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Advances in Brief

The DNA Repair Activity of Human Redox/Repair Protein APE/Ref-1 Is Inactivated by Phosphorylation[1]

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Abstract

The human DNA repair protein apurinic/apyrimidinic endonuclease (APE) is a dual-function protein that has important roles in both the repair of baseless sites that arise in DNA and in regulating the redox state of a number of proteins (Ref-1). Although previous attention has been focused on how the human *APE/Ref-1* gene may be regulated at the DNA level, we have instead examined if APE/Ref-1 is phosphorylated, and if so how it may affect DNA repair activity. We demonstrate here that APE/Ref-1 is indeed a substrate for phosphorylation by the serine/threonine casein kinases (CK) I and II and protein kinase C. Notably, although phosphorylation by CKI and protein kinase C had no effect whatsoever on the ability of APE/Ref-1 to act at abasic sites in DNA, phosphorylation by CKII completely abolished DNA repair activity. That phosphorylation was responsible for the loss of abasic repair activity was concluded from experiments showing that inactive APE/Ref-1 could be reversed to an active DNA repair protein with phosphatase treatment. These results may help to explain the mechanism by which APE/Ref-1 switches from one unrelated function to another.

Introduction

The major enzyme in humans, at least quantitatively, for the repair of AP[3] sites was originally purified and characterized as a single monomeric protein (1). A human cDNA encoding the DNA repair enzyme (2, 3) was subsequently identified (APE) and found to encode a protein of M_r 35,500 that cleaves phosphodiester bonds 5′ and adjacent to an AP site. APE is part of the *Escherichia coli* exonuclease III-like family of APEs based upon the conservation of protein sequences (2). On a functional level, APE also shares with exonuclease III a surprising array of substrate specificities including ribonuclease H and 3′-repair activity, although the latter represents only a modest activity when compared to exonuclease III (4). It also does not appear to contain robust 3′–5′ exonuclease activity resembling that of the *E. coli* enzyme.

The importance of APE expression in human cells for the repair of DNA damage has been shown by using antisense RNA to deplete endogeneous APE activity, which rendered HeLa cells sensitive to a variety of DNA-damaging agents (5), and a yeast two-hybrid analysis demonstrated that excision repair of an AP site involves a coordinated pathway involving protein-protein contact between APE and mammalian DNA polymerase β (6).

A completely independent line of investigations has led to the finding that APE is also involved in transcriptional regulation. This was originally discovered by the cloning of a human gene, the encoded product of which is involved in the redox stimulation of the DNA-binding activity of several proteins such as FOS and JUN and NF-κB (7). The protein responsible for the redox activity (Ref-1) was found in the same study to be identical to APE. More recent studies have shown that APE/Ref-1 also participates in the redox status of the tumor suppressor protein p53, converting inert p53 to an active form (8).

The redox and DNA repair activities of APE/Ref-1 appear to reside in two distinct domains (9), with the NH_2 terminus primarily involved in redox control and the COOH-terminus active in the repair of AP sites. Scattered throughout the protein are a number of potential sites that are susceptable to phosphorylation (Fig. 1) that we reasoned might be important in the control of APE/Ref-1 activity. Here we report that CKII, but no other kinases that were included in this study, completely abolished the ability of APE/Ref-1 to cleave DNA substrates containing an abasic site.

Materials and Methods

Cloning and Expression of APE. The full-length human APE/Ref-1 cloning into pGEX has been described previously (10). The GST-APE/Ref-1 fusion contained a factor X cleavage site so that GST and APE/Ref-1 could be proteolytically separated from one another. The conditions for factor Xa (NEB) cleavage were as described previously by us (11). These constructs were overexpressed in an *E. coli* strain (RPC501) deficient for the 5′-acting APEs exonuclease III (*xth*) and endonucleases IV (*nfo*; Ref. 12). The conditions for overexpression and purification are by procedures published previously (13), except that recombinant bacteria were grown at 37°C instead of room temperature.

Conditions for Phosphorylation and Phosphatase Treatment of APE. APE/Ref-1 was phosphorylated *in vitro* using CKI and CKII (New England Biolabs) or PKC (catalytic subunit; CalBiochem, San Diego, CA). Typically, APE/Ref-1 was phosphorylated in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 200 μM ATP, 200 μCi [γ − ^{32}P]ATP (300 cpm/pmol), 5 μg of APE/Ref-1, and 10 units of kinase. One unit (U) of kinase activity is defined as the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to peptide substrate in 1 min under the conditions described above. For cold phosphorylation, unlabeled 1 mM ATP was used. After incubation for 30 min at 30°C, SDS-PAGE sample buffer was added, samples were boiled for 10 min, and protein samples were separated by SDS 12% PAGE. The gel was stained, destained, dried, and subjected to autoradiography or PhosphorImager analysis. For dephosphorylation, *in vitro* phosphorylated APE/Ref-1 was diluted 20 times in λ-PPase buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM DTT, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 μg/ml BSA, and 5 units of λ-PPase (New England Biolabs). One unit of Lambda phosphatase (λ-PPase) is the amount of enzyme that hydrolyzes 1 nmol of *p*-nitrophenyl phosphate (50 mM) per min at 30°C under the conditions described above. Samples were analyzed by SDS-PAGE as described above.

Activity on an Abasic Site-containing DNA. A 37-bp 5′ ^{32}P end-labeled duplex DNA fragment (AP-37mer) was used as described previously (13). Reaction mixtures (10 μl) contained approximately 1 pmol of 5′ end-labeled AP-37mer, 50 mM HEPES (pH 7.5), 50 mM KCl, 1 μg/ml BSA, 10 mM $MgCl_2$,

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[3] The abbreviations used are: AP, apurinic/apyrimidinic; APE, AP endonuclease; CK, casein kinase; GST, glutathione *S*-transferase; PKC, protein kinase C; λ-PPase, lambda protein phosphatase.

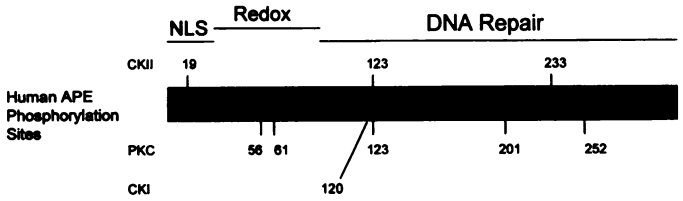

Fig. 1. Potential phosphorylation sites possessed by APE/Ref-1. The amino acid sequence of APE/Ref-1 was searched for protein motifs using GeneRunner software (version 3.02; Hastings Software, Inc., Hastings, NY, 1994). The motif analysis used the PROSITE database, which is based on the multiple alignment of proteins by EMBL. Over 924 motifs were searched, and only the CKI, CKII, and PKC sites identified.

0.05% Triton X-100, and 100 pg of GST-APE/Ref-1 or APE/Ref-1 alone. The DNA reaction products were separated on a 16% polyacrylamide gel containing 7 M urea. Dried gels were subjected to autoradiography for visualization or PhosphorImager (Molecular Dynamics) analysis using ImageQuant Software.

Results

Potential Phosphorylation Sites Present in APE/Ref-1. We have identified a number of potential phosphorylation sites that are scattered throughout the APE/Ref-1 protein (Fig. 1). There are three CKII sites (ST-2aa-DE), two of which reside in the presumptive DNA repair domain of APE/Ref-1. There is a single CKI site (S-2aa-ST), and it resides in the DNA repair portion of APE/Ref-1. Lastly, we have identified five PKC sites (ST-1aa-RK), three of which reside in the DNA repair domain of APE/Ref-1. Notably, one of these (position 123) appears to overlap with a CKII site that also exists at 123.

Inactivation of APE Activity with CKII. We tested three different kinases that are specific for serine/threonine residues, i.e., CKI, CKII, and PKC. Notably, all three kinases were found to phosphorylate APE/Ref-1, as revealed by PhosphorImager analysis of an SDS-PAGE gel used to separate the phosphorylated APE/Ref-1 proteins (Fig. 2*B*). It should be noted that GST alone is not subject to phosphorylation (data not shown), so that all the phosphorylation revealed by the analysis depicted in Fig. 2*B* is due to that occurring on the APE/Ref-1 portion of the fusion. Importantly, the phosphorylation carried out by CKII resulted in the total inactivation of the APE activity possessed by APE/Ref-1 (Fig. 2*A*). Conversely, the other two serine/threonine kinases tested had no effect whatsoever on the ability of APE/Ref-1 to cleave AP-37mer, although they were able to phosphorylate APE/Ref-1.

Reactivation of Inactive, Phosphorylated APE/Ref-1. If indeed CKII phosphorylation was the sole ingredient that led to the inactivation of APE activity possessed by APE/Ref-1, then the inactivation process should be reversible with phosphatase treatment of the inactive, phosphorylated form of APE/Ref-1. As can be seen in Fig. 3, a 30-min incubation combining λ-PPase together with CKII-phosphorylated APE/Ref-1 resulted in almost complete restoration of the ability of the DNA repair protein to act on the AP-37mer (*Lane 3*), and by 60 min (*Lane 4*), there were more than enough active molecules of AP/Ref-1 to completely process the abasic DNA substrate. On the other hand, if a 60-min incubation takes place combining λ-PPase and its specific inhibitor (*Lane 1*), APE/Ref-1 remained unable to act on the AP-37mer.

As a means of demonstrating that λ-PPase was in fact removing phosphate groups from CKII phosphorylated APE/Ref-1, a PhosphorImager analysis of phosphorylated and phosphastase-treated APE/Ref-1 separated on SDS-PAGE was conducted (Fig. 4). As can be seen, the λ-PPase (Fig. 4, *Lane 2*) completely removed all radioactive phosphate from CKII-phosphorylated APE/Ref-1 (Fig. 4, *Lane 1*).

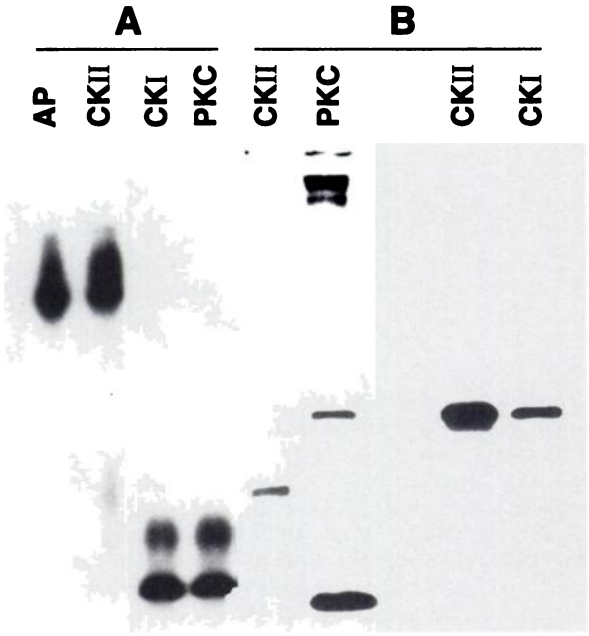

Fig. 2. Phosphorylation of APE/Ref-1 and the consequences on its ability to cleave AP-37mer. *A*, activity on AP-27mer. GST-APE/Ref-1 was phosphorylated with ATP (1 mM), and then the redox/repair protein (100 pg) was tested for activity using AP-37mer as a substrate. Reactions were separated on a DNA sequencing gel and analyzed by autoradiography. *B*, GST-APE/Ref-1 were phosphorylated with the individual serine/threonine kinases and [γ-^{32}P]ATP. Approximately 100 ng of phosphorylated protein were loaded per lane and subjected to SDS-12% PAGE and analyzed by a PhosphorImager. The *far left lane* depicting CKII phosphorylation is that of APE/Ref-1 cleaved from GST. The source of the lower molecular weight band produced by PKC phosphorylation is most likely a proteolysis product of APE/Ref-1.

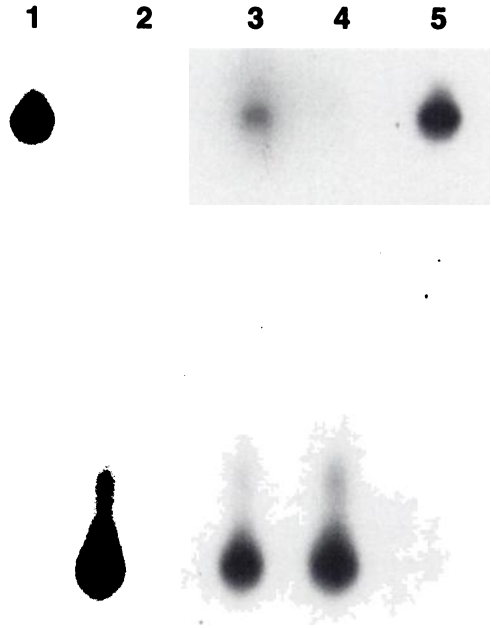

Fig. 3. Phosphorylase treatment restores activity of phosphorylated APE/Ref-1. Conditions for phosphorylation are described in "Materials and Methods" and the legend to Fig. 2*A*. These phosphorylated proteins in turn were used as substrates for λ-PPase. APE/Ref-1 (100 pg) was then tested for activity using AP-37mer as a substrate, and the results were analyzed by autoradiography. *Lane 1*, GST-APE/Ref-1 (100 pg) phosphorylated with CKII, then treated with 0.1 unit of λ-PPase (60 min) in the presence of its inhibitor sodium orthovanadate (2 μM). *Lane 2*, GST-APE/Ref-1 phosphorylated with PKC. *Lane 3*, CKII-phosphorylated GST-APE/Ref-1 treated with λ-PPase for 30 min or 60 min (*Lane 4*). *Lane 5*, AP-37mer alone.

Fig. 4. Phosphatase treatment dephosphorylates radioactive GST-APE/Ref-1. Phosphorylation of GST-APE/Ref-1 (*Lane 1*) used [γ-^{32}P]ATP as described in Fig. 2*B* and "Materials and Methods." Dephosphorylation (*Lane 2*) with λ-PPase was for 60 min. Proteins were then subjected to SDS-PAGE and PhosphorImager analysis.

Discussion

APE/Ref-1 was originally characterized as a DNA repair enzyme that was highly efficient in initiating the repair of AP sites in DNA. Considering that these lesions are noninstructive for DNA replication, their presence has been shown to be both mutagenic and lethal if they are allowed to persist in cellular DNA. That APE/Ref-1 has a major role in the repair of these sites is clearly suggested from results gathered recently showing that the depletion of APE activity renders cells sensitive to DNA-damaging agents (5) and that APE/Ref-1 and mammalian DNA polymerase β exist in a protein-protein complex at an abasic site (6).

APE/Ref-1 is also important for stimulating the DNA-binding activity of several transcription factors such as FOS, JUN, and NF-κB. More recently, it has been shown that APE/Ref-1 appears to be part of a redox cascade in which the direct association of thioredoxin and APE/Ref-1 (14) leads to the reduction of redox-sensitive cysteine residues present in the AP-1 proteins such as FOS and JUN.

Redox regulation mediated by APE/Ref-1 also appears to be important in p53 activity (8). Previous studies have shown that p53 is converted from an inactive to active state by phosphorylation, antibody binding, redox conditions, and the presence of short single strands of DNA (15). The primary outcome of this increased expression of p53 is either apoptosis or the induction of G_1 arrest, both of which are thought to protect or destroy a cell that has undergone DNA damage. It now appears that this cellular protection cascade can also be initiated by APE/Ref-1. In a manner similar to how it controls the expression of AP-1 proteins, it appears that APE/Ref-1 converts inert, oxidized p53 to an active form by changing its redox potential (8).

In view of the abundance of APE/Ref-1 molecules in human cells, the conversion of p53 to an active form could clearly have deleterious consequences on a cell that has not been challenged by some form of stress. As noted by Jayaraman *et al.* (8), one way of protecting the cell from the unwarranted consequences of p53 expression is the possible posttranslational regulation of APE/Ref-1.

As shown here, APE/Ref-1 is subject to phosphorylation *in vitro*. What effect this would have on the redox activation carried out by APE/Ref-1 has yet to be established. However, it is clear that the APE activity of APE/Ref-1 can clearly be inactivated by CKII. Thus, posttranslational events that affect the redox functions of APE/Ref-1 seems reasonable and perhaps warranted in view of its interaction with p53. Conversely, it is unclear what advantage it might be to the cell for the inactivation of a major DNA repair activity, although more than likely only a small proportion of APE/Ref-1 molecules are subject to this modification *in vivo*.

One scenario in which APE inactivation may be important is when APE/Ref-1 acts as repressor of promoter sites containing a negative Ca^{2+}-response element (16). The promoter of APE/Ref-1 contains a negative Ca^{2+}-response element sequence,[4] where recent evidence indicates that APE/Ref-1 may act as a repressor for its own expression (17). Under these conditions of protein-DNA contacts, it may be deleterious to promoter regulation to have the repair portion of APE/Ref-1 distracted by its potential to act on an abasic site. The redox functions of APE/Ref-1 involve protein-protein interactions, where the modulation of APE activity would not appear to be necessary. Nevertheless, the phosphorylation event itself may serve in the capacity to direct APE/Ref-1 as a repressor of negative Ca^{2+}-response element sequences.

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[4] We have data demonstrating negative regulation of the *APE/Ref-1* gene following an increase in intracellular Ca^{2+} (M. R. Kelley, W. A. Deutsch, and K. A. Robertson, manuscript in preparation).