Implication of SUMO E3 ligases in nucleotide excision repair

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Abstract Post-translational modifications alter protein function to mediate complex hierarchical regulatory processes that are crucial to eukaryotic cellular function. The small ubiquitin-like modifier (SUMO) is an important post-translational modification that affects transcriptional regulation, nuclear localization, and the maintenance of genome stability. Nucleotide excision repair (NER) is a very versatile DNA repair system that is essential for protection against ultraviolet (UV) irradiation. The deficiencies in NER function remarkably increase the risk of skin cancer. Recent studies have shown that several NER factors are SUMOylated, which influences repair efficiency. However, how SUMOylation modulates NER has not yet been elucidated. In the present study, we performed RNAi knockdown of SUMO E3 ligases and found that, in addition to PIASy, the polycomb protein Pc2 affected the repair of cyclobutane pyrimidine dimers. PIAS1 affected both the removal of 6–4 pyrimidine pyrimidone photoproducts and cyclobutane pyrimidine dimers,

whereas other SUMO E3 ligases did not affect the removal of either UV lesion.

Keywords SUMOylation · SUMO E3 ligase · Nucleotide excision repair · UV damage

Introduction

DNA damage arises continually from endogenous and environmental agents, which causes genomic instability leading to cancer. Therefore, DNA-damage response mechanisms, including various repair and cell cycle control pathways, protect organisms against genomic insults. Ultraviolet (UV) is one of the agents that causes DNA damage, and is divided into three groups (UVA, UVB, and UVC) according to its wavelength range. Since UVC and almost all UVB, which are harmful to organisms, are blocked by the ozone layer, they do not reach the surface of the earth (Budden and Bowden 2013; Biverstål et al. 2008). However, a decrease in ozone levels has led to an increase in unabsorbed and harmful UV radiation on the surface of the earth (Schuch et al. 2013; Coldiron 1992). UV damages skin, resulting in wrinkles/changes in texture and pigmentation as well as cancer. Therefore, understanding the repair mechanism of UV-induced DNA damage is of importance.

Nucleotide excision repair (NER) is the most important mechanism that can repair UV-induced DNA

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damage. This repair pathway is divided into two subpathways; global genome NER (GG-NER) and transcription-coupled NER (TC-NER). Mutations in various proteins involved in GG-NER have been shown to cause Xeroderma pigmentosum (XP), which is an autosomal recessive genetic disorder of DNA repair. The ability of patients with XP to repair UV-induced DNA damage is deficient, and they are at high risk of skin cancer. The proteins in which mutations cause XP have been designated XPA ~ XPG. UV is known to induce different types of damage including cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidone photoproducts (6-4PPs), which alter the conformation of DNA, and inhibit DNA replication and transcription. Except for the first step of lesion detection, common pathways remove CPDs and 6-4PPs. Of the XP proteins, XPC, XPA, XPF, and XPG have been implicated in the common process of repairing UV damage with many other proteins. DDB2 (XPE) primarily recognizes and binds CPDs, followed by the recruitment of XPC, whereas XPC can independently recognize 6-4PPs (Fitch et al. 2003; Sugasawa et al. 2005). Previous studies reported the involvement of over 30 proteins in NER (Li 2012). Some of these proteins are subject to post-translational modifications such as phosphorylation, ubiquitination, and poly(ADP-ribosylation). Modifications of the small ubiquitin-related modifiers (SUMOs) have also been reported.

SUMOylation appears to regulate these distinct processes by modulating the specific protein–protein interactions of pre-existing proteins (Gareau and Lima 2010; Johnson 2004; Geiss-Friedlander and Melchior 2007; Capili and Lima 2007). Three SUMO isoforms are expressed in mammals: SUMO1, SUMO2, and SUMO3 (Vertegaal et al. 2006). SUMO is generally covalently attached to proteins through the ϵ -amino group of a specific lysine residue that contains the consensus sequence ψ KXE (ψ , large hydrophobic residue) (Gareau and Lima 2010; Geiss-Friedlander and Melchior 2007; Capili and Lima 2007; Rodriguez et al. 2001). The conjugation of SUMO is similar to that of ubiquitin. There is only one known heterodimeric SUMO-activating E1 enzyme (Aos1/Uba2) and SUMO-conjugation E2 enzyme (Ubc9). An E3 ligase enhances the transfer of SUMO molecules from E2 to substrates. To date, several structurally unrelated proteins have been identified as SUMO E3 ligases. Although the PIAS family of SUMO E3 ligases is

similar to RING containing ubiquitin E3 ligases, the SUMO ligases RanBP2 and Polycomb group protein 2 (Pc2) are unrelated to ubiquitin E3 ligases (Gareau and Lima 2010; Johnson 2004).

We previously reported that DDB2 was SUMOylated by the E3 ligase PIASy in response to UV irradiation, and PIASy knockdown reduced the removal of CPDs, but not 6-4PPs (Tsuge et al. 2013). In the present study, we performed RNA interference (RNAi) screening on SUMO E3 ligases to reveal the framework of SUMOylation and its importance in NER. We found that, in addition to PIASy, Pc2 was implicated in the repair of CPDs. On the other hand, PIAS1 knockdown had a clear effect on the repair of both CPDs and 6-4PPs.

Materials and methods

Cell culture and UV irradiation

HeLa cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in DMEM high glucose medium (Sigma-Aldrich Japan, Tokyo, Japan) containing 10 % fetal bovine serum (FBS, Biological Industries, Beit-Haemek, Israel), 100 U/ml penicillin G (Wako Pure Chemical Industries, Osaka, Japan), and 100 μ g/ml streptomycin (Wako Pure Chemical Industries). To induce photo lesions, exponentially growing cells were washed with phosphate-buffered saline (PBS) and irradiated with UV using a germicidal lamp (GL-10, Hitachi, Japan) calibrated to deliver a dose of 0.5 J/m² per second. Cells were incubated in growth medium following UV exposure to allow the repair of DNA lesions for a defined period.

Knockdown of E3 ligases

To assess the influence of SUMO E3 ligase knockdown against CPD and 6-4PP removal, 40 nM of siRNAs were transfected to semi-confluent HeLa cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), and UV (5 J/m² for CPDs and 15 J/m² for 6-4PPs) was irradiated at 48 h post-transfection. The siRNA sequence for PIASy was 5'-GGAGUAAGAGUGGACUGAA-3'. The sequences of other siRNAs used were described previously (Tsuge et al. 2013).

Cell fractionation

Cells (1×10^6) were washed with PBS, harvested, and resuspended in 10 mM HEPES–KOH (pH 7.9), 1.5 mM MgCl_2 , and 10 mM KCl, followed by incubation on ice for 20 min. Cell lysates were mixed and centrifuged at 15,000 rpm, 4 °C for 10 s. The supernatants were discarded and the nuclear pellets were resuspended in 20 mM HEPES–KOH (pH 7.9), 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, and 25 % glycerol (Wako Pure Chemical Industries). After incubation on ice for 20 min, cells were centrifuged at 15,000 rpm, 4 °C for 2 min. The pellets were resuspended in 1 x SDS sample buffer (65 mM Tris–HCl pH 6.8, 3 % SDS, 10 % glycerol and 5 % β -mercaptoethanol (Wako Pure Chemical Industries).) (chromatin fractions), and the proteins modified by SUMO1 and SUMO2 were detected by western blotting. SUMO1 and 2 antibodies were generated by immunizing rabbits with affinity purified proteins. The mouse β -actin antibody was purchased from Applied Biological Materials Inc. Goat anti-rabbit IgG–HRP and goat anti-mouse IgG–HRP (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies.

ELISA

Genomic DNAs were purified from UV-irradiated cells using MagExtractor-Genome (TOYOBO, Osaka, Japan). DNAs (10 ng in PBS) were denatured and distributed to protamine sulfate (Wako Pure Chemical Industries)-precoated 96-well assay plates (Asahi Glass, Tokyo, Japan), which were then dried completely. Plates were then washed with PBS containing 0.05 % Tween-20, followed by blocking with 2 % FBS. After washing, either mouse monoclonal anti-CPD or mouse monoclonal anti-(6-4)PP antibodies (Cosmo Bio, Tokyo, Japan) diluted in PBS were distributed to DNA-coated plates, which were then incubated at 37 °C for 30 min. After washing, these plates were incubated with the Biotin-XX F(ab')₂ fragment of anti-mouse IgG (H+L) (Invitrogen Life Technologies) at 37 °C for 30 min, followed by incubation with Horseradish Peroxidase–Streptavidin (Invitrogen Life Technologies). Captured CPDs and 6-4PPs were visualized using BD OptEIA™ Substrate Reagents A and B (BD Biosciences, San Jose, CA).

Quantitative PCR

Total RNA was prepared from transfected HeLa cells, and 3 μg of RNA was subjected to reverse transcription by reverse transcriptase (ReverTra Ace; TOYOBO) to generate cDNAs according to the manufacturer's instructions. Primers specific to each protein are described in Supplementary Table 1. As a control, PCR was performed with the same samples using primers specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Samples were analyzed using THUNDER-BIRD™ SYBR qPCR Mix (TOYOBO) and LightCycler (Roche Diagnostics, Tokyo, Japan) for quantitative PCR (qPCR).

Results

Chromatin-bound SUMOylated proteins increased in a UV-dependent manner

Previous studies showed that many cell stresses linked to SUMOylation; heat shock, osmotic stress, and oxidative stress, increased global SUMOylation by the SUMO-2/3 isoforms (Tempé et al. 2008; Bossis and Melchior 2006; Saitoh and Hinchey 2000). In addition, some NER proteins were found to be SUMOylated after UV irradiation in *Saccharomyces cerevisiae* (Psakhye and Jentsch 2012; Silver et al. 2011). The NER proteins, XPC and centrin-2, were also reported to be the substrates of SUMOylation in mammalian cells (Wang et al. 2005; Klein and Nigg 2009). To determine the importance of the SUMOylation of NER proteins, chromatin fractions were obtained from UV-irradiated HeLa cells, and SUMOylated proteins were detected using either the anti-SUMO1 or anti-SUMO2 antibody, with anti- β -actin being used as a loading control. Global analysis of SUMOylation revealed that many SUMOylated proteins were detected in a high molecular weight range from 90 kDa to 200 kDa with the SUMO1 and SUMO2 antibodies shortly after UV irradiation (Fig. 1). When total cell lysates were analyzed in the same manner, a clear increase was not observed with either SUMO1 or SUMO2 (data not shown). This result indicated that UV-induced SUMOylation occurred on proteins in chromatin or SUMOylated proteins were recruited to chromatin upon UV irradiation.

Fig. 1 SUMOylated proteins accumulated on chromatin in a UV-dependent manner. The chromatin fraction was obtained from HeLa cells 5 min after UV irradiation (20 J/m²), and SUMO1 or SUMO2 proteins were detected by the anti-SUMO1 or anti-SUMO2 antibody

Several SUMO E3 ligases affected repair of CPDs and 6-4PPs in HeLa cells

The results shown in Fig. 1 demonstrated that many proteins in chromatin were SUMOylated; however, the implication of these proteins and their SUMOylation remains unclear. We previously demonstrated the involvement of PIASy in NER (Tsuge et al. 2013). Only six typical SUMO E3 ligases have been reported so far (Johnson 2004). Therefore, we investigated whether another five SUMO E3 ligases were implicated in the repair of UV-induced DNA damage.

For the measurement of NER efficiency, we chose HeLa cells because the functional NER-complex was originally isolated from this cell line and the cells have been widely used as mammalian NER-proficient model (Groisman et al. 2003; Wang et al. 2005; Fei et al. 2011). HeLa cells transfected with the siRNAs of SUMO E3 ligases were subjected to UV irradiation, and 6-4PPs and CPDs were measured in the HeLa genome by ELISA (Fig. 2a). The expression levels of all E3 ligases were less than 30 % of the original levels by qPCR (Fig. 2b). Approximately 20 % of 6-4PPs and 35 % of CPDs remained in mock transfected control cells 6 h and 24 h post UV irradiation, respectively, as reported previously (Wakasugi et al. 2002; Fei et al. 2011; Pines et al. 2012). In other words, approximately 80 % of 6-4PPs and 65 % of CPDs were repaired 6 h and 24 h post UV irradiation, respectively. The repair efficiencies of 6-4PPs and CPDs were not affected by the knockdown of PIAS2, PIAS3, or RanBP2 (Fig. 2). The depletion of Pc2 had

no effect on the excision of 6-4PPs, but hampered the repair of CPDs; 60 % of CPDs remained 24 h post irradiation, similar to PIASy knockdown as described previously (Tsuge et al. 2013). The knockdown of PIAS1 led to a significant reduction in the repair of both 6-4PPs and CPDs; 35 % of 6-4PPs remained 6 h post UV irradiation, while 60 % of CPDs remained 24 h post irradiation. In these experiments, the growth of siRNA-treated cells was not significantly different from that of the control cells (data not shown). These results revealed that, in addition to PIASy, Pc2 and PIAS1 are also implicated in NER.

Discussion

The SUMOylation of proteins implicated in DNA-damage repair systems appears to play a key role in advancing the entire process; however, the mechanisms involved have not been completely elucidated (Moschos and Mo 2006; Bergink and Jentsch 2009). Many proteins involved in DNA damage repair were shown to be SUMOylated in yeast, and the interaction of these proteins through SUMO and SIM (SUMO interacting motif) was supposed to be important for an effective repair reaction (Psakhye and Jentsch 2012). As shown in Fig. 1, many proteins were SUMOylated upon UV irradiation. Among the SUMO E3 ligases, PIAS2 and 3, and RanBP2 did not affect the repair of either CPDs or 6-4PPs. On the other hand, Pc2, a subunit of Polycomb repressive complex 1 (PRC1), reduced the repair of CPDs, while PIAS1 affected the removal of both CPDs

Fig. 2 Knockdown of SUMO E3 ligase and its effects on 6-4PP and CPD repair. **a** Remaining DNA lesions. Asterisk indicates significant differences by the Student's *t* test ($p < 0.05$). Data

and 6-4PPs (Fig. 2). Since Pc2 has intrinsic SUMO E3 activity, CPD-repair specific proteins such as DDB2 were expected to be SUMOylated by Pc2. However, we could not detect the SUMOylation of DDB2 by Pc2 under physiological conditions (Tsuge et al. 2013). This does not rule out the possibility that an unidentified protein involved in the repair of CPDs was SUMOylated. By now, the precise mechanism for the

are expressed as mean value with standard deviation of three independent experiments. **b** Efficiency of knockdown measured by real-time PCR

involvement of Pc2 in CPD repair still remains unclear. However, Pc2 was shown to SUMOylate the other PRC1 subunit Bmi1, and the SUMOylation is prerequisite for the recruitment of Bmi1 to damaged sites for double strand-break repair (Ismail et al. 2012). Bmi1 subsequently recruited Ring1B for ubiquitination of the histone H2A (Ismail et al. 2010), which may be essential for progress of the repair reaction. UV-dependent

ubiquitination of the histone H2A has also been reported (Bergink et al. 2006). Thus, it is possible that a similar mechanism is involved in the repair of UV lesions.

Pc2 is known to SUMOylate centrin-2, which is a component of the XPC complex in a UV-independent manner (Klein and Nigg 2009). XPC is a sensor protein of 6-4PPs that is also essential for the identification of CPDs in cooperation with DDB2. Our result showing that Pc2 only affected CPD repair suggests that the SUMOylation of centrin-2 may be dispensable for the repair reaction.

Although we tried to measure changes in the bulk SUMOylation in the PIAS1-knockdown cells, we could not detect obvious changes in both UV-dependent and independent SUMOylation (data not shown). This result suggests that SUMOylation of specific target proteins may be important, which does not influence the bulk SUMOylation. Since the knock-down of PIAS1 blocked the repair of both 6-4PPs and CPDs, the E3 ligase appeared to modify proteins downstream of the XPC reactions such as XPC itself, XPF, XPA, XPB, and XPG. ATP-dependent DNA helicase XPB (mammalian orthologue of yeast Ssl2) has been identified as a candidate for SUMOylation in yeast (Silver et al. 2011). Therefore, we examined whether XPB could indeed be SUMOylated by PIAS1 in mammalian cells. In fact, when FLAG-XPB, HA-SUMO1, and PIAS1 were transiently expressed in HeLa cells, SUMOylated XPB was detected depending on PIAS1 (Tsuge et al. unpublished results). Now, we are trying to identify target proteins of PIAS1 including XPB.

In the present study, we found that, in addition to PIASy, PIAS1 and Pc2 also affected DNA repair. These findings are consistent with Siz1 and Siz2, orthologues of mammalian PIAS family SUMO E3 ligases, being necessary for efficient GG-NER in *Saccharomyces cerevisiae* (Silver et al. 2011). Further studies are needed to clarify the detailed mechanism and importance of SUMOylation in NER.

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