A Quantitative Assay Reveals Ligand Specificity of the DNA Scaffold Repair Protein XRCC1 and Efficient Disassembly of Complexes of XRCC1 and the Poly(ADP-ribose) Polymerase 1 by Poly(ADP-ribose) Glycohydrolase*

Received for publication, November 7, 2014, and in revised form, December 3, 2014 Published, JBC Papers in Press, December 4, 2014, DOI 10.1074/jbc.M114.624718

In-Kwon Kim1, Roderick A. Stegeman, Chris A. Brosey, and Tom Ellenberger2

From the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Background: The DNA repair scaffold XRCC1 binds to poly(ADP-ribose)ylated PARP1 at damaged chromatin.

Results: XRCC1 preferentially binds to poly(ADP-ribose) chains longer than 7 ADP-ribose units in length.

Conclusion: We identify specific determinants of XRCC1-PARP1 complex assembly, and disassembly by PARG.

Significance: Our TR-FRET assay is useful for investigating turnover of posttranslational modifications and for identifying inhibitors by high-throughput screening.

The posttranslational modification of proteins with poly-(ADP-ribose) (PAR) regulates protein-protein interactions in DNA repair, gene expression, chromatin structure, and cell fate determination. The PAR polymerase PARP1 binds to damaged chromatin and synthesize PAR chains to signal DNA damage and recruit the DNA repair scaffold, XRCC1. Pharmacological blockade of PARP1 enzymatic activity impairs XRCC1-dependent repair of DNA damage and selectively kills cancer cells lacking other DNA repair functions. As such, PARP inhibitors are promising new therapies for repair-deficient tumors such as BRCA mutated breast cancers. Although the XRCC1-PARP1 complex is relevant to the proposed therapeutic mechanism of PARP inhibitors, the physical makeup and dynamics of this complex are not well characterized at the molecular level. Here we describe a fluorescence-based, real-time assay that quantitatively monitors interactions between PARylated PARP1 and XRCC1. Using this assay, we show that the PAR posttranslational modification by itself is a high affinity ligand for XRCC1, requiring a minimum chain length of 7 ADP-ribose units in the oligo(ADP-ribose) ligand for a stable interaction with XRCC1. This discrete binding interface enables the PAR glycohydrolase (PARG) to completely disassemble the PARP1-XRCC1 complex without assistance from a mono(ADP-ribose) glycohydrolase. Our quantitative, real-time assay of PAR-dependent protein-protein interactions and PAR turnover by PARG is an excellent tool for high-throughput screening to identify pharmacological modulators of PAR metabolism that may be useful therapeutic alternatives to PARP inhibitors.

Reversible protein posttranslational modifications (PTMs)3 increase the functional diversity of proteins and enable rapid switching of protein activities between “on” and “off” states (1). The majority of these PTMs alter binding activities, regulating either enzyme-substrate complexes or protein-protein interactions. PARP1 is a poly(ADP-ribose) (PAR) polymerase that is activated by binding to DNA strand breaks, causing PARylation of PARP1 and other substrates including histones, chromatin-remodeling enzymes, and DNA repair factors (2). PARP1 synthesizes branched PAR chains that can be hundreds of nucleotides in length, rivaling the native size of many proteins that are modified by PARP1. The PAR PTM generates a nidius of negative charge that can interfere with protein functions such as the DNA binding activity of PARP1, while creating new binding sites for PAR-specific binding proteins that mediate many of the downstream responses to this posttranslational modification. The PAR glycohydrolase (PARG) hydrolyzes PAR chains into mono(ADP-ribose) and oligo(ADP-ribose) products (3). The potential roles of oligo(ADP-ribose) chains generated during PAR turnover as signaling molecules and effectors of cell death are an active area of research (4). The terminal enzymatic products of the PARG reaction are ADP-ribose and a mono-(ADP-ribose)ylated protein, which is a substrate for recently identified mono-(ADP-ribose) glycohydrolases (5).

The synthesis and turnover of PAR by PARP1 and PARG, respectively, are required for normal responses to DNA damage and the maintenance of genomic integrity (6). Auto-modified PARP1 recruits a DNA repair scaffold XRCC1 to sites of DNA damage in a PAR-dependent manner (7). The PARP1-XRCC1 complex is subsequently disassembled to marshal the DNA repair activities associated with XRCC1, whereas PARP1 is polyubiquitinated and degraded (8, 9). PARylated PARP1

*This work was supported, in whole or in part, by National Institutes of Health Grant R01 GM052504 (to T. E.) and by the Structural Cell Biology of DNA Repair Program (Grant P01 CA92584 to John Tainer, The Scripps Research Institute).

1To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2974; Fax: 314-362-7183; E-mail: inkwon@biochem.wustl.edu.

2To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2974; Fax: 314-362-7183; E-mail: tome@biochem.wustl.edu.

3The abbreviations used are: PTM, posttranslational modification; PAR, poly-(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PARG, poly(ADP-ribose) glycohydrolase; PBM, poly(ADP-ribose)-binding motif; BRCT, BRCA1 C terminus; TR-FRET, time-resolved fluorescence resonance energy transfer; MAR, mono(ADP-ribose); Ni-NTA, nickel-nitrilotriacetic acid; iso-ADPr, iso-ADP-ribose; CK2, casein kinase 2.
Quantitative Assay for Poly(ADP-ribose) Binding and Turnover

binds to a conserved PAR-binding motif (PBM) located in one of two BRCA1 C terminus (BRCT) domains of XRCC1 (see Fig. 1A) (7, 10, 11). Pharmacological blockade of PARP1 by small molecule PARP inhibitors slows the repair of DNA single- and double-strand breaks, selectively killing tumors with genetic defects in homology-directed repair including BRCA-deficient breast cancer cells (12–14). A deficiency in PARG glycohydro-lase activity prolongs DNA damage foci, containing PAR, and similarly delays DNA repair, causing hypersensitivity to DNA-damaging agents and selective killing of BRCA-deficient cancer cells in a manner similar to PARP inhibition (15, 16).

XRCC1 and other PAR-binding proteins mediate many of the downstream responses to PARP1 activation in the face of DNA damage. Several types of PAR-selective binding domains have been identified (3), and hundreds of PAR-interacting proteins have been reported in proteomic studies (10, 17). Our understanding of the kinetics and thermodynamics of these PAR binding interactions is rudimentary (18), yet this information is essential for a mechanistic description of PAR-dependent regulation of DNA damage responses and other biological processes.

Here, we describe a kinetic assay using time-resolved fluorescence resonance energy transfer (TR-FRET) to quantitatively monitor the interaction of XRCC1 with automodified PARP1 and the disassembly of the protein complex by PARG (see Fig. 1B). We find that the PAR PTM by itself supports high-affinity binding to XRCC1 and requires a PAR chain length of at least 7 ADP-ribose units. PARG rapidly reverses the PARylation of PARP1 and efficiently disassembles the PARP1-XRCC1 complex, thereby uncoupling the DNA repair scaffolding activities of XRCC1 from PARP1, which is targeted for proteasomal degradation after recruiting XRCC1 to sites of DNA damage (9). Our TR-FRET assay enables studies of PAR binding specificity and the kinetics of PAR chain degradation. Its highly reproducible readout in a multi-well format makes the assay suitable for high-throughput screening to identify modifiers of these PAR-dependent activities.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The DNA-binding domain (residues 1–374) of human PARP1 and the PARP1 catalytic domain (residues 375–1014) were cloned in pET28a (Novagen) with an N-terminal His tag. The proteins were expressed in *Escherichia coli* Rosetta host cells and purified as described previously (19). The GST-tagged PARP1 construct in pGEX-6p1 (GE Healthcare) was expressed in *E. coli* Rosetta cells and then purified by affinity capture on a GSH-Sepharose column (GE Healthcare). After elution with a buffer containing 10 mM glutathione, the GST-PARP1 protein was further purified on a Superdex 200 size-exclusion column (GE Healthcare) in buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM dithiothreitol, and 5% glycerol. The wild type and catalytically inactive mutant (E752N) of rat PARP1 (residues 385–972) were expressed and purified from *E. coli* Tüner (DE3) cells co-expressing the GroESL chaperone, as described previously (19).

The GST-tagged BRCT1 domain of human XRCC1 (residues 294–417; cloned in pGEX-6p1) was expressed in *E. coli* Rosetta cells and then purified by glutathione affinity chromatography. Following cleavage of the GST tag with PreScission protease (GE Healthcare), the BRCT1 domain was purified on a Sephacryl 100 (GE Healthcare) size-exclusion column. XRCC1ΔN (residues 294–633) was cloned in pET32a (Novagen) with an N-terminal thioredoxin and His tag and expressed in *E. coli* Rosetta cells. XRCC1ΔN was purified by Ni-NTA (Qiagen) affinity chromatography. The protein was eluted from Ni-NTA with 250 mM imidazole and then loaded onto a heparin column (GE Healthcare) and eluted with a 0–1 M NaCl gradient. The thioredoxin/His tag was removed from XRCC1ΔN with PreScission protease before purification on a Superdex 200 column. Phosphorylated XRCC1ΔN was prepared by co-expression with human casein kinase 2α (CK2α) in *E. coli* Rosetta cells followed by purification using the same protocol as for XRCC1ΔN. The 15 sites of phosphorylation were confirmed by LC-MS/MS. The BRCT2 domain of human XRCC1 (residues 538–633) was cloned into pET28a with an N-terminal His tag, expressed in *E. coli* Rosetta cells, and then purified using a Ni-NTA affinity column followed by Superdex 200 chromatography.

**Biotinylation of the XRCC1 BRCT1 Domain**—The BRCT1 domain of XRCC1 (residues 294–405) was cloned in pGEX-6p1 with a C-terminal biotin acceptor peptide tag and co-expressed with the BirA biotin ligase (pACYC184-BirA plasmid; Avidine) in *E. coli* BL21 (DE3) cells. This design placed the biotin acceptor peptide tag adjacent to the predicted binding site for poly(ADP-ribose) (PAR motif) to optimize FRET efficiency when bound to FITC-labeled PARP1. The biotinylated BRCT1 was purified using the same protocol as the GST-BRCT1 protein (residues 294–417) described above. Efficient biotinylation of BRCT1 was confirmed by mixing biotin-labeled and unlabeled BRCT1 (2 μM) with increasing amounts of streptavidin (1–10 μM) followed by a 20-min incubation at 4 °C and analysis by SDS-PAGE. The electrophotoreceptor mobility shift assay confirmed that virtually all of the purified BRCT1 could be bound to streptavidin.

**Fluorescein Labeling of Poly(ADP-ribose)** of PARP1—FITC was incorporated into enzymatically auto-modified PARP1 in a reaction containing PARP1C (2 μM), the PARP1 DNA-binding domain (2 μM), a 24-mer nicked DNA oligonucleotide (2 μM), and a mixture of unlabeled NAD⁺ (Sigma) and FITC-NAD⁺ (Trevigen) substrates (total NAD⁺ concentration of 100 μM). After incubation for 1 h at 37 °C, PARylated PARP1 was passed through a PD-10 (GE Healthcare) desalting column in a buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.01% NP-40. This strategy specifically labels the PAR chains of PARylated PARP1 without altering its XRCC1 binding activity as compared with unlabeled PARylated PARP1 (see Fig. 2A).

**GST Pulldown Assay**—PARylated GST-PARP1C (2 μM) was immobilized by incubating with GSH-Sepharose beads for 10 min at 4 °C in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, and 10% glycerol. The beads were washed extensively with the binding buffer, and then 10 μM of a His-tagged XRCC1 domain was added (BRCT1, BRCT2, or biotinylated BRCT1 conjugated to streptavidin in a 1:1 ratio) and incubated for 1 h at 4 °C. After washing beads four times with the binding buffer, samples were analyzed by SDS-PAGE followed by staining with SYPRO Ruby staining (Invitrogen) and fluorescence imaging (GE Healthcare Typhoon).
Poly(ADP-ribose) Synthesis and Purification—Poly(ADP-ribose) chains were prepared by a published protocol (20). PAR polymers were synthesized in a 20-ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 12.5 µg/ml calf thymus DNA, 2 µM full-length PARP1, 4 µM PARP1C, and 2 mM NAD⁺. PARylated PARP1 was precipitated in 10% ice-cold TCA, and the pellet was resuspended in 1 M KOH and 50 mM EDTA and then incubated for 1 h at 60 °C to detach PAR polymers from the denatured PARP1. The PAR chains were recovered on a dihydroxyboryl Bio-Rex 70 column and then size-fractionated on a DNA-Pac PA100 (Dionex) ion-exchange column. PAR polymers were divided into pools representing different size fractions based on the HPLC elution profile (20), dried under vacuum, and then desalted into water using a PD-10 desalting column. Purified PAR chains were dried under vacuum and dissolved in water at a final concentration of 2–6 mM. The molar concentration of PAR (expressed as the concentration of ADP-ribose units) was estimated as follows: 

\[
[\text{PAR}] = \left( \frac{A_{260} \text{ (cm}^{-1})}{13,500 \text{ cm}^{-1} \text{·m}^{-1}} \right) \times 10^3 \text{ (mol/L)}
\]

UV absorbance at 260 nm (A₂₆₀) was measured with a NanoDrop 1000 (Thermo Scientific). Iso-ADPr was prepared as reported previously (21) with the following modifications. PAR chains (1 mg) were digested with snake venom phosphodiesterase (10 units; Worthington) in a reaction buffer of 50 mM Tris-HCl, pH 8.8, 0.1 M NaCl, and 15 mM MgCl₂ during an overnight incubation at room temperature. Iso-ADPr was further purified on a DNA-Pac PA100 ion-exchange column and desalted with water using a Sephacryl 100 size-exclusion column. Purified iso-ADPr was dried under vacuum and dissolved in water to 5 mM final concentration.

TR-FRET Assay for Poly(ADP-ribose) Binding and Turnover—TR-FRET experiments were performed in a final volume of 30 µl in 384-well polystyrene flat bottom microplates (Corning) using a Synergy2 microplate reader (Bio-Tek). TR-FRET assays were performed in triplicate in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.01% NP-40. The Tb³⁺ donor was excited at 340 nm with the fluorescein acceptor intensity measured at 520 nm, with a 100-µs delay after donor excitation.

To monitor the binding interaction of PARP1 and XRCC1, the biotin-tagged BRCT1 domain of XRCC1 (5 nM) was conjugated with an equimolar concentration of Tb³⁺-chelated streptavidin (Invitrogen) in TR-FRET assay buffer. The Tb³⁺-labeled BRCT1 domain was subsequently titrated with FITC-labeled PARP1. In control reactions, FITC-labeled PARP1 or biotinylated BRCT1 were omitted. For the TR-FRET competition binding assay, the preformed FRET pair consisting of FITC-labeled PARP1 (42 nm) and Tb³⁺-BRCT1 (5 nm) was challenged with unlabeled XRCC1 constructs (BRCT1, BRCT2, XRCC1N, or XRCC1N-p) or small ligands (ADP-ribose (Sigma), iso-ADPr, and PAR polymers) at the indicated concentrations. The EC₅₀ values were determined by fitting the data \( F_{520}/F_{495} \) to Equation 1.

\[
Y_{(F_{520}/F_{495})} = Y_{\text{min}} + \frac{Y_{\text{max}} - Y_{\text{min}}}{1 + \left( \frac{X}{EC_{50}} \right)^{\text{Hillslope}}} \quad \text{(Eq. 1)}
\]

The data for all competitor concentrations were globally fitted to the maximum value \( Y_{\text{max}} \) in Equation 1, which generated consistent minimum values \( Y_{\text{min}} \).

To monitor PAR turnover using the TR-FRET assay, FITC-labeled PARP1 (42 nm) was preincubated with Tb³⁺-BRCT1 (5 nm) before the addition of the indicated concentrations of rat PARG (0–800 nM) at time 0 min. The TR-FRET signal was subsequently monitored for 90 min. A \( K_{50} \) value for PARG-dependent disassembly of the PARP1-XRCC1 complex was determined by pretreating FITC-labeled PARP1 (42 nm) with rat PARG (0–375 nm) for 2 h and then adding Tb³⁺-labeled BRCT1 (5 nm) and immediately measuring the TR-FRET intensity ratio. Control reactions in the absence of PARG (100%) or BRCT1 (0%) were used for normalization of the TR-FRET intensity ratio. The data were plotted as a function of PARG concentration and fit to a hyperbolic function.

LC-MS/MS Identification of CK2-dependent Phosphorylation Sites of XRCC1—XRCC1ΔN-p (20 µg) was resuspended in 50 mM ammonium bicarbonate and incubated with trypsin (2 µg) for 16 h at 37 °C. The sample was dried in a SpeedVac (Thermo Fisher) and resuspended in 20 µl of 5% acetonitrile/0.1% formic acid. Peptides were analyzed with a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) coupled with nanoLC Ultra (Eksigent). Samples (5 µl) were injected into a trap column (C₁₈ PepMap100, 300 µm × 1 mm × 5 µm, 100 Å; Dionex) at a flow rate of 4 µl/min for 5 min. Peptide separation was carried out on a C₁₈ column (Acclaim PepMap C18, 15 cm × 75 µm × 3 µm, 100 Å; Dionex) at a flow rate of 0.26 µl/min. Peptides were separated with an 85-min linear gradient ranging from 2 to 40% B (mobile phase A, 0.1% formic acid; mobile phase B, 0.1% formic acid in acetonitrile). The mass spectrometer was operated in positive ionization mode. The MS survey scan was performed in the Fourier transform cell, recording a mass range of 300–2000 m/z. The resolution was set to 60,000 at 400 m/z, and the automatic gain control was set to 1,000,000 ions. Collision-induced dissociation fragmentation was used in the MS/MS scan, and the 20 most intense signals in the survey scan were fragmented. Fragmentation was performed with an isolation window of 1.5 m/z, a normalized collision energy of 35%, and an activation time of 30 ms. Dynamic exclusion was performed with a repeat count of 1 and exclusion duration of 12 s. The minimum MS signal for triggering MS/MS was set to 5000.

The data were processed using Mascot Distiller v2.3 and searched using Mascot Demon v2.3 (Matrix Science). The search was performed against the sequence of XRCC1 included in a customized database. Data were further analyzed with Scaffold v3.0.9.1 and Scaffold PTM v1.1.0.1 (ProteomeSoftware) to calculate the Ascore for the identified phosphorylation sites. Ascore measures the probability of correct phosphorylation site localization based on the presence and intensity of site-determining ions in MS/MS spectra.

RESULTS

A Novel, Quantitative Poly(ADP-ribose) Binding Assay—For real-time monitoring of the PARP1-XRCC1 interaction, both proteins were labeled with fluorescent groups that constitute a FRET pair (Fig. 1). The lanthanide terbium (Tb³⁺) was chosen...
as the FRET donor because its long-lived excited state is compatible with TR-FRET, a protocol that decreases interference from auto-fluorescence of biological samples or organic small molecules (22). Tb3⁺-chelated streptavidin was bound to the biotinylated BRCT1 domain of XRCC1, which harbors the PBM (11). The FRET acceptor, FITC, was incorporated into auto-modified PARP1 by enzymatic synthesis of PAR chains using a mixture of unlabeled NAD⁺ and FITC-NAD⁺ substrates (Fig. 1B). A protein construct comprising the auto-modification, WGR, and catalytic domains of PARP1 (PARP1C; residues 1–1014; Fig. 1A) was auto-modified in an enzymatic reaction containing the DNA-binding domain of PARP1, a nicked DNA oligonucleotide, and NAD⁺. Pulldown experiments with GST-tagged PARP1C confirmed that the BRCT1 domain binds tightly to auto-modified PARP1C, but not to unmodified PARP1C (Fig. 2A). This result demonstrates a direct interaction between XRCC1 and automodified PARP1, extending previous findings in which BRCT1 was co-immunoprecipitated with PARP1 from mammalian cell extracts (23). The fluorescent labels did not interfere with the protein-pro-
tein interaction, even at high concentrations of FITC-NAD\(^+\) in the PARylation reaction (Fig. 2A).

The amount of FITC-NAD\(^+\) incorporated into PARP1 was optimized at a 1:7 ratio of FITC-NAD\(^+\):NAD\(^+\), which saturates the FRET intensity ratio (\(F_{520}/F_{495}\)), using a minimal amount of labeled NAD\(^+\) substrate in the PARylation reaction (Fig. 2B). Assembly of the PARP1-BRCT1 complex generates more than a 10-fold increase in the TR-FRET signal in comparison with control reactions lacking the donor or the acceptor (Fig. 2C). Under FRET conditions, the intensity of Tb\(^3+\) emission (\(F_{495}\)) decreases slightly with increasing concentrations of labeled PARP1, whereas the intensity of FITC fluorescence (\(F_{520}\)) increases (data not shown). The TR-FRET signal resulting from PARP1-XRCC1 complex formation is sensitive to increasing salt concentration (Fig. 2D) as expected for an electrostatic interaction of the negatively charged PAR chains with basic residues of the PBM (10, 11). The high specificity of the XRCC1-PARP1 binding interaction is evident from the saturable binding isotherm and the low background fluorescence in the absence of biotinylated BRCT1 or FITC-labeled PARP1 (Fig. 2C).

**BRCT1 Is Sufficient for Binding to PARylated PARP1 with a Nanomolar Affinity**—To examine the binding specificity of PARylated PARP1 in more detail, the isolated BRCT1 and BRCT2 domains of XRCC1 were tested as ligands in competition binding experiments. When overexpressed in mammalian cells, the BRCT1 domain of XRCC1 was co-immunoprecipitated preferentially with auto-modified PARP1 (23), whereas BRCT2 lacks the PBM motif and instead interacts specifically with the BRCT domain of DNA ligase III\(^+\) (24, 25). The recombinant BRCT1 and BRCT2 domains purified from *E. coli* (Fig. 3A) were titrated against a fixed concentration of the labeled PARP1-XRCC1 complex. BRCT1 caused a concentration-dependent inhibition of the TR-FRET signal with an EC\(_{50}\) of 25 nM (Fig. 3B). BRCT2 did not compete for binding to PARP1 at concentrations up to 1 \(\mu M\) in the TR-FRET assay (Fig. 3B) and did not interact with PARylated PARP1 in GST pulldown experiments (data not shown).
Quantitative Assay for Poly(ADP-ribose) Binding and Turnover

The linker region separating the BRCT1 and BRCT2 domains of XRCC1 (residues 418–537; Fig. 1A) contains binding sites for the DNA repair factors polynucleotide kinase and aprataxin (24, 26). Phosphorylation of the linker by CK2 stimulates binding of polynucleotide kinase, and accelerates the recruitment of XRCC1 and repair of DNA strand breaks (27, 28). An XRCC1 construct spanning both BRCT1 and BRCT2 (XRCC1ΔN; Figs. 1A and 3A) binds to auto-modified PARP1 with an affinity (EC\textsubscript{50} of 62 ± 9 nM) comparable with BRCT1 alone (Fig. 3B), indicating that the PAR-binding site is wholly contained in the BRCT1 domain of XRCC1.

To explore the potential contribution of XRCC1 phosphorylation to PARP1 binding, the phosphorylated XRCC1ΔN (XRCC1ΔN-p) protein was prepared by co-expression with human CK2α in E. coli and tested for binding to auto-modified PARP1. Mass spectrometry of XRCC1ΔN-p confirmed the identities of 15 CK2-dependent phosphorylation sites within the linker and BRCT2 domain (27) (Fig. 3, C and D). XRCC1ΔN-p binds to PARP1 with 3-fold lower affinity (EC\textsubscript{50} of 196 ± 29 nM) than unphosphorylated XRCC1ΔN (Fig. 3B), raising the possibility that phosphorylation of XRCC1 by CK2 may regulate the binding affinity for auto-modified PARP1 and contribute to the release of XRCC1 once it is recruited by PARP1 to a site of DNA damage. The extent of phosphorylation of XRCC1ΔN-p is unknown, and the observed decrease in binding affinity may be a minimal estimate of the effect of XRCC1 phosphorylation.

**XRCC1 Has Strict Ligand Specificity to PAR Chains Longer than 7 ADP-ribose Units**—The PAR binding specificity of the PBM has not been characterized for XRCC1 or any other protein (3). We performed TR-FRET binding competition experiments to determine the minimum length of PAR oligonucleotide that binds effectively to XRCC1. Oligo(ADP-ribose) chains were prepared by enzymatic synthesis and purified with ion-exchange HPLC as reported previously (20). BRCT1 binds weakly to ADP-ribose (EC\textsubscript{50} of 1.39 ± 0.06 mM), whereas oligo(ADP-ribose) ligands with a chain length greater than 7 ADP-ribose units in poly(ADP-ribose) ligands longer than 7 ADP-ribose units in exchange HPLC as reported previously (20). BRCT1 does not bind to the MARylated PARP1 product of the PARG (Fig. 5A). Pretreatment of auto-modified PARP1 with PARG prevents binding to XRCC1 (Fig. 5B and C), showing that enzymatic turnover of the PAR PTM is required for disassembly of the PARP1-XRCC1 complex. Pretreatment of auto-modified PARP1 with PARG prevents binding to XRCC1 (Fig. 5B and C), further evidence that XRCC1 does not bind to the MARylated PARP1 product of the PARG digest. It was recently reported that XRCC1 binds equally well to poly(ADP-ribose) and to ADP-ribose based on Kd, measurements from isothermal titration calorimetry (29). However, our results show that XRCC1 binds to poly(ADP-ribose) and not mono(ADP-ribose). This binding selectivity for oligo(ADP-ribose) chains is corroborated by the significant loss of affinity for short chain PAR oligomers as compared with longer chains (Fig. 4), and the ability of PARG to regulate the PARP1-XRCC1 interaction by converting PARylated PARP1 into MARylated PARP1, which retains a terminal ADP-ribose modification but does not bind to XRCC1 (Fig. 5). Binding partners for MARylated PARP1 have not been identified, but mutations of the MAR hydrodase (32). Because XRCC1 binds weakly to ADP-ribose and iso-ADP-ribose and iso-ADP-ribose (Fig. 4), we hypothesized that PARG enzymatic activity should be sufficient to disassemble the PARP1-XRCC1 complex, leading to a time-dependent loss of TR-FRET signal (Fig. 5A). To test this hypothesis, the TR-FRET signal from the labeled PARP1-BRCT1 complex was monitored during challenge with increasing concentrations of PARG (Fig. 5, B and C).

The addition of PARG causes a time-dependent loss of the TR-FRET signal, with the rate of loss dependent upon the concentration of PARG (Fig. 5C). The addition of the catalytically inactive mutant PARG\textsuperscript{E752N} does not alter the TR-FRET signal (Fig. 5, B and D), showing that enzymatic turnover of the PAR PTM is required for disassembly of the PARP1-XRCC1 complex. Pretreatment of auto-modified PARP1 with PARG prevents binding to XRCC1 (Fig. 5E), further evidence that XRCC1 does not bind to the MARylated PARP1 product of the PARG digest.

**PAR Turnover by PARG Efficiently Disassembles PARP1-XRCC1**—PARG degrades the PAR PTM by a combination of exo- and endo-glycohydrolase activity (19, 30, 31), leaving a single ADP-ribose moiety attached to PARP1 that is a substrate for the recently identified mono(ADP-ribose) (MAR) hydrodase (3). We performed TR-FRET binding competition experiments with an affinity (EC\textsubscript{50} of 62 ± 9 nM) comparable with BRCT1 alone (Fig. 3B), indicating that the PAR-binding site is wholly contained in the BRCT1 domain of XRCC1.
that removes the terminal mono(ADP-ribose) are linked with a severe, inherited neurodegenerative disease (5).

The rates of PARG activity obtained using the TR-FRET assay were corroborated by monitoring the PARylation status of FITC-labeled PARP1 by SDS-PAGE analysis, in reactions performed under identical conditions. PARG caused a time- and concentration-dependent loss of FITC fluorescence associated with PARP1, on a time course matching the kinetics of the TR-FRET signal decay (Fig. 6). These results indicate that the enzymatic activity of PARG alone can disassemble the PARP1-XRCC1 complex.

**DISCUSSION**

We have developed a quantitative, real-time TR-FRET assay that measures both PAR binding and turnover by PARG in the context of a protein covalently modified by PAR (Fig. 1). The TR-FRET assay is less cumbersome than existing methods for PAR binding and turnover, such as radiometric assays using protein-free $^{32}$P-labeled PAR chains (31, 33). In a multi-well format, the TR-FRET assay is compatible with high-throughput screening to identify small molecule regulators of PARG- and PAR-mediated protein-protein interactions. More generally, it is compatible with monitoring a variety of conditional protein-protein interactions that are regulated by PTMs, as well as real-time assays of the enzymes that turn over these modifications.

**FIGURE 5.** PARG efficiently disassembles the PARP1-XRCC1 complex. **A**, schematic of the TR-FRET assay for PAR turnover. The addition of PARG to the labeled PARP1-XRCC1 complex removes the PAR posttranslational modification from PARP1, causing the complex to fall apart with the loss of the TR-FRET signal. **B**, a SDS-PAGE gel image of purified PARG proteins (1 μg) that were used for the TR-FRET PARG activity assay. **C**, a real-time monitoring of the disassembly of the PARP1-XRCC1 complex by PARG. The rates of complex disassembly, measured by the loss of the TR-FRET signal, are comparable with the rates of PAR hydrolysis monitored by SDS-PAGE for similar reaction conditions, demonstrating that the TR-FRET signal is correlated with the PARylation status of PARP1. **D**, PARG enzymatic activity is required for disassembly of the PARP1-XRCC1 complex. A time-dependent loss of the PARP1-XRCC1 complex by PARG was observed with increasing concentrations of PARG for 2 h, and the TR-FRET signal was measured by the addition of Tb$^{3+}$-BRCT1. The loss of interaction after complete digestion of PARylated PARP1 with PARG confirms that XRCC1 does not bind to mono(ADP-ribose)ylated PARP1.

**FIGURE 6.** A gel-based assay of PARG activity validates the TR-FRET activity assay. PARG activity was monitored by SDS-PAGE analysis of FITC-labeled, PARylated PARP1C. Removal of the PAR modification from PARP1C is evident from the loss of FITC label and the higher electrophoretic mobility of PARP1C as the PAR chains are hydrolyzed by PARG. The rate of loss of PARylation measured in this assay is in good agreement with the loss of the TR-FRET signal in similar reaction conditions shown in Fig. 5B.
Quantitative Assay for Poly(ADP-ribose) Binding and Turnover

Our data show that XRCC1 has a strict binding preference for poly(ADP-ribose) over mono- or di(ADP-ribose), with the length of the PAR chain as the main determinant of the PARP1-XRCC1 interaction (Fig. 4). The $K_m$ values of BRCT1 (25 ± 4 nm), XRCC1ΔN (EC$_{50}$ of 62 ± 9 nm), and poly(ADP-ribose) (EC$_{50}$ of 20 nm) determined by the TR-FRET assay are in good agreement with a published $K_m$ value (36 nm) for the interaction between poly(ADP-ribose) and the full-length XRCC1 measured by the surface plasmon resonance (34). This agreement is a validation of the TR-FRET assay as an accurate method for comparing binding affinities of proteins and ligands. The comparable, nanomolar affinities of protein-free PAR chains (Fig. 4) and the BRCT1 domain (Fig. 3) suggest that the PARP1-XRCC1 interaction is dominated by contacts with the PAR PTM, but not with the PARP1 protein itself. We conclude that XRCC1 interacts primarily with the PAR PTM of PARylated PARP1 with few, if any, binding contacts to the PARP1 protein. As such, protein-free PAR chains and the BRCT1 domain of XRCC1 are equipotent as competitive inhibitors of the PARP1-XRCC1 interaction.

Both PARP1 enzymatic activity and PARG enzymatic activity are required for DNA repair function of XRCC1 (15). PARG alone can efficiently disassemble the PARP1-XRCC1 complex (Fig. 5) by degrading the PAR chains to lengths that no longer bind XRCC1 (Fig. 4). Under our assay conditions, XRCC1 rapidly dissociates from PARP1 once PARG is added to the reaction. It was previously reported that the catalytic efficiency of PARG depends on the length of the PAR chain substrate (30, 31, 35). Long PAR polymers are efficiently hydrolyzed ($K_m$ = 1 μM) by a combination of endo- and exo-glycosidic activity (35), whereas smaller PAR oligomers are poor substrates for PARG ($K_m$ > 10 μM). This substrate length dependence of PARG activity may fine-tune the temporally dissociation of XRCC1 from PARP1 during active PAR synthesis by PARP1 and degradation by PARG.

While PAR chains are being hydrolyzed by PARG, PARylated PARP1 is polyubiquitinated by CHFR, a PAR-dependent RING E3 ubiquitin ligase, to target PARP1 for degradation by the proteasome (9). Withstanding turnover of PAR and proteasomal degradation of PARP1, XRCC1 is retained at damaged chromatin to coordinate DNA repair. The retention of XRCC1 in the face of PARP1 removal may indicate that XRCC1 is handed off to a new binding partner to continue the process of DNA repair. The PARG-catalyzed release of XRCC1 from PARP1 could facilitate a handoff of XRCC1 to another chromatin-associated factor (15) or to bind DNA by virtue of the DNA binding activities of repair factors stably associated with XRCC1 (36).

Acknowledgments—We thank Mark Glover kindly for kindly providing us with the pGEX-CK2 plasmid. We also thank Drs. Leslie Hicks and Sophie Alvarez for providing expert mass spectrometry analysis of proteins at the Proteomics & Mass Spectrometry Facility of the Donald Danforth Plant Science Center (St. Louis, MO).

REFERENCES

1. Walsh, C. (2006) Posttranslational Modification of Proteins: Expanding Nature’s Inventory, pp. 1–33, Roberts and Co. Publishers, Englewood, CO
2. Schreiber, V., Dantzer, F., Ame, J.-C., and de Murcia, G. (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol. 7, 517–528
3. Zaja, R., Mikoč, A., Barkauskaite, E., and Abel, I. (2012) Molecular Insights into poly(ADP-ribose) recognition and processing. Bioinorganic Chemistry 1, 1–17
4. Heerens, J. T., and Hergenrother, P. J. (2007) Poly(ADP-ribose) makes a date with death. Curr. Opin. Chem. Biol. 11, 644–653
5. Sharifi, R., Morra, R., Appel, C. D., Tallis, M., Chioza, B., Jankevicus, G., Simpson, M. A., Matic, I., Ozkan, E., Golia, B., Schellenberg, M. J., Weston, R., Williams, J. G., Rossi, M. N., Galehdari, H., Krahn, J., Wan, A., Trembath, R. C., Crosby, A. H., Abel, D., Hay, R., Ladurner, A. G., Timinszky, G., Williams, R. S., and Abel, I. (2013) Deficiency of terminal ADP-ribose protein glycohydrolase TARG1/Csor130 in neurodegenerative disease. EMBO J. 32, 1225–1237
6. Hakmæ, A., Wong, H.-K., Dantzer, F., and Schreiber, V. (2008) The expanding field of poly(ADP-ribose)ylation reactions: "Protein Modifications: Beyond the Usual Suspects" Review Series. EMBO Rep. 9, 1094–1100
7. El-Khamisy, S. F., Masutani, M., Suzuki, H., and Calcott, K. W. (2003) A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Res. 31, 5526–5533
8. Kang, H. C., Lee, Y.-J., Shin, J.-H., Andradi, S. A., Chi, Z., Gagné, J.-P., Lee, Y., Ko, H. S., Lee, B. D., Poizier, G. G., Dawson, V. L., and Dawson, T. M. (2011) Iduna is a poly(ADP-ribose) (PAR)-dependent E3 ubiquitin ligase that regulates DNA damage. Proc. Natl. Acad. Sci. U.S.A. 108, 14103–14108
9. Liu, C., Wu, J., Paudyal, S. C., You, Z., and Yu, X. (2013) CHFR is important for the first wave of ubiquitination at DNA damage sites. Nucleic Acids Res. 41, 1698–1710
10. Gagné, J.-P., Isabelle, M., Lo, K. S., Bourassa, S., Hendzel, M. J., Dawson, V. L., Dawson, T. M., and Poizier, G. G. (2008) Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. Nucleic Acids Res. 36, 6959–6976
11. Pleschke, J. M., Kleczkowska, H. E., Strohm, M., and Althaus, F. R. (2000) Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. J. Biol. Chem. 275, 40974–40980
12. Curtin, N. J., and Szabo, C. (2013) Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. Mol. Aspects Med. 34, 1217–1256
13. He, J.-X., Yang, C.-H., and Miao, Z.-H. (2010) Poly(ADP-ribose) polymerases inhibitors as promising cancer therapeutics. Acta Pharmacol. Sin. 31, 1172–1180
14. Telli, M. L., and Ford, J. M. (2010) PARP inhibitors in breast cancer. Clin. Adv. Hematol. Oncol. 8, 629–635
15. Wei, L., Nakajima, S., Hsieh, C.-U., Kanno, S., Masutani, M., Levine, A. S., Yasui, A., and Lan, L. (2013) Damage response of XRCC1 at sites of DNA single strand breaks is regulated by phosphorylation and ubiquitination after degradation of poly (ADP) ribose. J. Cell Sci. 126, 4414–4423
16. Fathers, C., Drayton, R. M., Solovieva, S., and Bryant, H. E. (2012) Inhibition of poly(ADP-ribose) glycohydrolase (PARG) specifically kills BRCA2-deficient tumor cells. Cell Cycle 11, 990–997
17. Gagné, J.-P., Pic, É., Isabelle, M., Krietsch, J., Ethier, C., Paquet, E., Kelly, I., Boutin, M., Moon, K.-M., Foster, L. J., and Poizier, G. G. (2012) Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress. Nucleic Acids Res. 40, 7788–7805
18. Fahrer, J., Kranaster, R., Altmeier, M., Marx, A., and Bürkle, A. (2007) Quantitative analysis of the binding affinity of poly(ADP-ribose) to specific binding proteins as a function of chain length. Nucleic Acids Res. 35, e143
19. Kim, I.-K., Kiefer, J. R., Ho, C. M. W., Stegeman, R. A., Classen, S., Tainer, J. A., and Ellenberger, T. (2012) Structure of mammalian poly(ADP-ribose) glycohydrolase reveals a flexible tyrosine clasp as a substrate-binding element. Nat. Struct. Mol. Biol. 19, 653–656
20. Tan, E. S., Krukenberg, K. A., and Mitchison, T. J. (2012) Large-scale preparation and characterization of poly(ADP-ribose) and defined length polymers. Anal. Biochem. 428, 126–136
21. Wang, Z., Michaud, G. A., Cheng, Z., Zhang, Y., Hinds, T. R., Fan, E., Cong, F., and Xu, W. (2012) Recognition of the iso-ADP-ribose moiety in poly(ADP-ribose) by WWE domains suggests a general mechanism for poly-
(ADP-ribosyl)ation-dependent ubiquitination. Genes Dev. 26, 235–240
22. Lakowicz, J. R. (2007) Principles of Fluorescence Spectroscopy, pp. 86–88, Springer-Verlag New York Inc., New York
23. Masson, M., Niedergang, C., Schreiber, V., Muller, S., Menissier-de Murcia, J., and de Murcia, G. (1998) XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. Mol. Cell Biol. 18, 3563–3571
24. Caldecott, K. W., McKeown, C. K., and Tucker, J. D. (1994) An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol. Cell Biol. 14, 68–76
25. Cuneo, M. J., Gabel, S. A., Krahn, J. M., Ricker, M. A., and London, R. E. (2011) The structural basis for partitioning of the XRCC1/DNA ligase IIIα BRCT-mediated dimer complexes. Nucleic Acids Res. 39, 7816–7827
26. Whitehouse, C. J., Taylor, R. M., Thistlethwaite, A., Zhang, H., Karimi-Busheri, F., Lasko, D. D., Weinfield, M., and Caldecott, K. W. (2001) XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. Cell 104, 107–117
27. Loizou, J. I., El-Khamisy, S. F., Zlatanou, A., Moore, D. J., Chan, D. W., Qin, J., Sarno, S., Meggio, F., Pinna, L. A., and Caldecott, K. W. (2004) The protein kinase CK2 facilitates repair of chromosomal DNA single-strand breaks. Cell 117, 17–28
28. Parsons, J. L., Dianova, I. I., Finch, D., Tait, P. S., Ström, C. E., Helleday, T., and Dianov, G. L. (2010) XRCC1 phosphorylation by CK2 is required for its stability and efficient DNA repair. DNA Repair (Amst.) 9, 835–841
29. Li, M., Lu, L.-Y., Yang, C.-Y., Wang, S., and Yu, X. (2013) The FHA and BRCT domains recognize ADP-ribosylation during DNA damage response. Genes Dev. 27, 1752–1768
30. Davidovic, L., Vodenicharov, M., Affar, E. B., and Poirier, G. G. (2001) Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. Exp. Cell Res. 268, 7–13
31. Braun, S. A., Panzelter, P. L., Collinge, M. A., and Althaus, F. R. (1994) Endoglycosidic cleavage of branched polymers by poly(ADP-ribose) glycohydrolase. Eur. J. Biochem. 220, 369–375
32. Feijs, K. L. H., Forst, A. H., Verheugd, P., and Lüscher, B. (2013) Macrodomain-containing proteins: regulating new intracellular functions of mono(ADP-ribose)lation. Nat. Rev. Mol. Cell Biol. 14, 443–451
33. Shah, G. M., Poirier, D., Duchaine, C., Brochu, G., Desnoyers, S., Lagueux, J., Verreault, A., Hoflack, J. C., Kirkland, J. B., and Poirier, G. G. (1995) Methods for biochemical study of poly(ADP-ribose) metabolism in vitro and in vivo. Anal. Biochem. 227, 1–13
34. Ahel, I., Ahel, D., Matusaka, T., Clark, A. J., Pines, J., Boulton, S. J., and West, S. C. (2008) Poly(ADP-ribose) binding zinc finger motifs in DNA repair/checkpoint proteins. Nature 451, 81–85
35. Hatakeyama, K., Nemoto, Y., Ueda, K., and Hayaishi, O. (1986) Purification and characterization of poly(ADP-ribose) glycohydrolase: different modes of action on large and small poly(ADP-ribose). J. Biol. Chem. 261, 14902–14911
36. Caldecott, K. W. (2003) XRCC1 and DNA strand break repair. DNA Repair (Amst.) 2, 955–969