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XPA is susceptible to proteolytic cleavage by cathepsin L during lysis of quiescent cells

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Abstract

The xeroderma pigmentosum group A (XPA) protein plays an essential role in the removal of UV photoproducts and other bulky lesions from DNA as a component of the nucleotide excision repair (NER) machinery. Using cell lysates prepared from confluent cultures of human cells and from human skin epidermis, we observed an additional XPA antibody-reactive band on immunoblots that was approximately 3–4 kDa smaller than the native, full-length XPA protein. Biochemical studies revealed this smaller molecular weight XPA species to be due to proteolysis at the C-terminus of the protein, which negatively impacted the ability of XPA to interact with the NER protein TFIIH. Further work identified the endopeptidase cathepsin L, which is expressed at higher levels in quiescent cells, as the protease responsible for cleaving XPA during cell lysis. These results suggest that supplementation of lysis buffers with inhibitors of cathepsin L is important to prevent cleavage of XPA during lysis of confluent cells.

Keywords

Nucleotide excision repair; UV radiation; XPA; proteolysis; cathepsin; quiescence; keratinocyte

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Competing financial interest

The authors declare that they have no conflict of interest.

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1. INTRODUCTION

The XPA (xeroderma pigmentosum group A) protein is essential for nucleotide excision repair (NER) in mammalian cells by coordinating the recruitment of other DNA repair factors at bulky DNA lesions [1,2]. Mutations in XPA and loss of XPA protein expression are found in patients with the disease xeroderma pigmentosum, which predisposes subjects to an elevated risk of skin cancer [3]. XPA expression has been reported to be regulated at the post-translation level by the HERC2 ubiquitin ligase [4,5] and at the transcriptional level by HIF-1α [6], HMGA1 [7,8], and the circadian transcriptional machinery [9,10]. Indeed, both XPA expression and NER activity have been shown to oscillate with a periodicity of 24 hr in several mouse tissues [4,9,11–13] such that the timing of UVB exposure over the course of the day impacts sunburn erythema and skin carcinogenesis [11,14]. Time-of-day differences in XPA protein expression and erythema have also been reported in human skin [15,16].

Monitoring changes in protein expression in cultured cells and tissue samples is commonly done through the immunological detection of the target protein. However, these approaches often have limitations [17,18]. For example, because of a lack of antibody specificity or the presence of multiple isoforms or modified forms of proteins, experiments employing SDS-PAGE and protein immunoblotting (or western blotting) with polyclonal antibodies frequently reveal the presence of multiple bands that can lead to the incorrect designation of a protein as the intended protein of interest. Thus, it is important to critically evaluate and validate antibodies for their intended use [19]. Moreover, the cropping of specific bands of interest during figure preparation may lead to the loss of potentially new and useful information to researchers, including other modified forms of proteins. Finally, the methods used for preparing cell and tissue lysates can also impact the ability to extract or solubilize a protein of interest and affect the stability and post-translational modifications of the protein [20]. Thus, the apparent expression of a protein of interest is affected by multiple experimental factors that can lead to an incorrect or incomplete understanding of the protein in its physiological context.

Here we characterized an unexpected XPA antibody-reactive band in cell lysates prepared from cultures of quiescent human cells. Our detailed analyses show that XPA is susceptible to cleavage by the endopeptidase cathepsin L when buffers lacking ionic detergents or cathepsin L inhibitors are used to lyse cultured cells or skin tissue. Finally, though we show that this XPA cleavage is correlated with increased cathepsin L expression in quiescent cells, we find no effect of cathepsin L inhibition on either UV survival or UV photoproduct removal. Thus, the use of cathepsin L inhibitors in lysis buffers is important for accurate detection of the XPA protein in confluent populations of human cells.

2. MATERIALS AND METHODS

Standard approaches for cell culture and protein immunoblotting were used in this work. Details of experimental materials and methods can be found in the supplementary file associated with this manuscript along with quantitative analyses of key experiments.

3. RESULTS

3.1 Analysis of XPA protein expression in quiescent cells reveals the presence of a lower molecular weight species

Using lysates from sub-confluent cells maintained in 10% serum (designated proliferating cells) or cells grown to confluence and then maintained for two days in 0.5% serum (designated quiescent cells), immunoblot analyses with four different anti-XPA antibodies revealed an additional series of bands (designated XPA*) located about 3–4 kDa below that of the presumed full-length form of XPA (Fig. 1A), which normally migrates as a series of 2–3 bands (due to protein oxidation) at approximately 40 kDa on Tris-glycine-SDS gels [26]. CRISPR/Cas9-mediated knockout XPA expression further validated the lower XPA* band is a form of the XPA protein (Fig. 1B). Furthermore, when cells were plated at 30–40% confluence and allowed to reach full confluence in normal growth medium (with 10% serum) over the course of several days, there was a clear time-dependent emergence of the lower molecular weight XPA species (Fig. 1C) such that nearly 80% of the total XPA protein signal was found in the lower molecular weight species 4–5 days after plating (Fig. S1A). Additional experiments counting cells over this growth period revealed that the highest fraction of lower molecular weight XPA was observed after cells reached full confluence (Fig. S1B). Importantly, when both XPA species were quantified by densitometry and signals combined as total XPA protein expression, no significant differences were noted over the 6 day incubation period (Fig. S1C). Moreover, there was also no significant change in the percentage of trypan blue-positive cells between the sub-confluent and confluent cells (Fig. S1D), indicating that apoptosis and/or other cell death processes are unlikely to be responsible for generating the lower molecular weight form of XPA.

3.2 Proteolysis of XPA is prevented by including a protease inhibitor cocktail or ionic detergents in the cell lysis buffer

We noted that these experiments did not involve the addition of protease inhibitors to the Triton X-100-containing cell lysis buffer (TLB) used to prepare the cell lysates. Thus, to determine whether the lower molecular weight species of XPA was occurring during the process of cell lysis, a protease inhibitor cocktail (PIC) was added to lysates at various time points after resuspending pellets of confluent HaCaT cells in TLB. As shown in Fig. 1D, we observed a clear time-dependent increase in the generation of the lower molecular weight form of XPA but only a small decrease in total XPA protein levels (Fig. S2A). Thus, these results indicate that XPA becomes cleaved by one or more proteases within minutes of lysing confluent cells.

Interestingly, XPA proteolysis did not occur when confluent cells were lysed in a RIPA (radioimmunoprecipitation assay buffer) lysis buffer, even when the PIC was omitted from the lysis buffer (Fig. S2B). Unique to the RIPA buffer and missing from the TLB was the presence of the ionic detergents sodium deoxycholate (NaDOC) and sodium dodecyl sulfate (SDS). Addition of NaDOC or SDS to the TLB eliminated the detection of the lower molecular weight form of XPA in the cell lysates (Fig. S2C). Time course experiments in which SDS was added at various times after lysing cells demonstrated that the early addition of SDS to the lysed cells had a similar effect as PIC addition in

limiting XPA proteolysis (Fig. 1E and Fig. S2D). Importantly, similar effects of SDS and PIC were obtained in quiescent osteosarcoma cells (Fig. S2E), in telomerase-immortalized keratinocytes maintained in either a quiescent or differentiated state (Fig. S2F), in human skin epidermis (Fig. S2G), and in confluent HaCaT and HeLa cells lysed with either a hypotonic lysis buffer or a buffer containing other non-ionic detergents (Fig. S2H–I). These results demonstrate that PIC- and SDS-sensitive proteolysis of XPA is not unique to keratinocytes and occurs with a variety of different cell lysis buffers.

3.3 XPA proteolysis during cell lysis occurs at the C-terminus and negatively impacts its interaction with the NER factor TFIIH

We next sought to better characterize XPA degradation using other forms of the XPA protein. A slightly higher molecular weight N-terminal Flag-tagged form of XPA expressed in XPA-knockout cells behaved nearly identically to endogenous XPA (Fig. S3A). Moreover, the degraded form of XPA could be detected with an anti-Flag antibody (Fig. S3B) and isolated with anti-Flag antibody-bound magnetic beads, indicating that the proteolysis of XPA did not impact the N-terminus of the protein that contains the Flag epitope (Fig. S3C).

To confirm these findings with an independent source of XPA protein, we purified a recombinant form of XPA from bacteria that contained maltose-binding protein (MBP) on its N-terminus (Fig. S3D). The TLB cell lysis buffer was then supplemented with MBP-XPA before resuspension of pellets of confluent HaCaT cells. As shown in Fig. 1F, a clear PIC-sensitive band could be observed by Coomassie staining and by immunoblotting with anti-XPA and anti-MBP antibodies. SDS also prevented cleavage of MBP-tagged XPA (Fig. S3E). Thus, recombinant, bacterially expressed XPA with an N-terminal MBP tag behaves identically to XPA expressed in cells in vivo.

XPA coordinates NER via interaction with several NER proteins, including TFIIH and RPA [1,27,28]. The C-terminal 16 amino acids of XPA were previously found to be essential for XPA to interact with the NER factor TFIIH [27]. We found that both RPA and the TFIIH protein component XPB that were present in lysates of confluent cells could co-precipitate with MBP-XPA when XPA was retrieved from the lysate with anti-MBP antibody magnetic beads (Fig. 1G). Interestingly, the omission of PIC from the lysis buffer did not impact the interaction of MBP-XPA with RPA, presumably because RPA interacts with N-terminal and central domains of XPA [1]. In contrast, the ability of the TFIIH subunit XPB to co-precipitate with XPA was severely impaired when MBP-XPA became cleaved in lysates lacking the PIC (Fig. 1G and Fig. S4A–C). Thus, these results further argue that XPA proteolysis during cell lysis occurs at the C-terminus of the protein.

3.5 Cathepsin L cleaves XPA during lysis of quiescent cells

To identify the protease(s) responsible for acting on XPA, confluent HaCaT cells were lysed using the individual protease inhibitors found in the PIC. As shown in Fig. 2A, addition of the cysteine protease inhibitor E-64 to the lysis buffer completely prevented the generation of the smaller molecular weight form of XPA. Treatment with the dual serine- and cysteine-protease inhibitor leupeptin also prevented XPA proteolysis, and EDTA partially reduced

XPA degradation (Fig. S5A). We conclude that a cysteine protease is primarily responsible for acting on the C-terminus of XPA.

Interestingly, previous studies have shown that the expression and/or activity of several cathepsin proteins, which are a major class of cysteine proteases [29,30], are elevated in quiescent and/or confluent cells [31,32]. Consistent with this data, we found that expression of both cathepsin B and L (CTSB and CTSL) were significantly elevated at the protein level in lysates from confluent HaCaT cells in comparison to sub-confluent, proliferating cells (Fig. 2B and Fig. S5B). Expression of both CTSB and CTSL gradually increased after cell plating and became maximal by day 5 after plating (Fig. 2C, Fig. S5C), which corresponds to approximately 2 days after the cells reached full confluence as measured by cell counting. Supplementation of the cell lysis buffer with a specific inhibitor of CTSL completely blocked XPA proteolysis, whereas CTSB and CTSK inhibitors had only a partial effect or no effect at all (Fig. 2D and Fig. S5D). Pre-incubation of the cells for 30 min in medium containing the CTSL inhibitor prior to cell lysis had a similar effect as supplementation of the lysis buffer in preventing XPA cleavage (Fig. 2E), and mixture of recombinant CTSL and MBP-XPA generated an XPA-immunoreactive band that was similar in molecular weight to that generated with lysates from confluent cells (Fig. 2F). CTSL activity towards MBP-XPA was also sensitive to inhibition with both the CTSLi and SDS, consistent with previous studies of recombinant CTSL [33,34]. Because CTSL is an endopeptidase and we observed only a single lower molecular weight species of XPA in our time course proteolysis experiments, its action on XPA likely involves a single proteolytic cleavage event near the C-terminus of the protein that results in release of a small 3–4 kDa peptide.

Our findings on XPA proteolysis presented thus far indicate that this cleavage by CTSL is specifically occurring during cell lysis when the lysis buffer lacks inhibitors of CTSL activity (SDS, E-64, or the specific CTSLi Z-FY-CHO). However, because this cleavage event impacts XPA interaction with TFIIH (Fig. 1G), and because mutation of the XPA C-terminus was previously shown to inhibit NER activity in cell-free extracts [27], we were curious whether XPA cleavage by CTSLi might be relevant to NER and cellular responses to UV radiation in cells in vivo. However, we observed that pre-treatment of confluent cells with CTSLi did not significantly impact UVB survival (Fig. 2G) or the rate of removal of UV-induced (6–4)PPs from genomic DNA (Fig. 2H). Moreover, pre-treatment of sub-confluent, proliferating HaCaT cells with CTSLi for either 0.5 or 24 hr similarly had little effect on UVB survival (Fig. S5E, F). Thus, we conclude that cleavage of XPA by CTSL is unlikely to occur in vivo and is instead limited to the process of lysing confluent cells in the absence of CTSL inhibitors.

4. DISCUSSION

The XPA protein has been widely studied by many groups over the past 30 years. Though an early study describing the purification of native XPA from HeLa cells reported observing an approximately 35-kDa XPA-immunoreactive band [42], to our knowledge, no one has previously characterized this XPA band. Presumably, this may be due to the routine use of SDS-containing lysis buffers (such as RIPA or direct SDS-PAGE loading buffer), protease inhibitor cocktails, and/or sub-confluent populations of cells. Nonetheless, we anticipate that

our finding that XPA is susceptible to degradation by CTSL may be relevant to studies that use confluent cells to either purify XPA or to prepare cell-free extracts for in vitro excision assays. Moreover, we expect that the sub-cellular localization of XPA, which has generated some debate in the field due to the use of different pre-extraction and cell fixation methods [43–45], could similarly be impacted by XPA cleavage by CTSL during sample preparation.

A fraction of CTSL has been reported to exist in the nucleus [35] where it has been reported to impact the expression and/or cleavage of several other proteins, including the double-strand break repair protein 53BP1 [36–38], the cell cycle transcription factor CDP/Cux [35], and histone H3 [39–41]. CTSL has also been shown to cleave topoisomerase I in cells induced to undergo necrosis by treatment with a caspase inhibitor and tumor necrosis factor α [34]. We therefore carried out several additional experiments to find evidence that CTSL could cleave XPA in cells in vivo, but exposure of quiescent cells to UVB radiation, heat stress, and hypoosmotic stress all failed to induce XPA cleavage prior to cell lysis (data not shown). However, it remains possible that CTSL cleaves XPA under certain, as-yet unknown physiological circumstances. Nonetheless, our results highlight the importance of including protease inhibitors during cell lysis when probing for XPA protein expression in confluent or quiescent populations of cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NER	nucleotide excision repair
XP	xeroderma pigmentosum
XPA	xeroderma pigmentosum group A
(6–4)PP	pyrimidine (6–4) pyrimidone photoproduct
CPD	cyclobutane pyrimidine dimer
TFIIH	transcription factor II-H
RPA	replication protein A

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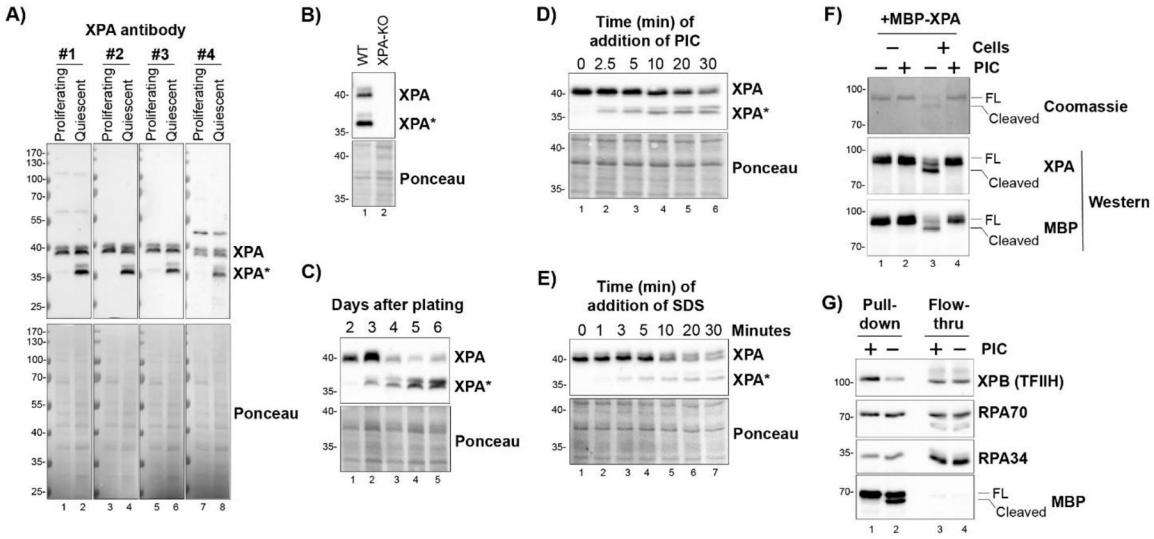

Figure 1. A proteolytically cleaved form of XPA is generated during lysis of quiescent cells and fails to interact with the NER factor TFIIH.

(A) Cell lysates were prepared from sub-confluent HaCaT cells (proliferating) and cells grown to confluence and then maintained for 2 days in low serum (quiescent). Lysates were analyzed by western blotting with four different anti-XPA antibodies (#1, Santa Cruz sc-28353; #2, Abcam ab65963; #3, Cell Signaling #14607; and #4, Santa Cruz sc-853). **(B)** XPA protein expression was detected in lysates prepared from wild-type (WT) HaCaT cells and a derivative line in which XPA was knocked out using CRISPR/Cas9 (XPA-KO). **(C)** Cell lysates prepared from cells after growth for the indicated periods of time were analyzed by immunoblotting for XPA. **(D)** HaCaT cell pellets were resuspended in lysis buffer and then protease inhibitor cocktail (PIC) was added to the lysis solution at the indicated time points. Lysates were analyzed by immunoblotting for XPA. **(E)** Cells were analyzed as in (D) except SDS was added at the indicated time points. **(F)** MBP-XPA was incubated in TLB in the absence or presence of confluent HaCaT cells and PIC and then examined by Coomassie staining or western blotting. **(G)** HaCaT cells were lysed in TLB containing MBP-XPA in the absence or presence of PIC. Anti-MBP magnetic beads were used to isolate the MBP-XPA from the cell lysates, which were then analyzed by western blotting with antibodies against the indicated proteins.

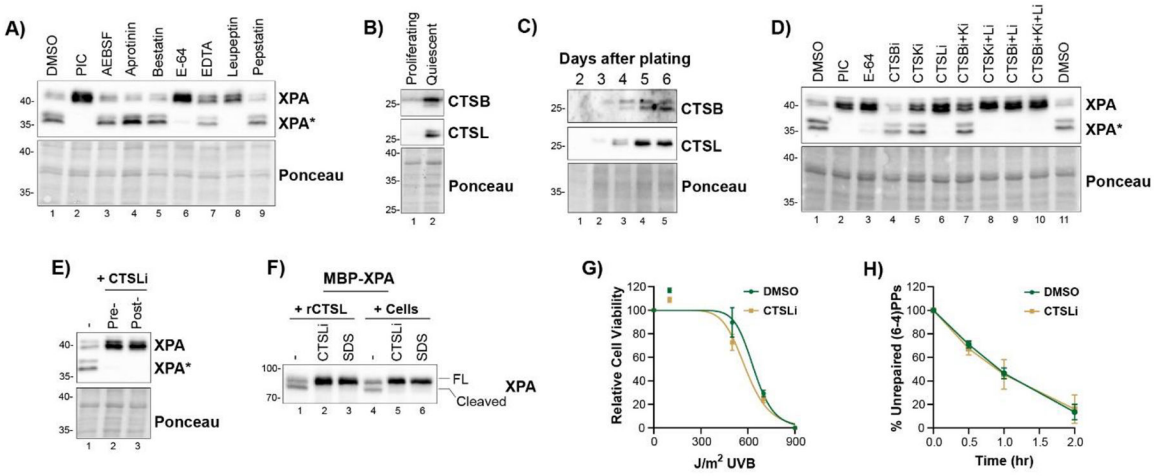

Figure 2. Cathepsin L is expressed at high levels in quiescent cells and is responsible for XPA cleavage but does not impact UVB survival or photoproduct removal from DNA.

(A) Confluent HaCaT cells were lysed in TLB containing the indicated protease inhibitor. **(B)** Lysates from proliferating and quiescent cells were analyzed by western blotting with antibodies against cathepsin B and L (CTSB and CTSL). **(C)** Lysates from HaCaT cells grown to confluence (Figure 2) were probed for CTSB and CTSL. **(D)** HaCaT cells were lysed in the presence of the indicated specific cathepsin inhibitors. **(E)** Quiescent HaCaT cells were treated with 10 μM CTSLi 30 min prior to cell lysis (Pre-) or at the time of cell lysis (Post-). **(F)** MBP-XPA was incubated with recombinant cathepsin L or confluent HaCaT cells in the absence or presence of SDS or cathepsin inhibitor (CTSLi). **(G)** Quiescent HaCaT cells were treated for 24 hr with 10 μM CTSLi before exposure to the indicated fluence of UVB radiation. MTT assays were performed 3 days later, and the graph shows results from 3 independent experiments (average and SEM). **(H)** Quiescent HaCaT cells were pre-treated with CTSLi for 30 min before exposure to 150 J/m2 UVB. Genomic DNA was purified from cells at the indicated time points and analyzed by DNA immunoblotting with an anti-(6–4)PP antibody. Results are the average and SEM from two independent experiments.