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Two Human Homologs of Rad23 Are Functionally Interchangeable in Complex Formation and Stimulation of XPC Repair Activity

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XPC-hHR23B protein complex is specifically involved in nucleotide excision repair (NER) of DNA lesions on transcriptionally inactive sequences as well as the nontranscribed strand of active genes. Here we demonstrate that not only highly purified recombinant hHR23B (rhHR23B) but also a second human homolog of the *Saccharomyces cerevisiae* Rad23 repair protein, hHR23A, stimulates the in vitro repair activity of recombinant human XPC (rhXPC), revealing functional redundancy between these human Rad23 homologs. Coprecipitation experiments with His-tagged rhHR23 as well as sedimentation velocity analysis showed that both rhHR23 proteins in vitro reconstitute a physical complex with rhXPC. Both complexes were more active than free rhXPC, indicating that complex assembly is required for the stimulation. rhHR23B was shown to stimulate an early stage of NER at or prior to incision. Furthermore, both rhHR23 proteins function in a defined NER system reconstituted with purified proteins, indicating direct involvement of hHR23 proteins in the DNA repair reaction via interaction with XPC.

Nucleotide excision repair (NER) is the main pathway for cells to remove DNA lesions caused by UV irradiation as well as various chemical mutagens (6). The molecular mechanism of NER has been extensively investigated in *Escherichia coli* (8, 12, 17, 32). Based on studies with this prokaryotic system, the NER reaction could be dissected into several steps: (i) recognition of DNA damage, probably accompanied by chromatin remodelling; (ii) introduction of asymmetric single-strand breaks on both sides of the injury; (iii) removal of the short oligonucleotide containing the lesion; (iv) gap-filling DNA synthesis by DNA polymerases; and (v) resealing of the resulting nicks by DNA ligase. Although some general features of the eukaryotic NER mechanism are similar to the prokaryotic system, there are many important differences. Only six *E. coli* proteins (i.e., UvrA, UvrB, UvrC, UvrD, DNA polymerase I, and DNA ligase) are required to accomplish the core of the NER reaction in vitro. The eukaryotic NER system, however, appears to be much more complex, involving at least 20 factors (13, 35).

Impaired NER activity has been found to be associated with several rare autosomal recessive human disorders, including xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (2). So far, seven XP (XP-A to -G), two CS (CS-A and -B), and three TTD (TTD-A, XP-B, and XP-D) NER-deficient complementation groups have been identified by cell fusion. Many mammalian NER genes have been cloned by using either mutant fibroblasts from patients or NER-deficient, UV-sensitive Chinese hamster ovary cells (3). For some of these genes, a possible role in the NER reaction has been suggested on the basis of their amino acid sequences as well as biochemical properties of the isolated gene products. On the other hand, additional factors involved in the mammalian NER have been identified from extensive analyses of cell-free NER systems, and some models for the eukaryotic NER mechanism have been proposed (see reviews in references 24 and 35). Several laboratories have recently succeeded in reconstituting the core of eukaryotic NER reactions with purified proteins (1, 10, 22).

XPC protein is involved in NER of DNA lesions on transcriptionally inactive sequences as well as the nontranscribed strand of active genes (33, 34) and is required for in vitro NER of many types of DNA lesions (20, 25). However, certain kinds of lesions could be repaired without XPC in vitro (21) and the precise role of XPC in the global genome repair NER subpathway remains to be elucidated. Human XPC protein was found to be tightly complexed in vivo with hHR23B (human homolog of Rad23), which shows overall homology to the *Saccharomyces cerevisiae* NER factor, Rad23 (20). Unlike yeast cells, a second Rad23 homolog, designated HR23A, has been identified in human (20) as well as murine (31) cells. The *RAD23* gene has thus been duplicated during eukaryotic evolution. Indirect immunofluorescence studies revealed that XPC and both hHR23 proteins are predominantly localized in the nucleus (30). Although XPC protein is bound to hHR23B in a quantitative manner, hHR23B is much more abundant than XPC so that a majority of hHR23B is present in a free form in vivo (28, 30). Both the *RAD23* gene duplication and the presence of two forms of hHR23B suggest that the protein may have multiple functions. Despite the overall sequence homology of the two human Rad23 homologs, almost all XPC

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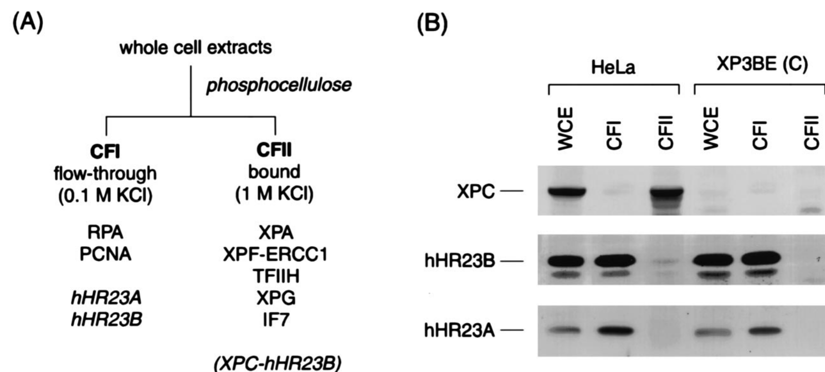

FIG. 1. Depletion of endogenous XPC and hHR23 proteins from human whole-cell extracts. (A) Scheme of phosphocellulose fractionation of human whole-cell extracts. (B) Whole-cell extracts from NER-proficient HeLa cells and XP-C cells [XP3BE(C)] were fractionated on phosphocellulose as illustrated in panel A. The presence of endogenous XPC, hHR23B, and hHR23A proteins in each fraction was determined by immunoblotting with polyclonal antibodies raised against each protein. Forty micrograms of whole-cell extract protein (WCE) and 20 μg of CFI and CFII were loaded onto each lane.

molecules appear to be complexed in vivo with hHR23B, not with hHR23A.

Using a reconstituted in vitro NER system devoid of endogenous XPC and hHR23B, we have previously shown that recombinant hHR23B protein (rhHR23B) significantly stimulates the activity of recombinant hXPC protein (rhXPC) (28). In the accompanying paper, we identified a domain in hHR23B that is responsible for XPC binding as well as its stimulatory activity (19). Because the amino acid sequence of this domain is well conserved between the two human Rad23 homologs, the XPC-binding and -stimulating activities of hHR23A have been of interest. Here we document and characterize the XPC-stimulating activity of both hHR23 proteins expressed in *E. coli.*

MATERIALS AND METHODS

Purification of repair proteins. rhXPC protein was expressed in an insect cell line, Sf9, by a baculovirus expression system and purified as described previously (28). Nontagged and His-tagged versions of rhHR23A and rhHR23B proteins were expressed in *E. coli* and purified as described in the accompanying paper (19). Recombinant human proliferating cell nuclear antigen (PCNA) was purified from *E. coli* BL21(DE3) harboring the pT7-PCNA plasmid (a generous gift from B. Stillman, Cold Spring Harbor Laboratory) as described previously (5). The final phenyl Sepharose fractions were loaded on a fast-performance liquid chromatography Mono Q HR5/5 column equilibrated with buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) containing 0.2 M NaCl and eluted with 15 ml of 0.2 to 0.7 M NaCl gradient in buffer A. Peak fractions were stored at −80°C. Human replication protein A (RPA) was also purified from recombinant sources by using *E. coli* BL21(DE3) pLysS transformed with p11d-tRPA (kindly provided by M. Wold, University of Iowa) as described previously (11). ERCC1-XPF complex was purified from Chinese hamster ovary group 1 mutant cells (43-3B) transfected with His-tagged human ERCC1 as described previously (27). XPA, XPG, TFIIH, and IF7 were purified as described previously (1, 7). For TFIIH, the fractions from the second heparin column were used for the reconstituted NER reactions (14).

Preparation and fractionation of whole-cell extracts. Lymphoblastoid cells (GM2248B) from an XP-C patient (XP3BE) were grown in suspension with RPMI 1640 medium containing 15% fetal calf serum and 20 mM HEPES-NaOH (pH 7.3). Whole-cell extracts were prepared as described previously (18, 36). For fractionation, whole-cell extracts (~50 mg of protein) were loaded onto a phosphocellulose column (1.2 [inside diameter] by 8 cm) equilibrated with buffer B (25 mM HEPES-KOH [pH 7.8], 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM PMSF) containing 0.1 M KCl. After an extensive wash with the same buffer, bound proteins were eluted with buffer B containing 1 M KCl. The peak fractions were pooled and dialyzed overnight against buffer containing 25 mM HEPES-KOH (pH 7.9), 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 20% sucrose, and 1 mM dithiothreitol. Insoluble material was removed by centrifugation, and the resulting supernatant fraction (column fraction II [CFII]) was stored at −80°C.

In vitro NER reactions. Standard reaction mixtures for in vitro NER (50 μl) contained the following components: 40 mM HEPES-KOH (pH 7.8), 7 mM $MgCl_2$, 70 mM KCl, 6.8% glycerol, 0.5 mM dithiothreitol, 2 mM ATP, 20 μM (each) dGTP, dCTP, and dTTP, 8 μM dATP, 23 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 18 μg of bovine serum albumin, 250 ng of each plasmid DNA substrate, pBluescript II KS+ (AAF [*N*-acetyl-2-aminofluorene]- or UV-damaged), and pHM14 (undamaged), and various amounts of fractionated cell extracts and/or purified NER proteins. After incubation performed at 30°C for various periods, 25 ng of PCNA and 74 kBq of [α-^{32}P]dATP (Amersham) (~110 TBq/mmol) were added and the mixture was further incubated at 30°C for 15 min. For reconstituted NER reactions with purified proteins, exonuclease-free *E. coli* DNA polymerase I (0.025 U; United States Biochemical) was used to carry out gap filling of DNA incised by the mammalian incision proteins.

Glycerol gradient velocity sedimentation. Stepwise glycerol gradients were made in polyallomer ultracentrifuge tubes (for the SW60 rotor; Beckman) by overlaying 340 μl each of 11-step glycerol solution (from 35 to 15%; 2% decrease in each step) in buffer C (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.3 M NaCl, 0.05% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM PMSF). The gradients were incubated overnight at 4°C before loading the samples. Protein samples were put in 50 μl of buffer C containing 10% glycerol and incubated at 4°C for 2 h. After the samples were loaded, the gradients were centrifuged at 2°C for 64 h at 49,000 rpm (Beckman SW60 rotor). Fractions (160 μl) were taken from the tops of the gradients. As marker proteins, egg white lysozyme (2.1S; 30 μg), bovine serum albumin (4.4S; 27 μg), and yeast alcohol dehydrogenase (7.4S; 40 μg) were loaded on parallel gradients and fractionated exactly as described above. The positions of the marker proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining.

Other methods. The rhXPC-binding assay was carried out as described previously (19). SDS-PAGE was performed as described by Laemmli (15). For immunoblotting, proteins separated on SDS gels were electrotransferred onto nitrocellulose membrane (BA85; Schleicher & Schuell) at 5 V/cm overnight in blot buffer (25 mM Tris, 193 mM glycine, 0.01% SDS, 15% methanol). XPC, hHR23A, and hHR23B proteins were detected with affinity-purified rabbit polyclonal antibodies raised against each protein and alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. The protein concentration was determined according to the method of Bradford (4), using Coomassie protein assay reagent (Pierce) and bovine serum albumin as a standard.

RESULTS

Both hHR23 proteins stimulate the activity of XPC protein in fractionated XP-C cell extracts. To detect XPC-stimulating activity of recombinant hHR23B protein, we have previously reported a reconstituted in vitro NER system from which endogenous XPC as well as hHR23B was depleted (28). This assay system is based on the fact that, when the XP-C whole-cell extract is fractionated on phosphocellulose as shown in Fig. 1A, the bound fraction (CFII) is devoid of both XPC and hHR23B (Fig. 1B). Although XP-C cells still express hHR23B protein as a free form, all of the hHR23B is recovered in the flowthrough fraction (CFI). Immunoblotting analysis revealed that hHR23A is also recovered in CFI and cannot be detected in CFII upon the same fractionation, regardless of the presence or absence of XPC expression (Fig. 1B). Because two purified NER proteins, RPA and PCNA, are sufficient to func-

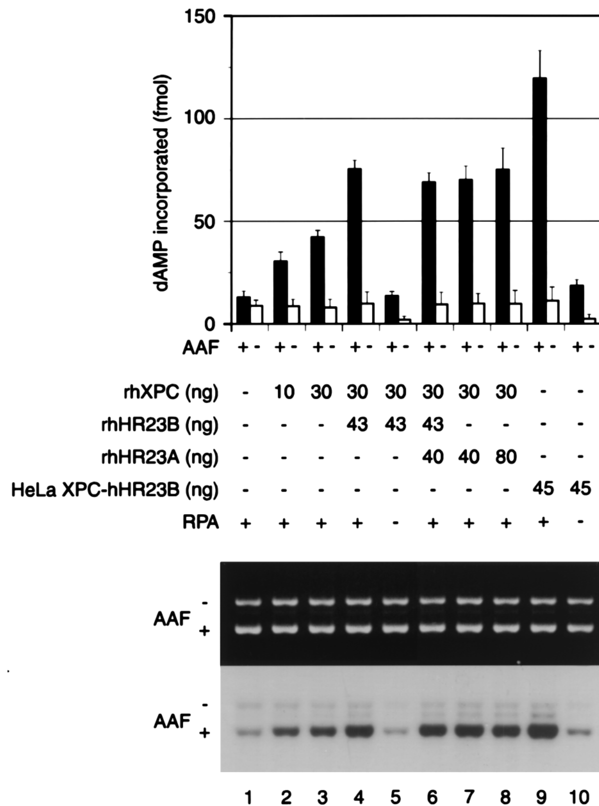

FIG. 2. Stimulation of in vitro NER reactions by rhHR23 proteins. Plasmid DNA substrates (AAF-damaged [+] and undamaged [−]; 250 ng each) were incubated at 30°C for 90 min with CFII from XP-C cells (100 μg) and the indicated purified proteins under standard conditions for in vitro NER reactions (see Materials and Methods). After the addition of PCNA (25 ng) and [α-^{32}P]dATP (74 kBq), incubation was continued at 30°C for 15 min. DNA samples were purified, digested with *Bam*HI, and then subjected to agarose gel electrophoresis. A photograph of the ethidium bromide-stained gel and an autoradiogram of the gel are shown. Incorporation of dAMP into each DNA band was calculated and depicted as a graph. The average values and standard errors were calculated from two independent experiments. Symbols: solid bars, incorporation in damaged plasmid; open bars, incorporation in undamaged control plasmid.

tionally substitute for CFI in reconstitution of the cell-free NER system (26), both endogenous hHR23 proteins present in the XP-C cell extracts can be depleted by this substitution. Thus, reconstitution of NER reactions with the XP-C CFII, purified RPA, and PCNA enabled us to test whether the purified recombinant hHR23A could functionally replace hHR23B in this reconstituted system. A two-stage reaction protocol was used for this experiment. A mixture of AAF-damaged and undamaged DNA substrates was incubated with CFII from XP-C cells and purified RPA in the presence or absence of rhXPC, rhHR23B, and rhHR23A. During this first incubation, oligonucleotides containing AAF adducts can be excised from plasmid DNA substrates when all incision proteins are present. In the second stage, PCNA and radioactive dATP were added to allow DNA repair synthesis by DNA polymerases and to label repair patches. As shown in Fig. 2, rhXPC alone supported DNA repair synthesis up to threefold over the background DNA synthesis (Fig. 2, lane 3). In the presence of the same amount of rhXPC, addition of nontagged rhHR23B gave a highly reproducible stimulation of repair synthesis, to sixfold over background (Fig. 2, lane 4), although the repair activity was significantly lower than that achieved with the authentic XPC-hHR23B complex purified from HeLa cells (Fig. 2, lane 9). As expected, the repair reaction was dependent on the presence of RPA (Fig. 2, lanes 5 and 10). Interestingly, rhHR23A could also stimulate the rhXPC activity to an extent similar to that stimulated by rhHR23B (Fig. 2, lanes 7 and 8). No further stimulation was observed when both hHR23 proteins were added in one reaction with rhXPC (Fig. 2, lane 6). These data suggest that hHR23A can functionally substitute for hHR23B in XPC stimulation in vitro.

hHR23 proteins stimulate XPC protein via complex formation. To assess complex assembly between rhXPC and rhHR23A, a His-tagged version of recombinant rhHR23 proteins (rhHR23-His) was used to coprecipitate rhXPC with nickel-chelating Sepharose. As shown in Fig. 3, rhXPC itself did not bind to the nickel beads (Fig. 3, lanes 1 and 2). In the presence of appropriate amounts of either rhHR23B-His or rhHR23A-His, however, rhXPC was coprecipitated almost completely (Fig. 3, lanes 4 and 6), indicating that rhHR23A is also able to form a physical complex with rhXPC. To compare the affinity of the two rhHR23 proteins for rhXPC, nontagged rhHR23 proteins were included as competitors in the binding reactions. When equimolar amounts of nontagged and His-tagged rhHR23B were incubated with rhXPC, about 50% of rhXPC was competed out from rhHR23B-His (Fig. 3, lanes 7 and 8). rhHR23A could compete for rhXPC binding with rhHR23B-His to a similar extent (Fig. 3, lanes 9 and 10), indicating that both rhHR23 proteins have comparable affinity for rhXPC. Since nontagged rhHR23A was hardly coprecipitated with the rhXPC-rhHR23B-His complex (Fig. 3, lane 10), formation of a ternary complex involving rhXPC, rhHR23B, and rhHR23A is unlikely. Instead, the two rhHR23 proteins bind to rhXPC in a competitive manner and, most likely, in a 1:1 ratio. Essentially the same results were obtained with rhHR23A-His (Fig. 3, lanes 11 to 14), although slightly more rhXPC was competed out by nontagged proteins when compared with rhHR23B-His.

The reconstitution of rhXPC-rhHR23 complexes was further analyzed by sedimentation through glycerol gradients. As

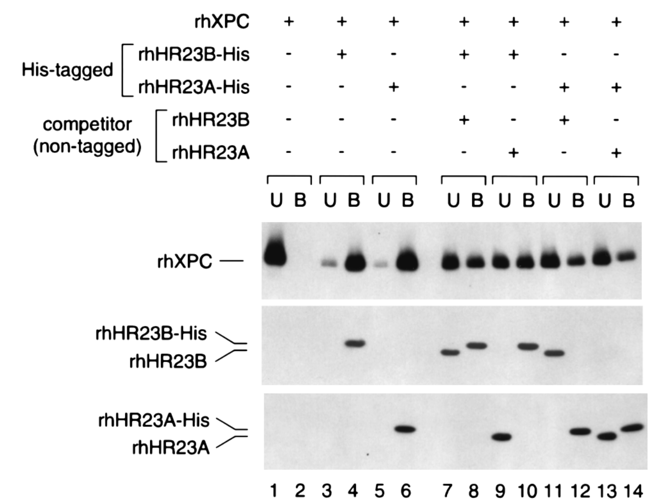

FIG. 3. Both rhHR23 proteins bind to rhXPC. rhXPC (0.25 pmol) was incubated on ice for 1 h with various combinations of 2.5 pmol each of His-tagged or nontagged rhHR23 proteins as indicated above the lanes. Nickel-chelating Sepharose was added, and His-tagged proteins were pulled down by centrifugation after an additional 1-h incubation. Unbound (U) and bound (B) materials were subjected to SDS-PAGE (9% polyacrylamide gels). The presence of XPC (upper gel), hHR23B (middle gel), and hHR23A (lower gel) in each fraction was assessed by immunoblotting. For the detection of hHR23A and hHR23B, antibodies raised against synthetic polypeptides corresponding to a unique sequence in each protein were used (19).

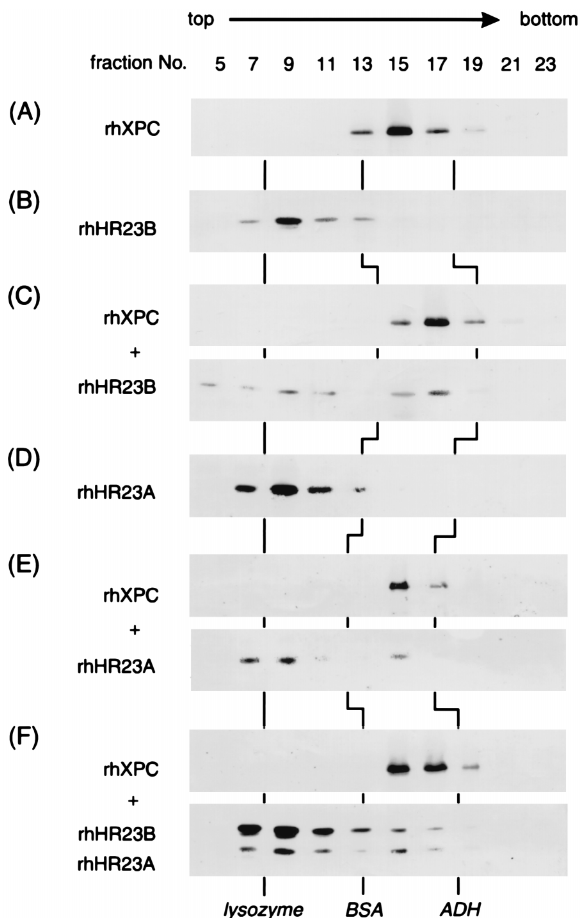

FIG. 4. In vitro reconstitution of rhXPC-rhHR23 complexes. The indicated protein samples were fractionated through 15 to 35% glycerol gradients as described in Materials and Methods. For mixtures of rhXPC and rhHR23, the samples were preincubated on ice for 2 h before being loaded on the gradients. Five microliters of each of the indicated fractions were subjected to SDS-PAGE (6% acrylamide gel), and the presence of each protein was visualized by immunoblotting with antibodies raised against each protein. The amounts of proteins loaded on each gradient were 7.5 μg (rhXPC), 2 μg (rhHR23B), and 1.7 μg (rhHR23A). Three marker proteins, egg white lysozyme (2.1S), bovine serum albumin (BSA; 4.4S), and yeast alcohol dehydrogenase (ADH; 7.4S), were centrifuged through parallel gradients, and their peak positions are indicated.

shown in Fig. 4, free rhXPC (Fig. 4A) and free rhHR23B (Fig. 4B) sedimented with peaks around 5.6 and 2.5S, respectively. When rhXPC was prebound to rhHR23B, the position of the rhXPC peak was slightly shifted toward the bottom of the gradient, up to around 6.3S (Fig. 4C). This is largely consistent with the S value determined for XPC-hHR23B complex purified from HeLa cells (6.2 [20] and 6.1S [25]). In the same gradient, rhHR23B was divided into two peaks, one coinciding with free rhHR23B and the other with rhXPC, indicating the formation of a physical complex between rhXPC and rhHR23B. Similar results were obtained with rhXPC and rhHR23A, as expected (Fig. 4D and E). When the three recombinant proteins were preincubated, both rhHR23 proteins were found in complex forms (Fig. 4F), in agreement with the results of the coprecipitation experiments shown in Fig. 3.

To assess whether the complex is responsible for the XPC stimulation, the peak fractions of free rhXPC and rhXPC-rhHR23 complexes were assayed for repair activity. As shown in Fig. 5A, these three peak fractions contained comparable concentrations of rhXPC. When the same volume of each of these fractions was assayed, however, both of the complex fractions showed significantly higher NER activity than did free rhXPC (Fig. 5B). Thus, the activity of rhXPC was enhanced by complex formation with rhHR23 proteins. As shown in Fig. 5C, 1 μl of rhXPC-rhHR23B complex fraction contained 3 to 10 ng of rhHR23B. On the other hand, when rhHR23B was titrated in the presence of 1 μl of free rhXPC fraction, approximately 10 times more (30 to 100 ng) rhHR23B was needed to obtain maximal stimulation (Fig. 5D), likely due to the fact that the efficiency of complex formation is dependent on the concentration of each component. As expected, addition of 100 ng of rhHR23B did not further stimulate the activity of preformed rhXPC-rhHR23B complex, although an excess of 1 μg of rhHR23B markedly inhibited repair reactions. These data indicated that the presence of rhHR23B is not enough; instead, complex assembly between XPC and hHR23 proteins is required for the stimulation of XPC activity.

hHR23 proteins stimulate NER in an early stage of the reaction. It has previously been shown that the XPC-hHR23B complex functions at or prior to incisions (25). When the XPC complex was included only in the second stage (i.e., the DNA repair synthesis stage), repair could hardly be detected. To determine which stages of NER are stimulated by hHR23 proteins, two-stage NER reactions were carried out in the presence of free rhXPC and rhHR23B was added at various time points. When rhHR23B was present only in the DNA repair synthesis stage, no stimulation was observed (compare Fig. 6A, lanes 6 and 7). Furthermore, when rhHR23B was added at different time points in the first stage of the reaction, the level of DNA repair declined in proportion to incubation time before the addition of rhHR23B (Fig. 6A, lanes 2 to 5). Thus, rhHR23B has to be present in the first stage of incubation to exhibit XPC-stimulating activity.

To see whether rhHR23B indeed stimulated NER at or prior to incision, DNA repair synthesis was uncoupled from the incision step. After the first incubation, DNA substrates were purified and the amount of excised DNA damage was subsequently measured by filling the repair gaps with T4 DNA polymerase and radiolabeled deoxynucleoside triphosphates. As shown in Fig. 6B, authentic XPC-hHR23B complex as well as reconstituted recombinant protein complex gave specific repair signals for damaged DNA (Fig. 6B, lanes 2 and 4) in an RPA-dependent manner (Fig. 6B, lane 3). Although free rhXPC gave some repair signal in this assay, coaddition of rhHR23B displayed clear stimulatory activity (compare Fig. 6B, lanes 5 and 6). These results demonstrate that hHR23B stimulates some early stages of the NER reaction before DNA repair synthesis.

hHR23 proteins stimulate NER reconstituted with purified proteins. It has previously been reported that damage-dependent incision reactions could be reconstituted in vitro with seven purified protein fractions: RPA, XPA, TFIIH, XPC-hHR23B, ERCC1-XPF, XPG, and incision factor 7 (IF7) (1). To test whether hHR23 proteins also show stimulatory activity in this defined NER system, the activity of rhXPC was assessed in the system with and without rhHR23 proteins. In this experiment, UV-damaged and undamaged DNA substrates were first incubated with purified incision proteins, and then radioactive dATP and Klenow fragment were added to allow DNA repair synthesis. In the presence of appropriate amounts of other incision proteins, the authentic XPC-hHR23B complex gave a considerable repair signal (Fig. 7, lane 9). Under the same conditions, rhXPC hardly supported repair reactions (Fig. 7, lanes 2 and 3) but the addition of either rhHR23B or rhHR23A resulted in strong stimulation of repair (Fig. 7, lanes

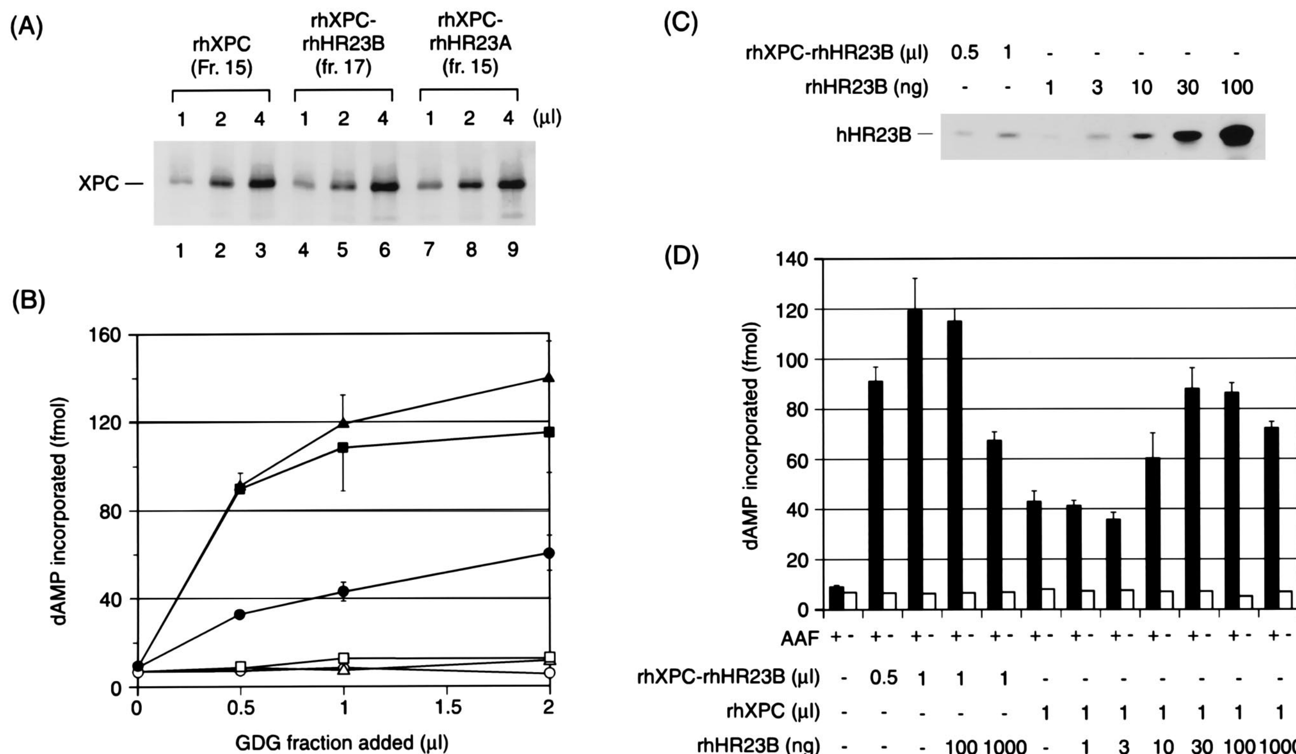

FIG. 5. Complex formation with rhHR23 enhances rhXPC activity. (A) The amounts of rhXPC in the glycerol gradient peak fractions of free rhXPC (Fig. 4A, fraction [Fr.] 15), rhXPC-rhHR23B (Fig. 4C, fraction 17), and rhXPC-rhHR23A (Fig. 4E, fraction 15) were compared by immunoblotting. (B) The three peak fractions in panel A were assayed for XP-C-correcting activity in the reconstituted in vitro NER system by using XP-C CFII and purified RPA. After 90 min of incubation at 30°C, PCNA and [α-^{32}P]dATP were added and the mixture was incubated for another 15 min. Radioactivity incorporated in damaged (closed symbols) and undamaged (open symbols) plasmids was quantified and depicted as a graph. Symbols: circle, free rhXPC; triangle, rhXPC-rhHR23B; square, rhXPC-rhHR23A. (C) The amount of rhHR23B in the peak fraction of rhXPC-rhHR23B was quantified by immunoblotting. (D) The glycerol gradient peak fractions of free rhXPC and rhXPC-rhHR23B complex were assayed in the presence of the indicated amounts of rhHR23B under the same conditions as used for panel B. Symbols: solid bar, incorporation in damaged plasmid; open bar, incorporation in undamaged plasmid. In panels B and D, the mean values and standard errors were calculated from two to four independent experiments.

4, 7, and 8). These repair signals were completely dependent on the presence of TFIIH (Fig. 7, lanes 5 and 10), confirming that the signals represented NER reactions. Again, the coaddition of rhHR23A and rhHR23B failed to give a higher repair signal than that achieved by each single rhHR23 protein (Fig. 7, lane 6). Finally, the clear effect of hHR23 proteins in the defined NER system made up of purified NER components provides a strong argument against the theoretical possibility that the stimulation of hHR23 proteins was in fact due to titrating out an inhibitory factor(s).

DISCUSSION

Functional interchangeability between the two Rad23 homologs. In the accompanying paper, a domain in hHR23B which is responsible for XPC binding was identified (19). This domain was shown to be required and even sufficient for stimulation of XPC repair activity in vitro. Because the amino acid sequence of the domain is well conserved between the two hHR23 proteins, it might be expected that hHR23A also binds to XPC and stimulates repair activity. In agreement with this, *E. coli*-expressed, highly purified rhHR23A exhibits an XPC-stimulating activity similar to that of hHR23B (Fig. 2). Furthermore, coprecipitation with His-tagged rhHR23 proteins (Fig. 3) and the sedimentation velocity experiments (Fig. 4) demonstrated that both rhHR23 proteins were capable of forming complexes with rhXPC. This complex assembly could be observed without any other protein fractions or any incubation at relatively high temperature, indicating that special posttranslational modifications, such as phosphorylation, of rhHR23 proteins are not necessary for the binding itself. Moreover, neither an energy source, such as ATP, nor a divalent cation was required.

The competition experiments shown in Fig. 3 revealed that both rhHR23 proteins can bind to rhXPC with comparable affinity under the conditions tested. Recently, possible interaction between XPC and hHR23A has been suggested with the yeast two-hybrid system (16). Furthermore, we have very recently detected trace amounts of hHR23A by immunoblotting in a highly purified XPC protein fraction from HeLa cells, indicating the existence of XPC-hHR23A complex in human cells (unpublished results). Because the vast majority of XPC is bound to hHR23B, one might expect that rhHR23B shows much higher affinity for rhXPC than does rhHR23A. However, this turned out not to be the case. It is thus still unclear why most of the XPC is bound to hHR23B. Although the content of hHR23B in the cells used seems to be higher than that of hHR23A, the difference is not large enough to explain preferential binding of XPC to hHR23B (unpublished observations). It is possible that specific modification of hHR23B increases its affinity for XPC or, alternatively, that auxiliary factors specifically target XPC to hHR23B. However, this is not absolutely necessary for the binding itself, as discussed above. Another possibility is that the subcellular localization of the two hHR23 proteins is different. We have recently shown by indirect im-

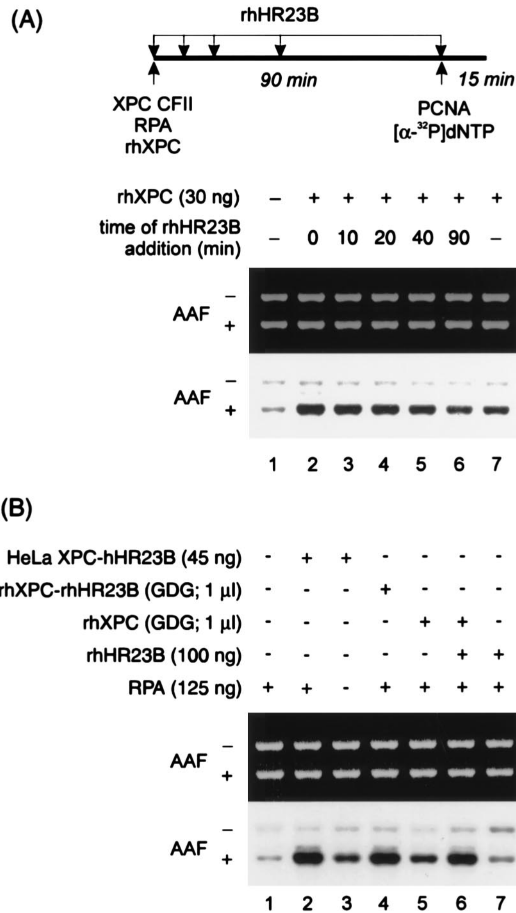

FIG. 6. rhHR23B stimulates early stages of NER. (A) In vitro NER reactions were carried out with XP-C CFII (100 µg of protein) and RPA (125 ng) in the presence (+) or absence (−) of rhXPC (30 ng). After first-stage reactions at 30°C for 90 min, PCNA (25 ng) and [α-^{32}P]dATP (74 kBq) were added and the mixture was incubated further for 15 min. rhHR23B (43 ng) was added at various time points as indicated above each lane. An ethidium bromide stain and an autoradiogram of the agarose gel are shown. (B) In vitro NER reactions were carried out in the presence of XP-C CFII and the indicated purified protein fractions. After incubation at 30°C for 90 min, DNAs were purified before being used for DNA synthesis reactions. The DNA synthesis reactions were performed at 37°C for 15 min in mixtures (50 µl) containing 40 mM HEPES-KOH (pH 7.8), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 µM (each) dGTP, dCTP, and dTTP, 8 µM [α-^{32}P]dATP (74 kBq), bovine serum albumin (18 µg), and T4 DNA polymerase (0.6 U; New England Biolabs). DNAs were purified again, digested with *Bam*HI, and then fractionated by agarose gel electrophoresis.

munofluorescence analysis that XPC as well as both hHR23 proteins is localized predominantly in the nucleus (30). From these experiments, however, it was difficult to determine which fraction of these proteins is present in the cytoplasm. One possibility is that a minor amount of hHR23B is localized in the cytoplasm and preferentially traps newly synthesized XPC protein before it is transported to the nucleus. Alternatively, the intranuclear localizations of hHR23A and hHR23B may be different. Further studies are needed to answer these questions.

Importance of complex assembly for XPC stimulation by hHR23 proteins. The correlation between XPC-binding and NER-stimulatory activities of various truncated rhHR23B proteins suggests that complex assembly is important for XPC stimulation (19). Here we demonstrate biochemically that complex assembly enhances the repair activity of rhXPC. Because sedimentation in glycerol gradients incompletely separates rhXPC-rhHR23 complexes from free rhXPC, it was difficult to determine which proportion of rhXPC was complexed with rhHR23s in each glycerol gradient. However, although not all rhXPC molecules may have formed complexes, we still observed a significantly higher activity of the complex fractions than of free rhXPC. Furthermore, when free rhXPC was assayed in the presence of various amounts of rhHR23B, a much higher concentration of rhHR23B was needed to obtain maximal stimulation, in comparison with amounts of rhHR23B present in the preassembled complex fractions (Fig. 5C and D). Since the presence of higher concentrations of rhHR23B should make complex formation more efficient, these data also support the importance of complex assembly for XPC stimulation by hHR23 proteins. In addition, Fig. 5D shows that the presence of a large excess of rhHR23B is inhibitory. Consequently, maximal stimulation is observed only within a certain range of rhHR23B doses. Thus, careful titration of rhHR23B is necessary for observing the stimulatory effect of rhHR23B.

Because the XPC-hHR23B complex was shown to function

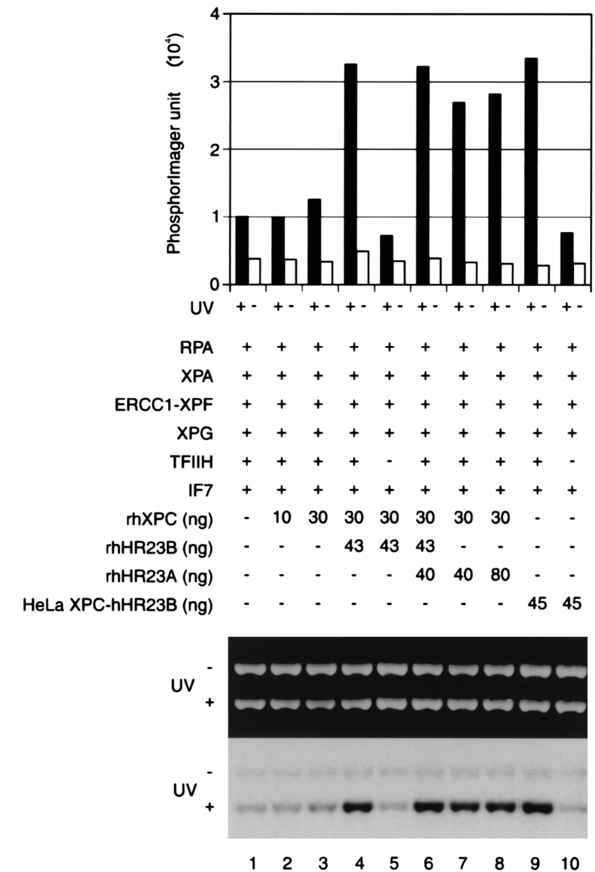

FIG. 7. rhHR23 proteins stimulate defined NER reactions reconstituted with purified proteins. Plasmid DNA substrates (UV damaged and undamaged) were incubated at 30°C for 60 min in the presence (+) of the indicated purified NER proteins. Exonuclease-free *E. coli* DNA polymerase I and [α-^{32}P]dATP were added, and incubation was continued at 30°C for 10 min. DNA samples were purified, linearized with *Bam*HI, and separated on a 1% agarose gel. A photograph of the ethidium bromide-stained gel and an autoradiogram of the gel are shown. The amounts of purified proteins used were as follows: RPA, 250 ng; XPA, 45 ng; ERCC1-XPF, 10 ng; TFIIH, 150 ng; XPG, 50 ng; and IF7, 500 ng. The gel was exposed to a PhosphorImager (Molecular Dynamics), and the relative radioactivity incorporated in each band was quantified. Symbols: solid bars, incorporation in damaged plasmid; open bars, incorporation in undamaged plasmid.

at an early stage of NER (25), it was of interest to determine which stage of NER is stimulated by hHR23 proteins. By carrying out DNA repair synthesis independently from incisions, we demonstrated that the stimulation by hHR23 proteins also takes place at or prior to incisions (Fig. 6B). This part of the NER reaction still encompasses multiple steps: damage recognition, local melting of double-stranded DNA, dual incisions, and removal of damage-containing oligonucleotides. Yeast Rad23 protein has previously been claimed (9) to mediate physical interactions with Rad14 as well as yeast TFIIH, raising the possibility that Rad23 promotes assembly of higher-order NER protein complexes, like "repairosomes" (29). However, similar interactions have not yet been established either in vivo or in vitro for hHR23 products and other mammalian NER proteins. Further studies are warranted to pinpoint the NER steps in which the hHR23 products exert their function(s).

Stimulation by rhHR23s in the defined NER system. Our rhHR23 proteins also showed XPC-stimulating activity in the defined NER system reconstituted with purified proteins, which was described by Aboussekhra et al. (1). Although some of the repair proteins (i.e., IF7, ERCC1-XPF, and TFIIH) used in these experiments were not purified to complete homogeneity, these observations strongly argue against a formal possibility that rhHR23s stimulate NER reactions indirectly, e.g., by titrating out some inhibitors present in crude fractions.

The results of the two reconstituted NER systems with XP-C CFII (Fig. 2) and the purified proteins (Fig. 7) were qualitatively similar, but we noted some quantitative differences as well. For instance, free rhXPC always exhibited some activity when added to the XP-C CFII, whereas the same amount of rhXPC hardly supported repair in the defined NER system (compare Fig. 3 and 7, lanes 2 and 3). Consequently, the stimulation by rhHR23s appeared to be more pronounced in the defined NER reaction. One possible explanation is that the XP-C CFII used in these studies still contains a trace amount of endogenous hHR23B and/or hHR23A. Alternatively, some repair proteins might be present as higher-order complexes in the CFII fractions, whereas all repair proteins should be free at the beginning of the defined NER reactions. If hHR23 proteins promote such supercomplex assembly by themselves or via change in a conformation of XPC, the defined system should be much more dependent on the presence of hHR23. Another possibility is that hHR23 proteins physically stabilize XPC via complex formation. In the defined NER system, which contains much lower protein concentrations than the crude system, free rhXPC might be more labile and thus depend more on the stabilizing effect of hHR23.

Generally, setting up defined reaction systems composed of multiple purified proteins needs special care: rate-limiting factors depend on the precise ratio of the different purified proteins used. Therefore, to determine the activity of XPC protein, it is important to set up reactions where XPC is rate limiting. Recently Reardon et al. have reported (23) that rhHR23B had no stimulatory effect in their reconstituted NER system. There are significant differences between the two reaction systems: one detects repair DNA synthesis on randomly damaged plasmid DNA, whereas the other detects dual incision on a singly damaged DNA substrate. Moreover, our defined NER system requires an extra protein fraction, designated IF7, which seems to be dispensable in the other system. Therefore, the discrepancy concerning the stimulation by rhHR23B could be attributed to some intrinsic differences between the two repair systems, including differences in the quality of the purified proteins used. As discussed above, however, stimulation of XPC might also be overlooked, especially in defined NER systems, unless all components are carefully titrated to set up conditions under which XPC is rate limiting. It is also important to carefully titrate rhHR23B, because a large excess of rhHR23B turned out to be inhibitory (Fig. 5D).

We are generating knockout mice of mHR23B as well as mHR23A. Because no natural mutant has so far been available for mammalian Rad23 homologs, these knockout mice and the derived mutant cells should give important clues to elucidate in vivo functions of this set of genes. Analysis of these mice is expected to give more definite answers to the current issues concerning the roles and possible functional redundancy of mammalian Rad23 homologs in NER, and it will complement the findings made in in vitro studies.

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