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Study of Interaction of XRCC1 with DNA and Proteins of Base Excision Repair by Photoaffinity Labeling Technique

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Abstract—The X-ray repair cross-complementing group 1 (XRCC1) protein plays a central role in base excision repair (BER) interacting with and modulating activity of key BER proteins. To estimate the influence of XRCC1 on interactions of BER proteins poly(ADP-ribose) polymerase 1 (PARP1), apurinic/apyrimidinic endonuclease 1 (APE1), flap endonuclease 1 (FEN1), and DNA polymerase β (Pol β) with DNA intermediates, photoaffinity labeling using different photoreactive DNA was carried out in the presence or absence of XRCC1. XRCC1 competes with APE1, FEN1, and PARP1 for DNA binding, while Pol β increases the efficiency of XRCC1 modification. To study the interactions of XRCC1 with DNA and proteins at the initial stages of BER, DNA duplexes containing a photoreactive group in the template strand opposite the damage were designed. DNA duplexes with 8-oxoguanine or dihydrothymine opposite the photoreactive group were recognized and cleaved by specific DNA glycosylases (OGG1 or NTH1, correspondingly), although the rate of oxidized base excision in the photoreactive structures was lower than in normal substrates. XRCC1 does not display any specificity in recognition of DNA duplexes with damaged bases compared to regular DNA. A photoreactive group opposite a synthetic apurinic/apyrimidinic (AP) site (3-hydroxy-2-hydroxymethyltetrahydrofuran) weakly influences the incision efficiency of AP site analog by APE1. In the absence of magnesium ions, i.e. when incision of AP sites cannot occur, APE1 and XRCC1 compete for DNA binding when present together. However, in the presence of magnesium ions the level of XRCC1 modification increased upon APE1 addition, since APE1 creates nicked DNA duplex, which interacts with XRCC1 more efficiently.

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Key words: XRCC1, base excision repair, photoaffinity labeling technique

XRCC1 protein does not posses any enzymatic activity; however, it plays a central role in coordination of base excision repair (BER) and single-strand break repair pathways. First, XRCC1 was shown to modulate activities of DNA polymerase β (Pol β) and DNA ligase IIIα (Lig IIIα) [1]. Later, the interaction of XRCC1 with enzymes acting at early stages of BER was shown. XRCC1 stimulates DNA glycosylase activity but does not change AP lyase activity of OGG1 [2]. Recently, interactions of XRCC1 with other DNA glycosylases: NTH1, NEIL1/2, and MPG were demonstrated [3]. XRCC1 promotes the AP endonuclease and 3′-dRPase activities of APE1 [4]. Moreover, XRCC1 interacts with PARP1 and PARP2. Efficiency of these interactions increases under poly(ADPribosyl)ation of PARP1/2 [5-7].

XRCC1 is not absolutely required for normal AP site repair. According to [1], XRCC1 inhibits Pol β strand displacement synthesis in the system reconstituted from uracil DNA glycosylase, APE1, Pol β, and Lig IIIα. The formation of triple complex containing XRCC1, Pol β, and incised AP DNA was shown [1]. Another group reported that XRCC1 stimulates incorporation of one nucleotide by Pol β in the absence of ATP and Lig IIIα [8]. XRCC1 is able to promote strand displacement DNA synthesis under conditions of ATP shortage when ligation

Abbreviations: APE1) human apurinic/apyrimidinic endonuclease 1; AP site) apurinic/apyrimidinic site; BER) base excision repair; dRP) deoxyribose phosphate residue; F) 3-hydroxyl-2-hydroxymethyltetrahydrofuran; FEN1) human flap endonuclease 1; NTH1) human DNA glycosylase, homolog of *E. coli* endonuclease III; OGG1) human 8-oxoguanine DNA glycosylase; 8-oxoG) 8-oxoguanine; PARP1) human poly(ADP-ribose) polymerase 1; Pol β) rat DNA polymerase β; XRCC1) human X-ray repair cross-complementing group 1 protein.

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cannot occur. Lig IIIα activity and ATP are required for inhibition of strand displacement activity of Pol β and the repair process is realized via short patch BER. XRCC1 and Lig IIIα normally exist in cells as a preformed complex [9]. The level of Lig IIIα polypeptide is reduced 4-fold and ligase activity is reduced 6-fold in Chinese hamster ovary (CHO) cells lacking XRCC1 (EM9) [10]. Experiments in systems reconstituted using purified recombinant proteins did not reveal the stimulation of Lig IIIα activity by XRCC1 [1, 8]. Parsons and colleagues studied dynamics of repair of an AP site-containing substrate by whole cell extracts (WCEs) of HeLa and mouse fibroblasts using formaldehyde cross-linking technique [11]. APE1 was cross-linked at the very early stages of repair. Pol β binds to an incised abasic site after APE1 dissociation and recruits the Lig IIIα–XRCC1 complex to facilitate repair.

However, the data on role of XRCC1 in base excision repair are contradictory. Wong and colleagues examined systematically the quantitative contribution of XRCC1 to specific biochemical steps of BER using WCEs from wild-type CHO AA8 cells and its XRCC1-deficient EM9 counterpart, as well as from EM9 cell line complemented with vector stably expressing wild-type human XRCC1 [12]. These studies reveal that XRCC1-deficient CHO extract exhibits normal base excision activity of damaged bases. Abasic site incision activity and one- and five-nucleotide gap filling were found to be normal in XRCC1-deficient cell extracts, while significant reduction in the nick ligation activity was observed in EM9 WCE. The results suggest that the primary biochemical defect associated with XRCC1 deficiency is associated with the ligation step of BER, and that XRCC1 plays no significant role in the repair of endogenous base damages and abasic sites [12].

The ability of XRCC1 N-terminal domain to bind gapped or nicked DNA has been shown earlier by electrophoretic mobility shift assay (EMSA) [13], but complexes of full-length XRCC1 with DNA were not found. Later, the interaction of XRCC1 with DNA was studied by fluorescence spectroscopy [14]. XRCC1 exhibits preferential binding to DNA with nick (5′-OH) or mononucleotide gap (5′-OH or 5′-p) with K_d values of 65, 34, and 52 nM, respectively. XRCC1 exhibits lower affinities for a duplex with a five-nucleotide gap, the intact duplex with no break, and a single stranded oligonucleotide with K_d values of 215, 230, and 260 nM, respectively.

This work was aimed at study of interaction of XRCC1 with DNA intermediates and proteins of base excision repair by the photoaffinity labeling technique to analyze the mechanism of coordination of BER stages.

MATERIALS AND METHODS

In this study we used dNTP, EDTA, Tris, N,N,N′,N′-tetramethylethylenediamine, imidazole, SDS, ammonium persulfate, and Coomassie G-250 and R-250 from Sigma (USA); $MgCl_2$, formamide, and NP-40 from Fluka (Switzerland); acrylamide and glycerol from ICN (USA); Hepes and β-mercaptoethanol from Serva (Germany); N,N′-methylene-bis-acrylamide from BioRad (USA); [γ-^{32}P]ATP (>110 TBq/mmol) and T4 phage polynucleotide kinase (PNK) from Biosan (Russia). Other reagents were of Russian production.

Recombinant proteins—XRCC1, NTH1, OGG1, FEN1, APE1, PARP1, Pol β—were isolated as described in [2, 3, 15-19], respectively. An exo-N-[2-N-(N-(4-azido-2,5-difluoro-3-chloropyridine-6-yl)-3-aminopropionyl)-aminoethyl]-2′-deoxycytidine-5′-triphosphate (FAP-dCTP), a photoreactive dCTP derivative, was synthesized according to [20] and kindly donated by S. V. Dezhurov (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (SB RAS)).

Synthetic oligonucleotides were obtained from Sigma-Genosys (Great Britain) or the Laboratory of Medicinal Chemistry (Institute of Chemical Biology and Fundamental Medicine, SB RAS). The sequences of oligonucleotides used in this study are shown in the table. Radioactive label was incorporated into the 5′-end of oligonucleotides with PNK, and the products were purified by electrophoresis in polyacrylamide gel in the presence of 7 M urea under denaturing conditions [21].

Incorporation of photoreactive derivative FAP-dCMP into the 3′-end of primer. A photoreactive dCTP analog was used as a substrate of Pol β to incorporate a photoreactive FAP-dCMP residue into the 3′-end of U1 primer in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, and 0.25 mM β-mercaptoethanol. The reaction mixture also contained 0.4 μM T1 template and 0.2 μM 5′-^{32}P-labeled U1 primer. To prepare DNA duplex, primer and template were annealed at ratio 1 : 2. After formation of DNA duplex (DNA0), Pol β and FAP-dCTP were added to the mixture to the final concentrations of 1 and 10 μM, respectively. The reaction was carried out for 30 min at 37°C. The DNA sequence provided dCMP incorporation. DNA was precipitated with ten volumes of 0.2% $LiClO_4$ in acetone, incubated for 40 min at –40°C, and then centrifuged. The pellet was washed with acetone, dried in air at room temperature, and dissolved in TE buffer. To form the substrates (DNA1-DNA6), the radioactively labeled photoreactive U2 oligonucleotide was annealed to T1 template and D1, D2, D3, or D4 oligonucleotides in a ratio of 1 : 2 : 2 in buffer containing 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl.

Design of DNA duplexes containing photoreactive analog opposite damaged base. Incorporation of photoreactive derivative of dCMP into one-nucleotide gap by activity of Pol β, the ligase reaction, and purification of photoreactive oligonucleotide were carried out according to [15]. Ligated oligonucleotide T3 was annealed with P1, P2, or P3 oligonucleotides to form duplexes DNA11, DNA12, or DNA13.

Structures of oligonucleotides used in this study

Sequences 5′→3′
T1 GGAAGACCCTGACGTTGCCCAACTTAATCGCC
U1 GGCGATTAAGTTGGG
* U2 GGCGATTAAGTTGGG**C**^{Az} (**C**^{Az} = photoreactive group)
D1 (OH)AACGTCAGGGTCTTCC
D2 (p)AACGTCAGGGTCTTCC
D3 (pF)AACGTCAGGGTCTTCC
D4 (p)ACGTCAGGGTCTTCC
D5 CATCCACAAACGTCAGGGTCTTCC
P1 (# = 8-oxoG), P2 (# = DHT), P3 (# = F) GCTTCATCGTTGTC#CAGACCTGGTGGATACCG
T2 CGGTATCCACCAGGTCTGCGACAACGATGAAGCC
* T3 CGGTATCCACCAGGTCTG**C**^{Az}GACAACGATGAAGCC
U3 CGGTATCCACCAGGTCTG
D6 (p)GACAACGATGAAGCC
T3 CATCGTTGTCGCAGACCTGGTGG

Types of DNA structures	Description of DNA duplex
DNA0 U1 : T1	3′-recessed DNA
DNA1 U2 : T1	3′-recessed DNA
DNA2 U2 : D1 : T1	nicked DNA (5′-OH)
DNA3 U2 : D2 : T1	nicked DNA (5′-p)
DNA4 U2 : D3 : T1	nicked DNA (5′-pF)
DNA5 U2 : D4 : T1	gapped DNA
DNA6 U2 : D5 : T1	flap structure
DNA7 P1 : T2 DNA11 P1 : T3	DNA duplexes containing 8-oxoG, DHT, or F
DNA8 P2 : T2 DNA12 P2 : T3	
DNA9 P3 : T2 DNA13 P3 : T3	
DNA10 U3 : D6 : T4	gapped DNA

Photoaffinity modification of proteins. The reaction mixtures (10 μl) contained photoreactive DNA (concentration range 30-100 nM) and proteins: APE1, FEN1, Pol β, PARP1, OGG1, NTH1, or XRCC1 (concentration range 30-300 nM), in buffer B1 (50 mM Tris-HCl, pH 8.0, 50 mM KCl) or B2 (50 mM Hepes, pH 7.7, 5% glycerol, 50 mM NaCl, 2 mM EDTA). After incubation on ice for 5 min, the reaction mixtures were UV-irradiated using a DRK-120 high-pressure mercury lamp and a UFS-6 light filter ($\lambda > 300$ nm, $W = 10^{-4}$ W/cm^2, distance 8 cm, irradiation time 5 min). Then the mixtures were analyzed by electrophoresis in 10 or 15% polyacrylamide gel according to Laemmli [22] with subsequent autoradiography. Radioactivity of the products was evaluated using Molecular Imager and QuantityOne software from BioRad (USA).

OGG1 and NTH1 assays. The reaction mixtures (10 μl) contained 10 fmol of the 8-oxoG-DNA or DHT-DNA and glycosylases OGG1 (0.3-30 nmol) or NTH1 (1.5-30 nmol), respectively. Reactions were carried out for 30 min at 37°C in buffer B2. Then NaOH was added (0.1 M final concentration). The samples were incubated for 5 min at 37°C. The products of the reactions were resolved by denaturing 20% PAGE [21].

Incision of F-containing DNA by AP-endonuclease 1. The reaction mixtures contained DNA substrate (concentration range 0.1-100 nM), APE1 (concentration range 0.01-1 nM), 50 mM Hepes, pH 7.7, 50 mM NaCl, 5% glycerol, 2 mM EDTA, and 8 mM $MgCl_2$. The reaction time was 30 min at 37°C. The products of the reactions were resolved by denaturing 20% PAGE [21].

RESULTS AND DISCUSSION

Study of interaction of BER proteins with DNA mimicking BER intermediates by photoaffinity labeling technique. The levels of photoaffinity labeling of XRCC1 using different DNAs mimicking BER intermediates were compared in this work. Six types of DNA structures were used: 3′-recessed DNA (DNA1), gapped DNA (DNA5), flap structure (DNA6), and nicked duplexes containing either hydroxyl (OH), phosphate (p), or furanophosphate (pF) (DNA2-DNA4, respectively). In these DNA duplexes, photoreactive FAP-dCMP was located at 3′-end and a radioactive label was at the 5′-end of the upstream primer. The structural formula of FAP-dCTP is presented in Fig. 1a. This dCTP derivative is highly photoactive [20]. Significant differences in the levels of photoaffinity labeling of XRCC1 using DNA3-DNA6 (Fig. 1b) were not observed. Lower level of cross-linking was observed for DNA1. The level of XRCC1 modification by nicked duplex containing 5′-OH was minimal. According to fluorescence titration assay, K_d values for complexes of XRCC1 with nicked (5′-OH) and gapped (5′-p) are 65 and 52 nM, respectively [14]. The level of XRCC1 cross-linking to DNA2 is 6-fold lower than that of DNA5, although these DNA are analogous in structures to those used in [14] and therefore have to display comparable K_d values. Such difference in the level of cross-linking cannot be explained by the differences in affinity of XRCC1 to these structures. The efficiency of protein cross-linking to DNA appears to reflect not only its affinity to DNA but also proper orientation of reactive moiety towards a potential acceptor amino acid. Unfortunately, we failed to estimate the affinity of XRCC1 to different DNA structures by electrophoretic mobility shift assay.

XRCC1 seems to be one of the main factors coordinating BER stages via its interaction with and modulating activity of BER proteins [23]. To estimate the influence of XRCC1 on interaction of BER proteins with DNA intermediates, photoaffinity labeling experiments with Pol β, APE1, PARP1, FEN1, and photoreactive DNA duplexes were carried out in the absence or presence of XRCC1. Decrease in the level of XRCC1 modification was observed in the presence of Mg^{2+}. An analogous decrease in cross-linking in the presence of Mg^{2+} was also observed for other proteins (compare Figs. 2a and 2b). This is probably due to the increase of ionic strength of the solution and, as a result, destabilization of protein–DNA complexes. The yields of covalent protein–DNA adducts varied substantially for different proteins. Efficiency of cross-linking of a particular protein to DNA reflects not only its affinity to DNA but also proper orientation of the reactive moiety towards a potential acceptor amino acid. The presence of XRCC1 significantly changes the levels of modification of BER proteins. The levels of cross-linking of Pol β to DNA2, DNA4, and DNA6 are higher than that of 3′-recessed DNA, which has a lower affinity to Pol

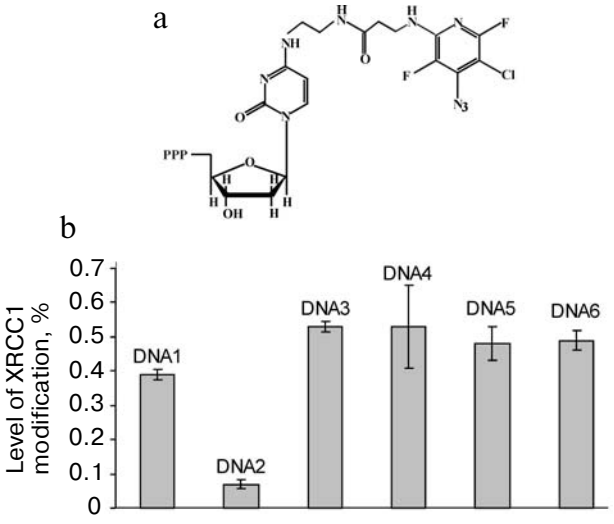

Fig. 1. a) Structural formula of FAP-dCTP. b) Levels of XRCC1 modification by different DNA duplexes. The type of DNA used is indicated on the figure. The reaction mixtures contained 30 nM photoreactive DNA substrate, 30 nM XRCC1, 0.1 mg/ml BSA, 50 mM Tris-HCl, pH 8.0, and 50 mM NaCl. Level of modification is defined as the portion of DNA cross-linked to protein (in %).

β (Fig. 2a, lanes *5-8*). The level of Pol β labeling decreases in the presence of XRCC1 with parallel increase in the efficiency of XRCC1 modification (Fig. 2). The effect is stronger in the presence of Mg^{2+} and depends on the type of DNA duplex. Formation of ternary complex XRCC1–Pol β–DNA, but not DNA–XRCC1, has been shown earlier by electrophoretic mobility shift assay [1].

Irrespective of the presence of Mg^{2+}, XRCC1 influences insignificantly the levels of PARP1 cross-linking (Fig. 2). But PARP1 inhibits XRCC1 modification (Fig. 2a, lanes *1-4* and *25-28*). PARP1 appears to displace XRCC1 from DNA. The most striking influence of XRCC1 was demonstrated for the APE1–XRCC1 pair. When present, XRCC1 fully abolished the cross-linking of APE1 to all DNA duplexes (compare lanes *9-12* and *21-24* in Fig. 2a, lanes *25-28* and *9-12* in Fig. 2b) with the level of XRCC1 cross-linking being decreased less than 3-fold. This effect may be a consequence of strong competition between APE1 and XRCC1 for DNA as well as shielding of APE1 by XRCC1 in the ternary complex APE1–XRCC1–DNA if the last is formed. On one hand, according to a current model of the BER process, XRCC1 is still bound to DNA from gap filling to ligation step. The data about high affinity of XRCC1 to gapped DNA [14] and formation of ternary complex XRCC1–Pol β–DNA [1] are in accordance with this model. In this case, XRCC1 can displace APE1 from the incised AP-site. Taking into account the data of protein–DNA formaldehyde cross-linking assay and affinity of BER proteins to the incised abasic site, other authors [11] suppose that Pol β binds to DNA alone and promotes

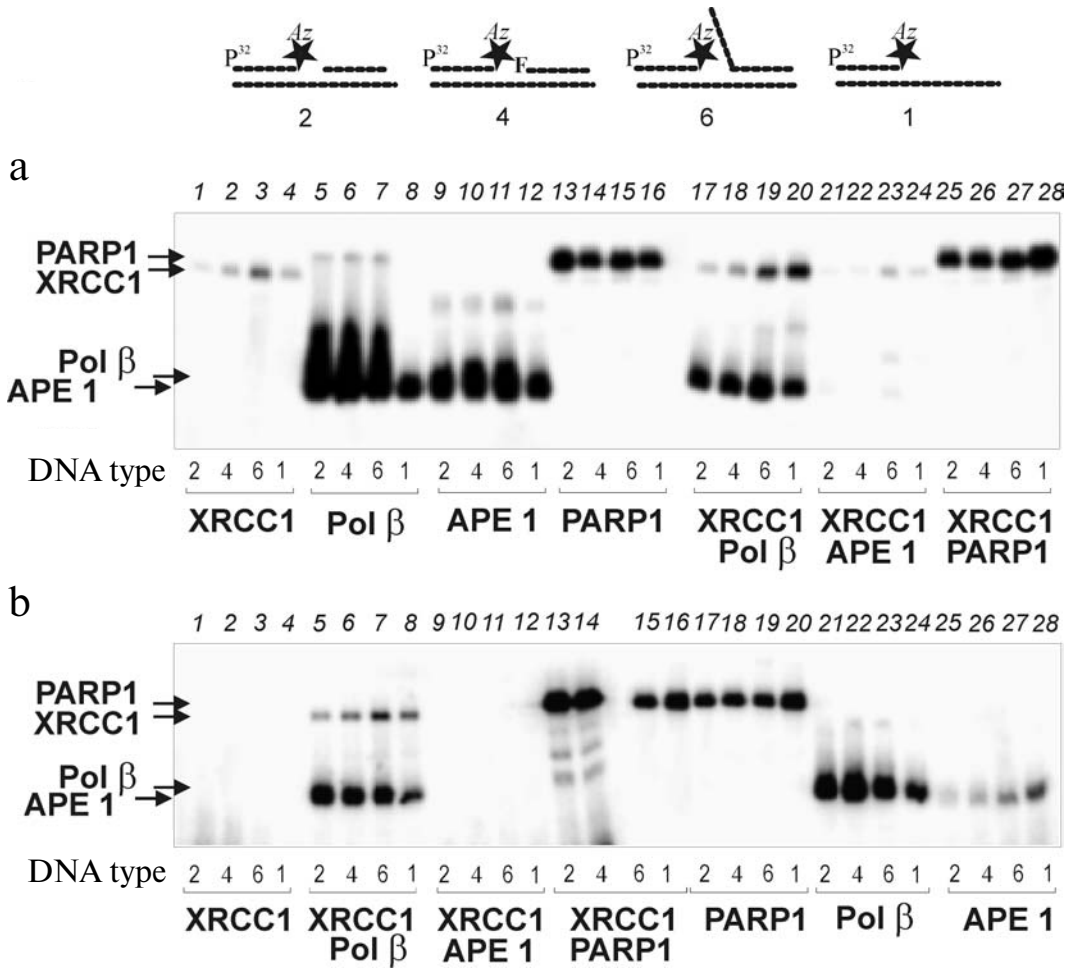

Fig. 2. Photoaffinity modification of the BER proteins. Autoradiograph of reaction products after separation in 10% polyacrylamide gel according to Laemmli. The reaction mixtures contained 0.1 μM DNA substrate, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and proteins at 0.3-μM concentration in the absence (a) or presence (b) of 10 mM $MgCl_2$.

recruitment of the XRCC1–Lig IIIα complex. On the other hand, it was shown earlier that XRCC1 interacts with APE1 and stimulates its AP endonuclease and 3′-dRPase activities [4]. This fact suggests the existence of ternary complex XRCC1–APE1–DNA. However, we failed to reveal the ternary complexes by EMSA. These complexes appear to be too unstable to be detected by non-equilibrium gel electrophoretic mobility shift assay.

Since XRCC1 cross-links to flapped DNA quite effectively, it was interesting to study the influence of XRCC1 on interaction of FEN1 with DNA. In the literature, there is no information about interaction of FEN1 with XRCC1. When added, XRCC1 decreases the level of FEN1 labeling for all DNAs (data not shown) with the level of XRCC1 modification being also decreased (data not shown). This can be explained by competition between proteins for DNA binding. However, XRCC1 influenced the FEN1 activity insignificantly (data not shown). FEN1 operates in long-patch BER, while XRCC1 is a factor acting in short-patch pathway. Since we failed to reveal an influence of XRCC1 on processing of DNA intermediates of long-patch BER, further experiments were carried out using analogs of DNA intermediates of short-patch and/or first stages of BER.

Study of interaction of XRCC1 with DNA and proteins in the first stages of BER. To study the interactions of XRCC1 with DNA and proteins in first stages of BER, DNA duplexes containing photoreactive group opposite the damage were designed. Oligonucleotide containing photoreactive dCMP moiety in the inner position of the chain was annealed to oligonucleotide bearing 8-oxoguanine (8-oxoG), dihydrothymine (DHT), or 3-hydroxyl-2-hydroxymethyltetrahydrofuran (F). First, the ability of enzymes to process photoreactive DNA duplexes was tested. The photoreactive DNA duplexes containing damaged bases (8-oxoG or DHT) opposite FAP-dCMP were recognized and excised by specific DNA glycosylases (OGG1 or NTH1, correspondingly), although the excision rate of

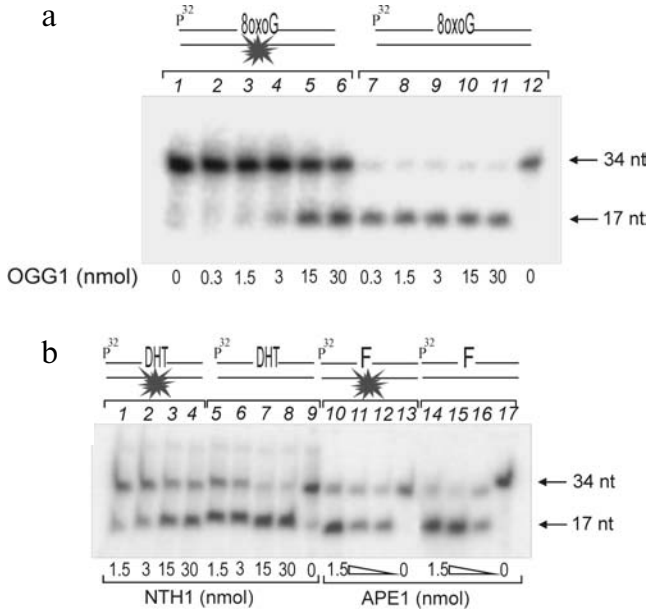

Fig. 3. Effect of the presence of photoreactive nucleotide in the DNA strand opposite the damage on activity of OGG1 (a), NTH1, and APE1 (b). Autoradiograph of reaction product after separation in 20% polyacrylamide gel under denaturing conditions.

photoreactive DNA structures was lower than that of normal substrates (Fig. 3). The presence of photoreactive derivative opposite 8-oxoG substantially decreases the activity of OGG1 (Fig. 3a). Even when the OGG1 concentration was increased by 100-fold, the level of excision of photoreactive DNA was still low. This result is in line with the data that OGG1 forms specific contacts not only with 8-oxoG but also with cytosine located opposite 8-oxoG [24]. The substitution of cytosine by other nitrogen bases results in decrease in DNA binding and rate of 8-oxoG excision by OGG1 [25]. NTH1 effectively processed both native and photoreactive substrates (Fig. 3b, lanes *1-9*). The presence of a photoreactive group in the template strand opposite to F residue did not alter the efficiency of incision of analog of AP site by APE1 (Fig. 3b, lanes *10-17*). These data demonstrate that the photoreactive DNA duplexes are recognized and processed by BER enzymes and can be used to study interactions of BER proteins.

The cross-linking experiments did not reveal the specificity of interaction of XRCC1 with photoreactive DNA duplexes containing damaged bases (8-oxoG or DHT) in comparison with undamaged duplex (data not shown). XRCC1 in itself apparently does not participate in recognition of damaged bases.

Influence of XRCC1 on interaction of DNA glycosylases with DNA substrates. XRCC1 is known to stimulate the DNA glycosylase activity of OGG1 and NTH1 [2, 3]. Addition of XRCC1 to the reaction mixture resulted in an increase in the borohydride trapping of OGG1 with 8-oxoG-containing DNA duplex [2]. Since the Schiff base is formed after or simultaneously to the excision of the base, these results suggest that XRCC1 stimulates either the binding of OGG1 to the damaged DNA or the catalytic stage itself. To study the influence of XRCC1 on interaction of OGG1 with the substrate, cross-linking experiment using wild type OGG1 (wt) and catalytically inactive mutant OGG1 (K249Q) was carried out in the presence of XRCC1. Mutant OGG1 with substitution K249Q contains glutathione-S-transferase. Under 2.5- and 10-fold excess of XRCC1 over OGG1, the levels of modification of neither wild type nor mutant OGG1 were changed (Fig. 4a, lanes *1-3* and *6-8*, correspondingly). Subsequent

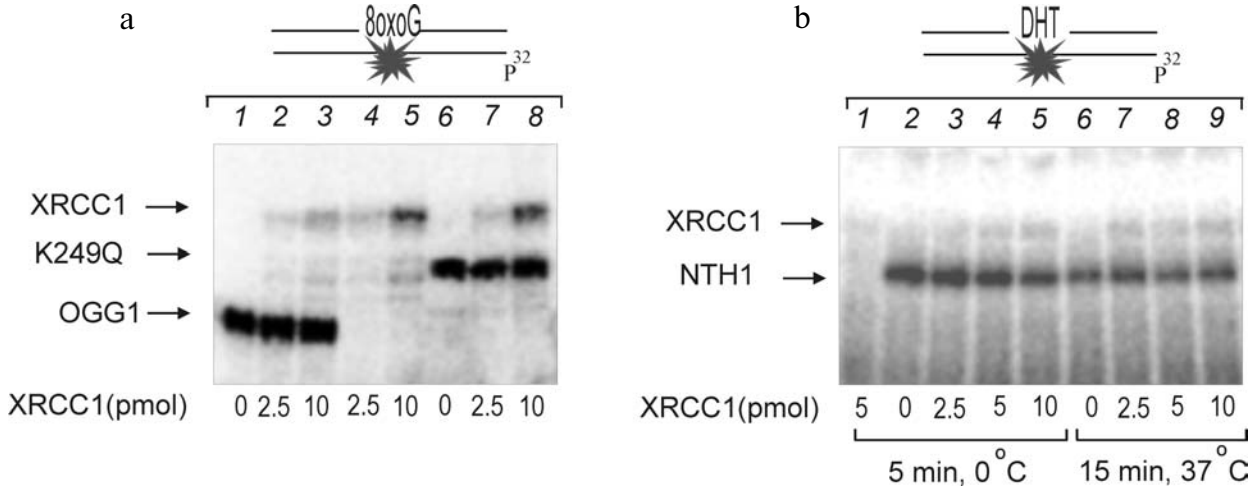

Fig. 4. Influence of XRCC1 on interaction of OGG1 and NTH1 with DNA substrates. a) The reaction mixtures 10 μl in volume contained 0.5 pmol of the 8-oxoG-DNA, 1 pmol OGG1 (wt or K249Q), and 2.5 or 10 pmol of XRCC1. b) The reaction mixtures 10 μl in volume contained 0.5 pmol of the DHT-DNA, 1 pmol NTH1, and 2.5, 5, or 10 pmol of XRCC1 were incubated as indicated in the figure and UV irradiated. The reaction products were separated in 15% polyacrylamide gel according to Laemmli.

increase in XRCC1 concentration decreases the level of OGG1 modification (data not shown). The level of XRCC1 modification is also decreased in the presence of OGG1 (Fig. 4a, lanes *3* and *5*), thereby indicating competition between these proteins. Recently, we have demonstrated competition between OGG1 and XRCC1 for interaction with DNA containing an AP site [26]. XRCC1 forms covalent complexes with AP DNA via a Schiff base. More efficiently, XRCC1 cross-links to AP DNA containing gap or nick irrespectively in what chain interruptions are located. When OGG1 and XRCC1 present in the reaction mixture at comparable amount, the level of cross-linking of XRCC1 to AP DNA is decreased [26].

An analogous experiment on photoaffinity labeling of NTH1 was carried out. To clarify the influence of XRCC1 on binding NTH1 to DNA, the reaction mixtures were UV-irradiated after incubation on ice, when the activity of NTH1 was low, or after incubation for 15 min at 37°C, when excision of DHT can proceed. Increase in XRCC1 concentration did not increase the yield of cross-linked NTH1. On the contrary, at low temperature in the presence of 10-fold excess of XRCC1, a decrease in modification of NTH1 was observed (Fig. 4b, lanes *2* and *5*) indicating competition between proteins. It should be noted that on all DNA duplexes, the level of XRCC1 modification was lower than that of glycosylases. This fact

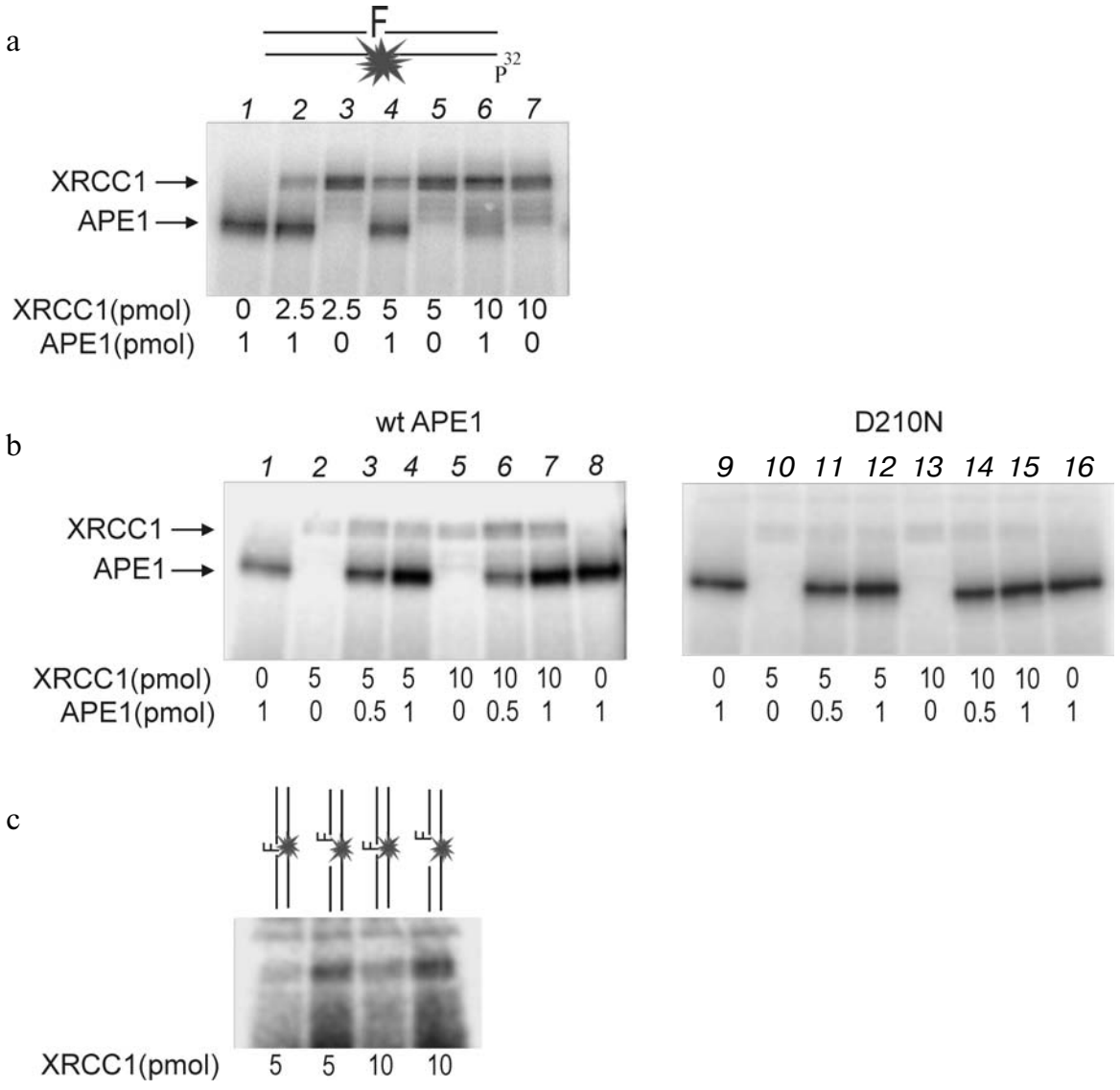

Fig. 5. Interaction of XRCC1 and APE1 with F-containing DNA duplex. The reaction mixtures contained 50 nM DNA substrate, buffer B3 (0.1 mg/ml BSA, 50 mM Hepes, pH 7.7, 5% glycerol, and 8 mM $MgCl_2$) (except (a)), and proteins as indicated in the figure. a) Photoaffinity modification of XRCC1 and APE1 in the absence of magnesium ions. b) Photoaffinity modification of XRCC1 and APE1 (wt and D210N) in the presence of magnesium ions. c) Photoaffinity modification of XRCC1 by DNA duplex containing analog of AP site unincised or incised by APE1. The reaction products were separated in 15% polyacrylamide gel according to Laemmli.

can be explained by less proper positioning of acceptor amino acids nearby DNA binding region of XRCC1. Thus, XRCC1 does not increase the levels of modification of DNA glycosylases (Fig. 4). The data suggest that XRCC1 stimulates the activity of glycosylases without affecting their binding with substrates.

Interaction of XRCC1 and APE1 with DNA duplex containing an analog of the AP site. XRCC1 was shown to promote the AP endonuclease and 3′-dRPase activities of APE1 [4] supposing the existence of triple complex AP DNA–APE1–XRCC1. Since the reaction of incision of AP site by AP endonuclease 1 is Mg^{2+} dependent, it was interesting to study interference of XRCC1 and APE1 both in the presence and absence of magnesium ions. At the absence of Mg^{2+}, when APE1 cannot incise DNA, the increase in XRCC1 concentration decreases the level of APE1 modification (Fig. 5a, lanes *1*, *2*, *4*, *6*). The presence of APE1 in the reaction mixture also decreases XRCC1 modification (Fig. 5a, lanes *2-7*). APE1 and XRCC1 seem to compete for DNA binding. Analogous result was observed earlier when we studied the interaction of these proteins with AP sites. Adding of APE1 to the reaction mixture resulted in decrease in cross-linking of XRCC1 to AP DNA [26]. On the contrary, in the presence of magnesium ions, APE1 increases the level of XRCC1 modification (Fig. 5b, lanes *2-7*). This effect may be due to either protein–protein interactions or formation of a DNA intermediate that binds by XRCC1 more effectively. Under the conditions used, DNA was incised completely. In the presence of inactive APE1 containing mutation D210N in the active site, no increase in XRCC1 modification was observed (Fig. 5b, lanes *10-15*) indicating that the effect observed is a consequence of DNA structure changing. The efficiency of XRCC1 interaction with the product of APE1 reaction was estimated. To create this structure, photoreactive F-containing DNA was incubated with APE1, and then the sample was heated to inactivate APE1 and cooled slowly to re-anneal DNA. Figure 5c represents a pattern of photoaffinity of XRCC1 modification by F-containing DNA before and after treating with APE1. The level of XRCC1 modification by duplex with F was lower than that of nicked DNA with 5′-furanophosphate group—the product of the endonuclease reaction. We have shown before that XRCC1 can interact with the 5′-dRP group, which is formed during incision of an AP site by APE1 [26]. When incised DNA was used, adding of APE1 to the reaction mixture did not influence the level of XRCC1 modification (data not shown). Thus, increase in XRCC1 modification in the presence of Mg^{2+} and APE1 is due to formation of nicked DNA structure, which interacts more efficiently with XRCC1. This observation is consistent with the data that affinity of XRCC1 to nicked or gapped DNA is 5-fold higher than to undamaged duplex [14].

Interaction of XRCC1 with DNA polymerase β. XRCC1 was shown to stimulate strand displacement DNA synthesis catalyzed by Pol β [8]. Formation of ternary complex XRCC1–Pol β–incised AP DNA was revealed by electrophoretic mobility shift assay [1]. Pol β has the maximal affinity to DNA with mononucleotide gap and less efficiently binds duplexes containing nick and short flap [27]. Pol β also binds DNA containing AP site [28]. Photoaffinity modification of XRCC1 and Pol β was carried out using F-containing DNA duplex before and after treatment with APE1. The level of cross-linking of Pol β to incised DNA was higher then to the un-incised one (Fig. 6a, lanes *5* and *10*). Pol β increases cross-link-

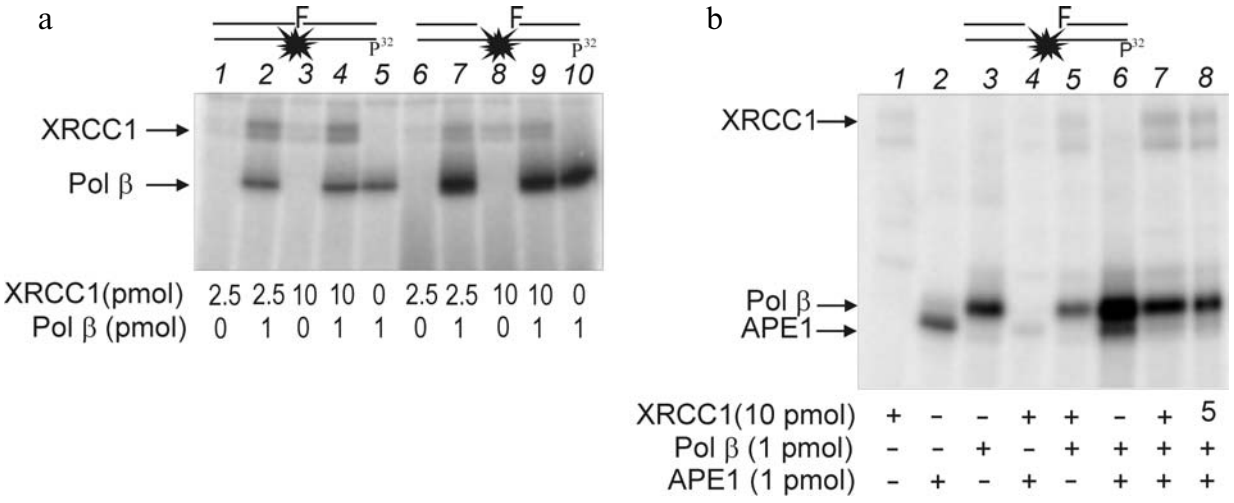

Fig. 6. Autoradiograph of reaction product after separation in 15% polyacrylamide gel according to Laemmli. a) Photoaffinity modification of XRCC1 and Pol β by F-containing DNA duplex un-incised or incised by APE1. The reaction mixtures contained 50 nM F-containing DNA duplex in buffer B3 treated (lanes *6-10*) or not treated (lanes *1-5*) with APE1. The amount of proteins in the reaction mixtures of 10 μl in volume is indicated in the figure. b) Mutual influence of XRCC1, APE1, and Pol β on the efficiency of their cross-linking to F-containing DNA duplex incised by APE1. The reaction mixtures contained 100 nM Pol β, 1 μM XRCC1, 100 nM APE1, and components indicated in (a).

ing of XRCC1 to both incised and un-incised DNA. The increase in XRCC1 modification in the presence of Pol β was also observed when the structures with photoreactive group at 3′-end of upstream primer were used (Fig. 2).

To reveal the interaction of XRCC1, APE1, and Pol β, we carried out photoaffinity modification of the proteins. Under the conditions used, APE1 incised the analog of AP site in 1 min. Thus, we used DNA incised at the position of F that allows us to compare samples with and without APE1. XRCC1 decreases the levels of modification of APE1 and Pol β (Fig. 6b, lanes *2* and *4*, *3* and *5*). APE1 considerably increases Pol β modification (Fig. 6b, lane *6*). Stimulation of Pol β binding to DNA by APE1 was shown earlier by electrophoretic mobility shift assay [29]. Addition of XRCC1 to the reaction mixture containing APE1 and Pol β results in decrease in Pol β modification to the initial level observed when Pol β is present alone. However, in the presence of APE1 the level remains higher than that in the system containing Pol β and XRCC1 (Fig. 6b, lanes *5*-*7*).

Two types of photoreactive DNA were used to study interaction of XRCC1 with DNA intermediates and proteins of base excision repair. One group consists of DNA structures with a photoreactive group at the 3′-end of the upstream primer. They mimic DNA intermediates of BER arising after incision of AP site by APE1. Another group covers DNA duplexes containing a photoreactive group in the template strand opposite the damaged bases that mimic intermediates of initial stages of BER. XRCC1 was shown to cross-link to flapped DNA quite effectively. Though according to the results of photoaffinity modification XRCC1 competes with FEN1 for DNA binding, we did not observed significant influence of XRCC1 on FEN1 activity. The role of XRCC1 in BER appears to consist in interaction with proteins of short-patch BER and modulation of their activity. We did not observe any specificity in binding of DNA duplexes with damaged bases by XRCC1. Apparently, XRCC1 itself does not participate in the recognition of damaged bases. All proteins used, except Pol β, decrease or do not change the levels of XRCC1 modification, while XRCC1 decreases the levels of modification of other proteins, except PARP1, probably by displacing them from DNA–protein complex. The increase in XRCC1 modification caused by the presence of Pol β agrees to a greater extent with the hypothesis that XRCC1 operates in BER beginning from the DNA synthesis step [11]. At the same time, we cannot exclude the participation of XRCC1 in earlier stages of BER. The stimulatory effect of XRCC1 on activity of BER proteins is most likely due to an affect on the catalytic step of the reaction, but not binding of enzymes with their substrates.

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