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XPC and human homologs of RAD23: intracellular localization and relationship to other nucleotide excision repair complexes

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ABSTRACT

The xeroderma pigmentosum syndrome complementation group C (XP-C) is due to a defect in the global genome repair subpathway of nucleotide excision repair (NER). The XPC protein is complexed with HHR23B, one of the two human homologs of the yeast NER protein, RAD23 [Masutani *et al.* (1994) *EMBO J.* 8, 1831–1843]. Using heparin chromatography, gel filtration and native gel electrophoresis we demonstrate that the majority of HHR23B is in a free, non-complexed form, and that a minor fraction is tightly associated with XPC. In contrast, we cannot detect any bound HHR23A. Thus the HHR23 proteins may have an additional function independent of XPC. The fractionation behaviour suggests that the non-bound forms of the HHR23 proteins are not necessary for the core of the NER reaction. Although both HHR23 proteins share a high level of overall homology, they migrate very differently on native gels, pointing to a difference in conformation. Gel filtration suggests the XPC–HHR23B heterodimer resides in a high MW complex. However, immunodepletion studies starting from repair-competent Manley extracts fail to reveal a stable association of a significant fraction of the HHR23 proteins or the XPC–HHR23B complex with the basal transcription/repair factor TFIIH, or with the ERCC1 repair complex. Consistent with a function in repair or DNA/chromatin metabolism, immunofluorescence studies show all XPC, HHR23B and (the free) HHR23A to reside in the nucleus.

INTRODUCTION

A complex network of DNA repair mechanisms protects the genetic information from continuous genotoxic pressure caused by the DNA-damaging effect of exogenous and genotoxic agents. Such damage can lead to inborn defects, cell death or neoplasia. Nucleotide excision repair (NER) is one of the most important DNA damage repair pathways, since this process recognizes a wide variety of lesions. Impaired NER activity has been extensively investigated in cells from three human disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (2,3). These genetic diseases are characterized by sun (UV) hypersensitivity, genetic instability and a marked clinical and genetic heterogeneity. Many genes involved in XP, CS and TTD complementation groups have been cloned using cells from human patients or from NER-deficient, UV-sensitive Chinese hamster ovary mutants (4). Extensive sequence homology between the mammalian and yeast NER proteins has become apparent, indicating that the NER pathway is strongly conserved in eukaryotic evolution (5).

At least two NER sub-pathways can be discerned: transcription-coupled repair (TCR) and global genome repair (GGR) (6). In contrast to all other xeroderma pigmentosum groups, group C patients are only defective in GGR (7). Previously, we reported the identification and cloning of two human homologs of the yeast NER gene *RAD23*: *HHR23A* and *HHR23B* (8). The yeast *Saccharomyces cerevisiae rad23* null mutants display an intermediate UV-sensitive phenotype (9), suggesting that the affected protein is not required for NER. The HHR23B gene product forms a tight complex with the XPC protein. This complex has a high affinity for ssDNA and both subunits were found to be indispensible for *in vitro* NER (10). Like the human XPC–HHR23B complex, the *S.cerevisiae* RAD4 and RAD23 protein homologs were also determined to be complexed with each other (11). XP-C cells harbour a specific defect in the repair of non-transcribed sequences of the genome, including the non-transcribed strand of active genes, whereas the NER subpathway that accomplishes the preferential repair of the transcribed strand of active genes is still operational (7,12). This implies a selective role for the XPC complex in the global genome

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NER system (13). Additionally, XP-C cells are claimed to be defective in the repair of rDNA (14). Recently it has been shown that *in vitro* repair of a cholesterol-substituted oligonucleotide does not require XPC protein (15).

RAD23 is a ubiquitin-like fusion protein displaying significant homology to ubiquitin at the N-terminus (8,16). A second link has been identified between RAD23 and the ubiquitin pathway, namely a twice repeated element, homologous to a C-terminal extension of a Class II ubiquitin-conjugating enzyme (E2) (17). The ubiquitin-conjugating pathway is involved in the proteolytic degradation of proteins, and in additional cellular processes such as DNA repair, chromosome condensation and decondensation, and cell cycle control (18,19). The link to these other DNA-metabolizing processes presumably comes from ubiquitin-mediated proteolytic degradation of key proteins involved in these events.

Here we present data on the purification and stable association of HHR23A, HHR23B and XPC proteins with other known NER factors. Additionally, the sub-cellular localization of HHR23A, HHR23B and XPC was determined by immunofluorescence.

MATERIALS AND METHODS

General procedures

Purification of nucleic acids, restriction enzyme analysis, gel electrophoresis of nucleic acids and proteins, transformation of *Escherichia coli*, etc. were performed according to standard procedures (20). RNA samples were separated on 1% agarose gels and transferred to Zeta probe membrane (Bio-Rad) as described (21). Labelling of DNA probes was carried out using the random priming protocol (22). Immunoblotting was performed as described elsewhere (23). HHR23A and HHR23B proteins were translated *in vitro* using a rabbit reticulocyte lysate system as recommended by the manufacturer (Promega) using 50 μCi of [^{35}S]methionine (1 mCi/mmol). After polyacrylamide gel electrophoresis (PAGE) and native gel electrophoresis, both labelled proteins were blotted and visualized by autoradiography.

For non-denaturing gel electrophoresis, a 4–15% gradient polyacrylamide gel in TBE buffer (90 mM Tris, 80 mM boric acid and 2.5 mM EDTA) and 12% glycerol was prepared. The gel was pre-run for 30 min at 70 V, loaded with samples and run for 2 h at 70 V followed by 16–20 h at 150 V. Proteins included as molecular mass standards used for estimation of the native molecular weight of HHR23A and HHR23B were ferritin (440 kDa), catalase (240 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa) (Boehringer Mannheim). The Western blot was stained with Ponceau-S to visualize the molecular weight markers for determination of the apparent MW of the HHR23 proteins. HHR23A and HHR23B proteins were detected by polyclonal antibodies and autoradiography.

Production of recombinant proteins and antibodies.

The full-length *HHR23A* and *HHR23B* cDNAs were cloned into the pET11D vector (Novagen), transferred into *E.coli* strain BL21(DE3), and gene expression was induced over 4 h by IPTG. Cells were homogenized in PBS and, after sonication, cleared by centrifugation. Approximately 20 g cells were disintegrated by sonication, followed by centrifugation to remove cell debris. Recombinant HHR23A and HHR23B proteins were purified by chromatography on a Q-Sepharose Fast Flow column (1× 12 cm, flow rate 18 ml/h). For HHR23A, the column was eluted with a linear gradient 0→0.5 M NaCl in 0.1 M NaCl, 10 mM K-phosphate pH 7, while for HHR23B a gradient 0→0.4M NaCl in 10 mM NaCl, 10 mM K-phosphate pH 7 was used. HHR23A protein eluted at 0.3 M, HHR23B protein at 0.11 M NaCl. For antibody production these proteins were subjected to SDS-PAGE. Bands were cut from Coomassie stained gels, electroeluted and concentrated with Centricon 30 concentrators (Amicon). The identity of the eluted proteins was verified by amino acid sequencing. For large scale purification, Q-Sepharose fractions were pooled, brought on to 20% ammoniumsulphate and loaded on a butyl Sepharose Fast Flow column (1 × 12 cm, flow rate 18 ml/h). Columns were eluted with a linear gradient 20→0% ammoniumsulphate in 0.1 M NaCl, 10 mM K-phosphate pH 7. Fractions containing HHR23A or HHR23B protein (eluted at 4.5% ammoniumsulphate) were dialysed and kept frozen after addition of 1/5 volume of glycerol. Polyclonal antibodies were raised in rabbits against the *E.coli*-overproduced human HHR23 proteins, as described (23). Affinity-purified antibodies were derived from precise elution of antibodies specifically bound to recombinant antigen immobilized on nitrocellulose after transfer from SDS-polyacrylamide gels (24).

The synthetic peptide (KTKREKKAAASHLFPFEKL), corresponding to the C-terminus of XPC, was used to produce a polyclonal antibody in rabbits. Prior to injection, the peptide was cross-linked to KLH carrier protein. Affinity-purified antibodies were derived by eluting XPC-peptide from a column to which it had been coupled. As a second antibody, alkaline phosphatase-conjugated goat anti-rabbit was used, the latter visualised by 5-bromo-4-chloro-3-indolyl phosphate. Immunoblots were incubated with monoclonal antibodies (Mab3C9) against the p62 subunit of TFIIH (generously provided by Dr J.-M. Egly, Strasbourg), as published earlier by Fischer *et al.* (25). A polyclonal antiserum raised against p89, a GST-ERCC3 fusion protein containing an internal part (amino acids 82–480) was used to detect the p89/ERCC3/XPB component of TFIIH.

Cell lines and extracts

HeLa cells and Chinese hamster ovary (CHO9) cells were grown in F10/DMEM medium (1:1) supplemented with 10% fetal calf serum, penicillin 100 U/ml and streptomycin 0.1 mg/ml. Cells were harvested and extracts were prepared from 2–5 ml of packed cell pellets by the method of Manley, as modified by Wood (26,27) dialysed in buffer A and stored at –80°C until use. XP-A and XP-C patient cell lines used in these experiments were CW12 (XP-A) and XP4PA (XP-C) (29,30). [NB. Cell line CW12 was originally described as belonging to XP group C (28). During our work, however, we found it to carry an XP-A defect, but to harbour no mutation in the XPC gene.] COS-1 SV40-transformed African green monkey kidney fibroblasts were seeded semi-confluent in 6-well plates, and grown on F10/DMEM medium (1:1) supplemented with 5% fetal calf serum, penicillin and streptomycin. For DEAE-dextran/chloroquine transfection, SV40 promoter-driven constructs were used (pSVL derived pSLM vector; Pharmacia biotech) containing full length *HHR23A* and *HHR23B* cDNAs. The empty pSLM vector was used as a negative transfection control in parallel with *HHR23A* and *HHR23B* genes. A 10% DMSO shock for 1.5 min was given 4 h after transfection. Transient expression of the corresponding HHR23A

and HHR23B protein was analysed by immunofluoresence 48 h after transfection.

Antibody depletion of NER-proficient extracts

Protein A-Sepharose CL-4B beads (Pharmacia-Biotech) (70 µg) were washed three times with PBS, then incubated with 10 µl anti-XPC antibodies or pre-immune serum for 15 min at 4°C. The beads were then washed three times in buffer A (25 mM HEPES-KOH pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$ 1 mM EDTA, 2 mM DTT and 17% glycerol) and added to a repair-competent HeLa extract for 30 min at 4°C. The supernatant obtained after spinning down the beads was used as a depleted HeLa extract and tested on immunoblots for co-depletion. After boiling the protein A-Sepharose beads, the depleted 'bound' fraction was analysed by immunoblot analysis.

Fractionation of whole-cell extracts

A HeLa cell free extract (14 µg/µl; 750 µl) was applied to a heparin–Sepharose column. Proteins were eluted with a linear 30 ml gradient from 0.15 to 1.15 M NaCl in PBS containing 10 mM 2-mercaptoethanol buffer.

Whole-cell extracts were prepared for fractionation on a phosphocellulose column. This 5 ml column equilibrated with buffer A (25 mM HEPES-KOH pH 7.9, 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM DTT and 0.25 mM PMSF) contained 0.2 M KCl. The column was washed with the same buffer and the adsorbed proteins were eluted with buffer A containing 1 M KCl. The peak fractions from the flow-through (CFI) and the eluate (CFII) were concentrated by dialysis against buffer A containing 0.1 M KCl and 20% sucrose, and stored at –80°C.

Size-fractionation of HeLa Manley extracts was performed on a Sephacryl S300-HR column. HeLa nuclear extracts were loaded on a 1 × 46.4 cm column with a flow rate of 0.92 ml/7.5 min and eluted with PBS (7.4 ml/h). The resulting fractions were concentrated. Protein profiles of HHR23A, HHR23B and XPC were visualized on immunoblots using alkaline phosphatase-labeled secondary antibodies. Proteins included as molecular mass standards used for estimation of the native molecular weight were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and albumin (67 kDa) (Boehringer Mannheim).

Immunofluoresence

HeLa, XP-A and XP-C cells were grown on slides in F10/DMEM medium supplemented with 10% fetal calf serum, penicillin and streptomycin, and washed prior to fixation in PBS. Cells were fixed for 10 min in 2% paraformaldehyde-PBS, followed by incubation with methanol at room temperature for 20 min. After extensive washing (3 × 5 min) with PBS supplemented with 0.15% glycine and 0.5% BSA (PBS^+) the slides were incubated with affinity-purified primary antibodies (1:100 dilution in PBS) for 1.5 h in a moist incubation chamber at room temperature.

Immunocytochemical controls were routinely included (omission of the primary antibody incubation step and incubation with pre-immune serum). Background was negligible.

Slides were washed in PBS^+ and incubated with goat anti-rabbit-FITC-conjugated antiserum (1:80 dilution) for 1.5 h. Slides were washed in PBS and preserved with vectashield™ mounting medium (Brunschwig). The DNA was stained with 4′-6 diamino-2-phenylindole (DAPI) whereas the fluorescein-labeled second antibody visualized the antigen of interest. Fluorescence microscopy was performed with an Aristoplan laser beam microscope. Image modification for figures was performed by using the Adobe Photoshop program on an IBM Compaq deskpro XE 560.

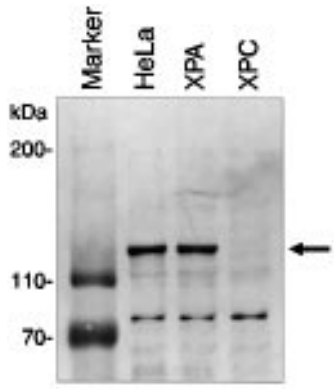

Figure 1. Specificity of XPC antiserum by immunoblot analysis in total cell extracts. Immunoblot analysis of repair-competent HeLa cell extract, XP-A (CW12) and XP-C (XP4PA) cells incubated with the affinity-purified anti-XPC antibodies. 10 µg protein was loaded in each lane and separated on a 6% SDS-PAGE gel. The cross-reacting band present in all three lanes indicates equal loading.

RESULTS

Characterization of HHR23A, HHR23B and XPC proteins

To characterize XPC, affinity-purified anti-XPC polyclonal antibodies were generated and tested by immunoblotting, using *in vitro* translated XPC protein, XPC protein purified from HeLa cells, and XP-C cells in total cell extracts. Figure 1 shows their specificity on HeLa, XP-A and XP-C protein extracts. A clear band of the expected molecular weight of 125 kDa as determined by *in vitro* translation and purified XPC (1) was observed in total cell extracts of repair-proficient HeLa and XP-A cells. The XP-C extract from patient XP4PA is useful for testing the specificity of the antibody. Due to a homozygous frameshift mutation that is predicted to result in a premature termination of the protein (30), this patient lacks the C-terminal XPC region encoding the part used to raise the antibodies. Further evidence for the specificity of the anti-XPC antibodies is derived from the immunofluoresence data depicted in Figure 8A.

Overproduction and purification of the two HHR23 proteins are described in the Materials and Methods section. Both recombinant HHR23 polypeptides behaved similarly during purification. *Escherichia coli* overproduced proteins were present in the soluble fraction of total sonicated extracts (Fig. 2, first lanes, upper panels). Due to their low iso-electric point, Q-Sepharose binds both HHR23 products efficiently. The presence of extensive hydrophobic stretches in the primary amino acid sequence suggested that a purification step based on hydrophobic interaction might also be successful. Figure 2 indicates that butyl Sepharose yielded very powerful purification (a full description of HHR23 purification will be described

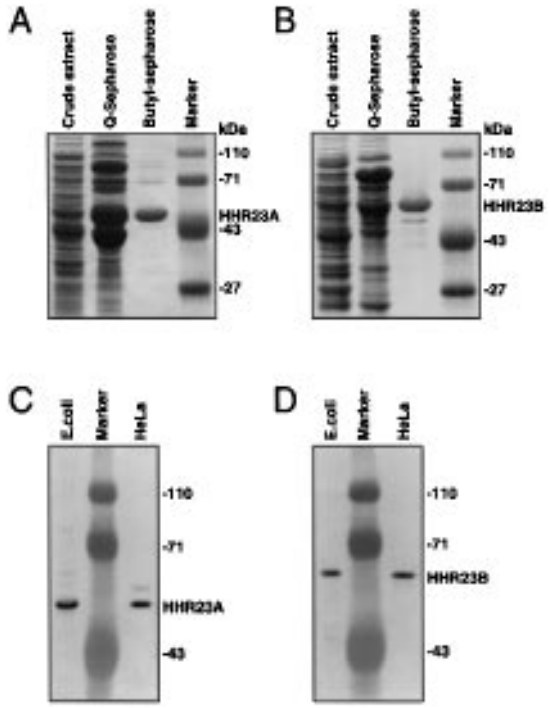

Figure 2. Protein purification and antibody characterization of HHR23A and HHR23B. The top panels show the purified CBB-stained 11% acrylamide gel containing crude, Q-Sepharose and butyl Sepharose purified fractions of the HHR23A and HHR23B proteins. The lower panels show affinity-purified HHR23A and HHR23B antibodies. 3 ng purified recombinant protein (butyl Sepharose) fraction and 10 µg HeLa extract were run on 8% acrylamide gels.

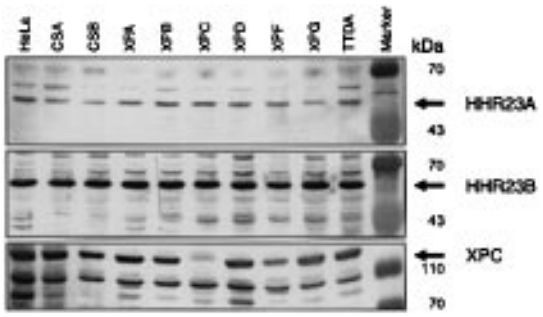

Figure 3. Immunoblot analysis of human NER syndromes. Immunoblotting of cells representative of different NER syndrome complementation groups analysed with crude anti-HHR23A, crude anti-HHR23B, and crude anti-XPC antibodies. The crossreacting bands provide an internal control for protein loading in the different lanes. Proteins were run on 11% polyacrylamide gels and blotted as described in Materials and Methods.

elsewhere). The specificity of affinity-purified polyclonal rabbit antibodies raised against the HHR23A and HHR23B proteins was checked on recombinant *E.coli*-overproduced human protein, repair-proficient HeLa extracts, and *in vitro* translated proteins.

In immunoblotting experiments, anti-HHR23A antibodies visualized a 50 kDa band in repair-competent Manley extracts of HeLa cells (Fig. 2C, third lane). The *E.coli*-overproduced recombinant HHR23A protein (Fig. 2A and C, first lane) and the *in vitro* translated protein (not shown) were of a similar size. The recombinant HHR23B protein migrated at 58 kDa, similar to the *in vitro* translated protein, the protein of a HeLa Manley extract (Fig. 2B and D, first and last lanes), and the protein from the purified XPC/HHR23B complex-containing fraction (8). Although the HHR23 gene products share 76% sequence similarity, polyclonal antibodies raised for each of the individual HHR23 proteins did not show any cross-reacting activity.

In addition to human NER-deficient patients, a second class of excision repair-deficient mutants is represented by laboratory-induced, UV-sensitive, rodent cell lines. Eleven complementation groups have been identified (31,32) which partially overlap with the genes defective in human NER syndromes (1,5). It is not known whether XPC or either of the HHR23 proteins is represented among these rodent mutants. Furthermore, defects in one product may lead to decreased stability of other proteins in the same complex. Previously, it was found that the amount of ERCC1 protein is significantly reduced in XP-F cells (33,34), although no primary defect in this gene is present in XPF. Since XPF and ERCC1 are known to form a protein complex (33,34), a mutation in the XPF protein most likely results in breakdown of ERCC1 protein due to instability (35). Therefore, Manley extracts of cells from all known repair-deficient mammalian complementation groups were checked for abnormalities of the XPC, HHR23A and HHR23B proteins, that could point to a possible involvement in any of these mutants. With the exception of XPC in XP group C, no alterations were observed for any of the proteins in the extracts from NER-deficient individuals. As an example, Figure 3 shows the human NER complementation groups. XP complementation group E, not included in this panel, also showed a normal expression pattern. In addition to this, no abnormalities were observed in any of the CHO groups analysed (data not shown). This is consistent with the idea that neither of the HHR23 proteins is implicated in the available NER mutants, and that XPC is not affected in the rodent mutants. Moreover, the HHR23B protein is not destabilized as a consequence of an XPC defect.

Behaviour of HHR23A, HHR23B and XPC in fractionation procedures

Previously, we determined XPC to be complexed with HHR23B protein (8). An association of XPC with TFIIH has been claimed (36). In an attempt to identify stable associations with other repair components or factors involved in the basal transcription machinery, systematically-purified protein fractions were tested for the presence or absence of HHR23A, HHR23B and XPC proteins. Purification protocols were used which are known to leave large stable protein complexes such as TFIIH intact (37).

To separate the components of the general transcription factors, a HeLa cell-free extract competent for *in vitro* repair and transcription was fractionated by heparin ultrogel column chromatography. Figure 4 shows the load and the elution fractions analysed with the affinity-purified HHR23A, HHR23B, XPC and TFIIH antibodies.

All detectable HHR23A protein was found to reside in the flowthrough fraction. The vast majority of the HHR23B protein also resided in the flowthrough, whereas ~10–20% was found in the XPC-containing fractions (0.62–0.72 M NaCl), consistent with the existence of an XPC–HHR23B complex. The elution profile of TFIIH (represented by the p89 and p62 proteins) partly overlaps with that of XPC, although the TFIIH elution profile is

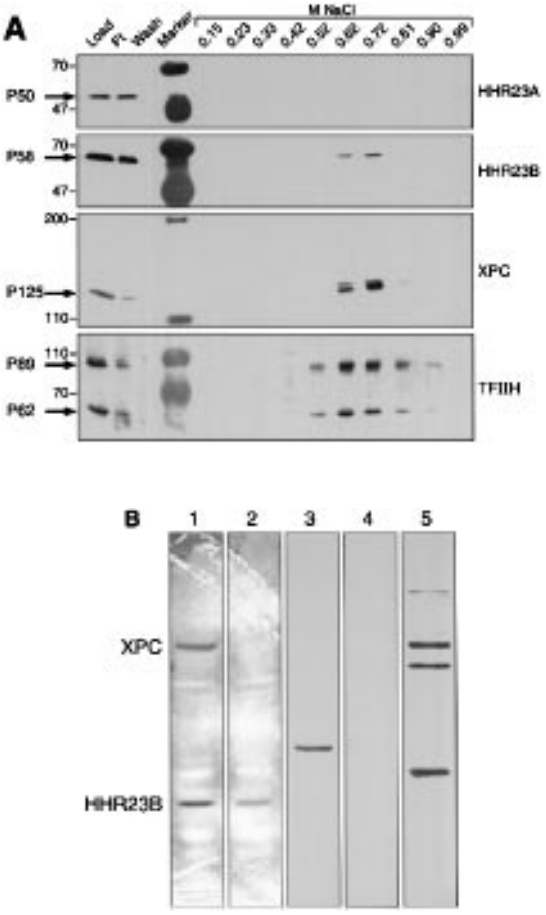

Figure 4. Heparin fractionation and immunodepletion. (**A**) Heparin-ultrogel fractions were assayed by immunoblotting with anti-HHR23A(p50), anti-HHR23B(p58), anti-XPC(p125) and anti-TFIIH (p89 and p62) antibodies. Affinity-purified polyclonal antibodies were visualized by alkaline phosphatase-labeled conjugates. The different bands of XPC probably represent different phosphorylation status. The minor amounts of XPC and TFIIH in the flow-through fraction are able to bind to the heparin upon a second application to the same column and thus do not represent a different species of these complexes. (**B**) HeLa extract (lane 1), HeLa extract depleted for XPC (lane 2), were both analyzed with anti-XPC and anti-HHR23B antiserum. HeLa extract depleted for XPC was analyzed with anti-p62 monoclonal antibody (lane 3). The protein fraction bound to the XPC-immunobeads was analysed by immunoblotting with anti-p62 antiserum (lane 4) and anti-XPC (lane 5), some of the extra bands are derived from the antibodies released from the beads.

slightly broader than that of XPC and the elution peaks are different. These findings support the existence of two subfractions of HHR23B and indicate that HHR23A is not detectably associated with XPC nor with TFIIH, but do not permit any conclusion concerning the existence of stable complex formation between XPC and TFIIH. Therefore, we performed immunodepletion experiments and other types of protein fractionation. A normal repair-competent extract depleted for XPC still contained p62 protein in addition to free non-complexed HHR23B protein, as determined by immunoblot analysis (Fig. 4B lanes 1–3). Moreover, the XPC-containing protein fraction bound to the protA beads showed no detectable co-depletion of the p62 subunit of the TFIIH complex (Fig. 4B, lanes 4 and 5). These data, together with other fractionation

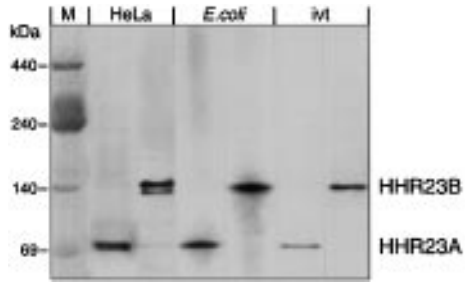

Figure 5. Non-denaturing gel electrophoresis of HHR23A, and HHR23B proteins. Migration pattern of HHR23A and HHR23B in HeLa Manley extracts as detected by immunoblotting, *in vitro* translated (ivt) HHR23 proteins and purified recombinant HHR23A and HHR23B *E.coli* proteins. Proteins were separated on a 4–15% polyacrylamide gradient gel.

studies including phosphocellulose (data not shown) and HA-His-tagged TFIIH complex (B. Winkler, unpublished results), strongly suggest that at least the vast majority of XPC–HHR23B complex is not stably associated with TFIIH in repair-active Manley extracts. Furthermore, highly purified (Sulphopropyl 5-PW) protein fractions containing ERCC1- correcting activity as described by van Vuuren *et al.* (38), showed no detectable amount of XPC or any of the HHR23 proteins, thereby excluding a stable association of significant quantities of XPC with the ERCC1 protein complex in the extracts used.

Non-denaturing gel electrophoresis

To detect possible complexes of HHR23 with other proteins, separation of repair-proficient HeLa Manley extracts under non-denaturing gel electrophoresis conditions was performed using wild-type cell extracts and anti-HHR23A and anti-HHR23B antibodies. The results are shown in Figure 5. The HHR23A protein was detected as a single band migrating at ~70 kDa. For HHR23B, two forms with approximate sizes of 140 kDa were distinguished in HeLa cell extracts. To determine the specificity of the apparent molecular weights of both HHR23 proteins, *in vitro* translated protein and recombinant *E.coli*-overproduced HHR23 proteins were run in parallel. After immunoblotting and autoradiography, all HHR23A protein samples were found to migrate at the same size, suggesting that the recombinant polypeptide has a similar conformation as the corresponding HeLa and *in vitro* translated proteins (Fig. 5). The lower HHR23B band observed in the HeLa lane migrates at the same position as the recombinant protein and the *in vitro* translated form. Thus, the upper band may represent a modified form of the HHR23B protein or binding of another small polypeptide. The notion that both bands are derived from HHR23B is supported by the observation that both signals disappeared when the antibodies were competed with excess recombinant HHR23B protein (data not shown).

Given the high sequence homology of both HHR23A and HHR23B proteins (57% identity, 76% similarity) a large apparent size difference was observed under native conditions (Fig. 5). From these data, it was concluded that HHR23A does not form a protein complex with HHR23B in detectable quantities. Furthermore, these data confirm that the majority of both HHR23 proteins is present in free non-complexed form in normal repair-competent extracts, and that the HHR23A and part of the

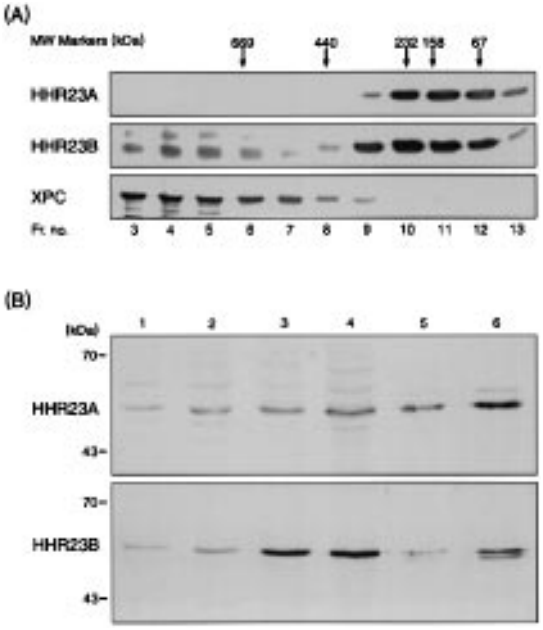

Figure 6. Size fractionation and titration experiments. (**A**) Size fractionation of HHR23 and XPC proteins was performed on a Sephacryl S300-HR column. Immunoblot analysis of fractions for the presence of HHR23A, HHR23B and XPC, respectively, using affinity-purified antibodies. The arrowheads indicate the size (kDa) of the marker proteins used as reference molecules. (Similar results were obtained using Sephacryl S500 fractionation.) Note that the bound HHR23B protein seems to migrate slightly differently from the free form. (**B**) Titration experiments to estimate the amount of HHR23 proteins per cell. 6 µg and 12 µg HeLa total cell extract (lanes 1 and 2), 6 µg and 12 µg HeLa Manley extract, and 3 ng and 10 ng recombinant protein (lanes 5 and 6) were analyzed by immunoblotting using anti-HHR23A and anti-HHR23B antibodies. Intensities were used to calculate the amounts of molecules per cell.

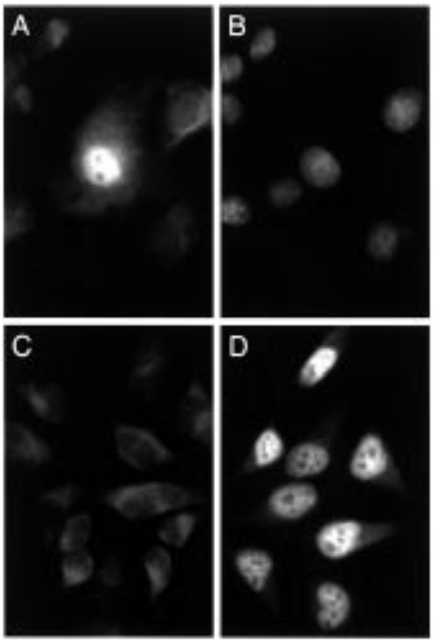

Figure 7. Nuclear subcellular localization of the HHR23 proteins. (**A**) The affinity-purified HHR23A antibody staining on COS-1 cells transfected with the HHR23A containing mammalian expression vector pSLM-HHR23A, visualized by FITC-labelled secondary antibodies. (**B**) The DAPI staining of the same cells. (**C**) Shows HeLa cells with the pre-immune serum of HHR23B (long exposure, to visualize the cells), whereas (**D**) shows the endogenous HHR23B protein in HeLa cells detected with affinity-purified antibodies.

HHR23B proteins do not undergo a gross post-translational modification. Analysis with anti-XPC antibodies revealed no detectable signal for XPC. Moreover, the XPC–HHR23B complex could also not be detected with the anti-HHR23B antibodies, suggesting a size or charge problem for migrating into the native gel.

To investigate the native size of XPC and the HHR23 proteins and their possible association with other factors, size-fractionation was performed using gel filtration. HeLa whole cell extracts, in which repair and transcription factors reside in an active configuration, were used as a starting point. The profiles of a Sephacryl S300 fractionation are shown in Figure 6A. Both XPC and part of the HHR23B proteins resided in similar overlapping fractions. However, the size of the XPC–HHR23B complex in this configuration is much higher than the apparent size of the purified complex as determined by glycerol gradient and Sephacryl fractionation (8). The large size provides a possible explanation for the failure to detect this complex under native gel electrophoresis conditions (see above). The size determined for the free form of HHR23B is slightly bigger than that for HHR23A, which is in agreement with the data of the native gel electrophoresis (as shown in Fig. 5).

The above findings indicate that the vast majority of the HHR23A and B molecules are in a free form. To determine how many A and B molecules reside in a cell, immunoblot titration experiments were performed using HeLa total cell extracts and standard amounts of recombinant HHR23A and HHR23B (Fig. 6B). From the data we calculate that ~2–4 $\times$ 10^5 molecules of each are present in a single HeLa cell. From the blots (e.g. see Figs 2 and 5) we estimate that a maximum of one fifth of HHR23B is complexed to XPC. In the absence of free XPC and on the assumption of a 1:1 stoichiometry in the complex, the XPC concentration is of the order of 4–8 $\times$ 10^4 copies per cell.

Intracellular localization of HHR23A, HHR23B and XPC

Analysis of HHR23A, HHR23B and XPC protein sequences for the presence of a DNA binding domain or a nuclear localization signal (39) revealed no clear matches conforming with the known consensus sequences. Moreover, we failed to detect DNA binding activity of the isolated recombinant HHR23A and HHR23B proteins (unpublished observations). To define the subcellular distribution of the free HHR23A, HHR23B protein molecules and the XPC–HHR23B complex, we performed indirect immunofluoresence in HeLa cells, COS-1 transfected cells, normal repair-proficient fibroblasts and XP-C patient fibroblasts. No labeling was seen after treatment with secondary antibodies alone or after competition with excess of the recombinant HHR23 proteins or the XPC peptide used for immunization. The specificity of the primary antibody was confirmed by the use of pre-immune sera in all experiments, included as a negative control. For both human equivalents, HHR23A and HHR23B, a clear nuclear localization was observed, and the protein appeared to be absent from the nucleoli (Fig. 7A and D). However, this absence can be due to the fixation procedure.

The XPC protein was predominantly localized in the nucleus of HeLa and xeroderma pigmentosum group A (CW12)

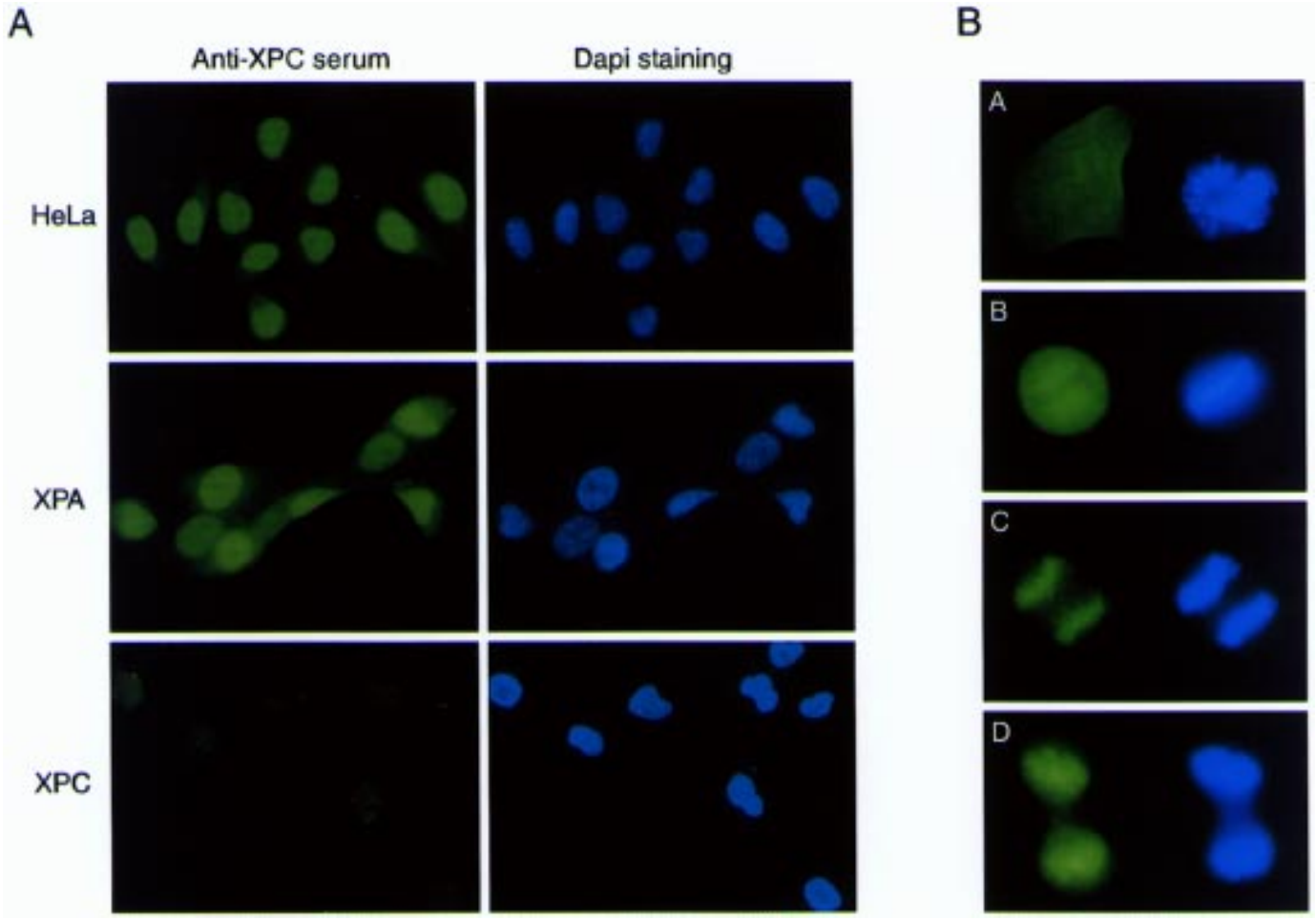

Figure 8. Subcellular localization of XPC protein. (**A**) Immunofluorescent localization of XPC in HeLa cells, xeroderma group A (CW12) and group C (XP4PA) fibroblasts. The left panel visualizes the FITC-conjugated anti-XPC antibodies whereas the right panel depicts DAPI staining of the same cells. (**B**) Different stages of mitosis analysed with affinity-purified anti-XPC antibodies. The panels represent (A) prophase, (B) metaphase, (C) late anaphase and (D) telophase. The green signals on the left represent XPC, visualized by FITC-conjugated secondary antibodies, whereas the blue signals on the right depict the DAPI staining of the DNA in the same normal repair-proficient fibroblasts cells. (**C**) and (**D**) represent the clear anaphase/telophase specific association of XPC protein with chromatin.

fibroblasts during interphase, as shown in Figure 8. The XP4PA (XP-C) cells gave no signal, confirming the absence of the intact XPC protein (as also shown by immunoblot analysis in Figures 1 and 2).

Additionally, we investigated the subcellular localization of the XPC and HHR23 protein during mitosis. This was carried out in human CW12, XP4PA fibroblasts and in HeLa cells. When the cell entered mitosis, XPC (and both HHR23) proteins relocalized in the cytoplasm. During metaphase, they were present diffusely throughout the whole cell, without particular association with chromatin. Unexpectedly, in the later stages of mitosis, during anaphase and telophase, the XPC protein became specifically associated with chromatin (Fig. 8B). This anaphase/telophase specific association was not observed for either of the HHR23 proteins. However, due to excess of the free HHR23B protein in the cell (see above), we cannot exclude whether this also holds for the small amount of HHR23B complexed with XPC. Cells from an XP-A patient (CW12) showed the same subcellular localization as HeLa. The HHR23B protein distribution in XP4PA (XP-C) cells was indistinguishable from that in HeLa or CW12 (XP-A).

DISCUSSION

This article describes the partial characterization of HHR23A, HHR23B and XPC proteins. Both XPC and HHR23B proteins are known to be specifically involved in global genome nucleotide excision repair. GGR deals with the repair of bulk DNA, including the non-transcribed strand of active genes (7,12,40) and is important for preventing carcinogenesis. Evidence for this comes from the lack of enhanced cancer risk in patients with the transcription-coupled NER disorder, Cockayne syndrome (41) and the high cancer predisposition when the GGR subpathway is defective as in XP-A and XP-C. Purification of the XPC-correcting NER activity revealed a heterodimeric protein complex consisting of XPC and HHR23B (8). However, the functional significance of the association of HHR23B with XPC is not known, and could be, for instance, stabilization of the XPC

protein. The yeast RAD23 protein also has a role in NER, and was recently found to form a protein complex with the yeast RAD4 protein (11), a structural homolog of XPC.

Except for potential phosphorylation sites, analysis of the primary amino acid sequence of XPC gave no clues about a particular function. The primary amino acid sequence of RAD23 protein and its mammalian homologues indicated that they are N-terminal ubiquitin-like fusion proteins (8,16). In addition, a second link with the ubiquitin pathway was observed. Two repeated domains in the RAD23 amino acid sequence shared homology to a C-terminal extension in a bovine ubiquitin-conjugating enzyme (E2-25kD) (17). This suggests that the RAD23 protein may have an involvement in the ubiquitin system, within the context of NER or in another process, implying a dual functionality. Other NER proteins have also been found to have dual functions. Examples include the XPB and XPD proteins in the multisubunit TFIIH transcription repair factor (42) and the RAD1–RAD10 complex, additionally involved in mitotic recombination (43).

In the present studies, we tried to find evidence for a stable association of XPC and HHR23 proteins with each other and with previously identified protein complexes which have defined enzymatic activity, involving endonuclease-mediated incision (ERCC1/ERCC4) or transcription initiation activity (TFIIH). Heparin fractionation experiments revealed that HHR23A and a large fraction of HHR23B resided in the flowthrough fraction (Fig. 4A). Native gel electrophoresis indicated that the vast majority of both HHR23 proteins was present in the free, non-complexed form (Fig. 5) a finding supported by the gel filtration experiments (Fig 6A). The heparin, phosphocellulose and size fractionation experiments as well as the immunodepletion studies all confirmed the complex formation of XPC protein with HHR23B protein (Figs 4A and B and 6A). No HHR23A protein could be detected in these purified heparin or phosphocellulose fractions containing XPC. From these findings we conclude that for HHR23B two forms exist: the majority is in a free form, whilst a small fraction is complexed with XPC. For HHR23A, we can only detect a free form, although it is not excluded that a fraction below our detection level is complexed with XPC or another protein. The absence of detectable quantities of HHR23A in the XPC-HHR23B containing high-salt fractions from the heparin (Fig. 4A) and phosphocellulose chromatography suggests that HHR23A may not be functionally fully equivalent to HHR23B. The small fraction of HHR23B that is complexed with XPC is necessary for NER (10). This raises the question whether HHR23A and the free form of HHR23B are involved in NER at all and/or whether they have an additional function. These proteins resided in the flow-through of the phosphocellulose fractionation. Previously, Aboussekhra *et al.* (44) showed that only the RP-A complex and the PCNA protein from this fraction are necessary for *in vitro* NER. These data therefore suggest that HHR23A does not play a role in the core NER reaction. However, the *in vitro* system might not reflect the step in which this protein plays a role *in vivo*. If only (the XPC-bound) HHR23B has a role in NER, one might wonder why no rodent or human mutants for HHR23B were found (Fig. 3). A possible explanation for the absence can be the dual function, that might give rise to an unexpected phenotype. Alternatively, HHR23A may bind to XPC when HHR23B is absent. A clear answer on what is the function of the free form of both mammalian RAD23 equivalents and whether they are functionally redundant should come from analysis of mutants generated by gene targeting and from *in vitro* reconstitution experiments (both experiments in progress).

Gel filtration studies suggested that the XPC protein can be part of protein complexes of large size (Fig. 6A). The purified XPC–HHR23B complex was previously determined to have a molecular weight of 500–550 kDa by gel filtration and a value of 110 kDa by glycerol gradients (8). Here we found in fractionated Manley-type cell extracts a molecular weight bigger than these previously determined values. This suggests that the XPC–HHR23B proteins are part of a bigger complex, that can easily fall apart during purification in a stable XPC–HHR23B subcomplex and other proteins. However, it cannot theoretically be excluded that XPC protein selectively multimerizes or aggregates. Therefore, it was investigated whether the large molecular weight XPC-containing complex also includes TFIIH and ERCC1 components, conforming with the 'repairosome' model reported by Svejstrup *et al.* (45) for yeast NER. Non-denaturing gel electrophoresis showed distinct bands for HHR23A (70 kDa) and HHR23B (150 kDa), for both the HeLa proteins as well as the *E.coli*-produced recombinant polypeptides. In this context, it should be noted that native molecular weight estimations themselves should be taken with caution, since these may strongly depend on the conformation of the proteins or protein complexes. However, bands migrating at a similar position can provide evidence for complex formation. Therefore, it is evident that both native molecular weights are different from the 280 kDa previously described for the ERCC1 complex (34) and the minimal molecular weight calculated for TFIIH.

Fractions containing highly purified ERCC1 complex described by van Vuuren *et al*. (38) were also checked for the presence of HHR23 proteins, and were found to be negative. This makes a tight association of these proteins with the ERCC1 complex highly unlikely.

From the data presented here one can also conclude that there is no stable complex of a significant fraction of XPC–HHR23B and TFIIH, under these conditions and in our (Manley) extracts. This is in conflict with the findings of Drapkin *et al.* (36), who after six purification steps for TFIIH components, still detected XPC protein in the purified fractions. However, their TFIIH complex is not completely pure, and no data are provided with respect to the yield and the fraction (percentage) of XPC present in the TFIIH preparation. Therefore, it is hard to identify whether this is a significant amount of XPC and whether cross-contamination is excluded. From our unpublished results, it appeared that the XPC complex by coincidence behaved in a similar way during several purification steps as TFIIH. Moreover, no physical interaction (e.g. immunodepletion) was shown by Drapkin *et al.* (36). On the other hand it should be stressed that our data on XPC/TFIIH interaction do not exclude a transient association, as reported by Bardwell *et al.* (46) for the *S.cerevisiae* system. The absence of any detectable interactions between these factors is of relevance in the context of the evidence for a 'repairosome' in *S.cerevisiae* (45), in which (almost) all NER components are represented in one super complex. A difference might exist between yeast and mammals. Alternatively, a 'NERosome' in mammals may be more delicate, and might dissociate sooner than its yeast counterpart. Therefore, the extract preparation procedure can be of crucial relevance. The Manley extracts are made from non-UV irradiated cells. Possible interactions of repair proteins might not be visible in extracts of non-damaged cells.

The subcellular localization of a protein can provide possible clues about its function. Immunofluoresence data displayed a

clear nuclear localization of the XPC protein on interphase cells (Fig. 7). This finding supports a function in DNA metabolism and is in agreement with the previously described ssDNA binding activity (8). The observation that during anaphase and telophase, XPC specifically associates with chromatin, suggests a role for XPC after the metaphase/anaphase transition, and is consistent with the DNA-binding activity of the XPC–HHR23B protein complex. However, such a function remains to be clarified.

Nuclear localization was also found for both HHR23 proteins, consistent with a role for these proteins in DNA or chromatin metabolism. These results are in accordance with previous *S.cerevisiae* data, in which the RAD23 protein was described to be nuclear (15). Both recombinant HHR23 proteins were found not to have specific affinity for ss or dsDNA, and, therefore, a direct role for these proteins in DNA damage recognition can be regarded as unlikely. On the other hand, recent analyses of dimer and 6-4 photoproduct repair in *rad23* deletion mutants in *S.cerevisiae* which are only intermediately UV-sensitive indicated that removal of both lesions is strongly impaired for both TCR and GGR (46). This implies an important role of this protein in both NER subpathways in yeast. However, it is unknown at present whether RAD23 is in excess over RAD4 and consequently whether a free form of RAD23 exists in *S.cerevisiae*.

Additional functions for both HHR23 proteins seem likely however, based on the excess of both HHR23 proteins in the cell compared to XPC, as deduced from the experiments presented in this paper.

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