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UV-DDB-dependent regulation of nucleotide excision repair kinetics in living cells

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ABSTRACT

Although the basic principle of nucleotide excision repair (NER), which can eliminate various DNA lesions, have been dissected at the genetic, biochemical and cellular levels, the important *in vivo* regulation of the critical damage recognition step is poorly understood. Here we analyze the *in vivo* dynamics of the essential NER damage recognition factor XPC fused to the green fluorescence protein (GFP). Fluorescence recovery after photobleaching analysis revealed that the UV-induced transient immobilization of XPC, reflecting its actual engagement in NER, is regulated in a biphasic manner depending on the number of (6-4) photoproducts and titrated by the number of functional UV-DDB molecules. A similar biphasic UV-induced immobilization of TFIIH was observed using XPB-GFP. Surprisingly, subsequent integration of XPA into the NER complex appears to follow only the low UV dose immobilization of XPC. Our results indicate that when only a small number of (6-4) photoproducts are generated, the UV-DDB-dependent damage recognition pathway predominates over direct recognition by XPC, and they also suggest the presence of rate-limiting regulatory steps in NER prior to the assembly of XPA.

1. Introduction

DNA is highly susceptible to damage caused by exposure to agents from both exogenous and endogenous sources. Unrepaired DNA lesions may induce mutations as well as chromosomal aberrations, thereby leading to cellular malfunctioning including cancer, and they may also cause cellular senescence or cell death implicated in damage-induced ageing. Multiple genome maintenance processes that counteract the deleterious effects of DNA lesions have evolved. The heart of this defense system is formed by several DNA repair mechanisms [1]. One of the most versatile DNA repair pathway is nucleotide excision repair (NER), which is able to eliminate a wide variety of lesions that destabilize the DNA double helix, such as ultraviolet light (UV)-induced (6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs) [2]. The severe clinical consequence associated with three human rare autosomal recessive diseases, xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) [3], which are based on inherited NER defects, illustrates the biological relevance of this process. So far, seven NER-deficient genetic complementation groups for XP (XP-A through XP-G), two for CS (CS-A and CS-B) and one for TTD (TTD-A) have been identified, and in all cases the responsible genes have been cloned [4,5]. On the other hand, one XP group, called a variant form of XP (XP-V), is exceptional since mutations confer defects in translesion DNA synthesis but not in NER [6,7].

NER consists of two subpathways: transcription-coupled NER (TC-NER), which removes DNA damage specifically from the transcribed strand of active genes, and global genome NER (GG-NER), which surveys the entire genome for damage. A major difference in the molecular mechanism of these two modes of NER is evident in the damage recognition step. While RNA polymerase II stalling at a damaged site is probably employed as a damage sensor in TC-NER [8], a complex containing the XPC protein, an XP-related gene product, plays an essential role in damage recognition in GG-NER [9–11]. Subsequent steps after damage recognition are thought to be shared by both subpathways: first, transcription factor IIH (TFIIH), which is composed of ten subunits containing two helicase proteins (XPB and XPD), unwinds the DNA duplex around the lesion in the

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presence of XPA, replication protein A and XPG [12–19]. An oligonucleotide of approximately 30 nucleotides including the lesion is then excised by two structure-specific endonucleases, ERCC1-XPF and XPG, which make incisions at sites 5′ and 3′ to the lesion, respectively [20–23]. The resulting single-stranded gap is filled by DNA polymerase (δ or ε) in conjunction with proliferating cell nuclear antigen (PCNA) and replication factor C, and the DNA strands are finally rejoined by DNA ligase I or DNA ligase III/XRCC1 [24–26].

XPC is part of a stable heterotrimeric complex with one of the two mammalian homologs of *Saccharomyces cerevisiae* Rad23 (RAD23A or RAD23B), which stabilizes and stimulates XPC, and centrin 2, which is also known as a centrosomal protein [9,27–33]. XPC is an essential protein for the initiation of GG-NER and binds various DNA lesions *in vitro* including UV-induced 6-4PPs [9,34]. Biochemical analyses revealed that XPC binds a certain structure of DNA, which is generated by the presence of lesions, probably enabling the recognition of structurally unrelated DNA lesions [35,36].

On the other hand, UV-damaged DNA binding protein (UV-DDB) also participates in damage recognition in GG-NER. UV-DDB was initially identified as a heterodimer consisting of the DDB1 and DDB2 subunits, the latter of which is implicated in XP group E [37–41]. Purified UV-DDB exhibits a much higher binding affinity and specificity than XPC for both major UV-induced photolesions CPDs and 6-4PPs [9]. Although XP-E cells show obvious defects in the GG-NER of CPDs, the same cells can remove 6-4PPs efficiently from genomic DNA, indicating that UV-DDB *in vivo* would be important particularly for the recognition of CPDs [42,43]. UV-DDB can be recruited to UV-damaged site even in the absence of XPC and it promotes recruitment of XPC [44–48]. Although UV-DDB is dispensable for cell-free NER reaction [24,49], *in vitro* reconstituted NER reaction was stimulated by the addition of UV-DDB under certain conditions [47,50,51]. Furthermore, UV-DDB physically interacts with XPC and the associated E3 ligase containing cullin 4A and Roc1 ubiquitylates XPC according with UV irradiation [52]. These findings suggest that UV-DDB is important for damage sensing *in vivo*.

Thus far, *in vitro* NER reactions have been successfully reconstituted with defined damaged DNA substrates and a set of highly purified proteins [24,49,50]. These studies have provided detailed insights into the reaction mechanism of GG-NER. However, it remains to be determined how the initial step of GG-NER, damage sensing, is regulated in time and space within a living mammalian cell nucleus. Since the initial steps of complex biological processes would be important for its regulation, it is expected that GG-NER may be regulated by initiating factors such as XPC and UV-DDB. To understand the molecular mechanism regulating GG-NER *in vivo*, we previously assessed the mobility and reaction kinetics of several GFP-tagged NER proteins in living cells by the fluorescence recovery after photobleaching (FRAP) technique, both in the presence and absence of UV irradiation [53–58]. However, how UV-DDB-dependent damage recognition of XPC is coordinated within GG-NER and how UV-DDB participates in the further assembly of other NER factors into functional NER complexes remain elusive.

To address these key issues, in the present study, we analyzed in further detail the *in vivo* dynamics of the GFP-tagged XPC protein. Our results not only provide novel insights into the concerted actions of XPC and UV-DDB in damage recognition, but also shed light on some previously uncovered aspects of regulation, including those governing the later step of NER in living cells.

2. Materials and methods

2.1. Cell culture

Simian virus 40-transformed human fibroblasts from a normal individual (WI38 VA13) or XP patients, XP2OSSV (XPA-deficient), XPCS2BASV (XPB-deficient), and XP4PASV (XPC-deficient), as well as stable transfectants were cultured at 37 °C in an atmosphere of 5% CO_2 with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS).

2.2. Establishment of cell lines

Two stably transformed cell lines, XP2OSSV cells expressing GFP-XPA and XPCS2BASV cells expressing XPB-GFP, were established as previously described [57,59]. The cDNA encoding GFP-XPC was cloned into the vector pIREShyg (Clontech), from which the GFP-XPC and hygromycin B resistance genes were transcribed as a single mRNA. Twenty micrograms of the construct was linearized and electroporated into a 100-mm dish of XP4PASV cells using Gene Pulser II (Bio-Rad). Stable transformants were selected initially with 200 μg/ml hygromycin B (Invitrogen), which gave rise only to clones overexpressing GFP-XPC. The concentration of hygromycin B in the culture medium was then gradually reduced to lower the selective pressure, which resulted in a natural drop of protein levels, thereby allowing isolation of a clone expressing GFP-XPC at nearly physiological levels.

2.3. Measurement of the repair rates of UV photolesions in vivo

To avoid dilution of lesions by DNA replication, cells (in 100-mm dishes) were treated for 2 h with 6 mM thymidine before each experiment. The cells were then irradiated with 10 or 40 J/m^2 of UVC (under a germicidal lamp with a peak at 254 nm) and further cultured for various time periods in the presence of 6 mM thymidine. Genomic DNA was purified with the QIAamp Blood mini kit (Qiagen), and the levels of remaining 6-4PPs and CPDs were measured by an enzyme-linked immunosorbent assay (ELISA) using the lesion-specific monoclonal antibodies 64M-2 and TDM-2, respectively [60].

2.4. Immunohistochemistry

Prior to immunostaining, 4×10^5 WI38 VA13 cells were cultured overnight in the presence of 0.01% (w/v) polystyrene microsphere beads (Polysciences) in a 25-cm^2 flask. After unincorporated beads were washed out with culture medium, the cells were mixed with an equal number of XP4PASV transformant cells expressing GFP-XPC and seeded in 35-mm glass-bottom dishes (MatTech) at a density of 1.5×10^5 cells/dish for immunohistochemistry. The cells were fixed with 1.6% (v/v) formaldehyde (Wako Pure Chemicals) for 15 min at 4 °C. The dishes were washed twice with ice-cold phosphate-buffered saline (PBS) and subsequently permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min on ice. The cells were again washed twice with ice-cold PBS and then incubated with 3% (v/v) FBS in PBS to block non-specific antibody adsorption. During the following procedures, the dishes were washed three times with PBS after each incubation. The cells were incubated at room temperature for 1 h with an anti-XPC (FL) antibody and then for 1 h with an anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (Molecular Probes, 1:500 dilution). Both antibodies were diluted with PBS containing 0.05% (v/v) Tween 20 and 0.5% (v/v) FBS. Fluorescence microscopy was performed using an Olympus IX71 instrument and Metamorph software (Mitani). For the analysis of localization of GFP-XPC, living cells were treated at 37 °C for 10 min with 10 μg/ml of Hoechst 33342 and analyzed with the same microscope system.

2.5. Preparation of cell lysates

For immunoblot analysis of the expression level of XPC or DDB2, cells in 60-mm dishes were washed twice with ice-cold PBS and