Poly(ADP-ribose) Polymerase 1 Modulates Interaction of the Nucleotide Excision Repair Factor XPC-RAD23B with DNA via Poly(ADP-ribose)ylation*

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Poly(ADP-ribose)ylation is a reversible post-translational modification that plays an essential role in many cellular processes, including regulation of DNA repair. Cellular DNA damage response by the synthesis of poly(ADP-ribose) (PAR) is mediated mainly by poly(ADP-ribose) polymerase 1 (PARP1). The XPC-RAD23B complex is one of the key factors of nucleotide excision repair participating in the primary DNA damage recognition. By using several biochemical approaches, we have analyzed the influence of PARP1 and PAR synthesis on the interaction of XPC-RAD23B with DNA. Free PAR binds to XPC-RAD23B with an affinity that depends on the length of the poly(ADP-ribose) strand and competes with DNA for protein binding. Using 32P-labeled NAD+ and immunoblotting, we also demonstrate that both subunits of the XPC-RAD23B are poly(ADP-ribose)lated by PARP1. The efficiency of XPC-RAD23B PARylation depends on DNA structure and increases after UV irradiation of DNA. Therefore, our study clearly shows that XPC-RAD23B is a target of poly(ADP-ribose)ylation catalyzed by PARP1, which can be regarded as a universal regulator of DNA repair processes.

Background: Poly(ADP-ribose)ylation of DNA repair proteins is essential for the regulation of DNA repair processes.

Results: Both subunits of the nucleotide excision repair factor XPC-RAD23B are poly(ADP-ribose)lated by PARP1.

Conclusion: PARP1 influences the interaction of XPC-RAD23B with DNA via PAR synthesis.

Significance: This study provides direct evidence for XPC-RAD23B belonging to the targets of poly(ADP-ribose)ylation catalyzed by PARP1.

Genetic stability of living organisms is substantially maintained by the action of the DNA repair systems. Regulation of the activity of the DNA repair systems is required to efficiently protect genomic DNA under genotoxic stress. One of the key mechanisms modulating the activity of DNA repair systems is the synthesis of poly(ADP-ribose) attached covalently to some repair proteins. Among the presently characterized proteins of the PARP3 family, cellular DNA damage response by PARP1 is mainly mediated by PARP1, which is the most abundant and well studied members of the PARP family (1, 2). Poly(ADP-ribose)ylation targets are certain nuclear proteins, including PARP1 itself. Poly(ADP-ribose)ylation of the proteins can modulate their interactions with DNA due to the linkage to the negatively charged PAR (3) and can also be regarded as a signal of DNA damage. The poly(ADP-ribose)ylation reaction is reversible; PAR is cleaved by the poly(ADP-ribose)glycohydrolase (PARG) that additionally regulates the cellular level of protein poly(ADP-ribose)ylation and PAR synthesis.

PARP1 was shown to interact with the proteins of base excision repair and to modulate their activities (4–7). Recent data suggest the participation of PARP1 not only in base excision repair but also in other DNA repair systems as well as in other pathways of DNA metabolism. In particular, data were reported on the involvement of PARP1 in chromatin decondensation at the sites of damage induced by UV irradiation and in the regulation of the interaction of the nucleotide excision repair (NER) factors with UV-damaged DNA (6–10). Thus, DNA damage-binding protein 2 (DDB2) that specifically recognizes UV-induced damages in DNA interacts with PARP1 and undergoes poly(ADP-ribose)lation (8, 10). Another NER factor, xeroderma pigmentosum protein A also interacts with PARP1 and PAR (10, 11). Moreover, PARP1 was identified as one of the main targets in HeLa cell extracts selectively labeled by a photoreactive DNA analogue of the NER substrate (12), suggesting the possible involvement of PARP1 in this repair process. There also exists proteomic data that XPC is one of the protein targets that are (ADP-ribose)lated upon induction of genotoxic stress (13, 14).

The XPC-RAD23B heterodimer is the major factor responsible for the primary damage recognition in DNA and initiates assembly of the NER complex. This protein is required for the global genome NER process that provides the removal of bulky lesions and UV-induced damages from the entire genomic DNA (15). Inhibition of PAR synthesis in the cell with PARP1

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§ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose)glycohydrolase; MBP, maltose-binding protein; NER, nucleotide excision repair; ICBFM SB RAS, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences.
inhibitors reduces the efficiency of XPC-RAD23B binding to UV-damaged DNA (8, 16). At the same time, no direct data exist concerning the interaction of XPC-RAD23B with PARP1 and PAR. In this study, we performed a detailed analysis of the influence of PARP1 and of PAR synthesis on the interaction of XPC-RAD23B with damaged DNA using biochemical approaches and the immunoblot assay. Noncovalent binding of XPC-RAD23B to PAR and poly(ADP-ribosyl)ation of both subunits of this heterodimer by PARP1 depending on DNA structure are demonstrated.

Experimental Procedures

Chemicals—Full anti-protease mixture was from Roche Applied Science (Mannheim, Germany).

Radioactive \( [\gamma^{32}P]ATP \) and \( [\alpha^{32}P]ATP \) were produced in the Laboratory of Biotechnology (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (ICBFM SB RAS)). BSA, NAD\(^+\) and \( \beta\)-nicotinamide mononucleotide were from Sigma. The reagents for electrophoresis and buffer components were from Sigma, Amresco, Panreac (Spain), or produced in Russia (extra pure grade).

Oligonucleotides—Synthetic oligonucleotides were produced in the Laboratory of Medicinal Chemistry (ICBFM SB RAS). An oligonucleotide bearing a fluorescein dUMP derivative was custom-synthesized by Nanotech-C (Russia). The sequences of the oligonucleotides are detailed in Fig. 1.

Proteins—Phage T4 polymerase kinase was from Biosan (Novosibirsk, Russia). Yeast nicotinamide mononucleotide adenyltransferase was a generous gift from S. Shram (IMG RAS, Moscow, Russia). A human PARP1 expression plasmid was kindly provided by M. Satoh (Laval University, Quebec, Canada); the His-tagged protein was overexpressed in \textit{E. coli} and purified as described (18). For the purification of the dimer FLAG-XPC-His-RAD23B, see below. The dimer MBP-XPC-His-RAD23B was kindly provided by O. Schäfer (State University of New York, Stony Brook, NY).

Purification of Recombinant FLAG-XPC-His-RAD23B—The FLAG-tagged XPC was overexpressed in High Five insect cells. The cells (6–8 g) were harvested, washed twice with ice-cold phosphate-buffered saline, and suspended in an 8-fold volume of lysis buffer containing 25 mM Tris-HCl (pH 8.0), 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 0.3 M NaCl, and 1 \( \times \) full anti-protease mixture. After incubation on ice for 30 min, the soluble fraction was recovered by centrifugation at 48,000 \( \times g \) for 1 h, completed with 4 mg of RAD23B, and dialyzed overnight against buffer A (20 mM sodium phosphate (pH 7.8), 10% glycerol, 1 mM EDTA, 0.01% Triton X-100, 10 mM 2-mercaptoethanol, 0.25 mM PMSF, and 0.1 M NaCl). Insoluble material was removed by further centrifugation at 150,000 \( \times g \) for 1 h, and the clarified extract was loaded onto a heparin-Sepharose 6 Fast Flow column (20 ml; Amersham Biosciences) equilibrated with buffer B (20 mM sodium phosphate (pH 7.8), 0.01% Triton X-100, 10 mM 2-mercaptoethanol, and 0.25 mM PMSF) containing 0.1 M NaCl. The column was sequentially washed with buffer B containing 0.1, 0.3, and 1 M NaCl, and the 1 M NaCl fraction was loaded onto an anti-FLAG M2-agarose column (2.5 ml; Sigma) equilibrated with buffer B containing 0.3 M NaCl. The column was sequentially washed with buffer B containing 1 and 0.3 M NaCl. Bound proteins were eluted with buffer B containing 0.3 M NaCl and 100 \( \mu \)g/ml FLAG peptide and loaded onto a Sephacryl 200 HR column (180 ml; Sigma) equilibrated with buffer C containing 25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.01% Triton X-100, 1 mM DTT, 0.25 mM PMSF, and 0.3 M NaCl. Protein-rich fractions were combined, divided into aliquots, and stored at \(-70^\circ\)C.

Preparation of Radioactive NAD\(^+\)—The reaction mixture (50 \( \mu l \)) containing 2 mM \( \beta\)-nicotinamide mononucleotide, 1 mM ATP, 0.25 mCi of \( [\alpha^{32}P]ATP \) (3000 Ci/mm), 5 mg/ml yeast nicotinamide mononucleotide adenyltransferase, 25 mM Tris-HCl (pH 7.5), and 20 mM MgCl\(_2\) was incubated for 1 h at 37 \(^{\circ}\)C. The enzyme was denatured at 97 \(^\circ\)C for 3 min and precipitated by centrifugation.

Preparation of \( 5'\)-\( [\alpha^{32}P]\)DNA Duplexes—Radioactive label was inserted into the 5'-end of oligonucleotides using phage T4 polymerase kinase as described (19). Labeled oligonucleotides were purified using MicroSpin\textsuperscript{TM} G-25 columns (Amersham Biosciences) or by electrophoresis under denaturing conditions followed by electroelution from the gel and acetone precipitation. DNA duplexes were obtained by hybridization of \( 5'\)-\( [\alpha^{32}P] \)-labeled oligonucleotides with complementary oligonucleotides in a 1:1 ratio. Oligonucleotide mixtures were incubated for 5 min at 95 \(^{\circ}\)C, then slowly cooled to 75 \(^{\circ}\)C, kept for 15 min at this temperature, and slowly cooled to room temperature. The hybridization degree was controlled by electrophoresis in 10% polyacrylamide gel (acylamide/bisacrylamide = 50:1). TBE buffer (50 mM Tris-HCl, 50 mM H\(_3\)BO\(_3\), 1 mM EDTA, pH 8.3) served as electrophoresis buffer.

Synthesis and Fractionation of \( 32P \)-Labeled PAR—The reaction mixture (900 \( \mu l \)) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 10% glycerol, 0.25 mM NAD\(^+\), 75 \( \mu \)Ci of
[125I]NAD\(^+\), 60 \(\mu\)g/ml natural mixture of bovine histones (Alexis Biochemicals, Heidelberg, Germany), 40 \(\mu\)g/ml activated cattle spleen DNA, 50 \(\mu\)M PARP1 was incubated for 30 min at 30 °C. The reaction was stopped by the addition of ice-cold TCA to a final concentration 20% (w/v). After incubation for 15 min on ice, the acid-insoluble material was collected by centrifugation at 16,000 \(\times g\) and 4 °C for 30 min, and the precipitate was resuspended sequentially with 5% ice-cold TCA and ethanol. The pellet was dissolved in 0.5 ml of 0.5 M KOH, 50 mM EDTA and incubated at 37 °C for 1 h to detach PARs from PARP1. The reaction mixture was then neutralized with glacial acetic acid, and the PARs were precipitated in ethanol and dissolved in water. For PAR fractionation, anion exchange chromatography on TSKgel DEAE-Toyopearl 650 M (500 \(\mu\)l, ToyoSoda, Japan) was used as described (20). The polymers were eluted using a multistep NaCl gradient in 25 mM Tris-HCl (pH 9.0). The fractions were collected manually, desalted by ethanol precipitation, and analyzed by electrophoresis in 14% denaturing gel.

**Binding of Proteins to DNA or PAR**—Complexes of the protein with 5'-[32P]DNA or [32P]PAR were analyzed by the electrophoretic mobility shift assay. The reaction was performed in a mixture (10 \(\mu\)l) containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl (or NaCl), 1 mM DTT, 0.6 mg/ml BSA, 1 mM DTT, and where indicated 20 nM 5'-[32P]DNA and 50 \(\mu\)M NAD\(^+\). The type of model of DNA, the concentration of the proteins used, and the PAR are indicated in the figure legends. The reaction mixtures were incubated at 37 °C for 10 min, and then 2.5 mM EDTA and PARG were added, if necessary, and the mixture was incubated for another 10 min. Loading buffer (0.2 volume) containing 20% glycerol and 0.015% bromphenol blue was then added to the samples. The DNA/PAR-protein complexes were analyzed by electrophoresis in 14% polyacrylamide gel (acrylamide/bisacrylamide = 60:1) in TBE buffer at 4 °C followed by phosphorimaging using a Molecular Imager FX Pro+ (Bio-Rad). The \(K_D\) values for the complexes of XPC-RAD23B with PAR were estimated using Equation 1 for bimolecular binding,

\[
K_D = \frac{(E_0 - ES)(S_0 - ES)}{ES}
\]

(Eq. 1)

where \(E_0\) and \(S_0\) represent the total concentration of XPC-RAD23B and PAR, respectively; \(ES\) corresponds to concentration of the PAR/XPC-RAD23B complex. The \(ES\) value is calculated from the experimental data. For PAR fractions 1–5, we used the data obtained for the XPC-RAD23B concentration equal to 552 \(\text{nm}\) and for the PAR fraction 6–276 \(\text{nm}\).

**Protein Poly(ADP-ribosylation)—Poly(ADP-ribosylation)** of the proteins was performed in a reaction mixture (15 \(\mu\)l) containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl (or NaCl), 1 mM DTT, 20 \(\mu\)M DNA, 78 \(\mu\)M PARP1, NAD\(^+\) ([32P]NAD\(^+\) if specified), and various concentrations of the proteins explored (XPC-RAD23B or RAD23B). Detailed information on the type of DNA structures, NAD\(^+\), and protein concentrations are indicated in the figure legends. The reaction mixtures were incubated at 37 or 25 °C for 20–30 min. Each reaction mixture was divided into two parts. One part (10 \(\mu\)l) was added with 0.2 volume of the loading solution containing 5% SDS, 5% 2-mercaptoethanol, 0.3 M Tris-HCl (pH 7.8), 50% glycerol, and 0.015% bromphenol blue and heated for 5 min at 95 °C. The reaction products were separated by 10 or 15% SDS-PAGE and visualized by phosphorimaging or staining with Coomassie Brilliant Blue G-250. Alternatively, the products were transferred onto nitrocellulose membranes (Hybond-ECL, Amersham Biosciences) using the semi-dry Western blotting technique and were probed with mouse antibodies to FLAG (1:2000; Asp-Asp-Asp-Asp-Lys, clone number G10, Abcam), PARP1 (1:1000; clone number CI110, Trevigen), or RAD23B (1:1000; polyclonal, Laboratory of Biotecnology, ICBFM SB RAS). Blots were then probed with horseradish peroxidase-coupled goat anti-mouse antibodies (1:15,000; produced in the Laboratory of Biotecnology, ICBFM SB RAS), and immunoreactivity was detected by chemiluminescence (Pierce ECL Western blotting Substrate, Thermo Scientific). The other part (5 \(\mu\)l) was added with 2 \(\mu\)l of the loading solution containing 90% formamide, 50 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, heated for 5 min at 95 °C, and subjected to 10% denaturing PAGE (acrylamide/bisacrylamide = 19:1), and visualized by phosphorimaging.

**UV Irradiation of Supercoiled pUC19 DNA**—The reaction mixtures (6 \(\mu\)l) containing 33 ng/\(\mu\)l pUC19 in water were irradiated for different times in an ice bath using a Bio-Link BXL-312 cross-linker from Vilber Lourmount (France), wavelength 312 nm, light intensity 5 mJ/cm²/s.

**Results**

**Influence of PAR Synthesis on XPC-RAD23B Binding to DNA**—Fig. 2 shows the data of the influence of PARP1 on XPC-RAD23B binding to a 48-mer DNA duplex containing one of the strands a fluorescein residue imitating a bulky lesion in the presence and absence of NAD\(^+\) (the substrate of the PAR synthesis reaction). In the presence of both PARP1 and NAD\(^+\) (the PAR synthesis conditions), the electrophoretic mobility of the XPC-RAD23B complexes with DNA is reduced (Fig. 2A, compare lanes 6, 7 and 2, 3). It should be noted that the mobility of the XPC-RAD23B-DNA complexes does not change in the presence of NAD\(^+\) alone (Fig. 2A, lanes 4 and 5) or PARP1 alone (lane 8). As the PARP1 or NAD\(^+\) concentration increases, both the electrophoretic mobility of the XPC-RAD23B complexes with DNA and its amount are reduced (see histograms on Fig. 2, B and C, respectively). At the same time, PARP1 complexes with DNA, which mainly locate in the top of gel, disappear, and the amount of free DNA increases when NAD\(^+\) is added (Fig. 2A, compare lanes 12 and 13), hence supporting the current hypothesis of the dissociation of poly(ADP-ribosyl)ated PARP1 from complexes with DNA (3). Poly(ADP-ribosyl)ated PARP1 does not compete with XPC-RAD23B for DNA binding due to low affinity of the PAR-modified (negatively charged) PARP1 for DNA. Nevertheless, the amount of XPC-RAD23B-DNA complexes in the conditions of PAR synthesis is lower than in the absence of both PARP1 and NAD\(^+\) (Fig. 2, B and C). Therefore, XPC-RAD23B is modified in the presence of PARP1 and NAD\(^+\), and this modification changes the affinity of this protein for DNA. Alternatively, XPC-RAD23B can bind to PAR tails of PARP1, so that PAR-PARP1 competes with DNA for XPC-RAD23B binding. It is very likely that the XPC-RAD23B-
DNA complexes with lower mobility are formed as a result of PAR polymer binding (covalently or noncovalently) to the protein. The fact that the mobility of the XPC-RAD23B-DNA complexes is practically re-established after treatment with PARG (Fig. 2A, lanes 9–11) speaks in favor of this assumption. At the same time, PARG treatment does not restore the normal binding capacity of both XPC-RAD23B and PARP1 (compare complex intensities in Fig. 2A, lanes 8 and 9–11), probably due to
incomplete hydrolysis of the PAR tails. PARG does not bind DNA under experimental conditions (Fig. 2A, lanes 14–16). Similar results were obtained when various DNA structures were used (an undamaged DNA duplex, a DNA duplex with unpaired bases, and a DNA duplex with the adduct of benzo[a]pyrene as a lesion; data not shown).

XPC-RAD23B Binding to Free PAR—It is known that some repair proteins, including the NER factor XPA, form noncovalent complexes with the PAR polymer (11). Moreover, XPC possesses a putative PAR-binding motif (21). To check whether XPC-RAD23B interacts with PAR, free [32P]PAR polymer was synthesized and subjected to fractionation depending on the length of the PAR strand (Fig. 3A) (20). The analysis of XPC-RAD23B binding to fractionated [32P]PAR showed that the protein forms complexes with each separated fraction (Fig. 3B). Meanwhile, the efficiency of binding increases as the strand lengthens; with PAR lengthening from 4 to 6 (Fig. 3B, lanes 1–3, fraction 1) to 45 to 50 ADP-ribose links (Fig. 3B, lanes 16–18, fraction 6) the affinity of XPC-RAD23B for PAR can increase 16-fold (Table 1). The long length PAR fractions compete with DNA for XPC-RAD23B binding, whereas short oligomers do not affect DNA-protein interactions (Fig. 3C).

Poly(ADP-ribosyl)ation of the XPC and RAD23B Polypeptides by PARP1—To examine whether XPC-RAD23B may be poly(ADP-ribosyl)ated by PARP1, [32P]NAD+ was used as substrate for PARP1 (Fig. 4). Because PARP1 itself is the target for poly(ADP-ribosyl)ation, and both the PARP1 and XPC polypeptides have similar apparent molecular masses (XPC, 125 kDa; PARP1, 113 kDa), the modified radioactive products of these two polypeptides were hardly discriminated by gel electrophoresis. To decrease the intensity of poly(ADP-ribosyl)ation, the NAD+ concentration in the reaction mixture was reduced from 50 to 2.5 μM, and the probes were incubated at 25 °C instead of 37 °C. Under these conditions, PAR tails are shortened allowing incomplete hydrolysis of the PAR tails. PARG does not bind DNA under experimental conditions (Fig. 2A, lanes 14–16). Similar results were obtained when various DNA structures were used (an undamaged DNA duplex, a DNA duplex with unpaired bases, and a DNA duplex with the adduct of benzo[a]pyrene as a lesion; data not shown).

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| Free PAR fraction | Average length of the PAR strand, ADP-ribose links | $K_d \times 10^7$ |
|-------------------|-----------------------------------------------|-----------------|
| 1                 | 4–6                                           | 20 ± 2          |
| 2                 | 11–12                                         | 9.7 ± 0.9       |
| 3                 | 13–14                                         | 6.8 ± 0.7       |
| 4                 | 20–22                                         | 6.5 ± 0.7       |
| 5                 | 32–35                                         | 3.3 ± 0.3       |
| 6                 | 45–50                                         | 1.2 ± 0.2       |

FIGURE 3. A, fractionation of 32P-labeled PAR using anion exchange chromatography on TSKgel DEAE-Toyopearl 650 M (500 μl, ToyoSoda, Japan). The polymers were eluted using a multistep NaCl gradient in 25 mM Tris-HCl buffer (pH 9.0). Fractions were analyzed by denaturing electrophoresis in a 14% PAGE and autoradiography of the dried gel. B, binding of XPC-RAD23B to fractionated PAR. The reaction was performed in the reaction mixture (10 μl) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.6 mg/ml BSA, 1 mM DTT, one of the fractions of [32P]PAR, and 276 or 552 nM XPC-RAD23B. The reaction products were analyzed by 5% native gel electrophoresis and autoradiography of the dried gels. C, free PAR competition with DNA for XPC-RAD23B binding. The reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.6 mg/ml BSA, 1 mM DTT, 20 nM 5′-32P-labeled DNA, 55 nM XPC-RAD23B, and increasing amounts of nonradioactive DNA or PAR competitor. UFlu-DNA-48-mer fluorescein contains DNA duplex (see Fig. 1 for the structure). Percentages of DNA bound were plotted against competitor concentrations. Averages and experimental errors were taken from at least five experiments.
us to improve the quality of product separation. The XPC-RAD23B heterodimer with a FLAG epitope (8 amino acids) in XPC (Fig. 4A, lanes 6 – 8) as well as the preparation in which this polypeptide is fused to maltose-binding protein (MBP, 42 kDa) (Fig. 4A, lanes 3–5) were used here. The data presented in Fig. 4A show that the pattern of the poly(ADP-ribosyl)ation products changes in the presence of XPC-RAD23B, especially in the case of the preparation with MBP-XPC where the band whose molecular mass corresponds to this polypeptide can be clearly observed. Moreover, in all the preparations examined, the modified product with a molecular mass of about 60 kDa corresponding to the mobility of the RAD23B subunit of the heterodimer can be detected. It should be noted that the RAD23B polypeptide alone is not modified by PARP1 in the presence of NAD\(^+\) (Fig. 4B). Therefore, modification of RAD23B occurs in the heterodimer.

Using low NAD\(^+\) concentrations, we could detect the major radioactive product of the modified PARP1 (Fig. 4A, lanes 1 and 2) whose mobility corresponds to the unmodified XPC (about 125 kDa). To verify that the new products correspond to poly-(ADP-ribosyl)ated XPC, it was necessary to determine the modified XPC with the PAR tail whose mobility is different from that of the unmodified protein. We replaced \[^{32}P\]NAD\(^+\) by nonradioactive NAD\(^+\) and increased its concentration to 50–500 \(\mu\)M. The XPC-RAD23B heterodimer with the FLAG epitope in XPC was used in these experiments. After their separation in 10% SDS-PAGE, the reaction products were stained with Coomassie (Fig. 5A) or transferred onto a nitrocellulose membrane and probed with various antibodies, namely the FLAG epitope, to PARP1 or to RAD23B (Fig. 5, B–D, respectively). In the conditions of PAR synthesis, the amount of all initial polypeptides decreases (Fig. 5A, compare lane 1 with 2, 3 for PARP1, lane 6 with 4, 5 for XPC, and lane 7 with 4, 5 for RAD23B), and smeared products with lower electrophoretic mobility than the unmodified XPC polypeptide appear (Fig. 5A, compare lane 6 with 4, 5). These products overlap with modified XPC (Fig. 5B, compare lane 4 with 2, 3) and modified PARP1 (Fig. 5C, compare lane 1 with 4 and 5). Moreover, the smeared products corresponding to the modified RAD23B polypeptide, invisible by Coomassie staining (Fig. 5A, compare

**FIGURE 4.** A comparison of poly(ADP-ribosyl)ation of MBP-XPC-RAD23B (lanes 3–5) and FLAG-XPC-RAD23B (lanes 6–8) preparations in the low NAD\(^+\) concentration conditions. The reaction was performed in the reaction mixture (10 \(\mu\)l) containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 1 mM DTT, 20 nm 48-mer DNA duplex containing a fluorescein residue, 77 nM PARP1, 2.5 \(\mu\)M \[^{32}P\]NAD\(^+\), and XPC-RAD23B at the indicated concentrations. The reaction mixtures were incubated at 25 °C for 20 min. The reaction products were separated by Laemmli electrophoresis in 10% SDS-PAGE and visualized by phosphorimaging. B, RAD23B is not poly(ADP-ribosyl)ated by PARP1. The reaction was performed as described in A.

**FIGURE 5.** Coomassie staining and Western blot analysis of the poly(ADP-ribosyl)ated XPC-RAD23B. The reaction was performed in 10 \(\mu\)l containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1 mM DTT, 20 nm 48-mer DNA duplex containing a fluorescein residue, 77 nM PARP1, 50 or 500 \(\mu\)M NAD\(^+\), and 276 nM XPC-RAD23B or RAD23B. The reaction mixtures were incubated at 25 °C for 20 min. The reaction products were separated by Laemmli electrophoresis in 10% SDS-PAGE and were stained with Coomassie Brilliant Blue G-250 (A) or were transferred onto a nitrocellulose membrane using the semi-dry Western blotting technique and were probed with mouse antibodies to FLAG (B), PARP1 (C), or RAD23B (D).
lane 6 with 4, 5), are also observed (Fig. 5D, compare lane 4 with 2, 3).

It was shown earlier that the reaction of protein poly(ADP-ribosyl)ation catalyzed by PARP1 is DNA-dependent and is stimulated in response to DNA damage; therefore, the effect of stimulation should depend on the DNA structure (22, 23). Indeed, both the protein poly(ADP-ribosyl)ation efficiency (Fig. 6A) and the amount of free PAR (Fig. 6B) are strongly reduced in the absence of DNA in the reaction mixture or in the presence of supercoiled pUC19 (Fig. 6, A and B, lanes 1, 2 and 5, 6, respectively) in comparison with 48-mer DNA duplex (Fig. 6, A and B, lanes 9, 10, 13, and 14). The introduction of a bulky lesion in the DNA duplex has little effect on the modifications of the protein and on PAR synthesis (Fig. 6, A, and B, compare lanes 9, 10 with 13, 14). This observation suggests that stimulation of the PAR synthesis reaction by the dsDNA ends (or dsDNA breaks) is higher than by a bulky lesion in the internal position of the DNA duplex. PAR synthesis is also influenced by XPC-RAD23B. In all cases, regardless of the DNA structure, the quantity of free NAD$^+$ clearly decreases, and the intensities of the PAR products increase in the presence of XPC-RAD23B (Fig. 6, B and C). At the same time, the influence of XPC-RAD23B on the intensity of the bands corresponding to the modified proteins depends on the structure of the DNA. In the absence of DNA or when pUC19 DNA was used, the bands corresponding to modified PARP1 (Fig. 6, A, lanes 1, 2 and 5, 6) disappeared in the presence of XPC (lanes 3, 4 and 7, 8). On the contrary, when a short linear DNA duplex was used, the amount of modified protein products increased in the presence of XPC (Fig. 6A, compare lanes 9 10 with 11, 12 and lanes 13, 14).
levels of PAR may increase 10–500-fold (2). Poly(ADP-ribo)sylation plays an important role in the regulation of many cellular processes, including DNA repair. As a rule, poly(ADP-ribo)sylation is considered as covalent joining of the PAR tail to proteins. However, some proteins interact with PAR noncovalently by forming stable complexes. In particular, XPA is well known to bind PAR (10, 11, 27).

In the literature there are preliminary data that XPC is able to be (ADP-ribosyl)ated upon induction of genotoxic stress (13, 14). These data were obtained by applying a mass spectrometry-based proteomics approach. XPC was revealed as one of a large number of proteins pulled down from cell extracts using single-stage chromatography to isolate ADP-ribosylated peptides with the subsequent mass spectrometry analysis. It is difficult to say with certainty that all proteins are really poly(ADP-ribo)sylated; they may be pulled down due to their strong affinity for other proteins interacting with them or with the PAR chain. Here, it was shown directly that indeed both subunits of the XPC-RAD23B dimer undergo poly(ADP-ribo)sylation, which in turn may matter in regulating the functioning of XPC-RAD23B in the cell. First of all, it influences the interaction of this protein with DNA. Adding a bulky negatively charged PAR tail to the protein may not only cause steric difficulties for DNA binding but may also dramatically change the charge of the protein and consequently prevent binding to negatively charged DNA. Moreover, poly(ADP-ribo)sylation may affect the amino acids, e.g. lysine, which usually contributes to direct contact with DNA (28). Along with the aspartic and glutamic acid residues, lysine residues are considered as main ADP-ribose acceptor sites (14, 29, 30).

We showed that XPC-RAD23B also forms noncovalent complexes with PAR, the affinity being dependent on the length of the PAR strand. In another study (8), robust synthesis of PAR chains was detected in nuclear regions of human fibroblasts containing DNA lesions induced by UV light. Such synthesis was completely suppressed by chemical inhibitors of PARP1. We demonstrated that XPC-RAD23B is poly(ADP-ribo)sylated by PARP1 in response to UV irradiation of the plasmid DNA. These findings are in line with observations that UV irradiation triggers both stimulation of PAR synthesis (31) and association of PARP1 with UV photolyses in chromatin (32). Free or covalently bound to other proteins (e.g. PARP1), PAR might serve as a target for the attraction of XPC-RAD23B to lesions in DNA. Indeed, treatment of human fibroblasts expressing XPC-GFP with PARP inhibitors suppressed the UV-induced immobilization of XPC. The levels of bound XPC at sites of DNA damage in human bone osteosarcoma cells were significantly higher after PARG knockdown, suggesting that PAR synthesis promotes lesion recognition by XPC (16). However, the authors link this fact with PARP-dependent DDB2-mediated chromatin remodeling, which increases XPC-RAD23B access to damaged DNA. Recruitment of the chromatin-remodeling enzyme ACL1 as a result of poly(ADP-ribo)sylation of UV-damaged chromatin through the activity of PARP1 may also promote chromatin decondensation (8). Therefore, the effect of PARP1 inhibition on XPC-RAD23B recruitment to the damaged site is indirect in the cell. However, we consider that direct interac-

### Discussion

Poly(ADP-ribo)sylation is a reversible post-translational modification found in higher eukaryotes. The structure of PAR is well characterized. The chain length of polymers is heterogeneous and may reach 200–400 units in length detected using *in vitro* and *in vivo* assays. Long polymers are branched in an irregular manner. Branching occurs with a frequency of approximately one branch per linear section of 20–50 units of ADP-ribose (25). The constitutive levels of PAR are usually very low in unstimulated cells. In the presence of DNA strand breaks, the
tion of XPC-RAD23B with PAR may also contribute to recruitment of this protein to the lesion.

In other studies (33, 34), it is postulated that long PAR chains may have a helical secondary structure as have RNA and DNA. Many antibodies against PAR recognize RNA and DNA and vice versa (35–37). Therefore, PAR can compete with DNA in binding to proteins. This was previously already shown for XPA and RPA (27, 38). We have confirmed that it is also the case for XPC-RAD23B, because this protein binds PAR. Moreover, long length PAR chains compete with DNA for XPC-RAD23B binding, whereas short chains do not (Fig. 3C). These data are in agreement with the prediction that DNA- and PAR-binding sites do not overlap in XPC structure (21). The competition effect for the long PAR chains may be a consequence of the steric hindrance to DNA binding.

Both covalent and noncovalent binding of PAR to XPC-RAD23B weaken the interaction of XPC-RAD23B with DNA. It may play a role in regulation assembly of the repair complex. The NER process is very complicated and proceeds through several stages. The minimal set for damage removal activity is composed of six repair proteins as follows: XPC-RAD23B, XPA, RPA, TFIIH, XPG, and ERCC1-XPF (39, 40). These proteins sequentially form several intermediate repair complexes and sequentially leave damaged DNA. The composition of NER complexes and the order of assembly are still under discussion. However, in experiments with pure individual proteins it was shown that XPC-RAD23B is not present in the penultimate and ultimate dual incision complexes (26). Binding of XPC-RAD23B to PAR may promote protein removal of XPC-RAD23B from the repair complex at a particular stage of this dynamic process.

Hence, the interaction of the nucleotide excision repair key factor XPC-RAD23B with free PAR and (ADP-ribosyl)ation of both subunits of this heterodimer catalyzed by PARP1 was shown. This mechanism can play an important role in regulating the NER process and explain the previously shown influence of PARP1 on the repair of UV-induced DNA damages in chromatin (8, 9).

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