Centrosome Protein Centrin 2/Caltractin 1 Is Part of the Xeroderma Pigmentosum Group C Complex That Initiates Global Genome Nucleotide Excision Repair

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Nucleotide excision repair (NER) is carried out by xeroderma pigmentosum (XP) factors. Before the excision reaction, DNA damage is recognized by a complex originally thought to contain the XP group C responsible gene product (XPC) and the human homologue of Rad23 B (HR23B). Here, we show that centrin 2/caltractin 1 (CEN2) is also a component of the XPC repair complex. We demonstrate that nearly all XPC complexes contain CEN2, that CEN2 interacts directly with XPC, and that CEN2, in cooperation with HR23B, stabilizes XPC, which stimulates XPC NER activity in vitro. CEN2 has been shown to play an important role in centrosome duplication. Thus, those findings suggest that the XPC-CEN2 interaction may reflect coupling of cell division and NER.

Centrin (caltractin) found in the centrosomes of a wide variety of organisms (1) is a member of the highly conserved superfAMILY of calcium binding EF-hand proteins. Centrin was first discovered in the flagellar apparatus of the unicellular green algae Chlamydomonas reinhardtii, where it is directly responsible for the contraction of calcium-sensitive structures (2–4). Analysis of a centrin mutant of green algae suggested that centrin is important for the proper segregation of the flagellar apparatus during cell division and for accurate basal body duplication and separation (5). The counterpart of centrin in Saccharomyces cerevisiae, Cdc31, was shown to be necessary for the initiation of spindle pole body (SPB) formation (6, 7).

In human, at least three centrin isoforms have been identified: centrin 1/caltractin 2 (CEN1) (8), centrin2/caltractin1 (CEN2) (9), and centrin 3 (CEN3) (10). Among these centrin isoforms, CEN1 displays tissue-specific expression, whereas CEN2 is expressed ubiquitously (11). A recent study showed that the expression and distribution of these three isoforms varies during human ciliated cell differentiation and proliferation in vitro (12). Moreover, CEN3, the closest homologue of S. cerevisiae Cdc31, has been suggested to have a distinct function in centrosome duplication (13). On the other hand, CEN2 and its isoforms have been detected in the nuclear fraction, although all centrin isoforms co-exist in the centrosomes of animal cells (14). The nuclear function of centrins has not yet been explored.

Xeroderma pigmentosum (XP) is a hereditary disease characterized by photosensitivity, a high incidence of sunlight-induced skin cancer, and in some cases, neurological complications. XP patient cells can be classified into seven different genetic complementation groups (XP-A to XP-G), all of which are defective in nucleotide excision repair (NER) (reviewed in Ref. 15), and into one variant group, XP-V, which is defective in translesion DNA synthesis (16, 17). NER is a versatile and universal DNA repair pathway that can eliminate most types of the lesions from DNA. NER reaction consists of four steps: 1) damage recognition, 2) excision of the damaged DNA by creating incisions on both sides of the lesion, 3) gap-filling by DNA polymerase activity, and 4) ligation (18). A group of recent studies revealed that XP gene products are involved in the early steps of the human NER reaction, before the gap-filling reaction begins (reviewed in Refs. 19 and 20). The DNA excision reaction can be reconstituted with six factors: XPA, the XPC complex, XPF-ERCC1, XPG, general transcription factor TFIH, and replication protein A (RPA) on naked DNA (21, 22) or minichromosomes (23). 24–32 base oligonucleotides containing the lesion are excised from duplex DNA in the cell-free NER reaction.

The XPC complex acts as a key component of global genome nucleotide excision repair (GGR), a NER subpathway, by functioning as the initial damage detector (24). This complex was isolated as a heterodimeric complex and consists of the XPC gene product (XPC) and the human homologue of Rad23 B (HR23B) protein (25). Further biochemical analyses showed that the XPC protein by itself preferentially binds to damaged DNA and single-stranded DNA (26) and elicits the translocation of another NER factor, TFIH, to damaged DNA (27). The second subunit of the XPC complex, HR23B stimulates the in vitro NER reaction only in the presence of XPC (28), and this stimulatory activity depends on the region of HR23B that mediates interaction with XPC (29). Further analyses showed that the association of HR23B with XPC was important for its ability to stimulate NER (30). However, the mechanism of NER stimulation by HR23B is still poorly understood (31).
In this article we report that the XPC complex contains another factor, CEN2, that is recruited via interaction with XPC. During the analyses of CEN2, we found that NER stimulatory activity of HR23B is due to stabilization of XPC protein. We show that CEN2 and HR23B cooperatively stabilize XPC and thereby stimulate NER in vitro. Thus, we conclude that CEN2 is a novel NER factor and suspect that the XPC complex may be involved not only in DNA repair but also in cell division.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell-free Extracts—**Lymphoblastoid cells (GM2248B, a kind gift of Dr. J. H. J. Hoeijmakers) from an XPC patient (XP3BE) were cultured at 37 °C with 5% CO2 in RPMI 1640 supplemented with 15% fetal bovine serum. For the in vitro NER assay, whole cell extracts were prepared as described previously (22, 39). HeLa cells were cultured in spinner flasks and harvested for protein purification as described previously (25).

**Purification of XPC-HR23B-CEN2 Complex from HeLa Cells—**The XPC-HR23B-CEN2 complex was purified from HeLa nuclear extracts as previously described with minor modifications (25). All procedures were carried out at 0–4 °C. HeLa nuclear extracts (1.49 g of protein), obtained from frozen stock of HeLa cells (236 ml of packed cell volume), were successively applied to a phosphocellulose column (Whatman P11 (90 ml), a single-stranded DNA cellulose column (Sigma (9 ml)), and a CM-cosmolgel column (Nalcadis Teseque (3.8 ml)). The fraction (1.2 mg) eluted from the CM-cosmolgel column with buffer 1 (20 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 0.1 M KCl in buffer 1. The column was washed with the equilibration buffer, and the adsorbed proteins were eluted with 30 ml of a linear salt gradient of 0–0.4M NaCl. CEN2-His eluted between 20 and 30% NaCl. The dialyzed fraction was applied to and eluted from the column three times. The fraction was collected and used as a source of CEN2-His.

**Construction of Plasmids for Protein Expression in Escherichia coli—**The human CEN2-coding sequence was obtained by PCR with first-strand cDNA synthesized using HeLa poly(A)+ RNA as template. The sequence of the oligonucleotides used was 5′-TTCTTCAGGCTGCTCCTAAC-3′ and 5′-AGAGATCTGGTATCAAGG-3′. This was performed with the reagent Mutan-K (Takara Shuzo). The resulting plasmid was digested by XhoI and self-ligated to fuse the C terminus of CEN2 in-frame with eight amino acids (LEHHHHHH). This construct was designated pET24-CEN2H. All constructs were verified by DNA sequencing using an ALF-Red DNA sequencer (Amersham Pharmacia Biotech).

**Expression and Purification of Recombinant CEN2 Proteins—**Recombinant nontagged CEN2 protein was expressed and purified essentially as described in the previous methods of Baron et al. (38). Ten milliliters of a fresh, full-grown culture of E. coli BL21 (DE3) carrying pET24-CEN2 was inoculated into 1 liter of Super Broth medium (5 g of NaCl, 32 g of Bacto-trypton, and 20 g of yeast extract/liter (pH 7.0)) containing 50 μg/ml of kanamycin. Cells were cultured at 30 °C with vigorous shaking to an absorbance of 600 nm of 0.4, and then induction of the recombinant protein was induced by further incubation in the presence of 0.5 mM isopropylthioligulose for 2 h at 30 °C. The cell pellet was washed with ice-cold 10% glycerol, suspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 0.4 μg/ml aprotinin, 0.4 μg/ml leupeptin, 0.2 μg/ml antipain, 0.1 mM EGTA, and 10 μg/ml lysozyme), and disrupted by freezing and thawing. The supernatant (129 mg of protein), obtained by centrifugation at 10,000 g for 15 min, was dialyzed against buffer 1 containing 0.15 M KCl. The dialyzed fraction was then loaded onto a MonoQ 1.6/5 column (Amersham Pharmacia Biotech (0.1 ml)) equilibrated with buffer 1 containing 0.15 M KCl using the SMART system (Amersham Pharmacia Biotech). The column was washed with the equilibration buffer, and adsorbed proteins were eluted with 2.5 ml of a linear gradient of 0.15–0.45M KCl in buffer 1. The eluted proteins were adsorbed proteins were eluted with 25 ml of a linear gradient of 0.15–0.45M KCl in buffer 1. The column was washed with the equilibration buffer, and the adsorbed proteins were eluted with 30 ml of a linear salt gradient of 0–0.2 M NaCl in buffer 4. CEN2 eluted around 0.2 M NaCl was collected and dialyzed against buffer 4 containing 1.5 M NaCl, 5 mM CaCl2, and 10% glycerol. The dialyzed extract was loaded onto a HiTrap DEAE column (Amersham Pharmacia Biotech (5 ml)) that was equilibrated with buffer 4 (50 mM Tris-34 mM HCl, and 1 mM DTT). The column was washed with the equilibration buffer, and the adsorbed proteins were eluted with 30 ml of a linear salt gradient of 0–0.2 M NaCl in buffer 4. CEN2 eluted around 0.2 M NaCl was collected and dialyzed against buffer 4 containing 1.5 M NaCl, 5 mM CaCl2, and 10% glycerol. The dialyzed extract was loaded onto a HiTrap Q column (Amersham Pharmacia Biotech (1 ml)) that was equilibrated with buffer 5 containing 0.6 M KCl. CEN2-His eluted between 20 and 30% KCl. The eluted fraction was applied to and eluted from the column three times. The eluates (2.6 mg) were combined, dialyzed against buffer 4, and loaded onto a MonoQ HR5/5 column (Amersham Pharmacia Biotech (1 ml)) equilibrated with the same buffer used for denaturation. The column was washed with the equilibration buffer, and the adsorbed proteins were eluted with buffer 5 (10 mM Tris, 6.8 mM HCl, 5 mM EGTA, 1 mM DTT, and 10% glycerol). Because a significant amount of CEN2 was retained in the flow-through fraction, the fraction was applied to and eluted from the column three times. The eluates (2.6 mg) were combined, dialyzed against buffer 4, and loaded onto a MonoQ HR5/5 column (1 ml) equilibrated with buffer 4. After washing the column with buffer 4 containing 0.1 M NaCl, the adsorbed proteins were eluted with 10 ml of a linear salt gradient of 0–1.4 M NaCl. CEN2 (2.3 mg) eluted around 0.25 M NaCl was pooled and stored at −80 °C.

Recombinant hexa-histidine-tagged CEN2 (CEN2-His) was expressed in E. coli cells transformed with pET24-CEN2H2 as described above. The cells were disrupted by sonication in buffer 6 (0.3 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.5 mM PMSF, 0.1% Nonidet P-40, 0.4 μg/ml aprotinin, 0.4 μg/ml leupeptin, 0.2 μg/ml antipain, and 0.1 mM EGTA) containing 10 μg/ml lysozyme. The lysate was clarified by centrifugation, and the supernatant was loaded onto an nickel-chelating Sepharose column (Amersham Pharmacia Biotech (1 ml)) equilibrated with buffer 6 containing 20 mM imidazole. The column was washed with the equilibration buffer, and the adsorbed proteins were eluted with a linear gradient of 20–250 mM imidazole. The protein-rich fractions were pooled, dialyzed against buffer 4. The dialyzed extract was loaded onto a Mono Q HR5/5 column (Amersham Pharmacia Biotech (1 ml)) equilibrated with buffer 4 containing 0.2 M NaCl. The column was washed with equilibration buffer, and the adsorbed proteins were eluted with a linear salt gradient of 0–0.4 M NaCl. CEN2-His eluted around 0.25 M NaCl was pooled and stored at −80 °C. Throughout the purification, column chromatographies were performed with fast protein liquid chromatography system, and the proteins were analyzed by SDS-PAGE.
Heat Inactivation Assay—To examine the stability of XPC protein, XPC was preincubated at various temperatures for 1 h in preincubation buffer (0.1 M NaCl, 25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.01% Triton X-100, 1 mM DTT, and 0.25 mM PMSF) in the presence or absence of CEN2 and/or HR23B. The residual activity was analyzed by the XPC-complementing assay and the damaged DNA binding assay. The XPC concentration during the preincubation was 12.5 nM and 50 nM for the XPC-complementing assay and the damaged DNA binding assay, respectively. In both cases, the molar ratios of CEN2 and HR23B were 10 times higher than that of XPC. In the 1 h preincubation, two tubes, the first one of which (tube A) contained XPC with or without CEN2 and/or HR23B and the second one of which (tube B) contained the proteins missing from the first tube, were incubated in a water bath. For the XPC-complementing assay, 2 μl of solution from tube A was combined with 2 μl of solution from tube B, added to the reaction mixture containing the SV40 minichromosome, and incubated at 30 °C for 3 h under standard conditions. For the damaged DNA binding assay, 4 μl of solution from tube A was combined with 4 μl of solution from tube B, added to the reaction mixture containing damaged or undamaged DNA, and incubated at 30 °C for 30 min. These procedures are summarized in Figs. 5A and 6A. At these amounts of XPC, both XPC-complementing activity and damaged DNA binding activity were dependent on XPC but not on CEN2 or HR23B, and the activity in both assays was not saturating but proportional to the amount of added XPC (data not shown). Thus, in both assays, the only limiting condition was the amount of XPC.

Cell-free NER Assay—For the XPC complementation assay, UV-irradiated (200 J/m²) simian virus 40 (SV40) minichromosomes were reacted with 80 μg of XPC-cell-free extract in reaction mixture (see "Experimental Procedures") in the presence or absence of 0.1 μl of a MonoQ fraction. A, schematic representation of the purification of the XPC complex from HeLa nuclear extracts. NER activity was determined by measuring 32P-labeled dCMP incorporation by endogenous DNA polymerases, which fill in the gaps generated by the damaged DNA excision reaction. Successively, SV40 DNA was purified from the reaction mixture, linearized by EcoRI digestion, and subjected to 1% agarose gel electrophoresis. ssDNA, single-stranded DNA; B, an autoradiogram of the gel. C, silver staining of the MonoQ fraction (fr). A portion (1 μl) of the MonoQ fraction was subjected to 8–18% SD PAGE and stained with silver. The proteins were identified by immunoblot analysis (XPC, HR23B, and HR23A) and amino acid sequencing (CEN2). Asterisks represent the XPC-derived degraded proteins. The indicated molecular weights were obtained using standard high molecular mass markers (Amersham Pharmacia Biotech).

RESULTS

XPC Forms a Trimeric Complex with CEN2 and HR23B—The XPC complex was purified from HeLa nuclear extracts after four column chromatographic steps (Fig. 1A). In this
Centrin in XPC Complex

study, we obtained a fraction that was approximately 10 times more concentrated than previous preparations (25). The addition of the MonoQ fractions to XPC cell extracts showed significant recovery of NER synthesis, whereas the extracts alone did not show any NER synthesis (Fig. 1B). The elution profile of XPC-complementing activity (NER activity) correlated with the presence of XPC and HR23B proteins as detected by silver staining SDS-polyacrylamide gels containing samples from the MonoQ fractions to XPC cell extracts showed significant amounts of CEN2 (data not shown). These results are consistent with the observation that only a small proportion of the XPC complex in the final purification step contained HR23A (Fig. 1C).

CEN2 Directly Interacts with XPC—To understand XPC complex formation, we studied the properties of complexes reconstituted from each component that were purified as a recombinant protein (see “Experimental Procedures”). CEN2-His was bound to nickel-chelating Sepharose resin, as detected by immunoblotting (Fig. 3, A and B), whereas neither XPC nor HR23B bound to the resin. In the presence of CEN2-His, however, XPC was retained on nickel-chelating resin (Fig. 3A) but HR23B was not (Fig. 3B), indicating that CEN2 interacts with XPC but not HR23B. CEN2 has four EF-hand domains that are known to be important for calcium binding. However, the XPC binding activity of the recombinant CEN2, which was purified in the absence of calcium, was not affected by the presence of 5 mM calcium (data not shown).

NER Activities of the XPC Complex Components—From the above studies, we surmised that CEN2 might have some function in NER because it is present in virtually all of the purified XPC complexes. To directly test the role of CEN2 in NER, we purified recombinant nontagged CEN2 as described under “Experimental Procedures” (Fig. 4A). NER activity was measured on UV-irradiated SV40 minichromosomes in the cell-free NER assay reconstituted with purified NER factors (23). As shown in Fig. 4, B and C, in reactions containing all factors except the XPC complex, the addition of recombinant XPC alone resulted in weak NER activity, as reported previously (30). In the absence of XPC, CEN2 alone, HR23B alone, or a mixture of CEN2 and HR23B indicated no significant NER activity. However, HR23B stimulated the NER activity of XPC, as previously reported (30). On the other hand, the addition of CEN2 either alone or in combination with HR23B failed to produce any significant effect on XPC-dependent NER activity in vitro.

HR23B Stabilizes XPC—As shown above, HR23B has an

Fig. 2. CEN2 is part of the XPC-correcting complex. A, MonoQ fraction 22 (Fig. 1) was subjected to glycerol density gradient centrifugation, and portions (16 µl) of the collected fractions were analyzed by 8–18% SDS-PAGE followed by silver staining. The sedimentation positions of marker proteins in a parallel gradient are indicated. A portion of the MonoQ fraction (Q) was subjected to SDS-PAGE as a control. The molecular weights were obtained using a 10-kDa ladder (Life Technologies, Inc.). BSA, bovine serum albumin. B, co-immunoprecipitation analyses of the XPC complex from HeLa whole cell extracts. HeLa whole cell extracts (200 µg) were incubated with protein A beads conjugated with affinity-purified anti-XPC, anti-HR23B, or anti-CSA antibody. The precipitates were subjected to 14% SDS-PAGE followed by immunoblotting with anti-CEN2(rat) antibody. Anti-CSA antibody was used for a negative control of the affinity-purified antibody. Ten ng of recombinant CEN2 protein was loaded onto the gel as a positive control. The recombinant CEN2 was prepared under a calcium-free condition, and it was reported that the lower band is a truncated version of CEN2 or another centrin isoform closely related to CEN2.

Fig. 3. CEN2 binds directly to XPC but not to HR23B. Hexahistidine-tagged recombinant CEN2 (1 pmol) was mixed with 0.2 pmol of recombinant XPC (A) or 0.2 pmol of recombinant HR23B (B) in binding buffer (80 µl) containing the nickel-chelating Sepharose resin. Unbound (U) and bound (B) materials were recovered, and a portion (16 µl) of each sample was subjected to SDS-PAGE (14% for CEN2 and 8% for XPC and HR23B). Proteins were detected by immunoblotting with anti-CEN2(rabbit), anti-XPC, or anti-HR23B.

XPC-complementing complex is 160 kDa, in good agreement with the calculated mass of these proteins (169 kDa).

To confirm that the XPC-HR23B-CEN2 complex is naturally present in the cells, we performed co-immunoprecipitation analyses. CEN2 was co-precipitated from HeLa cell extracts by resin containing anti-XPC antibody or by anti-HR23B antibody, but not by anti-CSA antibody (Fig. 2B). Quantitative analyses revealed that almost 100% of CEN2 in the cell extract was co-precipitated with the anti-XPC antibody. Thus, most of the CEN2 in this extract was complexed to XPC. The anti-HR23B resin precipitated lower amounts of CEN2 than did the anti-XPC resin (Fig. 2B). This was also true for the anti-HR23A antibody, which could also precipitate low but nonetheless detectable amounts of CEN2 (data not shown). These results are consistent with the observation that only a small proportion of the XPC complex in the final purification step contained HR23A (Fig. 1C).

NER Activities of the XPC Complex Components—From the above studies, we surmised that CEN2 might have some function in NER because it is present in virtually all of the purified XPC complexes. To directly test the role of CEN2 in NER, we purified recombinant nontagged CEN2 as described under “Experimental Procedures” (Fig. 4A). NER activity was measured on UV-irradiated SV40 minichromosomes in the cell-free NER assay reconstituted with purified NER factors (23). As shown in Fig. 4, B and C, in reactions containing all factors except the XPC complex, the addition of recombinant XPC alone resulted in weak NER activity, as reported previously (30). In the absence of XPC, CEN2 alone, HR23B alone, or a mixture of CEN2 and HR23B indicated no significant NER activity. However, HR23B stimulated the NER activity of XPC, as previously reported (30). On the other hand, the addition of CEN2 either alone or in combination with HR23B failed to produce any significant effect on XPC-dependent NER activity in vitro.
XPC-dependent NER stimulatory activity in defined conditions. Though the mechanism of NER stimulation by HR23B has not been well understood, several observations suggested that HR23B might function by stabilizing XPC. Previous studies showed that the XPC binding region of HR23B was necessary and sufficient for the stimulation of XPC-dependent NER activity in a semi-reconstituted reaction (29) and that the interaction of HR23B with XPC was important for the stimulation of NER by HR23B (30). Recently, we observed that the capacity of HR23B to stimulate XPC-dependent NER activity was greater during long incubations than short ones (data not shown). Taken together, these observations suggested that HR23B might function by protecting XPC protein from heat denaturation during the reaction.

To examine this possibility more directly, we designed a heat inactivation assay. As shown in Fig. 5A, XPC, CEN2, and HR23B were preincubated at various temperatures either alone or in various combinations. After preincubation, the proteins were combined and added to the XP-C whole cell extracts, and NER activity was measured (Fig. 5, B and C). When XPC was preincubated in the absence of either CEN2 and HR23B, ~75% activity was lost during a 1-h incubation at temperatures ≥37 °C. With a preincubation in the presence of HR23B, XPC retained most of its activity at 37 °C and showed a certain activity at higher temperatures. After a 1-h preincubation at 30 °C, a small but reproducible increase in XPC activity was observed in the presence of HR23B. These observations strongly suggest that the NER stimulatory activity of HR23B is due mainly to its ability to stabilize XPC. CEN2 alone did not show a significant effect on XPC stabilization. During the 42 °C preincubation, however, CEN2 increased the stability of XPC protein when HR23B was concomitantly present in the preincubation mixture, suggesting that CEN2 may help HR23B to stabilize XPC (see below).

To determine the reason for the loss of XPC function during the preincubation, we examined XPC binding to damaged DNA after preincubation (Fig. 6A). After preincubation, the components of the XPC complex were incubated with both UV-irradiated DNA and unirradiated DNA in reaction mixtures, and the DNA-XPC complexes were immunoprecipitated with resin conjugated to XPC antibodies (see “Experimental Procedures”). The DNA present in the precipitated complexes was purified and analyzed by Southern blotting (Fig. 6, B and C). The results showed that specific binding activity of XPC to damaged DNA was more resistant to preincubation at high temperatures in the presence of HR23B than in its absence. Moreover, CEN2 had a small but reproducible effect on XPC stabilization activity at 42 °C when combined with HR23B, whereas CEN2 alone had no significant effect on XPC stabilization. Finally, there was good correlation between the heat inactivation profiles of XPC as assayed by the NER assay and the DNA binding assay (compare Fig. 5C and 6C). We could not exclude the possibility that highly denatured XPC protein was not co-precipitated as efficiently as native XPC by the anti-XPC antibody conjugated resin. Therefore, the precipitated DNA may not reflect the real DNA binding activity of XPC for damaged DNA. However, we examined the DNA binding affinity of XPC for either damaged or nondamaged DNA in a filter binding assay and found that DNA binding activity decreased with increasing preincubation temperature. We also observed that CEN2 and HR23B prevented heat inactivation of XPC as measured by a filter binding assay (data not shown). Thus, CEN2 and HR23B are important for the stability of XPC protein and, especially, for the preservation of the binding activity of XPC for damaged DNA, which is essential for NER.

CEN2 Stabilizes XPC in Co-operation with HR23B—As shown in Figs. 5 and 6, CEN2 enhanced, albeit weakly, the ability of HR23B to stabilize XPC. To confirm this point, we performed the heat inactivation assay at different temperatures. As shown in Fig. 7, XPC preincubated only with HR23B
and lost more than 55% of its activity at 42 °C, 89% of its activity at 44 °C, and all of its activity at 46 °C compared with the activity of the control incubated at 0 °C. On the other hand, XPC preincubated with both CEN2 and HR23B retained nearly 69% of its activity at 42 °C, 47% of its activity at 44 °C, and 7% of its activity at 46 °C compared with the activity of the control incubated at 0 °C. These results further support our conclusion that CEN2 contributes to NER by binding to and stabilizing XPC.
tions for CEN2 did not show significant activity under standard conditions. Actually, CEN2 contributed to XPC stability, these observations strongly suggest that CEN2 has a role in XPC complex assembly from HeLa cells (data not shown). (Figs. 1 and 2) that directly interacts with XPC (Fig. 3). In experiments, the percentages of DNA recovered by the anti-XPC antibody. Symbols: square, XPC was preincubated in the presence of HR23B; circle, in the presence of both CEN2 and HR23B. Open symbols represent the percentage amounts of UV-irradiated plasmid bound to XPC, and solid symbols represent those of unirradiated DNA. Averages and experimental errors were calculated from two independent experiments.

**DISCUSSION**

**Roles of CEN2 in the XPC Complex**—In this study, we have demonstrated that CEN2 is a component of the XPC complex (Figs. 1 and 2) that directly interacts with XPC (Fig. 3). In contrast, we could detect neither XPC nor HR23B in a partially purified centrosome fraction from HeLa cells (data not shown). These observations strongly suggest that CEN2 has a role in XPC stability, thereby increasing the efficiency of NER (Fig. 5), whereas CEN2 did not show significant activity under standard conditions for in vitro NER. CEN2 stabilized XPC in vitro in cooperation with HR23B. Although the direct interaction was not observed between CEN2 and HR23B, it is possible that both proteins interact on XPC protein. It is also likely that the binding of CEN2 to XPC confers a proper interaction between XPC and HR23B. We suspect that CEN2 might be important for XPC complex formation in vivo, although CEN2 contributes relatively little to XPC stabilization in vitro (Fig. 5–7). Unprotected XPC is quite labile (Figs. 5 and 6) and susceptible to degradation during purification (Fig. 1C). Consequently, we believe that XPC is stabilized in human cells by virtue of association with CEN2 and HR23B.

Moreover, the complex reconstituted from the purified recombinant proteins showed lower activity in in vitro NER than the XPC complex purified from HeLa cells (data not shown). From our previous studies, we know this is mainly due to incomplete complex assembly in vitro (30). To examine the role of CEN2 in complex assembly, we purified XPC complexes from insect cells infected with recombinant baculoviruses expressing XPC and HR23B with or without CEN2 and measured XP-C complementation activity. The XPC complexes containing both CEN2 and HR23B showed slightly higher NER activity and solubility than XPC complexes containing HR23B alone. These observations support the results shown in this paper and strongly suggest that CEN2 plays a role in XPC complex assembly within cells.

**Roles of HR23B in the XPC Complex**—During the analyses of CEN2 function in in vitro NER, we found that HR23B stimulates NER mainly by stabilizing XPC and that CEN2 supports HR23B in this function. This is the first paper that clearly shows how HR23B stimulates in vitro NER by XPC-dependent manner, but we cannot rule out the possibility that HR23B has additional functions for NER in vivo (discussed below).

Studies in *S. cerevisiae* led to the idea that Rad23, the yeast counterpart of HR23B(A), has a function carried out by its N terminus ubiquitin-like (UbL) domain via interaction with proteasome. Previous studies showed that the UbL domains of Rad23 are important for cell survival after UV irradiation (44) and that Rad23 interacts with the 26S proteasome through its UbL domain (45). Recently, two completely different models have been proposed to explain the function of the UbL region of HR23B. Russell *et al.* (46) show that the proteasome subunit interacts with XPC and HR23B. Russell *et al.* (46) show that the proteasome subunit interacts with XPC, and that the ATPase activity, but not the proteolytic activity of the 19S subunit, is essential for NER. They suggest that the molecular chaperone function, but not the proteolytic function, of the 19S subunit is important for NER. However, Ortolan et al. (47) find that Rad23 can inhibit the degradation of proteosome substrates and show that a proteosome subunit mutation can partially rescue the UV sensitivity of a Rad23 deletion mutant. Taken together, they proposed that the UbL domain of Rad23 negatively regulates the degradation of repair factors after DNA damage, which may promote NER events. In neither report is there direct evidence of how an interaction between Rad23 and the proteosome acts to promote survival after UV irradiation. In human cells, a protein-protein interaction between HR23B and the 26S proteasome was observed (48), indicating that the above-mentioned mechanisms might be conserved in human cells.

Although most of the XPC-complementing complex contains CEN2 and HR23B, we also purified an XPC-HR23A-CEN2 complex (Fig. 1C) that was detected by co-immunoprecipitation (data not shown). HR23A was first identified as another human homologue of Rad23 by PCR methods (25) and was subsequently shown to functionally redundant to HR23B in NER both in the cell-free system (30) and in mouse embryonic fibroblast cells derived from knock out mice. Thus, we expect that HR23A can stabilize in a manner similar to HR23B. However, the functional differences between these two isoforms and the mechanism that generates the alternative complex still remain unclear.

**Centrosome Duplication and the XPC-HR23B-CEN2 Complex**—Genetic studies in yeast suggest that XPC complex might have a role in centrosome duplication in human cells. In *S. cerevisiae*, mutations in the gene encoding a counterpart of human centrin, CDC31, were also isolated as *dsk1*, dominant suppressors of the *kar1-Δ17* allele, which produces defects in SPB duplication (49). Another dominant suppressor of *kar1-Δ17* is *dsk2*, which encodes a protein that is very similar to Rad23 within the N-terminal UbL domain. The double deletion mutant, *Δrad23 Δdsk2* shows temperature-sensitive lethality

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3 M. Araki, C. Masutani, and F. Hanaoka, unpublished observations.

4 J. H. J. Hoeijmakers, personal communication.
caused by defects in SPB duplication, although neither UbL protein is essential for cell growth (50). These observations suggest that RAD23 has a role that is redundant to DSK2 in yeast SPB duplication and that RAD23 engages in an indirect genetic interaction with CDC31. Our findings appear to support the yeast genetic studies, and we suspect that Rad4 (the XPC counterpart in S. cerevisiae) interacts with Cdc31. Therefore, the Rad4 complex might have a role in SPB duplication in yeast.

In human cells, the intracellular localization of XPC changes dramatically during mitosis (51). During anaphase and telophase, the XPC protein becomes preferentially associated with chromatin, whereas it is distributed throughout the cell during metaphase. Thus, we can imagine that localization of XPC (Rad4 in yeast) complex is strictly regulated via CEN2 (Cdc31 in yeast) during mitosis and that the failure to regulate the Rad4 complex might cause some defect in SPB duplication in yeast. Further studies may show that XPC cancer-prone phenotypes are partly due to defects in the mechanism that couples cell division to NER and/or in the repair reaction involving the XPC-HR23B-CEN2 complex.

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