Biochemical Analysis of the Damage Recognition Process in Nucleotide Excision Repair*

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XPA, XPC-hHR23B, RPA, and TFIIH all are the damage recognition proteins essential for the early stage of nucleotide excision repair. Nonetheless, it is not clear how these proteins work together at the damaged DNA site. To get insight into the molecular mechanism of damage recognition, we carried out a comprehensive analysis on the interaction between damage recognition proteins and their assembly on damaged DNA. XPC physically interacted with XPA, but failed to stabilize the XPA-damaged DNA complex. Instead, XPC-hHR23B was effectively displaced from the damaged DNA by the combined action of RPA and XPA. A mutant RPA lacking the XPA interaction domain failed to displace XPC-hHR23B from damaged DNA, suggesting that XPA and RPA cooperate with each other to destabilize the XPC-hHR23B-damaged DNA complex. Interestingly, the presence of hHR23B significantly increased RPA/XPA-mediated displaced placement of XPC from damaged DNA, suggesting that hHR23B may modulate the binding of XPC to damaged DNA. Together, our results suggest that damage recognition occurs in a multistep process such that XPC-hHR23B initiates damage recognition, which was replaced by combined action of XPA and RPA. XPA and RPA, once forming a complex at the damage site, would likely work with TFIIH, XPG, and ERCC1-XPF for dual incision.

Nucleotide excision repair (NER)1 is one of the major repair pathways for removal of DNA damage caused by UV irradiation and a wide variety of bulky helix-distorting lesions such as cisplatin (1–4). In mammals, NER requires over 20 polypeptides, including damage recognition and/or structure distortion factors (XPA, XPC-hHR23B, replication protein A (RPA), and a transcription factor, TFIIH), strand separating helicases to create an open preincision complex (TFIIH containing XPB and XPD DNA helicases), two structure-specific endonucleases (ERCC1-XPF and XPG), and the enzymes needed for gap filling (DNA polymerase α/ε, proliferating cell nuclear antigen, replication factor C, and RPA).

Both RPA and XPA are also known as damage recognition proteins because they preferentially bind to cisplatin- or UV-damaged DNA (5–10). Both proteins may also play a role in subsequent steps in NER through interaction with other repair proteins (8, 11–15). The XPA-DNA interaction is relatively weak and characterized by rapid dissociation, whereas RPA formed a much more stable complex with UV-damaged DNA (16). XPA physically interacts with RPA, which is necessary for efficient NER action (14). Wild-type RPA, but not a mutant lacking the XPA interaction domain, led to stabilization of the XPA-damaged DNA complex, implicating a unique role for RPA in stabilizing the XPA-damaged DNA complex. The XPA-damaged DNA interaction is also likely necessary for recruiting other DNA repair proteins such as XPG, ERCC1-XPF, and TFIIH to the damaged site (8, 15, 17). RPA may also be involved in the later stage of NER, gap-filling, that requires proliferating cell nuclear antigen, replication factor C, and DNA polymerase δ (or ε) (18).

XPC-hHR23B is a human homolog of yeast Rad4 and Rad23 proteins, respectively, and forms a stable complex in solution. XPC-hHR23B exhibits the strongest affinity for damaged DNA (19–21), as does the yeast counterpart, Rad4-Rad23 (22). Rad23 without Rad4 does not show any DNA binding activity, suggesting that Rad4 is solely responsible for recognition of damaged DNA. Rad23 is essential for XPC function in NER and may also be necessary for the solubility of Rad4 (23). XPC-hHR23B showed a remarkable preference to UV-damaged DNA particularly in the presence of nondamaged competitor DNA and has been suggested as the initiator of global genome NER (19, 24). A recent immunohistochemistry study also strongly supports a role for XPC as a global initiator in repair (25), while suggesting a role for XPA and RPA as repair mediator proteins. XPC-hHR23B is also involved in the recruitment of TFIIH to damaged DNA (26). TFIIH, once recruited, may play a role in distinguishing the damaged strand from the nondamaged one (27) as well as local unwinding of the damaged DNA region. TFIIH with its DNA helicase activity likely generates a junction between single-stranded DNA and duplex DNA that is recognized by two structure-specific endonucleases, XPG and ERCC1-XPF, for dual incision of damaged strand.

An ongoing challenge is to understand how DNA damage is recognized and distinguished from nondamaged sites. In mammalian cells, XPC-hHR23B, XPA, RPA, and TFIIH factors may all have roles in damage recognition during the early stage of NER. In this study we carried out a comprehensive analysis on the interaction between damage recognition proteins and their assembly on damaged DNA. We found that XPC-hHR23B, like
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RPA, physically interacted with XPA. However, the XPA-XPC interaction, unlike the XPA-RPA interaction, failed to stabilize the XPA-damaged DNA complex. Instead, XPA cooperates with RPA to promote the destabilization of the XPC-hHR23B-damaged DNA interaction. This finding supports a notion that the damage recognition process occurs in a stepwise manner such that XPC-hHR23B initiates damage recognition, which was replaced by the combined action of XPA and RPA.

MATERIALS AND METHODS

Preparation of Platinum-induced Damaged DNA—To study the interaction between damaged DNA and damage recognition factors, we constructed a duplex DNA with the cisplatin lesion at a specific site (Fig. 1A). Oligonucleotides containing an intrastrand (ITR-60) cross-link were prepared according to the previously described procedure with some modifications (28). For cross-linking, the top strand was first incubated with cisplatin (in TE, pH 8.0, 2-fold molar excess) at 37 °C in the dark for 48 h, and then ethanol-precipitated. The damaged DNA was purified by 15% denaturing polyacrylamide gel electrophoresis and annealed to the bottom strand (5-fold molar excess). The duplex DNA was purified by 15% native polyacrylamide gel electrophoresis. Purified duplex DNA, 5'-32P-labeled at the 5'-end, was run on an agarose gel under non-denaturing conditions, and then ethanol-precipitated. The purified DNA was resuspended in buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl, 2 mM MgCl2, 0.005% polysorbate-20, and 1 mM dithiothreitol). Each experiment was repeated at least twice to assure reproducibility.

RESULTS

Interaction of Damage Recognition Factors with DNA—To get insight into the molecular mechanism of how damage recognition proteins are assembled and working together at the damaged DNA site, we carried out a comprehensive analysis on the interaction of XPA, RPA, and XPC-hHR23B with damaged DNA. Binding affinity of damage recognition factors to a duplex DNA containing an intrastrand platinum cross-link (ITR-60, Fig. 1) was measured using an electrophoretic mobility shift assay. As previously reported (Refs. 1–4 and references therein), both RPA and XPC-hHR23B showed preferential binding to the cisplatin-damaged DNA over the nondamaged one, whereas the XPA-DNA interaction was observed only in the presence of excess amounts (Fig. 2A). On the other hand, surface plasmon resonance kinetic analysis indicated that XPC-hHR23B compared with RPA exhibited much higher affinity to the damaged DNA (Fig. 2B). This finding is in keeping with the previous observation that XPC has a considerable preference for binding to the damaged DNA over nondamaged competitor DNA (24, 25). XPC-hHR23B also differs from RPA in its preferential binding to dsDNA over ssDNA (35), whereas RPA has a much higher affinity to ssDNA than to dsDNA (Fig. 2, A and B).

XPC Physically Interacts with XPA, but Not with RPA—Because all three proteins (RPA, XPA, and XPC-hHR23B) independently bind to the damaged DNA, we examined a possibility whether all three damage recognition proteins form a complex on the damaged DNA site (11–13). Initially, we examined the interactions among damage recognition factors without DNA. Because we know that XPA and RPA interact with each other in solution as well as on damaged DNA (14, 17), our focus was on the interaction of XPC-hHR23B with RPA or XPA (Fig. 3). GST-XPC or GST-XPC-hHR23B were incubated with an increasing amount of XPA (or RPA), pulled down with glutathione-Sepharose beads for co-precipitation of XPA (or RPA), and analyzed by Western blot (Fig. 3). XPA was co-precipitated with GST-XPC or GST-XPC-hHR23B complex, but not with GST (Fig. 3A), suggesting that XPA physically interacts with XPC-hHR23B. In the XPA-XPC interaction, GST-XPC was almost comparable with GST-XPC-hHR23B (Fig. 3A, lanes 3–4 versus lanes 5 and 6), indicating that XPC hHR23B physically interacts with XPA. In keeping with this, hHR23B without XPC failed to interact with XPA (data not shown). Unlike XPA, however, RPA had no physical interaction with GST-XPC-hHR23B or GST-XPC in solution (Fig. 3B).

Because both RPA and XPC interact with XPA (Refs. 11–13 and Fig. 3), it is possible that the binding of XPC (or RPA) to XPA may competitively exclude RPA (or XPC) from the complex. If so, we may not be able to see a complex containing all three damage recognition proteins. To examine this possibility, XPA was incubated with GST-XPC-hHR23B in the presence of an increasing amount of RPA and analyzed for the XPA-XPC interaction. Addition of a molar excess of RPA had little or no effect on the XPA-XPC interaction as determined by the GST-XPC-hHR23B pull-down assay (Fig. 3C), suggesting that RPA and XPC-hHR23B may recognize two separate domains of XPA. Nonetheless, we were not able to detect RPA in the GST-XPC pull-down assay in the presence of XPA and RPA (Fig. 3C).

Presence of Damaged DNA Significantly Inhibits the XPA-XPC Interaction—The XPA-damaged DNA interaction is weak, but markedly stimulated in the presence of a high affinity DNA-binding protein, RPA (14). The stimulatory effect of RPA

Protein-Damaged DNA Interaction: Surface Plasmon Resonance Analysis—Interactions of XPA, RPA, and XPC-hHR23B with DNA were measured using a surface plasmon resonance biosensor instrument, Biacore 3000 (Biacore) as described previously (16, 34). For preparation of the biosensor surface with DNA, 5’-biotinylated 60-mer duplex DNA (prepared in a buffer containing 10 mM sodium acetate, pH 4.8, and 1.0 mM NaCl) was manually injected onto a streptavidin-coated surface of a BIAcore sensor chip to the desired density in different flow cells. One flow cell was left derivatized to allow for refractive index change correction. Proteins were diluted in the running buffer containing 10 mM Hapes, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.005% polysorbate-20, and 1 mM dithiothreitol. Each experiment was repeated at least twice to assure reproducibility. 
on the XPA-damaged DNA interaction occurs through the RPA-XPA interaction (14, 16). Similar to RPA, XPC-hHR23B exhibits a high affinity to damaged DNA (19–23) and physically interacts with XPA (Fig. 3). We therefore examined whether XPC-hHR23B affects the XPA-damaged DNA interaction. In the GST-XPA pull-down assay, XPA had very little or no bind-
ing to the damaged DNA (Fig. 4A, lane 2), which was significantly stimulated by addition of RPA (Fig. 4A, lanes 3–5). In contrast, XPC-hHR23B showed no effect on the XPA-damaged DNA interaction (Fig. 4A, lanes 6–8). Interestingly, the stimulatory effect of RPA on the XPA-damaged DNA interaction was markedly reduced in the presence of XPC-hHR23B (Fig. 4A, lane 9), suggesting that XPC-hHR23B, unlike RPA, may not form a stable complex with XPA on damaged DNA. To investigate this further, we examined the effect of damaged DNA on the XPA-XPC interaction (Fig. 4B). XPA was successfully co-precipitated with GST-XPC in the pull-down assay in the absence of damaged DNA (Fig. 4B, lanes 4 and 5). In the presence of damaged DNA, however, the amount of XPA co-precipitated with GST-XPC was significantly reduced to an undetectable level (Fig. 4B, lanes 6 and 7), suggesting that the XPA-XPC interaction is destabilized in the presence of damaged DNA.

XPC-hHR23B Is Displaced from Damaged DNA by the Combined Action of RPA and XPA—Our result (Fig. 4) strongly suggests that XPA, RPA, and XPC-hHR23B do not form a
stable complex on the damaged DNA. Instead, the damage recognition process may occur in a stepwise manner such that XPC-hHR23B interacts with damaged DNA first and then is replaced by XPA and/or RPA. To test this, biotin-labeled cisplatin-damaged DNA was first incubated with XPC-hHR23B, followed by the addition of RPA (Fig. 5A, lanes 3–5), XPA (lanes 6–8), or XPA + RPA (lanes 9–11) to the reaction mixtures. The streptavidin-Sepharose pull-down assay revealed that XPC-hHR23B formed a stable complex with damaged DNA (Fig. 5A, top panel, lane 2) and, the addition of RPA (lanes 3–5) or XPA (lanes 6–8) showed very little effect on the XPC-hHR23B-damaged DNA interaction. In the presence of both XPA and RPA, however, the XPC-hHR23B-damaged DNA interaction was significantly inhibited (Fig. 5A, top panel, lanes 9–11). In contrast to XPC, the amount of XPA co-precipitated with damaged DNA was markedly increased in the presence of RPA (Fig. 5A, second panel, lanes 9–11), whereas the RPA-damaged DNA interaction was hardly affected by the presence of XPA (Fig.
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Panel A. Western blot analysis of individual damage recognition factors associated with biotin-labeled damaged DNA. Biotin-labeled damaged DNA (ITR-60; 2 pmol) was preincubated with streptavidin-Sepharose prior to the addition of GST-XPC-hHR23B (800 ng in lanes 1–11), RPA (2, 5, and 10 pmol in lanes 3–5, respectively), XPA (2, 5, and 10 pmol in lanes 6–8, respectively), and XPA + RPA (2, 5, and 10 pmol each in lanes 9–11, respectively). Following the pull-down of beads, proteins were analyzed by Western blot using an anti-GST (for GST-XPC), -XPA, -RPAp70, or RPAp34 antibody. In lane M, purified protein (GST-XPC, XPA, or RPA for p70 and p34) was included. Panel B, glutathione-Sepharose beads (25 μL) were mixed with GST-XPC-hHR23B (400 ng) and fluorescence (TET)-labeled damaged DNA (2 pmol), and gently rocked for 20 min at room temperature. After challenging the complex with either XPA (2, 5, and 10 pmol in lanes 3–5, respectively) or XPA + RPA (2, 5, and 10 pmol each in lanes 6–8, respectively), beads were pulled down and analyzed by 10% SDS-PAGE for TET-labeled damaged DNA using fluorescence image scanner (top panel) or Western blot using an anti-GST antibody (bottom panel). Relative amounts of TET-labeled damaged DNA are indicated at the bottom of the figure. Panel C, effect of RPA and XPA on the interaction between XPC and damaged DNA in a gel mobility shift assay. GSTXPC-hHR23B (20 ng) was incubated with 100 fmol of 32P-platinum-damaged DNA (ITR-60) and incubated for 20 min at room temperature. Where indicated, XPA (20 ng in lanes 4, 10, 12, and 14; 50 ng in lanes 5, 11, 13, and 15), RPA (20 ng in lanes 7, 10, 12, and 14; 50 ng in lanes 8, 11, 13, and 15), or an anti-GST antibody (5 μL; 100 μg/ml) was added to the mixtures and further incubated for 20 min at room temperature prior to gel electrophoresis. The protein-DNA complex was analyzed by 4% polyacrylamide gel in 0.5× TBE (acrylamide:bisacrylamide = 43:2:0.8). GST/XPC-damaged DNA complex and its supershifted complexes are indicated by arrows. No protein was included in lane 1.

Fig. 5. XPC-hHR23B is displaced from the damaged DNA by a combined action of XPA and RPA. Panel A, Western blot analysis of individual damage recognition factors associated with biotin-labeled damaged DNA. Biotin-labeled damaged DNA (ITR-60; 2 pmol) was preincubated with streptavidin-Sepharose prior to the addition of GST-XPC-hHR23B (800 ng in lanes 1–11), RPA (2, 5, and 10 pmol in lanes 3–5, respectively), XPA (2, 5, and 10 pmol in lanes 6–8, respectively), and XPA + RPA (2, 5, and 10 pmol each in lanes 9–11, respectively). Following the pull-down of beads, proteins were analyzed by Western blot using an anti-GST (for GST-XPC), -XPA, -RPAp70, or RPAp34 antibody. In lane M, purified protein (GST-XPC, XPA, or RPA for p70 and p34) was included. Panel B, glutathione-Sepharose beads (25 μL) were mixed with GST-XPC-hHR23B (400 ng) and fluorescence (TET)-labeled damaged DNA (2 pmol), and gently rocked for 20 min at room temperature. After challenging the complex with either XPA (2, 5, and 10 pmol in lanes 3–5, respectively) or XPA + RPA (2, 5, and 10 pmol each in lanes 6–8, respectively), beads were pulled down and analyzed by 10% SDS-PAGE for TET-labeled damaged DNA using fluorescence image scanner (top panel) or Western blot using an anti-GST antibody (bottom panel). Relative amounts of TET-labeled damaged DNA are indicated at the bottom of the figure. Panel C, effect of RPA and XPA on the interaction between XPC and damaged DNA in a gel mobility shift assay. GSTXPC-hHR23B (20 ng) was incubated with 100 fmol of 32P-platinum-damaged DNA (ITR-60) and incubated for 20 min at room temperature. Where indicated, XPA (20 ng in lanes 4, 10, 12, and 14; 50 ng in lanes 5, 11, 13, and 15), RPA (20 ng in lanes 7, 10, 12, and 14; 50 ng in lanes 8, 11, 13, and 15), or an anti-GST antibody (5 μL; 100 μg/ml) was added to the mixtures and further incubated for 20 min at room temperature prior to gel electrophoresis. The protein-DNA complex was analyzed by 4% polyacrylamide gel in 0.5× TBE (acrylamide:bisacrylamide = 43:2:0.8). GST/XPC-damaged DNA complex and its supershifted complexes are indicated by arrows. No protein was included in lane 1.

5A, third and fourth panels, lanes 9–11). Together, this result suggests that XPC-hHR23B can be effectively displaced from damaged DNA by the combined action of RPA and XPA.

We further analyzed the effect of XPA and/or RPA on the interaction between XPC-hHR23B and damaged DNA by measuring damaged DNA co-precipitated with GST-XPC-hHR23B in the glutathione-Sepharose pull-down assay. Fluorescence (TET)-labeled damaged DNA was first incubated with GST-XPC-hHR23B before the addition of either XPA or XPA + RPA and analyzed quantitatively following GST-XPC pull-down assay (Fig. 5B). Addition of both XPA and RPA drastically reduced the amount of damaged DNA interacting with GST-XPC-hHR23B, whereas XPA alone marginally affected the XPC-damaged DNA interaction (Fig. 5B), suggesting that...
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hHR23B Is Necessary for XPA/RPA-mediated Displacement of XPC from Damaged DNA—XPC (Rad4) forms a stable complex with hHR23B (Rad23), which appears to be essential for NER (23). Nonetheless, damaged DNA binding activity of the XPC-hHR23B complex belongs to the XPC subunit (23) and the exact role for hHR23B in repair is unclear. In an effort to explore the role for hHR23B in DNA repair, we examined whether the presence of hHR23B affects RPA/XPA-mediated displacement of XPC from the damaged DNA. GST-XPC-hHR23B (or GST-XPC) was incubated with fluorescence (TET)-labeled damaged DNA in the presence of RPA and XPA, and the amount of damaged DNA precipitated with GST-XPC was measured (Fig. 6A). Both XPC and XPC-hHR23B showed strong interaction with damaged DNA (Fig. 6A, lanes 2 and 5). In the presence of RPA + XPA, however, the damaged DNA-XPC-hHR23B complex, not the damaged DNA-XPC complex, was significantly reduced (Fig. 6A, lanes 3 and 4 versus 6 and 7), suggesting that hHR23B is somehow involved in the displacement of XPC from damaged DNA in the presence of RPA + XPA. To further investigate this, purified hHR23B was added to the reaction mixtures containing GST-XPC for its effect on the XPC-damaged DNA interaction in the presence of RPA + XPA (Fig. 6B). GST-XPC without hHR23B formed a stable complex with damaged DNA (Fig. 6B, lane 2), which was hardly affected by RPA + XPA (Fig. 6B, lanes 3 and 4). In contrast, addition of purified hHR23B to the reaction mixtures significantly decreased the binding of XPC to the damaged DNA in the presence of RPA + XPA (Fig. 6B, lanes 6 and 7). This result strongly suggests that hHR23B is necessary for the RPA/XPA-mediated displacement of XPC from damaged DNA.

The RPA-XPA Interaction Is Essential for the Displacement of XPC-hHR23B from Damaged DNA—Interaction between RPA and XPA is not only required to stabilize the XPA-damaged DNA interaction, but also necessary for NER activity (14, 16). Because both RPA and XPA are essential for the displacement of XPC from damaged DNA, we examined whether the RPA-XPA interaction is necessary for it. For this, wild-type RPA and two RPA mutants (RPAnp34C33 lacking the C terminus of p34 subunit (XPA interaction domain); ZFM4, a mutant with cysteine to alanine substitution at the zinc finger domain of the p70 subunit) were compared in the displacement of XPC-hHR23B from damaged DNA. Although both mutants poorly supported NER activity in vitro (14, 36), ZFM4 supported the displacement of XPC-hHR23B from damaged DNA, whereas RPAnp34C33 did not (Fig. 7). This result not only

After rocking for 20 min at room temperature, equimolar amounts of RPA + XPA (2 pmol (lanes 3, 6, and 9) or 5 pmol (lanes 4, 7, and 10)) were added to the mixtures, and beads were pulled down and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the bottom of the figure.
suggests that RPA and XPA cooperate with each other in the displacement of XPC-hHR23B from damaged DNA. GST-XPC-hHR23B (400 ng) was mixed with glutathione-Sepharose beads and TET-damaged DNA (2 pmol), and rocked for 20 min at room temperature. The mixtures were then challenged with increasing amounts of XPA (2 pmol (lanes 3, 5, and 7) or 5 pmol (lanes 4, 6, and 8)) in the presence of wild-type RPA (lanes 3 and 4), a mutant lacking C terminus of p34 (lanes 5 and 6), or a RPA zinc finger mutant (ZFM4) (lanes 7 and 8). Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the bottom of the figure.

Displacement of XPC-hHR23B from Damaged DNA Is Not Affected by the Order of Assembly of Damage Recognition Proteins.

The components indicated inside the boxes were incubated together for 20 min at room temperature prior to the addition of the remaining components. Where indicated, 400 ng of GST-XPC-hHR23B, 2 pmol of TET-labeled damaged DNA, and equimolar amounts of XPA + RPA (2 pmol (lanes 3, 6, and 9), 5 pmol (lanes 4, 7, and 10), or 10 pmol (lanes 5, 8, and 11)) were added. Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the bottom of the figure.

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suggests that RPA and XPA cooperate with each other in the displacement of XPC-hHR23B from damaged DNA, but also supports that the RPA-XPA interaction may be necessary for such cooperation.

Displacement of XPC-hHR23B from Damaged DNA Is Not Affected by the Order of Protein Assembly on Damaged DNA—Our finding that XPA and RPA cooperate with each other to displace XPC-hHR23B from the damaged DNA supports a notion that XPC-hHR23B is the initiator of global genome NER (19, 23, 24). To investigate this further, we examined whether the order of protein assembly at the damaged DNA site affects the XPC-hHR23B-damaged DNA interaction (Fig. 8). The 5'-fluorescence (TET)-labeled damaged DNA (ITR-60) was incubated with GST-XPC-hHR23B (Fig. 8, lanes 3–5), XPA + RPA (Fig. 8, lanes 6–8), or all three proteins (Fig. 8, lanes 9–11), and 20 min later, the remaining factor(s) were added to the reaction mixtures. The mixtures were then analyzed by the GST-XPC pull-down assay for the interaction of GST-XPC-hHR23B with damaged DNA. Regardless of the order of assembly, all assembly groups showed remarkably similar patterns in that the addition of increasing amounts of XPA + RPA proportionally displaced XPC-hHR23B from damaged DNA (Fig. 8). This result not only supports the multistep damage recognition in NER, but is also in keeping with the notion that XPC-hHR23B cooperates with RPA and XPA. **Fig. 7.** A mutant RPA lacking the XPA interaction domain did not support the displacement of XPC from damaged DNA. GST-XPC-hHR23B (400 ng) was mixed with glutathione-Sepharose beads and TET-damaged DNA (2 pmol), and rocked for 20 min at room temperature. The mixtures were then challenged with increasing amounts of XPA (2 pmol (lanes 3, 5, and 7) or 5 pmol (lanes 4, 6, and 8)) in the presence of wild-type RPA (lanes 3 and 4), a mutant lacking C terminus of p34 (lanes 5 and 6), or a RPA zinc finger mutant (ZFM4) (lanes 7 and 8). Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the bottom of the figure.

**Fig. 8.** Displacement of XPC-hHR23B from damaged DNA is not affected by the order of assembly of damage recognition proteins. The components indicated inside the boxes were incubated together for 20 min at room temperature prior to the addition of the remaining components. Where indicated, 400 ng of GST-XPC-hHR23B, 2 pmol of TET-labeled damaged DNA, and equimolar amounts of XPA + RPA (2 pmol (lanes 3, 6, and 9), 5 pmol (lanes 4, 7, and 10), or 10 pmol (lanes 5, 8, and 11)) were added. Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the bottom of the figure.
with a notion that XPC-hHR23B is the global initiator in the damage recognition process.

**DISCUSSION**

Recognition of damaged DNA is a complex process involving a number of proteins (XPA, RPA, XPC-hHR23B, and TFIIH), all of which can independently bind to the damaged DNA (Refs. 1–4 and references therein). Although some biochemical properties of damage recognition proteins are known, the molecular mechanism of how these proteins function at the damaged DNA site is not clear. In this study we carried out a comprehensive biochemical analysis on the interaction of damage recognition factors themselves and with damaged DNA.

**XPC as a Global Initiator in NER**—Recent in vitro studies strongly point to a role for XPC-hHR23B as the initiator of global genomic repair (25). This is primarily based on the findings that: 1) preincubation of UV-damaged plasmid DNA with XPC was preferentially repaired in an in vitro kinetic experiment, and 2) XPC shows a considerable preference for binding to UV-damaged DNA in the presence of nondamaged competitor DNA (24, 25). On the other hand, a separate in vitro study demonstrated that preincubation of damaged DNA with RPA and XPA, compared with that of XPC-hHR23B, led to a faster repair (10).

From the study described here and the previous studies by others (9, 16, 19–23), it is evident that XPC-hHR23B and RPA share basic properties in damage recognition: 1) preferential binding to the damaged DNA, and 2) the physical interaction with XPA (11–13) (Fig. 3), which makes both XPC-hHR23B and RPA eligible for a global initiator in NER (10, 19). On the other hand, these two proteins exhibit quite different biochemical characteristics: first, XPC has a considerable preference for binding to UV- or cisplatin-damaged DNA in the presence of nondamaged competitor DNA (24, 25) (Fig. 2B), whereas RPA retains only a moderate preference to damaged DNA over nondamaged DNA (16). Second, RPA has a significant preference to ssDNA over dsDNA, whereas XPC (or XPC-hHR23B) shows a higher affinity to dsDNA over ssDNA (35) (Fig. 2). The latter finding suggests that XPC-hHR23B functions at an early stage of duplex DNA damage recognition, whereas RPA is involved in a later stage of damage recognition including a structural distortion of damaged DNA that leads to an unwinding of duplex DNA (or a generation of ssDNA). Third, XPC-hHR23B and RPA differ in their interaction with XPA such that the RPA-XPA interaction stabilizes weak binding of XPA to the damaged DNA (14, 16) (Fig. 4) necessary for NER action (14), whereas the interaction between XPC-hHR23B and XPA did not contribute to a stability of the XPA-damaged DNA complex (Fig. 4). Instead, the presence of XPC-hHR23B interferes with the formation of the RPA-XPA-damaged DNA complex (Fig. 4). Moreover, the XPA-XPC interaction may eventually lead to the displacement of XPC-hHR23B from damaged DNA (Fig. 5). The difference between XPC-hHR23B and RPA in their biochemical characteristics described above not only reflects their unique role(s) in the early stage of NER, but also supports the role for XPC-hHR23B as a global initiator in NER.

**Multistep Damage Recognition Process in Early Stage of NER**—XPC physically interacted with XPA (Fig. 3), but the interaction was significantly inhibited by the presence of damaged DNA (Fig. 4). This observation suggests that the interaction between XPA and XPC may be necessary to recruit XPA to the damaged DNA site. The XPC-hHR23B, once binding to the damaged DNA, likely recruits RPA (Fig. 3) and other repair factors such as TFIIH and XPG (38) to the damaged DNA site (Fig. 9). XPC-hHR23B is also involved in the recruitment of TFIIH to damaged DNA (26). The XPA-RPA, once introduced to the damaged DNA site, cooperates with each other to destabilize the XPC-hHR23B-damaged DNA interaction. A recent in vitro study also supported the absence of XPC-hHR23B in the final incision complex (39). Displacement of XPC-hHR23B from the damaged DNA likely requires a physical interaction between XPA and RPA on damaged DNA because a mutant RPA lacking XPA interaction domain poorly functioned in the displacement of XPC from damaged DNA (Fig. 7). The XPA-RPA interaction on the damaged DNA would likely force XPC to dissociate itself from XPA and the damaged DNA (Figs. 5 and 9). These lines of evidence suggest that XPA, RPA, and XPC-hHR23B do not form a stable three-protein complex at the damaged DNA site. Instead, it supports a notion that the damage recognition process occurs in a stepwise manner. However, we cannot rule out a possibility that XPA, XPC-hHR23B, and RPA form a transient complex on the damaged DNA prior to the displacement of XPC-hHR23B (Fig. 9) because RPA and XPC likely recognize two separate domains of XPA (Fig. 3). We should also point out that the in vitro study described here did not include TFIIH, a key damage recognition protein involved in distinguishing the damaged strand from the nondamaged one (27) as well as local unwinding of the damaged DNA region. The interaction between XPC and TFIIH appears to be essential for nucleotide excision repair (40). Nonetheless, addition of TFIIH appeared to have no effect on the RPA/XPA-mediated
displacement of XPC-hHR23B from damaged DNA.²

Role for hHR23B in Damage Recognition—The XPC forms a stable complex with hHR23B (41, 42). Although the XPC subunit is solely responsible for the binding of the XPC-hHR23B complex to the damaged DNA (23) and the interaction with XPA (Fig. 3), hHR23B (Rad23) is essential for XPC function in NER (23). Very large amounts of XPC without hHR23B showed some repair activity, but equimolar hHR23B led about 10-fold higher activity (20). Rad23 contains a ubiquitin-associated domain that may play a role in controlling NER through proteosome-mediated degradation of repair factors (33, 43). In addition, hHR23B interacts with the base excision repair protein, N-methylpurine-DNA glycosylase, suggesting that it may have a role in mediating various repair pathways (37). In the study we found that hHR23B is necessary for XPA/RPA-mediated displacement of the XPC-hHR23B complex from damaged DNA (Fig. 6). hHR23B does not directly interact with XPA or RPA; we do not know what specific role hHR23B plays in the displacement of XPC from damaged DNA. A recent study showed that the DNA binding domain of XPC overlaps with the hHR23B interaction domain (40), suggesting that hHR23B may facilitate the displacement of XPC through the modulation of its DNA binding activity in the presence of XPA + RPA (Fig. 3).

Physiologic significance of the displacement of XPC from damaged DNA in NER is yet to be determined, however, it at least provides a crucial information that damage recognition occurs in an ordered, multistep process. Our finding that the order of protein assembly had no effect on the displacement of XPC from damaged DNA (Fig. 8) supported a notion that XPC-hHR23B is the initiator of global genomic repair (19, 24). Because XPC, compared with RPA and XPA, exhibited exceptionally strong affinity to damaged DNA (24), it is quite possible that the role for XPC-hHR23B is to effectively identify DNA damage in vivo. RPA-XPA-mediated displacement of XPC may be necessary for the formation of the stable XPA-RPA complex at the damaged site, which would allow a proper positioning of the endonucleases (XPC and ERCC1-XPF) for accurate and efficient incisions. It should be pointed out, however, that the RPA-XPA-mediated displacement of XPC from damaged DNA could be a part of the alternative global genomic NER. For example, although XPC-HR23B does not preferentially bind to CPD DNA (24), repair of cis-syn cyclobutane dimer-containing DNA was dependent on XPC-hHR23B, suggesting that there may be alternative pathways for the global genomic NER that requires XPC-HR23B but can be replaced by XPA and RPA (30). Further functional analysis would be necessary to validate the role for damage recognition complex in NER action.

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² J-S. You and S-H. Lee, unpublished data.