Functional analysis of DCAF7 and ERCC1-XPF interaction identified by affinity-purification and mass spectrometry

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学位論文要旨

Summary

The ERCC1-XPF complex is a structure-specific endonuclease, which is involved in various DNA repair pathways including nucleotide excision repair and also telomere maintenance. The heterodimer formation is essential for their endonuclease activity as well as the stability of each protein. However, the detailed mechanism of how a cellular level of ERCC1-XPF is regulated still remains elusive. In this study, to identify novel interacting factors, I conducted proteomic analysis after tandem purification and obtained massive numbers of candidates. Among them, I have focused on DDB1- and CUL4-associated factor 7 (DCAF7, also known as WDR68/HAN11). Immunoprecipitation experiments suggested its dominant association with XPF but not ERCC1. Interestingly, siRNA-mediated knockdown of DCAF7, but not DDB1, significantly attenuated the cellular level of ERCC1-XPF. The depletion of TCP1α, one of components of the molecular chaperon TRiC/CCT known to interact with DCAF7 and promote its folding, also reduced ERCC1-XPF level. Furthermore, I found that the depletion of DCAF7 causes inefficient repair of UV-induced (6-4) photoproducts, which can be rescued by ectopic overexpression of XPF. Altogether, this study strongly suggests that DCAF7 is a novel regulator of ERCC1-XPF protein level and cellular nucleotide excision repair activity.

Introduction

ERCC1-XPF is a structure-specific endonuclease involved in multiple DNA maintenance systems (McNeil and Melton, 2012; Manandhar et al., 2015); nucleotide excision repair (NER), interstrand crosslink repair, DNA double-strand break repair, and telomere maintenance. The ERCC1-XPF complex makes a nick at the junction of double-stranded to single-stranded DNA transition with a polarity of 5' to 3' direction. As a result, DNA substrates for this endonuclease contain a special structure such as a splayed arm, stem loop, bubble, or 3' flap. ERCC1 and XPF interact with each other via their C-terminal helix-hairpin-helix (HhH) regions. XPF protein contains a catalytic domain, although the structural support of ERCC1 is required for its endonuclease activity.

The heterodimer formation of ERCC1 and XPF is also necessary for their stability (Biggerstaff et al., 1993; Reardon et al., 1993; van Vuuren et al., 1993). XP-F patients carry a missense mutation in at least one allele of XPF/ERCC4 genes and show marginal or undetectable level of XPF protein, whereas ERCC1 level is also markedly reduced. Similarly, ERCC1-mutated cells showed low XPF level and ERCC1 knockdown attenuated XPF protein level without affecting mRNA level. ERCC1 was also shown to fold only in the presence of XPF in vitro, suggesting a possible role of XPF as a scaffold for proper ERCC1 folding. However, the detailed mechanism of how cellular levels of ERCC1 and XPF are regulated still remains elusive. In this study, I set a goal to identify novel interacting factors with ERCC1-XPF, and uncover their functions to obtain new knowledge

regarding regulatory mechanisms of ERCC1-XPF heterodimer.

Materials and Methods

For tandem affinity purification, I used Flp-In T-REx 293 cells conditionally expressing FLAG- and 6xHis-tagged ERCC1 or XPF upon tetracycline or doxycycline treatment. The double-tagged bait proteins were isolated by Ni-NTA agarose and subsequently by anti-FLAG antibody resin. Bound fractions eluted with FLAG peptide were concentrated and analyzed by mass spectrometry. Immunoprecipitation was conducted using HEK293T cells by transfecting with various combinations of expression plasmids and analyzing co-immunoprecipitation of partner proteins with epitope-tagged bait proteins. For RNA interference, U2OS or HeLa cells were transfected with various siRNAs using Lipofectamine™ RNAiMAX Transfection Reagent and basically incubated for 72 h. Cellular NER activity was evaluated using ELISA by measuring (6-4) photoproducts in genomic DNA isolated from the cells irradiated with 10 J/m^2 of UV-C and incubated for repair.

Results and Discussion

Identification of novel interacting factors with ERCC1-XPF.

To explore a novel interacting protein(s) of ERCC1-XPF, I utilized Flp-In T-REx 293 cells conditionally expressing FLAG- and 6xHis-tagged ERCC1 or XPF. After 48-h treatment with tetracycline, cell lysates were processed for tandem purification with Ni-NTA agarose and then anti-FLAG antibody resin. Mass spectrometry showed 699 candidates including 14 kinds of E3 ligases and 5 kinds of deubiquitinases. After co-immunoprecipitation analysis and siRNA-mediated knockdown analysis, I identified DDB1- and CUL4-associated factor 7 (DCAF7, also known as WDR68/HAN11) as an interacting protein with ERCC1-XPF, which potentially regulate the cellular level of ERCC1-XPF. I decided to focus on the functional analysis of DCAF7 and ERCC1-XPF interaction.

Functional analysis of DCAF7 and ERCC1-XPF interaction.

We first asked which subunit of the heterodimer associates with DCAF7. I conducted co-immunoprecipitation analysis using XP2YO(SV) cells from XP-F patient, in which mutant XPF protein is undetectable and ERCC1 level is also very low due to its destabilization. As a result, DCAF7 was shown to interact with XPF but not ERCC1, suggesting indirect interaction with ERCC1 via a direct association with XPF.

To examine a functional role of DCAF7 and ERCC1-XPF interaction, U2OS cells were transfected with DCAF7 siRNA. Depletion of DCAF7 reproducibly causes significant reduction of ERCC1-XPF level, whereas the cellular levels of other NER factors (DDB1, DDB2 and XPB) were not affected. The molecular chaperon TRiC/CCT, which plays an important role in proper folding of many proteins (Spiess et al., 2004; Horwich et al., 2007), has been reported to interact with DCAF7

and promote its folding and nuclear localization (Miyata et al., 2014). I examined the impact of depletion of TCP1α, one of eight subunits of TRiC/CCT, on the protein level of ERCC1-XPF. As expected, TCP1α knockdown similarly reduced ERCC1-XPF level, but not DDB2 and XPA levels, although less efficiently compared to DCAF7 knockdown. Taken together, these results suggest that DCAF7 is required for maintaining the normal cellular levels of ERCC1-XPF.

DCAF7 is known to form a complex with Cul4-DDB1 and to function as a substrate receptor that determines the substrate specificity of the E3 ligase complex. I wished to know whether the depletion of DDB1 also causes the downregulation of ERCC1-XPF. However, transfection of DDB1 siRNA affected neither the basal levels of ERCC1-XPF nor the downregulation of ERCC1-XPF upon DCAF7 depletion, indicating that DDB1 and probably also Cul4 are unlikely to participate in this mechanism.

ERCC1-XPF is an indispensable factor for the dual incision reaction of NER. Given that DCAF7 depletion reduces the protein level of ERCC1-XPF, I wished to know the impact of DCAF7 depletion on cellular NER activity. DCAF7-depleted HeLa cells showed significantly slower repair kinetics of UV-induced (6-4) photoproducts, compared with undepleted cells. Importantly, the attenuated NER activity was rescued by doxycycline-induced overexpression of exogenous XPF in Flp-In T-REx 293/FLAG-XPF-3xHis cells. Collectively, these results suggest that DCAF7 plays an important role in maintaining normal NER activity by stabilizing XPF and hence ERCC1-XPF heterodimer.

In this study, I demonstrated that DCAF7 is a novel interacting protein of ERCC1-XPF and plays an important role in maintaining the cellular level of ERCC1-XPF and hence nucleotide excision repair activity. These findings provides a new insight into the regulatory mechanism of cellular stability of NER factors, which is crucial for sufficient NER activity. In addition, the cellular level of ERCC1-XPF is well known to influence the efficacy of cisplatin-based anticancer chemotherapy. The knowledge from this study may be applicable to new strategy for controlling ERCC1-XPF level in cancer cells.

Reference

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審査結果の要旨

川原弘明氏から提出された学位論文について、上記 5 名の審査委員による査読後、2019 年 7 月 26 日に口頭発表会が行われ、同日の最終審査委員会で審議した結果、以下のとおり判定した。

本研究で注目している ERCC1-XPF は、ヌクレオチド修復（NER）をはじめとする複数の DNA 修復機構で働く重要なエンドヌクレアーゼであるが、ヘテロダイマーの相互依存的安定性や細胞内レベルの調節メカニズムは未解明のままである。本研究では、タンデムアフィニティ精製と質量分析を組み合わせて ERCC1-XPF の新規相互作用因子の探索を行い、候補の中から DCAF7 に注目して詳細に解析した。まず、免疫沈降実験から DCAF7 が主に XPF と相互作用することを示し、siRNA によるノックダウンで ERCC1-XPF の細胞内レベルが顕著に減少することを見出した。また、分子シャペロン TRiC/CCT 複合体の構成因子である TCP1α のノックダウンでも同様の減少が見られ、DCAF7 は TRiC/CCT も関与して ERCC1-XPF を安定化していると考えられた。一方、DCAF7 の既知機能である Cul4-DDB1 E3 リガーゼ複合体の基質受容体としての働きは関与せず、プロテアソーム系の関与も小さいことが示された。重要なことに、DCAF7 のノックダウンはヒト細胞の NER 活性を低下させ、この低下は XPF の過剰発現で回復することを明らかにし、DCAF7 は ERCC1-XPF の安定化を通して NER 活性の維持に寄与していると考えられた。

本研究は、細胞内の ERCC1-XPF レベルと NER 活性を制御する新規調節因子として DCAF7 を同定したものであり、細胞内の NER 調節メカニズムに新たな視点をもたらしたものと評価され、博士（創薬科学）の学位に値すると判定した。