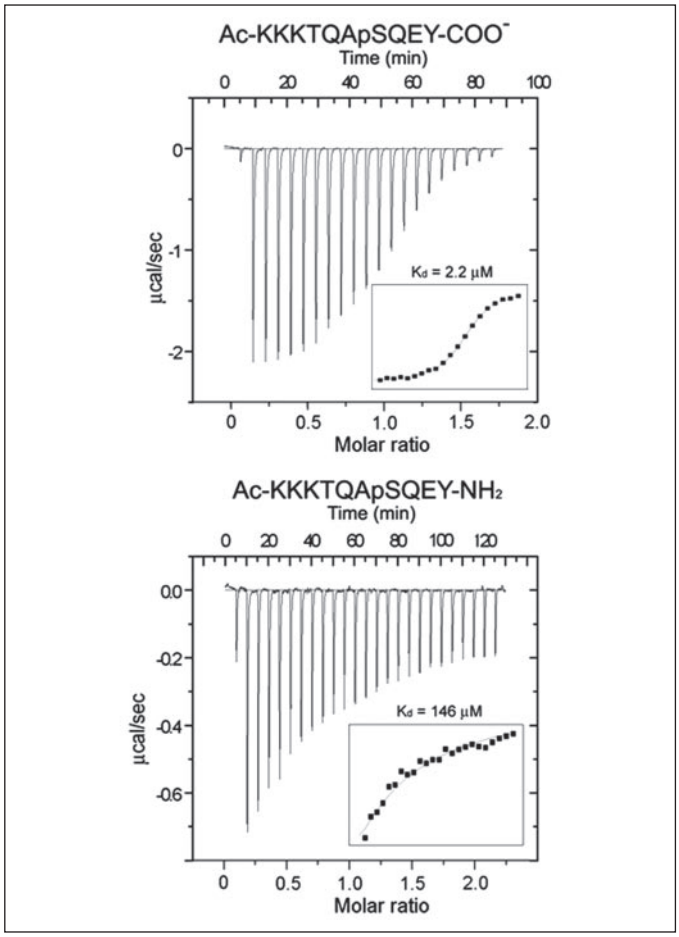

FIGURE 1. **The MDC1 BRCT domain specifically recognizes the free carboxyl terminus of the γ-H2AX tail.** Shown are sample ITC experiments showing titration of the MDC1 BRCT domain with the γ-H2AX peptide bearing a free carboxylate at the COOH terminus (*top panel*) or an amidated COOH terminus (*bottom panel*). The titration curves (*insets*) were used to determine the dissociation constants. The K_d values shown are the average of three independent experiments.

and synthetic phosphopeptides corresponding to the γ-H2AX tail, with or without an amide group blocking the carboxyl terminus (Fig. 1). The results show that MDC1 binds to the γ-H2AX tail with a free COOH terminus with high affinity (K_d = 2.2 ± 0.2 μM), similar to binding affinity of the BRCA1 BRCT for Ser(P)-*X*-*X*-Phe targets (5, 10). The binding reaction is driven by favorable enthalpic (ΔH = −6.3 kcal/mole) and entropic (ΔS = 4.8 cal/mol/degree) contributions. In contrast, the same peptide with an amidated, and therefore uncharged, COOH terminus showed ~100-fold weaker binding for MDC1 (K_d ~150 μM). This reduction in binding affinity could be due to the loss of charge on the COOH-terminal residue and/or steric repulsion between MDC1 and the terminal amide. This result demonstrates that, unlike BRCA1, the MDC1 BRCT domain recognizes the COOH-terminal carboxylate of its phosphopeptide target with a high degree of specificity.

To begin to understand the structural basis for MDC1 phosphopeptide recognition, we determined the x-ray crystal structure of the tandem BRCT repeats of MDC1 using multiwavelength anomalous diffraction and a selenomethionine-substituted protein (TABLE ONE). The structure reveals that MDC1 bears a structure that is strikingly similar to that of the tandem BRCT repeats of BRCA1, despite the fact that these two protein domains are only 15% identical at the sequence level. Both proteins share many of the same major secondary structure elements and the NH_2- and COOH-terminal BRCT repeats pack in the same head-to-tail manner seen in both BRCA1 (2) and 53BP1 (3, 4) (Fig. 2*A*).

To compare the putative peptide binding surface of MDC1 with that of BRCA1, we aligned the two structures based on a superimposition of key peptide-binding residues in BRCA1 with those in MDC1 (Fig. 2*B*). The superimposition reveals that the NH_2-terminal BRCT repeat of MDC1 contains a phosphoserine recognition pocket that is nearly identical to that of BRCA1, despite the fact that a critical serine in BRCA1 (Ser^{1655}) is a threonine in

TABLE ONE

Crystallographic data collection, phasing and refinement statistics

Data collection		Se-methionine MDC1	Native MDC1
Space group		P1	P1
Cell dimensions			
	a (Å)	44.411	42.051
	b (Å)	62.266	44.440
	c (Å)	73.437	61.942
Cell angles	α (°)	80.38	72.95
	β (°)	85.63	87.54
	γ (°)	73.85	61.80
	λ1 (peak)	λ2 (remote)	
Wavelength (Å)	0.9795	1.0199	1.1159
Resolution range (Å)	30–2.7	30–2.7	30–1.45
Observations	368,546	354,008	170,850
Unique reflections	20,877	20,208	60,647
Data coverage total/final shell (%)[a]	97.9 (90.5)	98.4 (94.1)	90.8 (75.9)
$\langle I/\sigma I \rangle$ total/final shell	13.0 (2.5)	16.6 (3.8)	14.5 (1.9)
R_{sym} total/final shell (%)[b]	13.5 (58.9)	11.3 (44.6)	4.8 (38.0)
Phasing statistics			
Resolution range(Å)	30.0–2.7		
No. of selenium sites	8		
FOM (solve)	0.34		
FOM (resolve)	0.67		
Refinement statistics			
Resolution range(Å)		30–1.45	
R_{work}/R_{free} (%)[c]		0.195/0.212	
No. of refined atoms	Protein	3001	
	Water	256	
Root mean square deviations	Bonds (Å)	0.010	
	Angles (°)	1.7	
Average *B*-factors(Å^2)	Protein	12.3	
	Water	16.9	
Ramachandran	Most favored	292 (93.3%)	
	Allowed	21 (6.7%)	
	Generously allowed	0	
	Disallowed	0	

[a] Final shell: λ1, 2.80–2.70 Å; λ2, 2.80–2.70 Å; MDC1, 1.5–1.45 Å.
[b] $R_{sym} = \Sigma|\langle I_{hkl}\rangle - \langle I\rangle|/\Sigma\langle I_{hkl}\rangle$, where I_{hkl} is the integrated intensity of a given reflection.
[c] $R_{work} = \Sigma_h|F_o(h) - F_c(h)|/\Sigma_h|F_o(h)|$, where $F_o(h)$ and $F_c(h)$ are observed and calculated structure factors. Data from wavelength λ1 were used during crystallographic refinement. R_{free} was calculated with 5% of all reflections excluded from refinement stages using the native data set. No $I/\sigma I$ cutoff was used in the refinement.

MDC1. The phosphate-binding ligands are the Oγ of Thr^{1898}, the main chain NH of Gly^{1899}, and the Nε of Lys^{1936}. Thr^{1898} is held in place by a hydrogen bond with Thr^{1934}, and the Nε of Lys^{1936} is held in place by a hydrogen bond with the main chain oxygen of Phe^{1897}.

In contrast, the region of MDC1 corresponding to the +3 specificity pocket in BRCA1 shows only limited conservation. Arg^{1699} is critical for phosphopeptide recognition in BRCA1, and mutations of this residue have been uncovered in breast cancer patients (8–10). This residue hydrogen bonds to the main chain of the +3 residue of the peptide target in BRCA1, helping the phenylalanine side chain to dock into the specificity pocket. In MDC1, this residue is conserved (Arg^{1933}) and adopts a nearly identical conformation to that seen in BRCA1. In both BRCA1 and MDC1, the guanidinium group of the arginine is held in place via a salt-bridging interaction with a conserved glutamic acid side chain (Glu^{2063} in MDC1, Glu^{1836} in BRCA1). The conservation of this critical arginine suggests that the +3 tyrosine of the γ-H2AX peptide will indeed bind at this site; however, the structure of the specificity pocket is otherwise quite

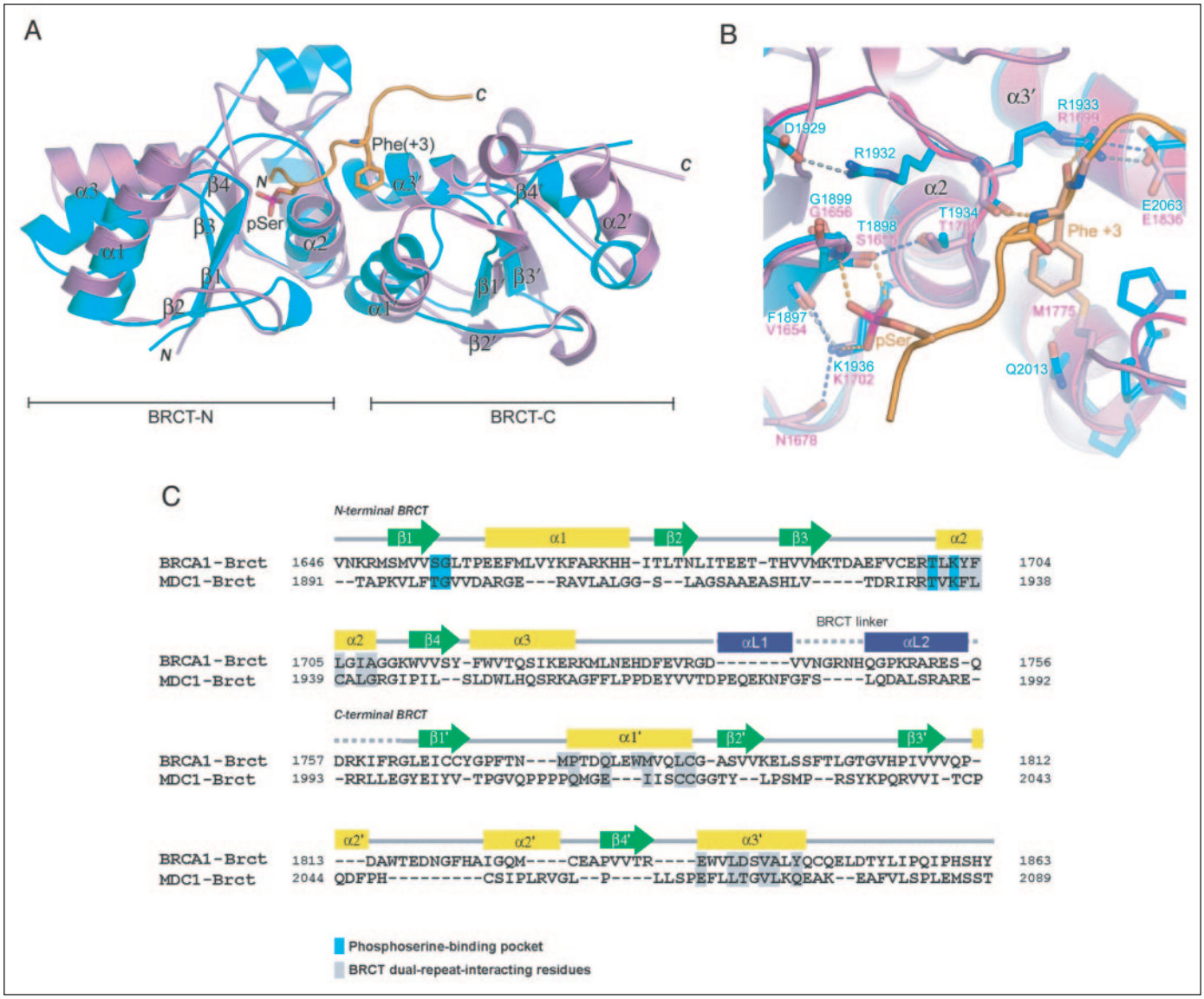

FIGURE 2. **Structure of the tandem BRCT repeats of MDC1.** *A*, overview of the MDC1 BRCT structure (*cyan*) aligned with the structure of the BRCA1 BRCT (*magenta*) bound to an optimized phospho-peptide target (*orange*) (Protein Data Bank accession code 1T2V). *B*, details of the phosphopeptide recognition surfaces of BRCA1 and MDC1, colored as in *A*. Residues involved in peptide binding are labeled and shown as *sticks*. *C*, sequence alignment of the MDC1 and BRCA1 BRCT repeats. The secondary structure of MDC1 is indicated, and residues conserved in the phosphoserine binding pocket are highlighted in *blue*. Residues involved in the head-to-tail packing of the NH_2- and COOH-terminal BRCT repeats are *shaded* in *gray*.

different in both proteins. A key residue for phenylalanine recognition in BRCA1 is Met[1775], which forms the base of the pocket and, when mutated to arginine, blocks peptide binding and is associated with cancer (8–10, 22, 23). No similar hydrophobic residue is in this position in MDC1, and indeed, the loop at this position adopts a quite different and rigid conformation, due to the presence of four consecutive proline residues, which are not conserved in BRCA1.

A comparison of the structure of the MDC1 BRCT with the structure of the BRCA1 BRCT-phosphopeptide complex suggests how the MDC1 specificity pocket may recognize the COOH-terminal tyrosine of γ-H2AX. We predict that the guanidinium group of the conserved Arg[1933] will contact the carboxyl terminus, but this must not be sufficient, otherwise the BRCA1 BRCT would also be specific for the COOH-terminal carboxylate of the +3 residue. MDC1 also contains Arg[1932], which could also form a salt bridge to the COOH-terminal carboxylate of γ-H2AX, although this would require a re-orientation of this side chain and a disruption of its salt bridging interactions with Asp[1929]. Arg[1932] could also come into proximity with the conserved glutamic acid of γ-H2AX at the +2 position and could explain the observed binding preference for glutamic acid at this position (13). The specificity for tyrosine over phenylalanine at the COOH terminus of γ-H2AX may be explained by the presence of Gln[2013], which is positioned to hydrogen bond with the tyrosine hydroxyl group, assuming this side chain is oriented in the pocket in a manner similar to that of the +3 phenylalanine in the BRCA1 structure. Thus, subtle differences between the specificity pockets of MDC1 and BRCA1 can explain the unique ability of the MDC1 BRCT to recognize the negatively charged COOH terminus of γ-H2AX and thereby be recruited to DNA double strand breaks.

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