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Role of Interaction of XPF with RPA in Nucleotide Excision Repair

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Nucleotide excision repair (NER) is a very important defense system against various types of DNA damage, and it is necessary for maintaining genomic stability. The molecular mechanism of NER has been studied in considerable detail, and it has been shown that proper protein–protein interactions among NER factors are critical for efficient repair. A structure-specific endonuclease, XPF–ERCC1, which makes the 5′ incision in NER, was shown to interact with a single-stranded DNA binding protein, RPA. However, the biological significance of this interaction was not studied in detail. We used the yeast two-hybrid assay to determine that XPF interacts with the p70 subunit of RPA. To further examine the role of this XPF–p70 interaction, we isolated a p70-interaction-deficient mutant form of XPF that contains a single amino acid substitution in the N-terminus of XPF by the reverse yeast two-hybrid assay using randomly mutagenized XPF. The biochemical properties of this RPA-interaction-deficient mutant XPF–ERCC1 are very similar to those of wild-type XPF–ERCC1 *in vitro*. Interestingly, expression of this mutated form of XPF in the XPF-deficient Chinese hamster ovary cell line, UV41, only partially restores NER activity and UV resistance *in vivo* compared to wild-type XPF. We discovered that the RPA-interaction-deficient XPF is not localized in nuclei and the mislocalization of XPF–ERCC1 prevents the complex from functioning in NER.

Introduction

UV light penetrates cells, causing damage to DNA including 6-4 photoproducts (6-4 PPs) and cyclobutane pyrimidine dimers. These lesions are removed by nucleotide excision repair (NER),[1–3] and if not repaired, they inhibit replication and induce mutations by incorporation of incorrect nucleotides across from the damage.[4] Defects in NER lead to a rare autosomal recessive disease, xeroderma pigmentosum. Patients with this disease are extremely sensitive to sunlight and have a predisposition to skin cancer.[5]

Six factors, XPA, RPA, XPC–HR23, TFIIH, XPG, and XPF–ERCC1, are essential to reconstitute the excision step of NER *in vitro*.[6–8] Protein–protein interactions among these six factors are crucial for the efficient accomplishment of the excision reaction.[9–14] NER machinery must recognize DNA damage with a high specificity. None of the six NER factors recognizes DNA damage with such a high specificity.[15–18] It has been recently proposed that

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Abbreviations used: NER, nucleotide excision repair; dsDNA, double-stranded DNA; NLS, nuclear localization signal; EDTA, ethylenediaminetetraacetic acid; CPV, cell pellet volume; 6-4 PP, 6-4 photoproduct.

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cooperative damage recognition by XPA, RPA, and XPC–HR23 coupled with the kinetic proofreading by TFIIH provides a requisite damage-specific recognition in NER.[16,19,20] XPA, RPA, and XPC-HR23 (and TFIIH through the interaction with XPC) form an unstable complex at the site of DNA damage.[16–18] This cooperative binding achieves a higher specificity for damage recognition.[15,16] TFIIH contains the two DNA helicases, XPB and XPD, and the ATP-dependent unwinding by these helicases opens the DNA around the lesion, forming a bubble-like structure.[21,22] This unwinding by TFIIH further verifies the specificity of damage recognition and also promotes entry of XPG into the complex to further stabilize the damage recognition complex (kinetic proofreading).[17] Lastly, XPF–ERCC1 is recruited through an interaction between ERCC1 and XPA,[12,14,23] and dual incision by XPG at the 3′ of the damage and XPF–ERCC1 at the 5′ of the damage removes a short oligonucleotide with the damage.[24]

Human RPA is involved in various DNA transactions including DNA replication, repair, and recombination.[25] It was shown that RPA interacts with XPF–ERCC1, suggesting a potential role for this interaction in NER.[7,26] This interaction alone does not seem to be sufficient for recruiting XPF–ERCC1 to the pre-incision complex;[9] however, it has been suggested that RPA helps position XPF–ERCC1 properly.[27] To define the role of the interaction between XPF–ERCC1 and RPA, we isolated a point mutant form of XPF that specifically lacks the interaction with RPA but retains other known biochemical properties. This XPF mutant can only partially restore NER activity and UV resistance in the XPF-deficient UV41 cells. Unexpectedly, cell fractionation studies revealed that the RPA-interaction-deficient XPF was mislocalized in cytosolic fraction. We conclude that cellular localization of XPF is controlled by the interaction with RPA.

Results

XPF interacts with p70 through its N-terminal region

To determine which subunit of RPA, p70, p34, or p14 interacts with XPF, we performed the two-hybrid assay. XPF, fused to the DNA binding domain of GAL4, and each RPA subunit, fused to the activating domain of GAL4, were co-expressed in the tester strain of yeast, CG1945. Interaction was detected as growth on a restrictive medium between XPF and the p70 subunit of RPA, to a lesser degree between XPF and the p34 subunit, and no interaction was detected with the p14 subunit (Fig. 1a). As a control, XPF was co-expressed in yeast with ERCC1 fused to the activating domain of GAL4. The binding between XPF and ERCC1 is very strong and did result in a positive signal in this assay (Fig. 1a).

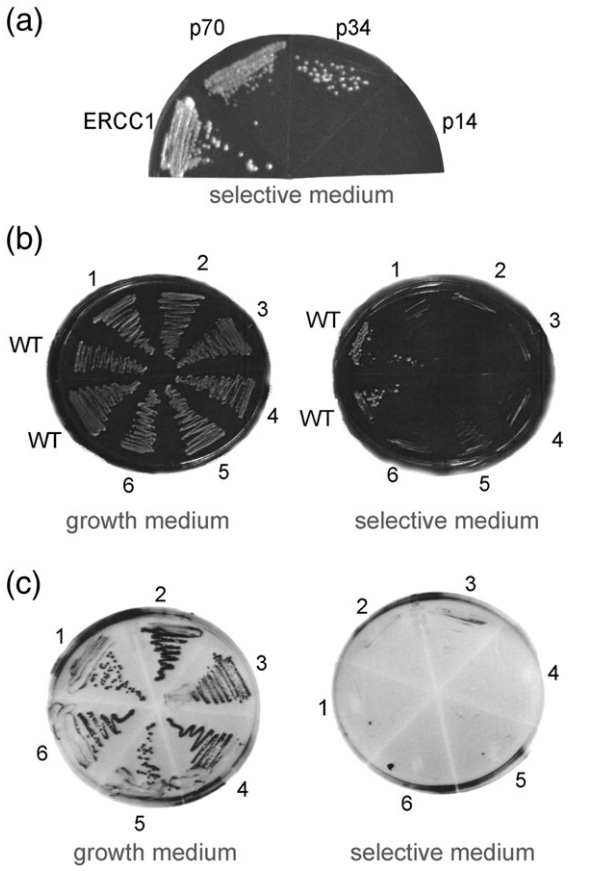

Fig. 1. Isolation of a mutant XPF that is defective in interaction with RPA. (a) XPF interacts with the p70 subunit of RPA. Wild-type XPF was co-expressed in CG1945 yeast with each of the RPA subunits or ERCC1 as a control and grown on synthetic growth medium (SD-Trp-Leu). Colonies were streaked on selective medium (SD-Trp-Leu-His) to detect an interaction. (b) Screening of RPA-interaction-deficient XPF mutants. Yeast colonies co-expressing randomly mutated XPF and p70 were streaked onto growth and selective media. Colonies that did not grow on the selective plates were taken from the growth medium, and the plasmid DNA was isolated. Two colonies co-expressing wild-type XPF and p70 were streaked as positive controls along with six colonies arising from co-expression of p70 and randomly mutated XPF, indicated as 1–6 in (b) and (c). (c) Identification of a mutation in the RPA-interaction-deficient mutant XPF. A repeat of the yeast two hybrid showing 6 of 12 candidate XPF genes that were re-cloned into fresh plasmids and co-transformed with the p70 gene.

To define the RPA interaction domain of XPF, we employed a reverse two-hybrid assay based on a screening strategy described by Krejci *et al.*[28] Random mutations were introduced into the XPF gene by propagating the XPF plasmid in a mutator