Isolation of human complexes proficient in nucleotide excision repair

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ABSTRACT

More than 20 polypeptides are required for the process of nucleotide excision repair (NER) in both human and yeast cells. This pathway of excision repair has most often been viewed as an ordered multi-step process involving steps of damage recognition, incision/excision and finally repair DNA synthesis. Here we present evidence for the existence of a complex of human NER proteins pre-assembled in the absence of damaged DNA. This multi-protein complex was initially isolated from HeLa cell extracts by affinity chromatography on a matrix containing the damage recognition protein XPA. Subsequent co-immunoprecipitation and gel filtration experiments demonstrated that a significant portion of the human NER proteins was present in the form of a high molecular weight complex and that these complexes, or repairosomes, were capable of performing all steps of NER in vitro. Consistent with studies indicating that DNA polymerases δ and ε can both function in NER, these two polymerases are found in these repairosome complexes.

INTRODUCTION

Previous reports have documented direct protein–protein interactions amongst many of the proteins involved in nucleotide excision repair (NER) (1–3). Since these direct interactions could lead to the sequential recruitment of the different NER proteins to sites of DNA lesions, the process of NER has most often been viewed as a stepwise series of reactions (3–6). Accordingly, DNA lesions would first be bound by two proteins, XPA and RPA, known to interact directly (7–9). Then, perhaps by virtue of an interaction between XPA and TFIIH (10), the DNA helicases XPB and XPD, as part of TFIIH, may be recruited to unwind DNA adjacent to the lesion. Interactions between the endonucleases ERCC1-XPF and XPA (11–14) and between the other endonuclease XPG and RPA (7) and/or TFIIH (15) may then lead to recruitment of these two enzymatic activities to the growing NER complex and help position them to make the 5′ and 3′ incisions on the damaged strand flanking the lesion (16). After displacement of the excised oligomer, the gapped DNA, possibly still complexed with one or more of these incision proteins (17), would become a substrate for the activity of PCNA-dependent DNA polymerase δ or ε (18,19). Since each of the documented protein–protein interactions has been detected in vitro in the absence of a damaged DNA substrate, it is conceivable that some or all of these NER proteins could exist in pre-assembled sub-complexes or even a single complex or repairosome. Indeed, such a repairosome complex containing TFIIH and a number of other NER proteins was partially purified from Saccharomyces cerevisiae cell extracts, although an ability of this yeast complex to perform NER was not reported (20). In this report we show that a complex of repair proteins can be isolated from human cell extracts by steps of XPA affinity chromatography followed by gel filtration and that these human complexes are capable of performing NER in vitro.

MATERIALS AND METHODS

Affinity chromatography

Frozen HeLa S3 cells were purchased from Cellex Biosciences (Minneapolis, MN). The XP-C cell lymphoblast line (GM02246C) was purchased from Coriell Cell Repositories and was grown in RPMI 1640 medium with 15% serum by Cellex Biosciences. The whole cell extracts were prepared according to Sopta et al. (21). Histidine-tagged XPA protein was expressed in and purified from Escherichia coli as described by Jones and Wood (22). Affinity chromatography was carried out virtually as described previously (7,23). XPA protein was coupled to Affigel 10 (BioRad) at 2 mg/ml. Aliquots of 1 ml HeLa cell extract in ACB buffer (7) containing 0.1 M NaCl were loaded onto 50 µl affinity columns. After washing with 200 µl ACB buffer containing 0.1 M NaCl, the columns were eluted with 200 µl ACB buffer containing 1.0 M NaCl. For the gel filtration chromatography, in vitro NER assays and immunoprecipitation experiments the bound fractions eluted from control or XPA columns were dialyzed against NER buffer (45 mM HEPES–KOH, pH 7.8, 70 mM KCl, 7.4 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 10% glycerol) and in some cases further concentrated 3-fold by centrifugation through Centricon-10 concentrators (Amicon).

Antibodies and Western blotting

The antibodies against RPA and XPA have been described previously (7). For making anti-XPG antibodies, a 519 bp fragment (encoding amino acids 455–627) was released by PstI digestion from pRAD2synthetic (a gift from Dr S.Clarkson) and subcloned into the vector QE-11 (Qiagen). Following the manufacturer’s instructions, histidine-tagged XPG polypeptide

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was expressed in and purified from *E. coli* and was used to generate rabbit anti-XPG serum. Additional antibodies used in these studies were obtained as follows: rabbit anti-p62, anti-RAP30 (24) and anti-TFIIB, J. Greenblatt; anti-XPC mAb (PC10), Santa Cruz Biotechnology Inc.; anti-RFC(140) mAb (26), B. Stillman; anti-ligase I mAb 7A12, G. Daly and T. Lindahl; rabbit anti-pol (C20) (27), H. Ochs and U. Hübscher; anti-pol ε mAb 93G1A (28), J. Syvaoja; rabbit anti-hMSH2/N20, Santa Cruz Biotechnology Inc. For Western blotting, aliquots of column fractions or whole cell extract were subjected to SDS–PAGE. After electrophoretic transfer onto nitrocellulose, the filters were probed with different antibodies. The immunoblots were then developed using an enhanced chemiluminescence procedure (Pierce).

### NER assay

The *in vitro* NER assay was performed essentially as previously described (29). DNA from plasmids pUC18 (2.7 kb) and pGEM-3Zf(+) (3.2 kb) was isolated by alkaline lysis and CsCl–ethidium bromide centrifugation. The pUC18 DNA was treated with *N*-acetoxy-2-acetylaminofluorene (AAAF) and purified on a 5–20% sucrose gradient as described by Wang et al. (30). Reaction mixtures (50 µl) contained 300 ng AAAF-treated pUC18 DNA and 300 ng control pGEM-3Zf(+) DNA, 45 mM HEPES–KOH, pH 7.8, 70 mM KCl, 7.4 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 20 µM each dGTP, dATP, TTP, 8 µM dCTP, 2 µCi [α-³²P]dCTP (3000 Ci/mmol), 2 mM ATP, 40 mM disodium phosphocreatine, 2.5 µg creatine kinase, 3.4% glycerol, 18 µg bovine serum albumin and the indicated amount of column fraction. Reactions were incubated at 28°C for 2 h. Plasmid DNA was then purified from the reaction mixtures, linearized by digestion with *Hind*III, electrophoresed on a 1% agarose gel and autoradiographed as described by Wood et al. (29). For the antibody inhibition experiments shown in Figure 2B, the indicated amount of pre-immune or anti-RPA antiserum was preincubated with the cell extracts for 15 min at 28°C before addition of reaction buffer and plasmid DNAs.

### Co-immunoprecipitation

Pre-immune or anti-XPA serum (500 µl) was incubated with 500 µl of a 1:1 mixture of Sepharose 4B–protein A beads (Sigma) in phosphate-buffered saline (PBS) at room temperature for 2 h. The beads were sedimented, washed three times with 10 µl PBS and four times with 0.2 M sodium borate, pH 9.0, and re-suspended in 10 ml 0.2 M sodium borate, pH 9.0. Antibodies were then covalently coupled to protein A–Sepharose by addition of solid dimethylpimelimidate at a final concentration of 20 mM and a further incubation at room temperature for 2 h. The reactions were then terminated by incubation with 0.5 M ethanolamine, pH 7.6. The beads (30 µl) were incubated at 4°C for 4 h with the dialyzed column fractions (30 µl), washed three times with 100 µl NER buffer and then bound proteins were eluted by boiling for 3 min with 50 µl SDS sample buffer.

### Sepharose CL-2B Chromatography

A Sepharose CL-2B (Pharmacia) column (0.7 × 15 cm, total volume 6 ml) was packed and equilibrated with NER buffer according to the manufacturer’s instructions. Samples of 250 µl dialyzed and concentrated bound fraction eluted from XPA columns were applied to the column and 300 µl fractions were collected and analyzed by Western blotting or by an *in vitro* NER assay after being concentrated 5-fold on Centricon-10 concentrators (Amicon). Aliquots of *E. coli* 70S ribosomes (a gift of M. Kiel) or high molecular weight markers (BioRad) were also chromatographed on the same column under similar conditions.

### RESULTS

#### NER proteins in HeLa cell extracts bind specifically to XPA columns

In this study we have used protein affinity chromatography as a means to isolate a complex of human NER proteins. Since the damage recognition protein XPA has been reported to bind directly to several other NER proteins, namely RPA (7–9), ERCC1 (11–14) and TFIIH (10), as well as to itself (14), we chose to make affinity columns using purified recombinant histidine-tagged XPA covalently coupled to an Affigel-10 matrix. Aliquots of HeLa cell extracts were chromatographed on the XPA
endonuclease XPG, which does not bind directly to XPA (7), was released from the XPA column, but not in control column eluates. The proteins by Western blotting (Fig. 1). Consistent with previous reports, XPA, RPA, ERCC1 and the TFIIH components XPB, XPG and p62 were each detected in the bound fraction eluted from the XPA column, but not in control column eluates. The endonuclease XPG, which does not bind directly to XPA (7), was also retained on the XPA column. The NER proteins binding to the XPA matrix, however, were not limited to just those involved in the early steps of NER, damage recognition and incision/excision. We also found that proteins involved in the DNA synthesis step of repair, namely the p140 subunit of RFC, PCNA and DNA ligase I, were in the XPA column eluate. By comparing the intensity of individual polypeptides in the input HeLa cell extracts, we estimate that ∼20–40% of the input RPA, ERCC1 and TFIIH subunits, each of which has been reported capable of binding XPA directly, was retained by the XPA column, whereas 10–20% of the input XPG, RFC or PCNA bound to the XPA column. The recovery of XPA appeared artificially high, since some of the column-bound XPA dissociated from the column in the high salt elution step. We have not determined the precise stoichiometry of the components in this XPA eluate. Since a number of these NER proteins have been previously shown capable of direct interaction with XPA, both free and repairosome complexed forms of these polypeptides were recovered by this affinity column procedure.

Since the pre-assembled RNA polymerase II holoenzyme also contains TFIIH as one of its important initiation factors (31,32), we also asked if other transcription initiation factors found in the RNA polymerase II holoenzyme also bound to the XPA column. RAP30, a subunit of TFIIH, and TFIIIB could be detected in the loaded HeLa cell extract, but no significant amounts of these two proteins were retained by either the control or the XPA column (Fig. 1). Thus the XPA column does not appear to be retaining the RNA polymerase II holoenzyme. Nor does this XPA column contain TFIIH as one of its important initiation factors (31,32), which has recently been implicated in the process of transcription-coupled excision repair (33).

### Figure 2

Figure 2. XPA column eluates are proficient in performing an in vitro NER reaction. The NER activity of column fractions described in Figure 1 was assessed by detecting DNA repair synthesis. The incorporation of radioactively labeled deoxynucleotides into control or 4AAF-treated DNA isolated from repair reaction mixtures was detected by autoradiography after agarose gel electrophoresis. In the upper panels the DNA was visualized by ethidium bromide staining; the lower panels show autoradiograms of the gels. (A) NER activity of 10 (lanes 1, 3, 5 and 7) or 15 µl (lanes 2, 4, 6 and 8) of the concentrated 1.0 M NaCl eluates from control (lanes 1, 2, 5 and 6) or XPA (lanes 3, 4, 7 and 8) columns loaded with HeLa cell extract (lanes 1–4) or an equal amount of an XP-C cell extract (lanes 5–8). (B) DNA repair synthesis was assessed in 15 µl of the concentrated 1.0 M NaCl eluate from an XPA column after a preincubation with 0 or 4 µl pre-immune serum (lanes 1 and 2) or 4 µl anti-RPA serum (lanes 3 and 4) in the presence of 0 (lanes 1–3) or 250 ng (lane 4) recombinant human RPA.

The XPA column eluate performs NER in vitro

The observation that many proteins required for NER were present in the bound fraction eluted from the XPA column prompted us to ask if this XPA eluate fraction could perform NER in vitro. As shown in Figure 2A, the XPA column eluates (lanes 3 and 4), but not the eluates from control columns (lanes 1 and 2), preferentially incorporated radiolabeled deoxynucleotides into 4AAF-damaged DNA during repair synthesis. Several lines of evidence suggest that the radiolabel incorporation in this assay reflects bona fide NER activity. First, when a cell extract made from a NER-deficient XP-C cell line was chromatographed on similar XPA affinity columns, although many of these same NER proteins were detected by Western blotting (data not shown) the XPA-bound fraction from this extract failed to perform repair synthesis (Fig. 2A, lanes 7 and 8). Secondly, the preferential incorporation of radiolabeled deoxynucleotides into 4AAF-treated DNA was inhibited by anti-RPA antibodies (Fig. 2B, compare lane 3 with 1) and this inhibition was reversed by addition of recombinant human RPA (lane 4). Inhibition of nucleotide incorporation into 4AAF-treated DNA was also seen with anti-XPG antibodies (data not shown). Quantitation by phosphorimaging of assays employing 15 µl of the 3-fold concentrated eluate from the XPA column indicated that typically 40–50 fmol dCMP were incorporated preferentially into the 4AAF-treated DNA. As observed by Aboussekka et al. (4), biochemical fractionation of HeLa cell extracts leads to a reduction in the efficiency of this NER reaction.

NER proteins in an XPA column eluate further co-purify as a complex

To test whether these repair proteins present in the XPA column eluates are complexed with each other, we used anti-XPA antibodies to immunoprecipitate XPA and any associated proteins within the bound fraction eluted from the XPA column. The majority of XPA protein was precipitated and other repair proteins representative of activities required at early as well as later...
steps of excision repair, namely RPA, XPG, the TFIIH subunit p62, PCNA and the p140 subunit of RFC, were also co-immunoprecipitated by anti-XPA antiserum as well (Fig. 3). Use of pre-immune serum in the same precipitation experiments failed to bring down these proteins (Fig. 3). The co-immunoprecipitation of these proteins is unlikely to be mediated by contaminating DNA, since the inclusion in the reaction of ethidium bromide, which has been shown previously to efficiently inhibit DNA-dependent protein interactions (34), did not affect precipitation of repair proteins by anti-XPA antibodies (data not shown). Similar co-immunoprecipitation of NER proteins was obtained when the bound fraction eluted from the XPA column was precipitated with either anti-RPA or anti-XPG antibodies (data not shown).

To provide additional evidence for the existence of a high molecular weight repairosome complex in the XPA column eluate, the eluate from an XPA column was subjected to gel filtration on Sepharose CL-2B. Fractions across the elution profile were assayed for the presence of a number of repair proteins by immunoblotting and for their ability to perform NER in vitro. The XPA, RPA, TFIIH (p62) and PCNA polypeptides each co-migrated on this sizing step, as evidenced by the Western blots shown in Figure 4A. A peak of NER activity was also detected in the same fractions in which these NER proteins appeared (Fig. 4B). These data provide further evidence that the excision repair proteins which co-eluted from the XPA column exist in the form of a high molecular weight complex, a human repairosome. We did not detect peaks of any free NER proteins in other fractions. Since the elution position of these NER proteins and of the in vitro repair activity is similar to that for purified 70S ribosomal particles on this Sepharose CL-2B column (data not shown), which fractionates proteins up to 40 MDa in size, we suggest that this human repairosome complex could be as large as 2.7 MDa. The predicted size of a complex containing all the human NER proteins needed for reconstituted repair reactions (4) is 2.1 MDa.

**Human repairosome complexes contain both DNA polymerase δ and ε**

Although mammalian DNA polymerase ε has been suggested to be most suitable for repair synthesis during NER (35), studies in *Saccharomyces cerevisiae* with mutations in each of four different DNA polymerases have indicated that either DNA polymerase δ or ε can function to repair the DNA damage induced by UV irradiation (36). We have examined which of these DNA polymerases are within the human complexes isolated by XPA affinity chromatography. As indicated in Figure 5, both DNA polymerase δ and DNA polymerase ε were detected in the bound fraction eluted from an XPA column, but not from a control column. Moreover, both DNA polymerases could be co-immunoprecipitated by anti-XPA, anti-RPA and anti-XPG antibodies (data not shown). Although we have not determined which DNA polymerase functions within this isolated repairosome complex, our results suggest that human cells, like yeast cells, may be able to use either DNA polymerase δ or ε for the gap filling step of DNA repair synthesis.

**DISCUSSION**

The results in this study suggest that a significant portion of each of the proteins required for NER in human cells can be isolated...
in assembled repairosome complexes in the absence of DNA damage. Unlike the complex of yeast NER proteins isolated after chromatography steps on Bio-Rex 70, phosphocellulose and Ni²⁺-NTA-agarose columns (20), these human NER complexes, isolated by XPA affinity and gel filtration chromatography, contain all of the components required to perform the reactions of NER in vitro. Proteins involved in the incision/excision reactions of NER as well as those involved in the later steps of repair synthesis are present in these human complexes. Details of the specific protein–protein contacts which interconnect the incision/excision and DNA synthesis groups of proteins are not yet clear. RPA has been reported to interact with DNA polymerase δ (37) and may also interact with DNA polymerase ε (38). Since RPA can make direct contacts with both XPA and XPG (7), this multi-functional protein could be serving as a central link within an NER complex.

The repairosome complexes we have characterized appear to exist in the absence of DNA lesions. Thus, during DNA repair an entire NER complex may be recruited in a single step to sites of DNA lesions. Since a significant portion of some of these NER proteins was not retained on our XPA columns, certain of the NER proteins almost certainly also exist free within the cell and perhaps in partial sub-complexes. It is difficult, therefore, to determine the contribution of each of these different pools of NER proteins to the NER process in vivo. Reports on the amount of free versus complexed forms of NER proteins purified in vitro may also reflect differences in individual procedures. Using cell extracts from a yeast strain carrying a tagged TFII B1 gene, Guzder et al. (39) recently failed to demonstrate a significant portion of the Rad1, Rad10 and Rad14 proteins associated with TFIIH and on that basis argued against the existence of assembled repairosome complexes in yeast. However, the chromatographic procedures used to partially purify the NER proteins used in this particular study differed significantly from those used previously by Svejstrup et al. (20) and may have led to the loss of important proteins such as Rad4 and RPA, which could be indispensable for the stability of any repairosome complex. Others have reported that certain of these NER proteins, as well as proteins implicated in double-stranded break repair, can be isolated together with a high molecular weight form of RNA polymerase II (40). The complexes described in our study do not contain significant quantities of RNA polymerase II (data not shown) and its general initiation factors (Fig. 1). Furthermore, the presence of NER proteins, apart from subunits of TFIIH, has not been documented in other preparations of the RNA polymerase II holoenzyme (31,32,41). Therefore, the relationship between the RNA polymerase II complexes reported by Maldonado et al. (40) and those reported here is not obvious.

Recent studies of the incision/excision steps of DNA repair using both purified human and yeast NER proteins have indicated that the steps of this portion of the NER process are tightly coupled (5,17,42). Moreover, there is an in vitro requirement for the simultaneous presence of many of the NER proteins. Thus, to make the 3' incision there is a requirement for XPG, as well as XPA, RPA, XPC, TFIIH, ERCC1-XPF and IF-7, although it is the endonuclease XPG that makes the actual cleavage (42). An attractive explanation for these observations would be the existence of a multi-protein repairosome complex. Moreover, that certain mutations in yeast PCNA are specifically defective in NER but not in DNA replication (43) could be an indication that PCNA is required to make a unique set of direct interactions with NER-specific proteins. The isolation of assembled human and yeast RNA polymerase II holoenzyme complexes has necessitated an altered view of the steps of initiation of transcription. This demonstration of human repair complexes assembled in the absence of damaged DNA may similarly prompt a different view of the process of nucleotide excision repair.

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