Overproduction, Purification, and Characterization of the XPC Subunit of the Human DNA Repair Excision Nuclease*

(Received for publication, April 4, 1996, and in revised form, June 19, 1996)

Joyce T. Reardon, David Mu‡, and Aziz Sancar§

From the Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Xeroderma pigmentosum complementation group C gene (XPC) encodes a protein of 125 kDa which is present in a tight complex with a 58-kDa protein encoded by the human homolog of the yeast RAD23 gene, HHR23B (Masutani, C., Sugawara, K., Yanagisawa, J., Sonoyama, T., U1, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P. J., Bootsma, D., Hoeijmakers, J. H. J., and Hanaoka, F. (1994) EMBO J., 13, 1831-1843). The XPC-HHR23B complex is required for excision of thymine dimers from DNA in a human excision nuclease system reconstituted from purified proteins. In order to understand the role of the XPC-HHR23B complex in excision repair, we have overexpressed each subunit alone and the heterodimer in heterologous systems, purified them, and characterized their biochemical properties. We find that both XPC and the heterodimer bind DNA with high affinity and UV-damaged DNA with slightly higher preference. Surprisingly, we find that the XPC subunit alone is sufficient for reconstitution of the human excision nuclease and that the HHR23B subunit has no detectable effect on the excision activity of the reconstituted system.

Nucleotide excision repair is a general repair system which is particularly suited for removing bulky DNA lesions such as thymine dimers and cisplatin-d(GpG) diadducts (Friedberg et al., 1994). The XPC-HHR23B complex is required for excision of thymine dimers from DNA in a human excision nuclease system reconstituted from purified proteins. In order to understand the role of the XPC-HHR23B complex in excision repair, we have overexpressed each subunit alone and the heterodimer in heterologous systems, purified them, and characterized their biochemical properties. We find that both XPC and the heterodimer bind DNA with high affinity and UV-damaged DNA with slightly higher preference. Surprisingly, we find that the XPC subunit alone is sufficient for reconstitution of the human excision nuclease and that the HHR23B subunit has no detectable effect on the excision activity of the reconstituted system.

* This work was supported in part by National Institutes of Health Grant GM32833. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Grant DRG-1319 from the Damon Runyon-Walter Winchell Foundation.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, CB# 7260, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260. Tel.: 919-962-0115; Fax: 919-966-2852.

§ The abbreviations used are: XP, xeroderma pigmentosum; bp, base pair(s); CFE, cell-free extract; ds-DNA, double-stranded DNA; DTT, dithiothreitol; ERCC, excision repair cross-complementing; HHR, human homolog of RAD; his, histidine tag; MBP, maltose-binding protein; nt, nucleotide(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RPA, replication protein A; ss-DNA, single-stranded DNA; TFIIH, transcription factor IIH.

are required for the dual incision (Mu et al., 1995, 1996; Guzder et al., 1995b). Furthermore, upon purification of XPC complementing activity, it was found that the 125-kDa protein encoded by XPC (Legerski and Peterson, 1992; Masutani et al., 1994) was in a complex with a protein of 58 kDa which is highly homologous to the yeast excision repair protein encoded by the RAD23 gene (Watts et al., 1993; Masutani et al., 1994). In humans there are two genes with sequence homology to RAD23, and these were named human homolog of RAD23 A and B (HHR23A and HHR23B), respectively; only the protein encoded by HHR23B is found in a complex with XPC protein (Masutani et al., 1994).

Although XPC and HHR23B appear to be tightly bound and the final purification step for XPC yields these two proteins in 1:1 stoichiometry (Masutani et al., 1994; Aboussehra et al., 1995; Mu et al., 1996), there was no genetic or biochemical data from mammalian systems indicating that HHR23B is involved in excision repair. To clarify the role of HHR23B in repair, we expressed XPC and HHR23B, separately and in a complex using baculovirus/insect cells and plasmid/E. coli expression systems, purified these proteins, and characterized them. We found that XPC and the XPC-HHR23B complex bind to DNA nonspecifically and with relatively high affinity and to UV-damaged DNA with slightly higher affinity. Both forms of XPC are capable of complementing cell-free extracts of XP-C mutants and are active in reconstituting excision nuclease activity in a completely defined system. We conclude that with naked DNA under our experimental conditions HHR23B does not play a direct role in excision repair.

MATERIALS AND METHODS

Plasmid Construction and Baculovirus Stock Establishment—Two constructs were used for protein expression in insect cells (Fig. 1A). For p2Bac.XPC-HHR23B, a 1.8-kb Smal-Dral fragment from pUC19.HHR23B (Masutani et al., 1994) was subcloned into the p2Bac vector (Invitrogen) at the Pvu II site under the control of the PH promoter (p2Bac.HHR23B), following subcloning of the 3.6-kb NotI fragment from pBS11SK(+).XPC (Masutani et al., 1994) at the NotI site under the control of the P10 promoter. For p2Bac.XPC, only the NotI fragment was subcloned into the p2Bac vector. SF21 cells were transfected with either p2Bac.XPC-HHR23B or p2Bac.XPC using the BaculGold Transfection Kit (Pharmingen). Recombinant virus stocks were established from single plaques and identified by polymerase chain reaction (PCR) amplification of viral DNA using a combination of p2Bac vector and gene-specific primers. Standard procedures (O'Reilly et al., 1994) were used for cell culture and infection by recombinant baculoviruses as well as for amplification and titering of the virus stocks.

To express recombinant proteins in bacterial cells, two constructs were used (Fig. 1B). For pHis.HHR23B, a 1.2-kb PCR fragment was subcloned into pQE-30 (Qiagen). The PCR primers were designed to introduce a 5'-BamHI site while simultaneously deleting the first two (Met-Gln) and substituting the 3rd and 4th amino acids from Val-Thr to Gly-Ser) and to generate a 3' -Sall site beyond the stop codon. For pHMR.XPC, a 2.5-kb PCR fragment was subcloned into pMAL-c2 (New England Biolabs). Primers were designed to generate a blunt end at the first ATG codon of pXPC-3 (Legerski and Peterson, 1992) and to introduce a 3' -Sall site beyond the stop codon. The insert was in-frame with
maltE sequences (data not shown), but only a truncated fusion protein was produced. Therefore, the pMBP-XPC DNA was digested with HindIII and BamHI, purified, and ligated to this plasmid. pMAL-c2 creating pMBP-XPC(445C), which encodes the maltE gene fused in-frame to the 445 amino acids from the carboxyl terminus of XPC.

Cell-free Extracts and Protein Purification—Mammalian cell-free extracts (CFE) were prepared from an XPC fibroblast line (patient 21RO, GM00709A, NIGMS Human Mutant Cell Repository, Camden, NJ) and from a rodent homolog of XPG (cell line UV35, CRL1867, ATCC Repository, Rockville, MD). Extracts were prepared according to the method of Manley et al. (1980), dialyzed into buffer A (25 mM Heps-KOH, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 12.5% glycerol), and stored at −80 °C. For immunoblot analyses, extracts were also prepared from XPC(1-16) at 4°C, and then the CFE-resin mixture was poured into a column (15 cm × 1 cm). Following extensive washing with buffer C (50 mM potassium phosphate, pH 6, 0.3 mM NaCl, 10% glycerol), bound proteins were eluted with a step gradient of 0.05 to 0.5 M imidazole in buffer C, with His-HHR23B eluting at 0.15–0.3 M imidazole. His-HHR23B-containing fractions were pooled, dialyzed into buffer A, and stored at −80 °C.

For purification of the MBP-XPC(445C) fusion protein, the initial processing was as described above for His-HHR23B except the 4-h induction was with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. Clarified extract (200 mg in 150 ml) was applied to an amylose column (15 ml, New England Biolabs) at 4°C with a gradient of 0.05 to 0.5 M KCl. Fractions containing XPC were pooled, dialyzed into buffer A, and stored at −80 °C.

DNA Binding Assays—Several oligonucleotides were prepared for the DNA binding assays, but all substrates within a particular category were prepared in essentially the same manner. Approximately 50 pmol of each oligonucleotide were 5'-end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (7000 Ci/mmol; ICN). Labeled oligonucleotides were either ethanol-precipitated alone (ss-DNA) or in the presence of unlabeled complementary oligomers (ds-DNA), and DNA was resuspended in annealing buffer (40 mM Tris, pH 7.4, 100 mM NaCl, 4 mM MgCl2). To obtain ds-DNA substrates, the coprecipitated oligomers were heated at 90–95 °C for 5 min and slow cooled to 30 °C (about 3 h). Both ss- and ds-DNA were separated from unincorporated [γ-32P]ATP or nonhybridized complementary strands by electrophoresis on 6–8% polyacrylamide gels, followed by electroblotting. Antigen-antibody complexes were detected using the alkaline phosphatase method (Promega) or enhanced chemiluminescence (Amersham).

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For the binding assays, DNA (0.3 nm) was mixed with the indicated amounts of protein in 15 μl of binding buffer (30 mM Heps-KOH, pH 7.9, 4 mM potassium phosphate, pH 7.5, 100 mM KCl, 3.2 mM MgCl2, 0.4 mM EDTA, 0.3 mM DTT, 0.1% Triton X-100, 2 mM ATP, 2% glycerol, 1 μg of bovine serum albumin). Following a 30-min incubation at 30 °C,
**Plasmids for recombinant protein expression.** Four constructs were used in this study. A, p2Bac.XPC and p2Bac.XPC-HHR23B were used for the expression and purification of recombinant proteins used in DNA binding and repair assays. B, protein expressed with pHis.HHR23B was used for both of these assays as well as for antisera production. Recombinant protein expressed with pMBP.XPC(445C) was used only for the generation of antisera. RecSeq indicates recombination sequences for integration into the viral genome; Amp is the ampicillin resistance gene, $\beta$-lactamase.

**Analysis of purified proteins.** Polypeptides were separated by electrophoresis through a 10% SDS-polyacrylamide gel and detected by staining with Coomassie Brilliant Blue (A, lanes 1–3) or by immunoblot analysis with alkaline phosphatase reagents (B, lanes 4–6). Lane 1, rXPC-HHR23B, 1.2 $\mu$g; and lane 4, 0.5 $\mu$g of the same fraction; lane 2, 0.6 $\mu$g; and lane 5, 0.25 $\mu$g of rXPC; lanes 3 and 6, 1.3 $\mu$g and 0.8 $\mu$g, respectively, of His-HHR23B. Numbers to the left show positions of size markers (Life Technologies, Inc. 10-kDa protein ladder).

**Western blot analyses of XPC cell-free extracts (CFEs) and purified protein fractions.** Polypeptides were separated by electrophoresis through a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antisera. A, immunoblot analysis (alkaline phosphatase reagents) of CFEs from HeLa (lane 1), normal lymphoblastoid (NL, lane 2), and normal fibroblast (NF, lane 3) cells compared with CFEs from various XPC strains (lanes 4–8). Approximately 50 $\mu$g of CFE were resolved in lanes 1–8 and 0.5 $\mu$g of his.HHR23B in lane 9. The large arrow indicates HHR23B while the smaller, upper arrow indicates a slower-migrating, cross-reacting material (X) unique to lymphoblastoid cells. B, immunoblot analysis (enhanced chemiluminescence) of purified proteins used in the types of experiments illustrated in Fig. 6. Lane 1 contained 15 ng of TFIIH, 20 ng of XPG, and 20 ng of XPF-ERCC1. Lane 2 contained 5 ng of XPC-HHR23B purified from HeLa cells. Lane 3 contained 20 ng of recombinant XPC-HHR23B purified from E. coli.

**RESULTS**

Purification of Recombinant XPC and XPC-HHR23B—We attempted to overproduce XPC and HHR23B in E. coli, but failed to express full-length XPC in E. coli either from its own initiation codon or in the form of a fusion protein with maltose-binding protein (MBP). Nevertheless, the MBP-XPC fusion protein containing the carboxyl-terminal 445 amino acids of XPC was expressed and used for generating XPC antibodies used in the present study.

To obtain functional recombinant XPC, we cloned the gene into the baculovirus expression vector p2Bac (Invitrogen) either alone or together with HHR23B. Additionally, HHR23B was cloned into an E. coli expression plasmid. Fig. 1 shows the plasmid constructs used in the current study. Full-length soluble proteins were expressed with these constructs. The XPC protein and XPC-HHR23B heterodimer were purified essentially as described by Masutani et al. (1994). The HHR23B
protein was expressed in soluble form and in full-length in E. coli and was purified from E. coli by nickel-affinity chromatography. Fig. 2 shows the analyses of purified proteins by Coomasie Blue staining and Western blotting.

Expression of HHR23B in XPC Mutant Cell Lines—Since one of the goals of this study was the investigation of the properties of the XPC protein in the absence of HHR23B, we wished to know whether or not the XP-C mutant cell-free extracts contained HHR23B protein at normal or reduced levels. Cell-free extracts from a variety of cell lines were tested by Western blotting. Fig. 3A shows that XPC mutants have normal levels of HHR23B. In fact, during purification of XPC complementing activity from HeLa cells, we found that HHR23B is in vast molar excess over the XPC polypeptide and separates from the heterodimer readily by ion exchange chromatography.

In addition, our purified repair factors were tested for the presence of HHR23B to ascertain that factors other than the XPC fraction were free of HHR23B, and, hence, complementation experiments done with recombinant XPC reflected the activity of this subunit alone. Fig. 3B reveals that the repair factors purified from HeLa cells contained no detectable HHR23B (<0.2 ng per excision assay) and hence any activity arising from the addition of XPC to the reconstituted system could safely be ascribed to XPC alone.

DNA Binding Properties—The natural as well as the recombinant XPC-HHR23B were purified through DNA-cellulose affinity chromatography and hence were known to be DNA-binding proteins. For quantitative analysis of DNA binding properties, we conducted electrophoretic mobility shift assays with various types of DNAs. The heterodimer (both recombinant and natural forms) binds to single-stranded and double-stranded DNA (60-bp duplex) with comparable affinities (Fig. 4A) and with a $K_D$ of $5 \times 10^{-9} \text{M}$ (calculated from the 50% binding point); heavily irradiated 60-bp duplex (about 3 photolyses per molecule) was bound with slightly higher affinity ($K_D = 4 \times 10^{-9} \text{M}$, Fig. 4B). The heterodimer bound to the 45-bp duplex with somewhat lower affinity and to a 26-bp duplex very weakly; no binding with 20-bp oligomer could be detected over the concentration range used (Fig. 4C); similar binding properties were observed when the probe was single-stranded DNA. As with recombinant heterodimer, 50% binding was at 5.2 nM while XPC bound 50% substrate at 2 nM. For all panels, the faster migrating free DNA species is single-stranded DNA.

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Fig. 5. Complementation of XPC mutant cell-free extracts by rXPC and rXPC-HHR23B. rXPC-HHR23B (5 ng, lanes 3–5) or rXPC (2.5 ng, lanes 6–8) were added to the indicated amounts of XPC CFE and tested for complementation of excision nuclease activity. Control reactions are in lane 1 (50 µg of XPC + 50 µg of XPG CFE) and lane 2 (100 µg of XPC CFE). The prominent 29-mer excision product is indicated.

Fig. 6. Reconstitution of excision nuclease with rXPC and the effect of rHHR23B. Assay conditions were as described under “Materials and Methods.” The amounts of XPC-HHR23B, rXPC, and rHHR23B (his-tagged) are indicated, and other repair factors included in all reaction mixtures were as follows: XPA, 25 ng; TFIIH, 2 ng; XPG, 4 ng; XPF-ERCC1, 9 ng; and RPA, 250 ng. The 29-mer excision product is indicated.

DISCUSSION

The yeast RAD23 gene belongs to the RAD3 epistasis group, whose members participate in excision repair. Null mutations of genes in this group, such as RAD1, completely abolish excision repair (Prakash et al., 1993). In contrast, deletion of RAD23 increases the UV sensitivity of yeast cells only modestly (Perazzo and Prakash, 1986; Watkins et al., 1993) indicating that, unlike RAD1 and other members of the RAD3 epistasis group, RAD23 is not essential for excision. Thus, extrapolating from yeast genetics, one would expect that the same would be true in humans. However, even though the yeast and human excision repair systems are highly homologous (Prakash et al., 1993; Friedberg et al., 1995; Sancar, 1996), certain significant differences have been found. In particular, it was found that the 3' nick of the dual incision could occur in the absence of XPF-ERCC1 in humans (Mu et al., 1996) but requires the entire complement of excision repair proteins in yeast (Guzder et al., 1995b). Thus, a priori, it was not possible to predict whether XPC without HHR23B would be sufficient to reconstitute the human excision nuclease. Indeed, it was reported that it was not possible to separate XPC from HHR23B without losing the XPC correcting activity (Masutani et al., 1994). The results presented in this report indicate that the HHR23B polypeptide is not required for the excision repair function of the XPC protein and that HHR23B modulates XPC activity, perhaps by interacting with and masking the DNA binding domain of XPC. It is possible that the stringent conditions used to separate XPC from HHR23B in the previous study inactivated the XPC protein.

In light of our results, it is reasonable to ask whether HHR23B plays any role in repair. To answer this question, in addition to the yeast rad23 phenotype, one must take the following findings into account. First, in humans, two RAD23 homologs were found which exhibit 57% sequence identity to each other and 30–34% sequence identity to RAD23. Second, of the two polypeptides, only HHR23B was found in a complex with XPC (Masutani et al., 1994). Third, RAD23 (Watkins et

heterodimer. We found that both forms are active in complementing XP-C cell-free extracts (Fig. 5). This experiment, which reveals that recombinant XPC is active in excision repair, indicates that the XPC polypeptide made in the absence of HHR23B folds properly. However, these experiments do not show whether XPC can function without HHR23B because XPC cells appear to have normal levels of HHR23B protein (Fig. 3A) which could reconstitute the XPC-HHR23B heterodimer upon addition of XPC to the cell-free extract. To address this particular question, we conducted experiments with a defined system of purified excision repair proteins not containing detectable levels of HHR23B (Fig. 3B). Fig. 6 shows that XPC alone is sufficient to reconstitute the excision nuclease to the same extent as the heterodimer purified from its natural source (HeLa cells). Supplementing HHR23B to the defined excision repair system reconstituted with XPC alone did not alter the rate of excision.

A previous study (Mu et al., 1996) showed that a cholesterol substituent attached with a shorter linker to the phosphodiester backbone (cholesterol-B) than the one used in the current work (cholesterol-A) was excised normally even in the absence of XPC-HHR23B. Therefore, it was conceivable that these excisions in the absence of HHR23B or even the XPC-HHR23B complex were peculiar to these unnatural substrates. Hence, we tested the classic substrate for human excision nuclease, cyclobutane thymine dimer, in our reconstituted system. As evident from Fig. 6, lanes 9 and 10, even the XPC polypeptide alone is sufficient for reconstitution and there is no detectable effect of HHR23B on excision under these experimental conditions.

Thus, we conclude that HHR23B does not play a direct role in excision repair but may modulate the XPC activity. Indeed, HHR23B may have a negative effect on both DNA binding and excision activity because we reproducibly observe better binding (Fig. 4D) and excision (Figs. 5 and 6) with rXPC alone compared to the heterodimer.
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al., 1993) and HHR23 belong to the ubiquitin-fusion family of proteins in that the NH₂-terminal 80 amino acids are highly homologous to ubiquitin (Masutani et al., 1994). The precise role of ubiquitin in these proteins is not known but is thought to function as a chaperone in assisting proper folding and assembly and thermostability (Finley et al., 1989; Garrett et al., 1994; Aso et al., 1995). Fourth, the Rad23 protein is not essential for, but promotes complex formation between TFIIH and Rad14 (XPA homolog) proteins (Guzder et al., 1995a). In humans TFIIH seems to be directly bound to XPA without the aid of other proteins (Park et al., 1995) although the possibility that HHR23B stimulates XPA-TFIIH interaction has not been ruled out. Fifth, the yeast homolog of XPC, the Rad4 protein, is required for global excision repair (Prakash et al., 1993) but not for excision repair of the transcribed strand of rDNA genes which are transcribed by RNA polymerase I (Verhage et al., 1996a). In contrast, in humans the template strand of the polymerase II-transcribed sequences is repaired in XP-C mutants at an essentially normal rate (Mellon et al., 1987; Kantor et al., 1990; Venema et al., 1990, 1991; Evans et al., 1993). In fact, it has been demonstrated in vitro that for certain lesions, even in the absence of transcription, excision repair occurs without XPC-HHR23B (Mu et al., 1996). Thus, not only HHR23B but even XPC is dispensable for excision nuclease activity under certain conditions or with certain substrates.

The following model is consistent with most of these observations. The HHR23B subunit of the heterodimer may be involved in nucleosome disassembly or reorganization which makes DNA accessible to the XPC subunit and other factors of the excision nucleosome system. Alternatively, HHR23B may modulate the activity of XPC and perhaps function as a chaperone molecule. Indeed, preliminary experiments suggest that HHR23B protects the XPC subunit from thermal inactivation as tested by the DNA binding assay. The role of XPC may be to stabilize the local unwinding that is thought to occur in the preincision complex. Thus, with naked DNA (such as the 140-mer substrate), there is no need for HHR23B. Similarly, in transcribed DNA where local unwinding is provided by the RNA polymerase stalled at a lesion or with certain lesions which intrinsically cause local unwinding, XPC is not needed and hence excision occurs at a near-normal rate in the absence of the XPC-HHR23B heterodimer. This model is in agreement with the finding of Miller et al. (1982) that yeast rad23 mutants removed pyrimidine dimers at about 60% of wild type levels. In contrast, McCready (1994) and Verhage et al. (1996b) using different methodologies did not find dimer removal from the genome overall or from transcriptionally active genes in rad23 mutants. Further studies are needed to establish the function of XPC, to determine whether there are bona fide differences between the roles of HHR23B in humans and RAD23 in yeast, and to reconcile these seemingly contradictory reports regarding the requirement of RAD23 for excision repair in yeast.

Acknowledgments—We thank Dr. Randy Legerstè (University of Texas) for pXPC-3 and Dr. Fumio Hanaoka (Osaka University) for his gift of the XPC and HHR23B clones.

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