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MULTI-AUTHOR REVIEW

Posttranslational modification of mammalian AP endonuclease (APE1)

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Abstract A key issue in studying mammalian DNA base excision repair is how its component proteins respond to a plethora of cell-signaling mediators invoked by DNA damage and stress-inducing agents such as reactive oxygen species, and how the actions of individual BER proteins are attributed to cell survival or apoptotic/necrotic death. This article reviews the past and recent progress on posttranslational modification (PTM) of mammalian apurinic/apyrimidinic (AP) endonuclease 1 (APE1).

Keywords DNA base excision repair · APE1 · Phosphorylation · Acetylation · Ubiquitination

Abbreviations

AP	Apurinic/apyrimidinic
APE	AP endonuclease
APE1	AP endonuclease 1
BER	Base excision repair
CKII	Casein kinase II
FEN1	Flap endonuclease 1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
MDM2	Mouse double minute 2
NAM	Nicotinamide
nCaRE	Negative calcium response element
NPM1	Nucleophosmin
PARP1	Poly(ADP-ribose) polymerase 1
PKC	Protein kinase C
Pol B	DNA polymerase beta
Ref-1	Redox factor 1
ROS	Reactive oxygen species
SSB	Single-strand break
PTM	Posttranslational modification
SNP	Single nucleotide polymorphism
TF	Transcription factor
TSA	Trichostatin A
XRCC1	X-ray repair cross complementation group 1

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Introduction

Oxidative DNA damage is continuously generated under physiological conditions [1]. These lesions include apurinic/apyrimidinic (AP) sites, base damage (e.g., 8-oxoguanine), and single-strand breaks (SSBs) [2]. They are repaired primarily via the DNA base excision repair (BER) pathway in which an AP-endonuclease (APE) plays an essential role in generating 3′-OH termini at the damaged sites [3–5].

Because of its pivotal role in DNA repair [4, 6], the intracellular APE1 level influences sensitivity of the tumor cells to therapeutic reagents. Koukourakis et al. examined the level of APE1 expression in head and neck cancer and found a correlation between high APE1 levels and resistance of cancer tissues to chemo/radiotherapy (CRT) [7]. Glioma cells with higher-than-normal APE1 levels also showed a poor outcome after CRT [8, 9]. APE1 levels are generally higher in cancer tissues than in their corresponding normal tissues (http://www.oncomine.org/) [8, 10, 11]. While the high APE1 level is advantageous to transformed cells to survive under the rapidly proliferating condition and therefore may jeopardize cancer treatment, it is not clear how cells loosen APE1 regulation in the course

of transformation. Understanding this regulation should provide ways to modulate APE1 in cancer cells, and would ultimately be beneficial for cancer management. As we will review later the mechanism of APE1 ubiquitination, it is possible that one way to regulate APE1 cellular level is via ubiquitination as we see a similarity to degradation of DNA polymerase beta (POLB) via polyubiquitination [12, 13].

The BER mechanism is coordinated where intermediate DNA lesions are processed in a stepwise fashion, until the entire repair reaction is complete [14]. Importance of the coordinated BER is clear, considering that the BER process converts DNA base lesions into SSBs; SSB accumulation may be far more deleterious than the base damage, partly because of cellular energy depletion caused by over-activation of Poly(ADP-ribose) polymerase 1 (PARP1) [15–17]. APE1 functions together with XRCC1 (X-ray repair cross complementing group 1) and PARP1 for the repair of SSBs, and interaction of APE1 with XRCC1, pol β, and others enhances BER [18–21]. It is also noteworthy that the tumor suppressor protein p53 interacts with APE1 for an efficient BER reaction [22] and a genetic link between APE1 and p53 was suggested in a mouse genetics study [23]. APE1 is a multifunctional protein. It not only plays a key role in BER, but also has at least two different gene-regulatory functions: APE1 is a redox-dependent transcription activator and a co-repressor responding to intracellular calcium influx [24–26]. Therefore, interaction of APE1 with transcription factors (TFs) such as cJun/Fos, p53, YB-1, and STAT3 [27–29] is equally crucial to maintain status quo in cells.

Recent studies have pointed out importance of the N-terminal 6-kDa segment of APE1 for the protein–protein interaction [19, 27–29]. The domain is highly conserved in the mammalian APE1 and non-mammalian vertebrates such as zebra fish [30], but does not exist in APEs of prokaryotes and lower eukaryotes [31–34], suggesting that the region provides its ability to cooperate with other cellular factors specific to mammals. Therefore, a post-translational modification in this region may have a profound effect on APE1's functions [35–37]. Furthermore, as the region is the target of acetylation which alters APE1's affinity for a regulatory DNA element [38], and is important for RNA binding with nucleophosmin (NPM1) interaction [39], modulation of the APE1 N-terminus is of considerable interest to study APE1:DNA/RNA interaction. This review article focuses on the previous and ongoing studies of the posttranslational modification (PTM) of APE1. These include APE1 phosphorylation, acetylation and ubiquitination. Although the redox modification of APE1 (as Ref-1) can be categorized as a PTM, a number of dedicated reviews [26, 40] including the one by Tell et al. in this issue should be referenced for the Ref-1 functions. We will also briefly touch on other proteins that undergo PTM, notably p53 and MDM2 (mouse double minute 2), which provide clues as to the role APE1 PTM plays in relation to DNA damage/stress-response network in cells.

APE1 variants in SNP and EST databases

First, we would like to summarize the available information for APE1 single nucleotide polymorphisms (SNPs) with previous studies and our unpublished analysis using the human EST database, because only a change of amino acid (a.a.) side chain could significantly affect the level of PTM. Several studies examined the effect of SNPs in the human APE1 gene. Hadi et al. [41] described seven types of SNPs in the APE1 gene. One allele, 148 Asp/Glu, is the most frequent variant in the APE1 gene (about 68% for Asp). While the D148E substitution did not result in any detectable difference in APE or nucleotide incision activity [41, 42], the SNP has been reported to affect cell growth and sensitivity to ionizing radiation if combined with the XRCC1 R399Q allele, and markedly increased the risk of breast cancer [43, 44]. Jiao et al. [45] later reported a predisposition of individuals carrying the SNP combination of APE1 D148 and XRCC1 R194 W to pancreatic cancer risk (odds ratio = 4.98). It is currently not clear how the particular SNP influences cell growth and cancer risk, although it is plausible that the position is important for APE1 and XRCC1 interaction. There have been no data which indicate that the a.a. substitutions might change the APE1 conformation and indirectly affect PTM efficiency.

In addition to D148E, Hadi et al. reported other SNPs which affected the APE activity (Table 1). However, in the SNP database of the National Cancer Institute, only two of the above mentioned SNPs, namely Q51H (allelic frequency Q 98.5%/H 1.5%) and D148E (D 50.5%/E 49.5%) were confirmed in cancer patient genomes (as in http://snp500cancer.nci.nih.gov).

We have developed a streamlined method to analyze the vast mRNA EST database. Notable variations were again found on Q51H (Q 98.7%/H 1.3%) and D148E (D 68%; E 32%). Although the EST database is known to be error-prone, the frequencies of variants on Q51 and D148 are similar to those of the SNP database. We thus applied the search results based on EST to other a.a. positions. Variants with notably high frequency in EST are listed in Table 1 and Fig. 1. We have summarized the possible variations of the APE1 a.a. residues of interest for the posttranslational modifications (Table 1) and will mention possible variants found in EST at specific positions of PTM in later sections.

APE1 phosphorylation

APE1 phosphorylation was reported by several groups and shown to affect its repair activity in vitro. Yacoub et al.

Table 1 APE1 variants found in SNP and EST databases

Type of PTM	Position	a.a.	Conservation[b]	SNP	APE activity[c]	Variants in EST[d]	
PTM							
Phosphorylation	19[a]	T	S (rat and mouse)	None reported	N/A	0/730	
	123[a]	S	Yes			2/420	F, P
	233[a]	T	Yes			2/280	H, R
Acetylation	6	K	Yes			1/718	E
	7	K	R (rat)			5/714	3N, Q, T
Ubiquitination	24	K	Yes			3/623	E, R, T
	25	K	Yes			2/625	2N
	27	K	Yes			2/626	2N
SNP	51	Q	Yes	H[e]	Normal	18/588	10H, K, L, 4R, T, V
	64	I	Yes	V	Normal	8/583	N, 7V
	104	L	Yes	R	56.7%	3/525	P, T, V
	126	E	Yes	D[e]	60.4%	3/420	2A, G
	148	D	Yes	E	94.36%	117/366	116E, N
	237	R	Yes	A	35.7%	5/281	3A, L, T
	241	G	Yes	R	108.7%	6/281	A, E, 2R, V, W
	306	G	Yes	A	107.2%	6/401	2A, D, 2R, V

[a] Prediction only

[b] Among human, bovine, mouse, rat

[c] Retained activity, % of the wild-type APE1 in the previous study (Hadi et al., 2000, Nucleic Acids Res, 28, 3871–9)

[d] Total 1,327 human EST cases were analyzed. Numbers of varied a.a. versus total EST deposits covering the positions were shown. Ambiguous sequence (DNA base = 'N') and nonsense mutations were excluded from the result

[e] Described in http://snp500cancer.nci.nih.gov

[46] showed that APE1 was phosphorylated by casein kinase II (CKII) and that the modification inactivated its AP endonuclease activity. Phosphorylation was predicted to occur at a.a. residues of 19T, 123S, or 233T. APE1 phosphorylation by CKII was also reported by another group [47]. In this report, however, the APE1 phosphorylation resulted in enhanced Ref-1 activity but did not affect its nuclease activity. APE1 phosphorylation in cells appears to be increased by DNA damage (alkylation), which may affect cellular redox gene regulation under stressful conditions. Hsieh et al. [48] later confirmed that APE1 phosphorylation activated its Ref-1 activity, although in this case protein kinase C (PKC) was responsible for the phosphorylation reaction. CKII was predicted to act on 123 Ser. APE1 also contains PKC consensus sites at 56S, 61T, 123S, 201S, 252S. Available SNP data and our analysis in the EST database suggest no variations at these sites (Table 1). More recently, APE1 phosphorylation activities were compared among PKC, mitogen-activated protein kinase 1 (Erk2), cGMP-dependent protein kinase (PKG), CK-I and CK-II in a biochemical study [49]. Although APE1 was a poor substrate for all the kinases tested, CKII showed the highest activity on APE1, followed by PKG, PKC, and CKI (Erk2 did not phosphorylate APE1).

Despite the potential significance of APE1 phosphorylation, its biological importance has not been investigated further. Variable results and conclusions in the above reports are also somewhat confusing. For example, APE1 phosphorylation activity in cells is very weak [49] and thus the presence of endogenously phosphorylated APE1 has not been confirmed. A methodology for sensitively detecting endogenously phosphorylated APE1, such as labeling with nonorganic radioactive phosphate, should confirm its modification in vivo, and warrant biological significance further in depth. Another key observation would be the identification of specific phosphorylation sites by site-directed mutagenesis, which would be useful for developing phosphor-specific antibodies to APE1. These studies should help us to understand the importance of APE1 phosphorylation in BER and gene regulation.

APE1 S-nitrosation

Recently Qu et al. [50] observed S-nitrosation of Cys 93 and 310 in APE1. The modification in cells, induced by S-nitrosoglutathione, triggered exclusion of APE1 from nuclei, while a double Cys mutant APE1, i.e., C93S/C310S was not affected by the treatment. The results provide a novel mechanism of APE1 nuclear export, and it will be

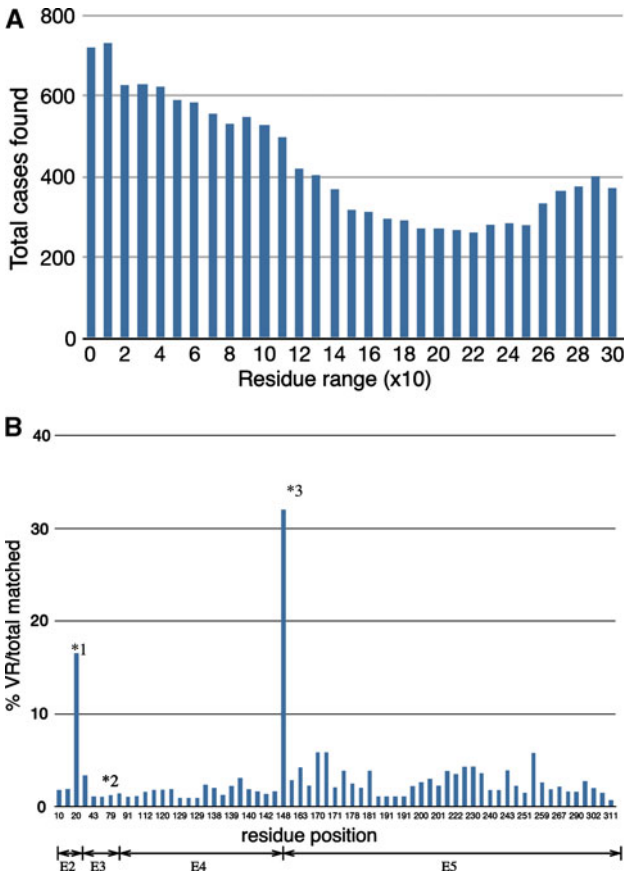

Fig. 1 Variant frequency found in EST database. A collection of APE1 EST sequences (total 1,327 cases from http://www.nih.ncbi.gov) was translated for all coding frames and compared with human APEX1 protein sequence (GenBank accession number D90373), and frequencies of a.a. substitutions are shown with total numbers of cases that cover particular regions. **a** Histogram of EST deposits that cover indicated a.a. regions. **b** Frequencies of individual a.a. substitutions. Those with more than 1.5% of variants are shown. **1* E20G appears due to a frame shift by mis-splicing at the junction between exon 2 and intron 2. Surprisingly, about 16% of all EST sequences contained the intron 2 but not the other introns; **2* Q51H omitted from the blot. Q51 dominant (98.3%) over H; **3* D148E (32.0%). The entire analyses were carried out with original scripts written in Ruby (http://www.ruby-lang.org/en/). Individual EST cases described here are available upon request

interesting to examine the level of S-nitrosation on endogenous APE1. This will give an idea whether S-nitrosation is an important determinant for the cytoplasmic distribution of APE1 that has been observed in some human tissues including prostatic glands epithelia [51].

APE1 acetylation

APE1 acetylation has been studied fairly extensively. In 2004, using a colon carcinoma cell line and a baby hamster kidney cell line, Bhakat et al. [38] showed that APE1 was acetylated by histone acetyl transferase (HAT) p300. The acetylation reaction occurred in the cells, as a fraction of cellular APE1 with a [^{3}H]-labeled acetyl group was detected. Their development of useful biological probes is noteworthy as these facilitated the characterization of acetylated APE1. The group was later successful in generating an antibody specific to acetylated APE1 and confirmed its endogenous presence in vivo [28]. Specific acetyl acceptor Lys residues were also found at K6 and K7 in the APE1 polypeptide using domain mapping and site-directed mutagenesis [38]. While either Lys could be acetylated at almost the same efficiency in cells, dual acetylation was not observed. The result indicates that a single acetyl group at either K6 or K7 may be inhibitory to another acetylation reaction. Because the two Lys are located adjacent to each other, there probably is little difference in the effect of acetylation at K6 or K7. Interestingly, K7R substitution is found in the rat APE1, and relatively a higher variation frequency was also found among the EST deposits (Table 1). It is thus possible that K7 is dispensable in which case K6 can be the unique acetyl group acceptor. More recently, Fantini et al. observed other Lys residues in the APE1N-terminal domain, including K27, K31, K32, and K35, could be acetylated in vivo (G. Tell, personal communication, [52]).

As K6/K7 residues are in the 6-kDa N-terminal region that is only conserved with mammalian APE1, it was not surprising that the acetylation did not affect its AP endonuclease (APE) activity. Rather, it is the gene repressor function of APE1 that was enhanced by the acetylation [38]. As a co-repressor, APE1 suppresses the parathyroid hormone (PTH) gene expression through the consensus DNA element nCaRE [25]. An additional observation, i.e., the direct interaction between APE1 and histone deacetylase 1 (HDAC1), provided a possible molecular mechanism of the gene suppression. Once acetylation takes place, APE1 increases its existence at the nCaRE regions in the genome, where nearby promoters are actively transcribed. Through the direct interaction with APE1, HDAC1 is recruited to the promoter region to deacetylate histones and to suppress gene expression. APE1 is released from the region as HDAC1 removes the acetyl group from APE1 thereafter. This feedback model ensures the temporary nature of the gene suppression. The p300 HAT activity may be enhanced by phosphorylation via calmodulin kinase [53], which explains the dependency of the PTH gene suppression on intracellular concentration of Ca^{2+}. An additional layer of complexity was recently added by Yamamori et al., who showed that APE1 was deacetylated by SIRT1 in addition to HDAC1 [52]. SIRT1 is a class III HDAC and thus inhibited by nicotinamide (NAM) but not by Trichostatin A (TSA), a potent inhibitor of HDAC1. In their condition, NAM had a greater inhibition of APE1 deacetylation than TSA. The results suggest not only that nCaRE-dependent gene suppression involves SIRT1 in

addition to HDAC1, but also that APE1 acetylation may influence gene regulation involved in energy metabolism and many other stress responses via SIRT1 [52].

APE1 acetylation is also a key modification that led to transcriptional activation of the multi-drug resistance gene MDR1 through the modulation of a transcriptional factor YB-1 [28]. It will be important to find out whether APE1 acetylation also affects the renin gene regulation via a similar nCaRE-dependent suppression [54, 55]. The K6/K7 Lys residues were shown to be essential in a mouse embryonic fibroblast cell line in which the APE1 gene was conditionally knocked down [36]. Because acetylation occurs at the two Lys residues, the results suggest that the modification has an essential effect on at least one of APE1's functions. While the effect of acetylation appears to be minimal to APE1's DNA repair activity, acetylation has been shown to occur on other BER proteins, i.e., DNA glycosylases including OGG1 and NEIL2 [56, 57]. In those cases, the modification indeed altered repair activities of these proteins. NEIL2 acetylation was identified on K49 and K153, and K153 acetylation eliminated the AP lyase activity of NEIL2 [56]. On the other hand, acetylation at K338 and K341 in OGG1 enhanced its activity due to increased turnover in the reaction [57]. Considering these findings, acetylation appears to be a general mechanism by which cells regulate BER.

APE1 ubiquitination

Brief introduction of ubiquitination

Ubiquitin is a highly conserved small-molecule modifier protein (76 a.a. residues, 8.5 kDa) that serves as a signal mediator for a number of cellular activities, including cell cycle and DNA repair, of which dysregulation is associated with cancer [58, 59]. Polymerization of ubiquitin (poly-ubiquitination) on a protein triggers degradation of the ubiquitinated protein by the 26S proteasome (through its Lys 48 polymerization) [60], while ubiquitin proteins are recycled with their removal from the target protein by deubiquitinases (DUBs) or ubiquitin-specific proteases (USPs) [61]. Ubiquitination is a three step-enzymatic reaction involving E1, E2, and E3 ubiquitin ligases. Targeting proteins for degradation is a highly specific process that is generally accomplished by the interaction between E3 ligases and the target proteins. A ubiquitin-driven degradation of the tumor suppressor protein p53 controlled by its ubiquitin E3 ligase MDM2 (mouse double minute 2) is worth mentioning [62, 63], because this well-studied system provides a mechanistic model of APE1 ubiquitination as described below. Under normal conditions, MDM2 ubiquitinates the p53 protein through a direct interaction. Phosphorylation at S15 and S20 of p53 by ATM under genotoxic stress interferes with p53:MDM2 interaction [64, 65], which in turn inhibits the ubiquitination reaction by MDM2. Therefore, p53 is stabilized and activated during stress responses. As a feedback, one of the transcriptional activation targets of p53 is the MDM2 promoter [66]. Thus, stabilized p53 will increase the transcription of MDM2, which degrades excess p53 and resumes the normal p53 level. This mainstream diagram of cancer biology explains the fact that many cancer tissues and cell lines are p53 deficient due to p53 mutations, MDM2 over-expression (which decreases the p53 level), or by disruption of CDKN2A $p14^{ARF}$ mutation (deficient in suppressing MDM2) [67].

In addition, ubiquitination is a critical signal mediator for fundamental cellular activities other than the salvation of amino acids through degradation. Some target proteins are ubiquitinated with only a single ubiquitin molecule (monoubiquitination). Because a tandem chain of at least four ubiquitin molecules is required for the 26S proteasome to recognize a target protein [68, 69], monoubiquitinated proteins can stably exist. As ubiquitin changes conformations and biochemical characteristics of target proteins, monoubiquitination can be a unique signaling mediator. For example, monoubiquitinated p53, known to exist in cells, can serve as a signal mediator for apoptosis. Marchenko et al. [70] showed that p53 was monoubiquitinated under DNA damage stress induced by camptothecin (CPT), a topoisomerase inhibitor and therefore DNA double strand break inducer. The modified p53 was specifically translocalized to mitochondria to destabilize the organelle. The Fanconi anemia (FA) protein FANCD2 is also modified by a single ubiquitination reaction [71] catalyzed by a complex consisting of the FA proteins and UBE2T E2 ligase [72].

Furthermore, ubiquitin can be polymerized on a target protein via its Lys 63 residue without being recognized by the proteasome complex. Proliferating cell nuclear antigen (PCNA), an essential DNA replication and repair cofactor, is known to be polyubiquitinated through K63 [73, 74]. Thus the ubiquitin moiety is crucial for the polymerase switching process during replication bypass by DNA polymerase iota.

APE1 is ubiquitinated

The examples above illustrate the influence ubiquitination has on intracellular levels of target proteins, their subcellular localization, and protein/DNA interactions. Given that APE1 interacts with DNA and other BER proteins and transcription factors, the addition of ubiquitin on APE1 likely causes a significant alteration to APE1 functionality. We recently characterized the mechanism of APE1 ubiquitination [75]. The reaction was detected with transiently

expressed APE1 and ubiquitin tagged by a histidine hexamer, which was used to enrich ubiquitinated proteins, followed by APE1 detection in immunoblot assays. In contrast, other ubiquitin-like modifiers, including SUMO, Nedd8, and ISG15 [76], did not modify APE1 using transient expressions of similarly His-tagged modifier genes. Thus, ubiquitin is likely a specific small-molecule modifier on APE1, although we cannot exclude the possibility that our assay was not sensitive enough to detect PTMs by others.

APE1 ubiquitination reaction was catalyzed by MDM2 as the MDM2 co-expression markedly enhanced APE1 ubiquitination [75]. Furthermore, a missense mutant of human MDM2 (C464A) failed the ubiquitination reaction, indicating that its ligase activity with the proper RING finger structure is directly involved in APE1 ubiquitination.

A small-molecule inhibitor, nutlin, is a p53 activator through the inhibition of the interaction between p53 and MDM2 by binding to MDM2 [77]. The mechanism of interference by this compound was characterized extensively by X-ray crystallography [77]. In a co-crystal structure of polypeptides derived from MDM2 and p53, a.a. 17–29 of p53 tightly bound to a region of MDM2 consisting of two alpha helices (M50-T63 and H96-N106) and two short loops (Q72-H73 and S92-H96) of MDM2 (Fig. 2). Three p53 side chains (F19, W23, and L26) are critical for the interaction with MDM2, and closely resemble the geometry of an MDM2-nutlin co-crystal structure. Unable to access to p53 in the presence of nutlin, the ubiquitin ligation reaction by MDM2 on p53 is much reduced, and thus the p53 level and activities increase.

The observation that nutlin significantly increased the ubiquitination of APE1 was unexpected and yet provided a few insights into the ubiquitination mechanism [75] (Fig. 2c). The fact that nutlin is not inhibitory for APE1 ubiquitination indicates that the segment (a.a. 50–106) of MDM2, where nutlin and p53 bind, is not required for APE1 ubiquitination. A physical interaction between APE1 and MDM2 was observed with immuno-pulldown experiments of APE1-FLAG [75]. The interaction was not hampered by the N-terminal truncation of 40 a.a. residues, indicating that the region surrounding the ubiquitin acceptor Lys residues (see below) is not required for the stable interaction between APE1 and MDM2. Rather, we suspect that C-terminal part of APE1 is involved in the interaction [75], although a more detailed domain mapping would be necessary to identify residues in both APE1 and MDM2 critical for the interaction. The information should be useful in controlling the activity of APE1 ubiquitination in the cells.

Activation of p53 by nutlin triggers transcriptional activation of the MDM2 gene, and consequently APE1 ubiquitination was increased, providing evidence that endogenously expressed MDM2 can ubiquitinate APE1 protein. Since nutlin did not block APE1 ubiquitination, it is unlikely that the a.a. M50-H96 of MDM2 is required for the interaction with APE1. An alternative possibility is that p53 is inhibitory for APE1 ubiquitination by interacting with MDM2 and sequestering catalytically important segments of MDM2. Nutlin may facilitate dissociation of p53 from MDM2, and effectively enhance APE1 ubiquitination.

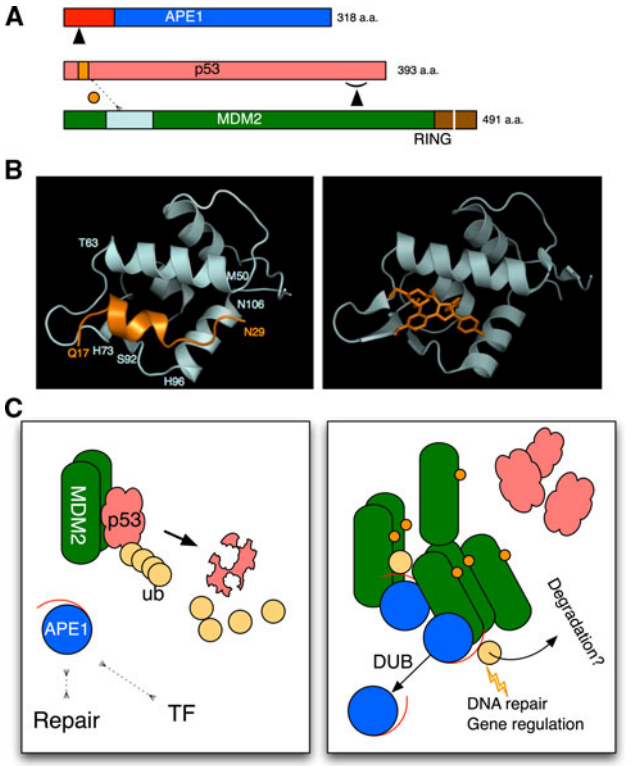

Fig. 2 APE1 ubiquitination regulated by p53 and MDM2. **a** Schematic diagram of APE1 (*blue*), p53 (*pink*), and MDM2 (*green*) and polypeptides. The N-terminal 6-kDa region in APE1 is shown in *red*. The RING finger domain and its core residue C464 in MDM2 are colored in *brown* and *white*, respectively. The interaction between p53 (*orange*) and MDM2 (*thin blue*) can be blocked by nutlin (*orange circle*). *Arrows* indicate ubiquitin acceptor sites in APE1 and p53. **b** *Left* the interaction interface of p53 (*orange*) and MDM2 (*thin blue*) revealed in a co-crystallographic study. *Right* the same region of MDM2 shown with nutlin-2 (*orange*). Generated using PyMol based on PDB coordinates 1YCR and 1RV1 [77, 101]. **c** Model of APE1 ubiquitination. (*Left*) In a normal condition, p53 is continuously ubiquitinated by oligomerized MDM2 (or with MDMX) through its RING finger motif [102] and thus both proteins as well as APE1 ubiquitination are maintained low. (*Right*) Interfering with the p53-MDM2 interaction by nutlin (*orange circles*) inhibits p53 ubiquitination, leading to p53 stabilization and transcriptional up-regulation of MDM2, favorable for APE1 ubiquitination. The effect of nutlin resembles p53 phosphorylation and stabilization by ATM and CHK1 at S15 and S20 triggered by DNA damage stress. The ubiquitination may affect APE1's subcellular localization and interaction with DNA/proteins, thereby alter its DNA repair and gene-regulatory role. Situations may exist that trigger APE1 polyubiquitination for rapid degradation via 26S proteasome, or revert the modification due to deubiquitinase reactions

Ubiquitin acceptor site and effect of the modification

An important question, as in the cases of phosphorylation and acetylation, was whether only specific residues of APE1 were ubiquitinated. Except for N-terminal and rare Cys, Ser, or Thr conjugation [78–80], ubiquitin can only be conjugated via isopeptide bonding between its carboxyl terminus and the ε-amino group of a Lys side chain [81]. Through a systematic domain mapping, we determined that three (24K, 25K, and 27K) out of 29 Lys residues in the entire APE1 polypeptide were ubiquitinated almost exclusively. The region surrounding the three Lys residues is completely conserved among mammalian APE1s. It has not been entirely clear why the region containing the three Lys is highly conserved. The structure and the exact function of the 6-kDa region are unknown; the domain could not be resolved in the X-ray crystallographic studies [82, 83] and is dispensable for the DNA repair activities [84]. The role of the N-terminal domain for Ref-1 activity [29] appears to be indirect, considering the fact that the region contains no Cys residues. While nuclear localization signals were found in the N-terminus, it only requires the first 13 a.a. [85], and thus would not explain the high conversation in the downstream a.a. sequence. In addition, the available SNP database reveals no variation in the Lys cluster region, which is consistent with our own analysis using the EST database (Table 1; only a few substitutions with Asn, Arg, Glu, and Thr found). It is thus likely that the N-terminal 20–60 a.a. residues form a distinct structure that is required for a proper ubiquitination reaction.

APE1's N-terminal 6-kDa domain is required to interact with other BER proteins (i.e., PolB and XRCC1) and transcription factors including YB-1 and STAT3 [19, 21, 27, 28]. Multiple ubiquitin-binding modules have been identified in many cellular proteins [86] that may specifically interact with ubiquitinated APE1.

It should also be noted that although it is not required for the endonucleolytic activity, several lines of evidence indicate that the 6-kDa domain can modulate APE1's affinity for DNA. The region is relatively basic with multiple Lys and Arg residues, implying that it may be able to bind to nucleic acids. Acetylation at K6/K7 enhanced specific DNA binding to the nCaRE consensus element. Finally, Vascotto et al. recently discovered that APE1 specifically binds to nucleophosmin (NPM1) to modulate "RNA cleansing" activity of APE1, and the N-terminal domain is indispensable for the binding [39, 87]. Therefore, it is likely that modification of APE1 with ubiquitin significantly influences its interaction with other proteins and nucleic acids. To elucidate the effect of ubiquitin on APE1's DNA binding activity, we created a ubiquitin-APE1 fusion protein such that ubiquitin is linearly fused to APE1's 24 Lys. We found that, in addition to being highly stable, the protein fully retained APE activity. Moreover, the addition of ubiquitin to APE1 appears to increase its stability and affinity for DNA, particularly in the presence of high salt concentration. This unpublished observation suggests that ubiquitination has important ramifications on both the DNA repair and gene-regulatory functions of APE1.

Subcellular localization of ubiquitinated APE1

Whether ubiquitination affects the subcellular distribution of APE1 is an important question, as both the DNA repair and gene-regulatory functions rely on its nuclear localization. We observed that ubiquitin-APE1 fusion proteins, transiently expressed in mouse fibroblast cells, were excluded from nuclei [75]. Unable to function properly in nuclei, ubAPE1 may be polyubiquitinated and degraded. Zhao et al. [88] reported that APE1 interacts with Bcl-2, an anti-apoptotic oncoprotein. The researchers observed that Bcl-2 was transferred into nuclei after genotoxic stress, interacted with and prevented APE1 from participating in BER, and thereby sensitized the cells. We postulate that ubAPE1 may interact with Bcl-2 at the mitochondrial membrane and alter cellular sensitivity to apoptotic signals.

Unlike ubiquitin-APE1 fusion proteins that are produced in the cytosol, de novo ubiquitination reactions may occur either in nuclei or cytosol. Since MDM2 is localized in nuclei, it is possible that APE1 ubiquitination takes place in nuclei. If this is indeed the case, other outcomes following ubiquitination are feasible. One possibility is that the ubAPE1 stays inside nuclei, presumably because the modification prevents transmembrane movement (the opposite was true with the nuclei-excluded ubiquitin-APE1 fusion protein). Ubiquitination may thus have profound effects on APE1's functions in nuclei. Determining its effects on the interactions with other BER components (Pol B, XRCC1), transcriptional regulators (AP-1, Pax8, NFkB, STAT3), and DNA (damaged and undamaged) should illuminate the biological significance of APE1 ubiquitination.

Karni-Schmidt et al. [89] investigated nucleoli with a fine resolution and concluded that MG132, a potent 26S proteasome inhibitor, can translocate p53 into the nucleolar fibrillar center, the central dense region of nucleoli. Moreover, the enrichment of p53 in nucleoli was observed in lung and prostate tumor tissues. As previously described, APE1 was also reported to be translocated to nucleoli after DNA damage induction [39]. Whether the nucleolar translocation of APE1 requires ubiquitination has yet to be tested.

After APE1 ubiquitination in nuclei, an alternative scenario is that ubiquitination serves as a signal for APE1 export. In regards to the p53-MDM2 analogy, MDM2

appears to shuttle in and out of nuclei with the ubiquitinated p53 in order for p53 to be exported out of nuclei [90, 91]. Thus the regulation of subcellular localization by ubiquitination is quite complex, and little is understood about its influence in cancer biology.

Consideration on amount of ubiquitinated APE1 in the cell

The ubiquitination assay described recently [75] uses exogenous expression of His-ubiquitin and APE1. While the assay was sufficient to detect the modified APE1 and to identify the ubiquitin acceptor sites (24, 25, 27K), it did not reveal the level of ubAPE1 in vivo. The relative level of ubAPE1 compared to intact APE1 appears to be much lower than the intact APE1, because endogenous ubiquitination was only detectable when MDM2 expression was activated by DNA damaging reagents or by nutlin. Therefore, it is not clear if the 26S proteasome has a significant effect on endogenous APE1 levels. However, we often observed a considerable amount of APE1 degradation under stress conditions induced by DNA damaging reagents such as H_2O_2. While APE1 is known as an abundant protein in cells, the APE1 level in mouse tissues appears to be lower than cells cultured in vitro and highly sensitive to protein degradation. A rapid decrease in APE1 protein after ischemia and reperfusion in a specific region of mouse brains (hippocampal CA1) was recently reported [92]. In this context, it is important to note that our studies have almost exclusively relied on transformed cancer cell lines cultured under 21% pO_2, while most human tissues regularly experience considerably lower pO_2 ranging 3–10% [93]. It is therefore plausible that during transformation and adaptation to the experimental cell culture conditions, cells tolerant to fluctuation of pO_2 are enriched, and the cell lines may lose the proper regulation of APE1. To understand the cellular regulatory mechanism of APE1 ubiquitination completely, various factors including the difference between primary cell cultures and established cell lines, a hypoxic/hyperoxic growth condition, and genotoxic stress induced by reactive oxygen species need to be considered.

Monoubiquitination as a predominant form of ubiquitinated APE1

As monoubiquitination and polyubiquitination trigger cellular activities completely different from each other, there are some important questions yet to be answered regarding the balance between mono- and polyubiquitination. In our assay, monoubiquitinated APE1 (muAPE1) is the major conjugation product while the polyubiquitinated form is less abundant. Recombinant ubiquitin-APE1 fusion protein (at APE1's 24th Lys) is a highly stable polypeptide with no obvious change in APE activities. It is thus likely that modified APE1 exists stably once monoubiquitination occurs. However, formation of higher-molecular-weight APE1 bands was also observed after a stress induction by hydrogen peroxide. It is therefore likely that APE1 is polyubiquitinated under genotoxic stress that may lead to apoptotic cell death. Therefore, while MDM2 was shown to be the major E3 ligase for APE1's monoubiquitination, an attractive model for APE1 polyubiquitination is that the polymerization reaction is catalyzed more efficiently by a set of E2 and/or E3 ligases other than MDM2. The dual reaction model ensures that muAPE1 has a specific function, while degradation can be initiated independently from the regulation of MDM2. This type of dual-ubiquitination mechanism has been reported for p53 ubiquitination. It is known that p53 also undergoes relatively stable monoubiquitination. A critical factor in determining the ratio of mono and polyubiquitinated p53 appears to be de novo concentration of MDM2. Li et al. [94] reported that p53 was polyubiquitinated rather than monoubiquitinated when the intracellular concentration of MDM2 was higher. Two E3 ligases (COP1 and PIRH2), unrelated to MDM2, also catalyze the polyubiquitination reaction for p53 [95, 96].

The stability of muAPE1 may be affected by a plethora of ubiquitin specific proteases (USP) or deubiquitinase (DUB). These enzymes remove ubiquitin molecules from the target proteins by specifically hydrolyzing the isopeptide bond between the ubiquitin C-terminus and the ε-amino group of Lys. In some particular cases, a DUB can stabilize the ubiquitin target proteins because it reverses polyubiquitin chain formation. Again as an example, p53 is regulated by a specific ubiquitin protease USP7 (or HAUSP) [97]. However, an opposing result was later reported, wherein disruption of the HAUSP gene caused p53 stabilization [98]. This result was initially perplexing; however, HAUSP can also hydrolyze ubiquitin chains from MDM2, which undergoes autoubiquitination and degradation. Therefore, it appears that the balance of three factors, i.e., the target (p53), ubiquitin ligase (MDM2), and the DUB (HAUSP) determines p53 stability. Considering the above ubiquitin pathway, the APE1 ubiquitination mechanism may be quite complex if similar diverse pathways are involved.

Key studies and molecular probes for understanding APE1 ubiquitination

The investigation of APE1 ubiquitination has just begun, and our attempt to understand its catalytic mechanism and effect on cellular response to environmental stress is at an infant stage. The aforementioned studies on APE1 phosphorylation and acetylation provide us several important and short-term objectives leading to a coherent strategy to

study the mechanism and significance of APE1 ubiquitination. We have already determined the ubiquitin acceptor Lys sites as described above, namely, 24K, 25K, and 27K. This not only indicates that the PTM reaction involves specific recognition of the polypeptide sequence, but also provides a number of methodologies thereafter. For example, ubiquitin-APE1 fusion protein could be generated by the direct cDNA fusion at the site. While the approach does not perfectly reproduce the actual ubiquitination via isopeptide bonding, its relatively small disturbance on the APE1 sequence should help us elucidate the effect of ubiquitin on APE1's DNA repair function. As identification of the acetyl group acceptor Lys (K6/K7) allowed the development of acetylation deficient APE1 (e.g., K6R/K7R), we should also be able to examine the effect of the mutants at the ubiquitin acceptor sites.

A fine methodology is required to enable sensitive detection of ubiquitination on endogenous APE1. Although nutlin can enhance APE1 ubiquitination, the assay still relies on the transient expression of His-tagged ubiquitin, and the amount of muAPE1 is still too low to detect in immunoblot without enrichment. A more sensitive assay would be to use ^{125}I-ubiquitin thioester [99]. While the method is commonly used in in vitro ubiquitination reactions with purified ubiquitin ligases, the radioisotope labeling cannot reveal the subcellular localization of ubiquitinated proteins. Because the ubiquitin conjugation site is known, an alternative approach would be to generate antibodies specific to the ubiquitin-APE1 conjugation sites, as a branched-out polypeptide with isopeptide bonding can be synthesized. Ubiquitin conjugation-specific antibodies, which detect the polyubiquitin conjugation sites, were successfully generated in the past [100]. In any case, such an antibody would be especially important: the molecular probe could be used not only for immunoblot assays but for immunocytochemistry and immunoprecipitation assays as well. Another important study would be to examine the effect of ubiquitination on APE1's interaction with other cellular factors including DNA repair proteins, transcription factors, and damaged/undamaged DNA. The multiple functions of APE1, which appear to be unrelated to one another (e.g., Ref-1 gene regulation and DNA repair), make the study highly complicated. Therefore, identification of a group of interactomes affected by APE1 and its ubiquitination should provide a better approach for molecular study. For example, a modification of APE1 in its interaction with a BER protein (i.e., PolB/XRCC1/FEN1) or with damaged DNA would strongly suggest that ubiquitination is important in modulating BER coordination. This parallels the advance in the APE1 acetylation study, i.e., the finding that APE1 acetylation altered its binding affinity for the nCaRE consensus elements. This helped establish APE1's gene-suppression function. Studying APE1 ubiquitination under the premise of the aforementioned issues should reveal a future research direction with comprehensive understanding of intracellular APE1 activity and its influences in carcinogenesis.

Conclusions

We have reviewed the past and ongoing investigations regarding posttranslational modification and their effects on the multiple functions of APE1, a cellular protein that plays multiple pivotal roles in cell defense systems. Ubiquitin and ubiquitination have been known for more than three decades, and yet its ever-increasing mechanistic complexity challenges advanced technology in modern molecular and cell biology. A number of issues regarding the mechanism and significance of APE1 ubiquitination remain to be solved. Fortunately, we are guided by past PTM studies, i.e., phosphorylation and acetylation on APE1 and ubiquitination of other factors involved in DNA repair and those important for tumor suppression and progression. We have described the p53 signaling network and its involvement of ubiquitin in particular, not only because the system is one of the best-studied subjects but also because genetic and functional links have been reported between APE1 and p53 in regards to their DNA repair and tumor-suppression functions. Careful elucidation of the p53 signaling networks and their involvement in the DNA repair pathway coordinated by APE1 is an important subject for molecular carcinogenesis.

Acknowledgments While we were in the final process of revision, Huang et al. newly reported that APE1 was phosphorylated at Thr 233, resulting in a decrease of the APE activity, particularly in brain tissues from patients with Parkinson's and Alzheimer's diseases (Nat Cell Biol 12:563–571, 2010). A significant question in relation to this review is if and how T233 phosphorylation affects the intracellular levels of acetylated and ubiquitinated APE1. This work was supported by NIH CA98664 (TI).

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