

# Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of Cysteines 93 and 310

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## ABSTRACT

**Apurinic/aprimidinic endonuclease 1/redox effector factor-1 (APE1/Ref-1, abbreviated as APE1) is a molecule with dual functions in DNA repair and redox regulation of transcription factors. Accumulated work has shown that the biological activities of APE1 are sensitive to oxidative stress; however, whether APE1 functions can be regulated by nitrosative stress remains unknown. In this investigation, we found that S-nitrosoglutathion (GSNO), a nitric oxide donor and also an S-nitrosating agent, effectively stimulated nuclear export of APE1 in a CRM1-independent manner. This nuclear-cytoplasmic translocation was dependent on S-nitrosation modification of APE1, as simultaneous mutation of S-nitrosation target sites Cys93 and Cys310 completely abrogated the cytoplasmic redistribution. The translocation process was reversal and specific, as it could be reversed by reductive reagents, but could not be mimicked by H<sub>2</sub>O<sub>2</sub>. In structure, the region aa.64–80 and the beta-strand aa.311–316 in proximity to Cys93 and Cys310 were important for GSNO-induced APE1 relocation. In addition, a defect of importin-mediated nuclear import pathway was found in the NO-insulted cells, and p50 and HDAC2 were identified as APE1 nuclear export inhibitory proteins. Together, this study may provide a novel molecular mechanism, which links nitrosative stress to APE1-associated physiological and pathological processes.**

## INTRODUCTION

Human apurinic/aprimidinic endonuclease 1/redox effector factor-1 (APE1/Ref-1) is a bifunctional oxidative-stress-responsive protein (1–5). On one hand, it acts as an apurinic/aprimidinic endonuclease, during

the second step of the DNA base excision repair pathway, which is responsible for the repair of cellular oxidative DNA damages. On the other hand, it plays a crucial role, as a coactivator for various transcription factors in controlling gene expression by redox-dependent mechanism. Therefore, the regulation of APE1 function is a very important issue (5–7). The cellular function of APE1 is coordinately controlled at several levels. Firstly, the expression of APE1 can be upregulated by a variety of reactive oxygen species (ROS) and ROS-generating systems (6,8–10). Secondly, APE1 can be modified by phosphorylation, acetylation, and redox modification, which are important for the regulation of its DNA-binding, transcriptional regulation, and DNA repair functions (3,11–14). Lastly, the APE1 subcellular distribution varies according to different cell types and environment stresses. (1) The expression pattern of APE1 is mainly nuclear, but cytoplasmic staining has also been reported (15–17). The latter is usually observed in highly metabolically active or proliferative cells, which may experience an increased oxidative stress (4,5). (2) Generally, stimuli that induce APE1 expression are also able to promote its intracellular movement. Various redox-related stimuli can induce the translocation of APE1 from cytoplasm to nucleus (9,18–20). The nuclear import process may be dependent on an N-terminal nuclear import sequence (NLS) that mediates the importin-dependent nuclear import of APE1 (21). (3) In B-lymphocyte, H<sub>2</sub>O<sub>2</sub> stimulation can induce a relocation of APE1 into mitochondria (22). Recently, a low abundance of mitochondrial-localized APE1 was found as the N-terminal 33 residues-truncated form (22,23). Although various subcellular localizations and intracellular trafficking of APE1 have been reported, little is known about how these phenomena are regulated. Particularly, the mechanism of the redox regulated localization change remains open.

Nitric oxide (NO) is a reactive free radical that plays a central role in diverse signaling pathways (24–27). Apart from the well-known cGMP-dependent signaling pathway of NO, there is also a cGMP-independent pathway

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that involves protein S-nitrosation. S-nitrosation is a ubiquitous redox-related modification of cysteine thiols by nitric oxide, which transduces the bioactivity of NO. S-nitrosation has been implicated in regulation of gene transcription (28), enzyme activity (29), and protein nuclear translocation (30). APE1 contains a redox-active domain and three redox-sensitive cysteine residues, and its subcellular localization seems redox-sensitive (4). However, whether APE1 can be modified by NO-elicited S-nitrosation and whether its subcellular distribution can be regulated by this redox modification have not been reported.

In this study, we reported that APE1 can inducibly translocate from nucleus to cytoplasm in response to nitric oxide stimulation in a CRM1-independent manner. This nuclear export process of APE1 is reversible and dependent on the S-nitrosation of its Cys93 and Cys310 sites. In structure, two antiparallel beta-strands close to Cys93 and Cys310 were identified to be required for NO-mediated export of APE1. In addition, it was found that the importin-mediated nuclear import pathway was repressed in NO-insulted cells, which may prevent cytosolic APE1 from re-transporting into the nucleus. Thus, we for the first time reveal a molecular event that coordinates S-nitrosation modification and nuclear-cytosolic shuttling of APE1. Since the disruption of APE1 subcellular localization may result in a defect in intra-nuclear DNA repair and transcriptional regulation functions, this finding may establish a novel role of APE1 in NO-related physiological and pathological processes.

## MATERIALS AND METHODS

### Reagents and plasmids

Leptomycin B (LMB), 1, 4-dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), cycloheximide (CHX) and other reagents, which were not specified, were purchased from Sigma. Methyl methanethionylsulphonate (MMTS) and *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide (Biotin-HPDP) were purchased from Pierce. Hoechst 33342 was purchased from Molecular Probes. PNRI-299 was a generous gift of Dr. Michael Kahn (University of Washington, USA) (31). S-Nitrosoglutathione (GSNO) was prepared as previously described (32). The HA-APE1 expression plasmid was kindly provided by Mark R. Kelley (Indiana University, School of Medicine, USA) (33). The APE1 mutants HA-APE1(C65S), HA-APE1(C93S), HA-APE1(C310S), HA-APE1(C93/310S), and HA-APE1(C65/93/310S) were generated by site-directed mutagenesis (Stratagene). The expression vectors encoding APE1-truncated mutants were generated by PCR amplification of APE1 cDNA fragments and subsequent cloning of the PCR products into the XbaI and HindIII sites of pcDNA3.1-myc expression vector (Invitrogen). The truncated mutants were named based on the amino acid residues retained in the constructs. For example, APE1 (43–318) contains amino acids 43 to 318, while APE1(1–305) contains amino acids 1 to 305. The APE1 ( $\Delta$ 64–80) was created by deleting amino acids 64–80 from the wild-type APE1.

GFP-I $\kappa$ B was kindly provided by Michael R.H. White (University of Liverpool, UK) (34); EGFP-XRCC1 and Flag-XRCC1 are kind gifts from Dr. Josiane M $\acute{e}$ nissier-de Murcia (Ecole Sup $\acute{e}$ rieure de Biotechnologie de Strasbourg Boulevard S, France) (35); GFP-GFP-NLS and GFP-GFP-M9 were kindly provided by Dr. Ralph H. Kehlenbach (Universit $\ddot{a}$ t Heidelberg, Germany) (36); CMV-p53, Flag-p53, HDAC2-Myc, and pcDNA4-Trx were generous gifts from Dr. Warner C. Greene (University of California, USA) (37), Dr. Kenji Fukasawa (Yamaguchi University, Japan) (38), Dr. Tony Kouzarides (Gurdon Institute and Department of Pathology, UK) (39), and Dr. J. Haendeler (University of Frankfurt, Germany) (40), respectively.

### Cell culture and transient transfection

Human HepG2 hepatoma cells and HEK293 embryonic kidney cells (ATCC) were cultured in a DMEM medium containing 10% fetal calf serum (FCS) at 37°C in a CO<sub>2</sub> incubator. Cell transfection was performed with JetPei (Polyplus) transfection reagent according to the manufacturer's instruction.

### Immunofluorescence analysis

Indirect immunofluorescence was performed as previously described (41). In brief, HEK293 cells were transfected with HA-APE1 or APE1-myc expression vector. 36 h later, cells were fixed with 3.7% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and then blocked with PBS containing 5% BSA and 10% FCS for 30 min. The cells were subsequently incubated with anti-HA antibody (Santa Cruz, sc-805) or anti-myc antibody (Santa Cruz, sc-40) overnight at 4°C, and flooded with Texas-Red-labeled secondary antibody (Molecular Probe) for 60 min. The cell nuclei were stained with Hoechst 33342. The fluorescence image of the stained nuclei and the immunofluorescence image of HA-APE1 were observed on the laser-scanning confocal microscope (Olympus FV500, Tokyo, Japan) equipped with appropriate filters.

### Cell fractionation

HepG2 cells growing on 35 mm plates were harvested in ice-cold PBS and pelleted by centrifugation at 2000 g for 1 min. Cell pellets were resuspended in ice-cold hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1% Triton X-100, and protease cocktail), vortexed for 15 sec, and incubated on ice for 15 min followed by centrifugation at 1000 g at 4°C for 5 min. The pellets were washed twice with hypotonic buffer, and then resuspended in SDS sample buffer (without dye or DTT) and boiled for 20 min. After a brief centrifugation, the supernatants were collected and used as the nuclear fractions. Both cytosolic and nuclear proteins were quantified by bicinchoninic acid protein assay (Pierce) and subjected to Western blot analysis.

### Biotin-switch assay

Cell lysates were prepared in lysis buffers (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid,

0.1% SDS, 1% NP-40, 0.1 mM neocuproine, and protease cocktail). The supernatant of cell lysis was assayed for protein concentration by BCA method, and then adjusted to the appropriate concentration. Blocking buffer (2.5% SDS, 20 mM MMTS in HEN buffer) was mixed with the samples and incubated for 30 min at 50°C to block free thiol groups. After removing excess MMTS by acetone precipitation, nitrosothiols were then reduced to thiols and biotinylated by reducing buffer (1% SDS, 10 mM ascorbate, 4 mM Biotin-HPDP). Then, the biotinylated proteins were pulled down by streptavidin-agarose beads, eluted by SDS sample buffer, and subjected to Western blot analysis.

### Western blot analysis

Western blot analysis was performed according to standard protocols. In brief, equal amount of protein fractions or agarose beads were mixed with SDS sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Pall). Then, rabbit anti-human APE1 polyclonal antibody (Santa Cruz, sc-334), rabbit polyclonal HA antibody (Santa Cruz, sc-805), mouse monoclonal tubulin antibody (Santa Cruz sc-5286), and mouse monoclonal Histone H1 antibody (Santa Cruz, sc-8030) were used as primary antibodies to detect APE1, HA-APE1, tubulin, and Histone H1, respectively. The horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence kit (Pierce) were used to visualize the separated proteins.

## RESULTS

### GSNO-induced nuclear export of APE1 in a CRM1-independent manner

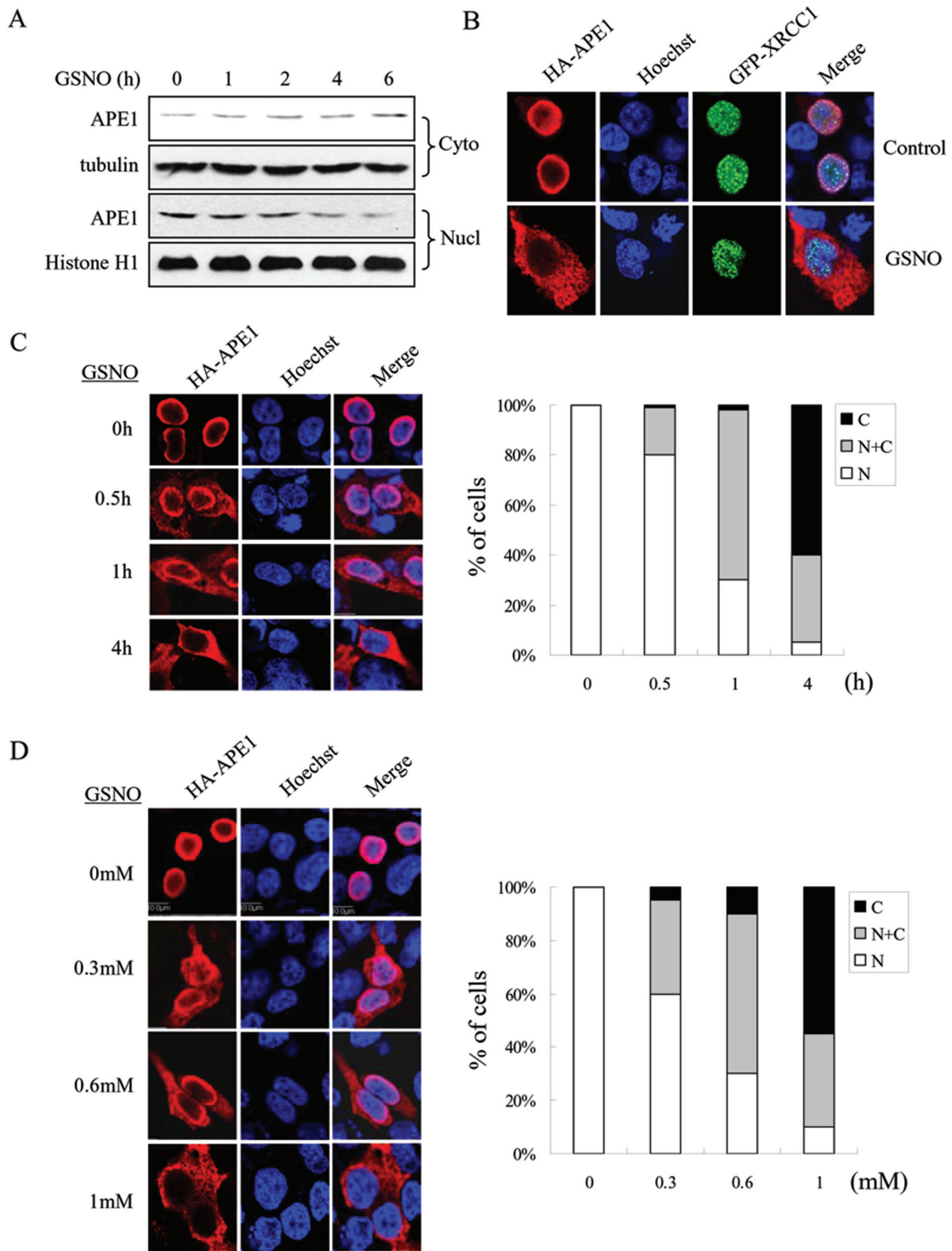
To investigate whether nitric oxide was able to affect the subcellular distribution of APE1, HepG2 hepatoma cells were treated with GSNO, a widely used nitric oxide donor and also an S-nitrosating agent (30,42). APE1 expressions in both the cytoplasmic and nuclear fractions were examined by immunoblotting analysis. As shown in Figure 1A, GSNO stimulation resulted in a significant decrease of nuclear distribution of APE1 in a time-dependent manner. At the same course, the cytosolic APE1 contents were markedly enhanced. As a control, H<sub>2</sub>O<sub>2</sub> did not result in significant change in nuclear or cytosolic APE1 content (data not shown). These observations suggest a possibility that GSNO could stimulate nuclear-to-cytoplasmic translocation of APE1 in HepG2 cells. To further confirm this phenomenon, HA-APE1 expression vector was transiently transfected into HEK293 cells together with GFP-fused XRCC1, another DNA-repair-related protein (35), and the subcellular distribution of APE1 was analyzed by immunofluorescence with anti-HA antibody. As shown in Figure 1B, the transfected APE1 was absolutely localized in the nucleus in the untreated cells. However, treatment of cells with 1 mM GSNO for 4 h resulted in a clear translocation of APE1 from the nucleus to the cytoplasm. In contrast, the intra-nuclear localization of GFP-XRCC1 could not be

affected by GSNO at all. A similar phenomenon was also observed in COS-7 cells (data not shown). Thus, it is suggested that GSNO selectively induced cytosolic translocation of APE1 but not all the nuclear proteins. To determine whether the GSNO-induced nuclear exclusion of APE1 was time or dose dependent, we stimulated the cells with 1 mM GSNO for different time. As Figure 1C shows, consistent with the endogenous APE1 in HepG2 cells, transfected APE1 time-dependently translocated into the cytosol in response to GSNO stimulation. About 60% of transfected APE1 was completely expelled to the cytoplasm within 4 h. It was also observed that GSNO induced nuclear export of APE1 in a dose-dependent manner. Treatment with up to 1 mM GSNO exerted the most significant effect on APE1 translocation (Figure 1D). To know whether GSNO-induced intracellular redistribution of APE1 was attributed to the synthesis of new proteins, CHX, a protein synthesis inhibitor, was used. It turned out that CHX could not prevent GSNO-induced APE1 nuclear export (Figure 2A).

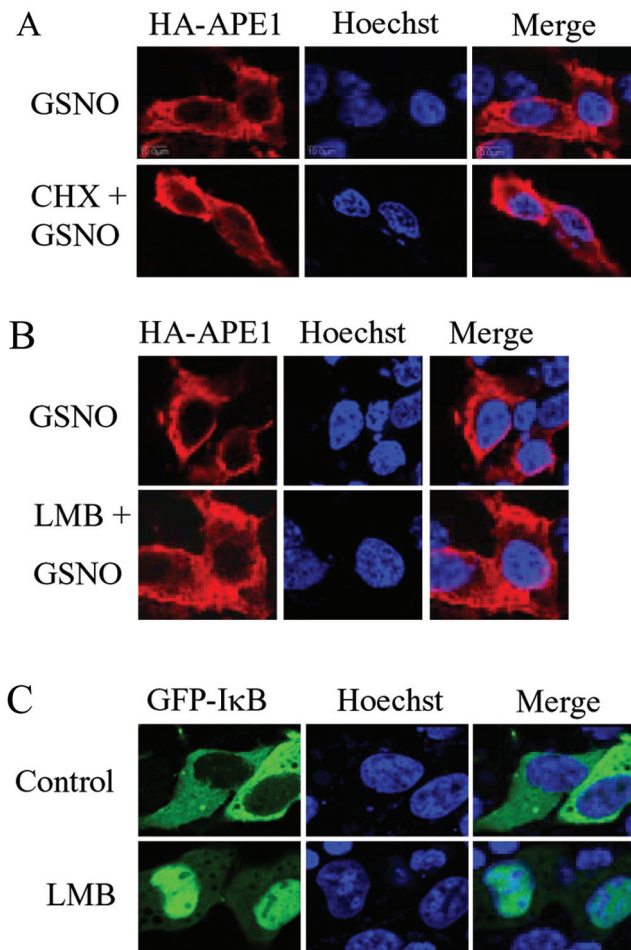
Most proteins that undergo nucleocytoplasmic trafficking depend on the nuclear export receptor CRM1 for nuclear export. It was speculated in a recent report that APE1 probably bore a CRM1-dependent nuclear export sequence (NES) (21). To test whether GSNO-induced nuclear export of APE1 was associated with the CRM1 pathway, HEK293 cells transfected with HA-APE1 were pre-incubated with LMB, a CRM1-specific inhibitor, and then stimulated with GSNO. Figure 2B showed that LMB failed to prevent GSNO-induced nuclear export of APE1. As a parallel control, LMB treatment markedly induced nuclear accumulation of transfected GFP-IκB (Figure 2C), whose nuclear export is dependent on CRM1 pathway (34,43). Therefore, this result indicated that the nuclear export of APE1 induced by GSNO may be not via a classical CRM1-dependent pathway.

### S-nitrosation of APE1 at cysteines 93 and 310 controlled its nuclear-cytoplasmic shuttling

S-nitrosation, as a post-translational modification, has been implicated in nitric-oxide-elicited multiple cellular effects (26,27). To investigate the possibility that GSNO-induced nuclear export of APE1 is regulated by nitrosation modification, we first examined whether transfected HA-APE1 could be S-nitrosated using the biotin-switch assay, in which all the S-nitrosated cysteines can be specifically identified on Western blots after replacing -SNO with a more stable biotin group by chemical reduction with ascorbate, as described previously (30,42). As shown in Figure 3A, treatment of HEK293 cells with 1 mM GSNO for 4 h significantly induced HA-APE1 S-nitrosation. In contrast, H<sub>2</sub>O<sub>2</sub> treatment did not induce such modification (data not shown), indicating the specificity of this method. To further confirm this modification, the cells were stimulated with GSNO for 4 h, followed by DTT or NAC for another 2 h. As shown in Figure 3A, the S-nitrosated form of APE1 was totally eliminated. Since both DTT and NAC have



**Figure 1.** GSNO stimulated the translocation of APE1 from nucleus to cytoplasm. **(A)** GSNO induced nuclear-cytosolic translocation of endogenous APE1 in HepG2 cells. Cells were stimulated with 1 mM GSNO for indicated time. Cytoplasmic and nuclear fractions were separated by SDS/PAGE and analyzed by immunoblotting with antibody against APE1. Antibodies against tubulin and histone H1 were used to ensure equal cytosolic and nuclear loading, respectively. A representative of three independent experiments with similar results is shown. **(B)** Representative fluorescence images of fixed HEK293 cells co-transfected with HA-tagged APE1 and GFP-XRCC1 in the presence or absence of 1 mM GSNO for 4 h treatment. HA-APE1 was detected with the anti-HA primary antibody and a Texas-Red-labeled second antibody. XRCC1 was visualized by GFP. The nuclei were stained with Hoechst 33342. The merged image was also shown. **(C)** Time-dependent translocation of HA-APE1 overexpressed in HEK293 cells in response to GSNO determined by immunofluorescence with anti-HA. Cells were stimulated with 1 mM GSNO for 0.5, 1, and 4 h, and the distribution patterns of HA-APE1 were scored for at least 100 cells and classified into three categories: C, cytoplasmic-dominant distribution; N+C, roughly equal distribution in nuclear and cytoplasmic compartments; and N, nuclear-dominant distribution. **(D)** Dose-dependent effect of GSNO on the nuclear export of HA-APE1. Cells were stimulated with GSNO at the indicated concentrations for 4 h.



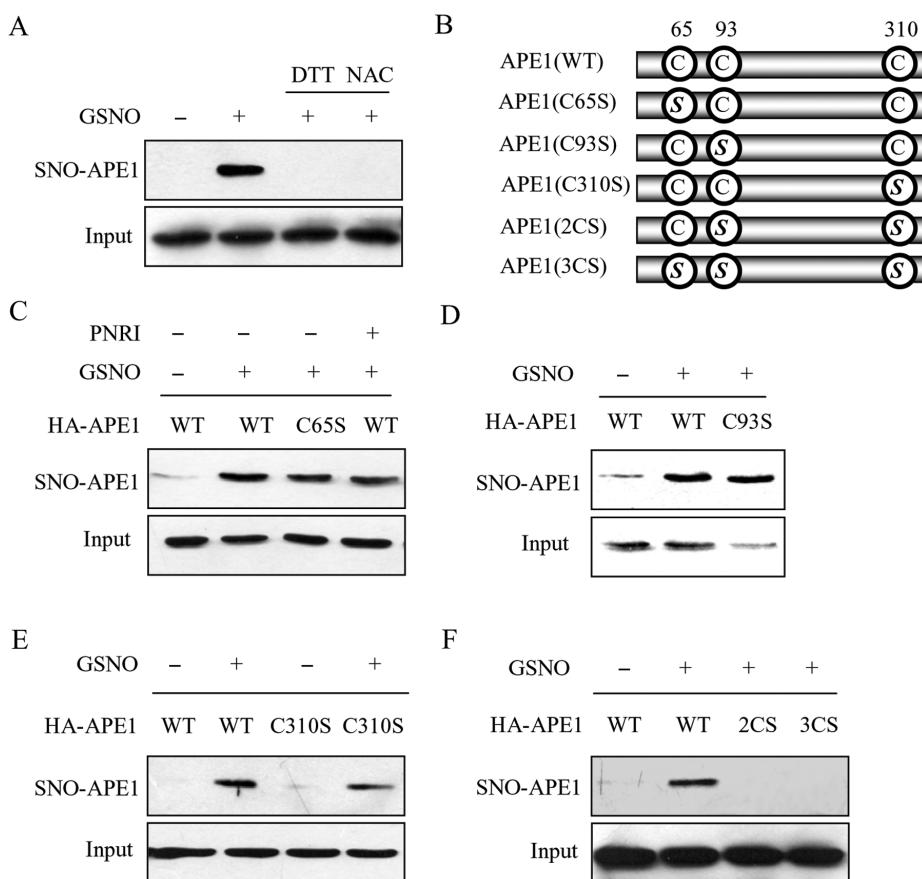
**Figure 2.** The effects of CHX and LMB on GSNO-induced cytosolic translocation of APE1. (A) Effect of CHX. 30 h after transfection, cells were treated with 1 mM GSNO for 4 h in the presence or absence of CHX, and then fixed and immunostained by HA antibody and a Texas-red labeled secondary antibody, counterstained with Hoechst 33342, and analyzed by fluorescence microscopy. (B) Effect of LMB. HEK293 cells transfected with HA-APE1 were treated with 5 ng/ml LMB for 4 h, followed by 1 mM GSNO for another 4 h, and then the subcellular distribution of HA-APE1 was determined. (C) Positive control of the effect of LMB on the localization of GFP-I $\kappa$ B expressed in HEK293 cells.

been reported to denitrosylate target proteins (30,44), these observations suggested that S-nitrosation of APE1 was a reversible process. Furthermore, we did observe that the endogenous APE1 could be effectively S-nitrosated in multiple cell types including HepG2, Eahy926, SH-SY5Y and RAW264.7 cells (unpublished data).

In the next series of experiments, we sought to identify the specific sites of APE1 S-nitrosation. APE1 contains seven cysteine amino acid residues, three of which, including Cys65, Cys93 and Cys310, are redox-sensitive. The Cys65 and Cys93 residues are located within the redox-active domain, and Cys65 has been speculated to be the redox-active site in the regulation of transcription factor activity. The Cys310 residue resides in the

endonuclease domain, and its oxidative modification has been implicated in regulation of the DNA-repair function (4). For this reason, we hypothesize that the three redox-sensitive cysteines were the potential targets of APE1 S-nitrosation. We first developed three single-point mutants APE1(C65S), APE1(C93S) and APE1(C310S), and used the biotin-switch method to test for their nitrosation. As demonstrated in Figure 3C, D and E, mutation of a single residue of the three cysteines to serine could not eliminate GSNO-induced APE1 S-nitrosation in transfected HEK293 cells. Consistently, PNRI-299, an APE1 Cys65 site blocker (31), could not affect APE1 nitrosation (Figure 3C). These data suggested that there may be more than one cysteine to be S-nitrosated. To test this hypothesis, we simultaneously mutated the Cys93 and Cys310 to serines. Interestingly, simultaneous mutation of Cys93 and Cys310 (the mutant was named "2CS") completely prevented GSNO-induced APE1 nitrosation (Figure 3F), although mutation of either one had no discernible effect. To further confirm the result, we subsequently examined whether mutation of all the three cysteines could affect the nitrosation of APE1. As expected, similar to the APE1 (2CS), the triple mutant APE1 (3CS) was refractory to NO-elicited nitrosation (Figure 3F). Taken together, these results indicated that both Cys93 and Cys310 of APE1 were S-nitrosated targets *in vivo*.

To explore the possibility that S-nitrosation modification may determine the nuclear export of APE1, we examined the effects of DTT and NAC on GSNO-induced APE1 nuclear-cytosolic translocation. As shown in Figure 4A, both DTT and NAC could effectively reverse the GSNO-induced nuclear export of APE1, which was consistent with their effects on APE1 S-nitrosation, suggesting that the nuclear export of APE1 may be an S-nitrosation-dependent reversible process. To exclude the possibility that GSNO-exerted regulatory effect on APE1 translocation was attributed to the outcome of unspecific oxidative stress, HEK293 cells expressing HA-APE1 were exposed to 100  $\mu$ M or 1 mM  $H_2O_2$ . As shown in Figure 4A,  $H_2O_2$  didn't cause marked nuclear export of APE1 as GSNO did (the data obtained with 100  $\mu$ M  $H_2O_2$  was not shown). This observation indicated that the cytosolic translocation of APE1 was attributed to its S-nitrosation rather than other oxidative modifications. To finally establish the causal relationship of APE1 S-nitrosation and its nuclear export, we examined the subcellular distributions of APE1 cysteine mutants in the presence of GSNO. As shown in Figure 4B, mutation of Cys65 did not prevent, but instead slightly enhanced the GSNO-induced APE1 nuclear export. Mutation of either Cys93 or Cys310 could partially repress APE1 redistribution in response to GSNO stimulation. However, significantly, the S-nitrosation-deficient APE1 mutants, APE1 (2CS) and APE1 (3CS), remained in the nucleus irrespective of the presence of GSNO. Collectively, these observations clearly indicate that S-nitrosation of APE1 determined its nuclear export.

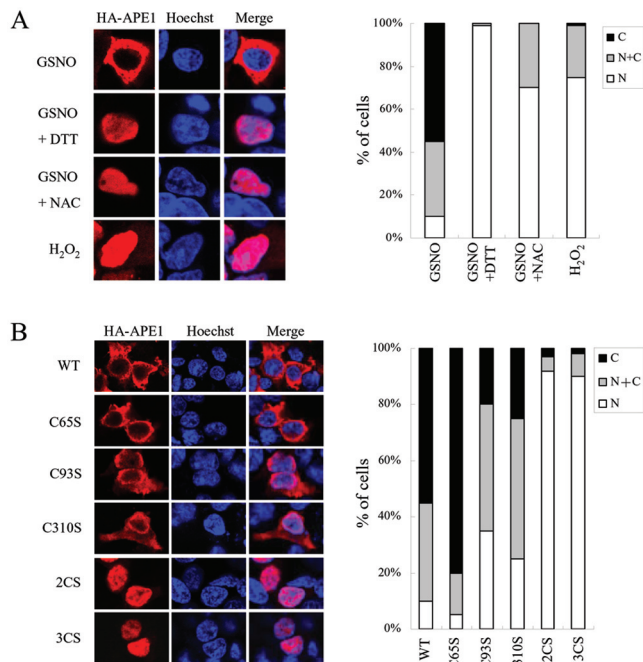


**Figure 3.** S-nitrosation of APE1 at Cys93 and Cys310 *in vivo*. (A) S-nitrosation of HA-APE1 expressed in HEK293 cells in the presence of 1 mM GSNO as determined by biotin-switch assay. For certain experiment, GSNO-challenged cells were treated with 10 mM DTT or 10 mM NAC during the last 2 h of incubation. All the S-nitrosated proteins were precipitated and detected by Western blotting with anti-HA antibody. (B) Schematic representation of APE1 and APE1 derivatives containing Cys-to-Ser mutations in various combinations. (C) GSNO-induced S-nitrosation of APE1 (WT) and APE1(C65S) in the presence or absence of PNRI. (D-F) S-nitrosation of APE1(C93S), APE1(C310S), APE1(2CS) and APE1(3CS).

### Two beta-strands in close proximity to Cys93 and Cys310 of APE1 were important for NO-induced conditional nuclear export

Except a flexible N-terminal 35-residues portion, APE1 forms a tight globular structure (45,46). In order to explain how NO-elicited nitrosation controlled nuclear export of APE1, we hypothesized that APE1 may undergo a conformational change upon S-nitrosation modification, which may lead to the exposure of a masked internal region that facilitates its nuclear export. For this consideration, we developed a series of N- and C-terminal-truncated APE1 mutants (Figure 5B), which were expected to unmask internal 'NES'. All these mutants were expressed in HEK293 cells as C-terminal Myc fusion. Their proper expressions in HEK293 cells were confirmed by immunoblotting (supplementary Figure 1A), and their subcellular localizations were examined by immunofluorescence with anti-Myc antibody. As Figure 5A shows, deletion of the N-terminal 43 residues of APE1, which contains the known NLS, effectively prevented APE1 from residing in the nucleus. Further deletion of N-terminal 123 residues led to

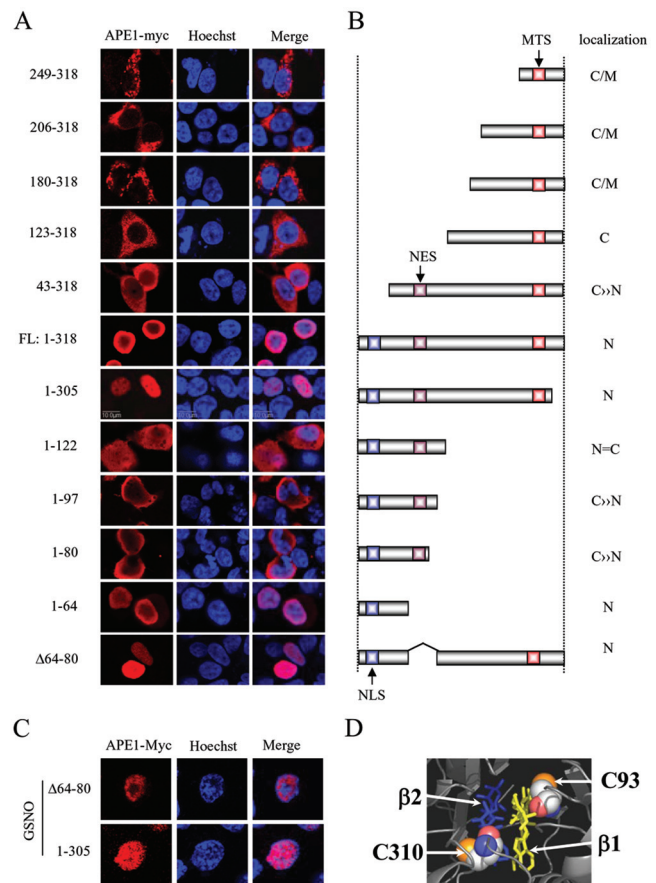
complete nuclear exclusion of APE1. Interestingly, the mutants with their N-terminal 180, 206 and 249 aa deleted demonstrated a marked mitochondrial localization. The immunofluorescence images of these mutants were overlapped with the co-transfected Mito-GFP, a mitochondrial indicator (data not shown). Although Chattopadhyay *et al.* identified the mitochondrial-localized APE1 as an N-terminal 33 residues-truncated form (22,23), the MTS of APE1 has not been mapped yet. Our work implied that the MTS is located in the C-terminal 69 amino residues of APE1 (Figure 5A). Interestingly, the MTS appears to be hidden by the N-terminal sequence of APE1 and, therefore, inaccessible to the protein surface, although the significance of the masked MTS is not known. Subsequently, to obtain the more direct information about the putative NES, the cellular localization of C-terminal-truncated mutants were investigated. Consistent with the full-length APE1, deletion of the C-terminal 13 aa did not alter the intranuclear distribution of APE1 (Figure 5A). However, surprisingly, further truncation up to N-terminal 122 aa resulted in a significant cytoplasmic distribution in



**Figure 4.** S-nitrosation of APE1 determined its nuclear export. (A) HA-APE1 subcellular distribution in transfected HEK293 cells determined by immunofluorescence after 4-h-treatment with 1 mM GSNO, followed by co-treatment with 10 mM DTT or 10 mM NAC for another 2 h, and 1 mM H<sub>2</sub>O<sub>2</sub>, respectively. (B) The subcellular localizations of HA-APE1 or its various mutants were determined in transfected HEK293 cells in the presence of 1 mM GSNO.

addition to its nuclear localization. Interestingly, the cytosolic proportion of APE1 increased with the deletion of more C-terminal aa residues. Notably, the mutant APE1(1-80) containing N-terminal 80 aa residues showed the least nuclear staining. However, once the region containing the 64-80 aa residues was deleted, it was relocalized in the nucleus (Figure 5A). Since the N-terminal 20 amino acids portion of APE1 contains NLS, these results indicated that the region (aa 64-80) might serve as an 'NES' when it is sufficiently unmasked. Interestingly, treatment with LMB did not result in nuclear accumulation of APE1(1-80) (data not shown), suggesting that the 'NES' may function in a CRM1-independent manner. In the next experiment, to evaluate the importance of the sequence (aa 64-80) in nitric-oxide-caused APE1 cytosolic translocation, we developed a mutant APE1( $\Delta$ 64-80), in which the potential NES region was deleted. Immunofluorescence experiment showed that this mutant completely lost the responsibility to GSNO-induced nuclear export (Figure 5C). It should be pointed out that the APE1( $\Delta$ 64-80) mutant could slightly interfere with cell viability, while all of the other APE1 mutants we used did not show any discernible effects (supplementary Figure 1B). In some sense, this observation perhaps supported the functionality of the aa.64-80 region.

Is there the possibility that S-nitrosation of APE1 leads to the exposure of "NES"? Analysis of APE1 ternary structure revealed that the putative NES is composed of



**Figure 5.** Mapping of the APE1 domains responsible for GSNO-induced nuclear export. (A) Immunofluorescence images depicting the subcellular localization of APE1 truncated mutants in transfected HEK293 cells. (B) Schematic representation of the N-terminal and C-terminal truncated mutants of APE1. The NLS and the predicted NES and MTS within the full-length protein are indicated. C, cytoplasmic staining; N, nuclear staining; M, mitochondrial staining. (C) The subcellular distribution of HA-APE1( $\Delta$ 64-80) and HA-APE1(1-305) determined by immunofluorescence in the presence of GSNO treatment. (D) Three-dimensional localization of the B1 (amino acids 61-69) and B2 (amino acids 311-317) beta-strands and the S-nitrosation sites C93 and C310 in the closed conformation of APE1. B1 and B2 form an antiparallel beta-fold. C93 and C310 are close to B1 and B2, respectively.

a part of beta-strand (B1) and a part of alpha-helix (H1) (45). Interestingly, Cys93 is adjacent to B1, whereas Cys310 is linked to another B1-associated beta-strand (aa 311-316, referred to B2). In structure, B1 and B2 form an antiparallel beta-fold residing between Cys93 and Cys310 (Figure 5D). To evaluate the importance of B2, we examined the intracellular localization of APE1(1-305) in the presence of GSNO. APE1(1-305) was chosen because it included neither Cys310 nor B2. Figure 5C showed that APE1(1-305) was rather insensitive to GSNO-induced nuclear export. Considering that APE1(C310S), the C310-inactive APE1 mutant, can still translocate to the cytosol in response to GSNO (Figure 4B), we concluded that B2 was another region that may facilitate GSNO-induced export of APE1 from nucleus. Thus, both the 'NES' and B2 beta-strand were critical for NO-elicited APE1 cytosolic translocation,

and the S-nitrosation of Cys93 and Cys310 may result in a conformational change that facilitates the two beta-strands to function.

### Nitric-oxide-repressed classical importin-dependent nuclear import pathway

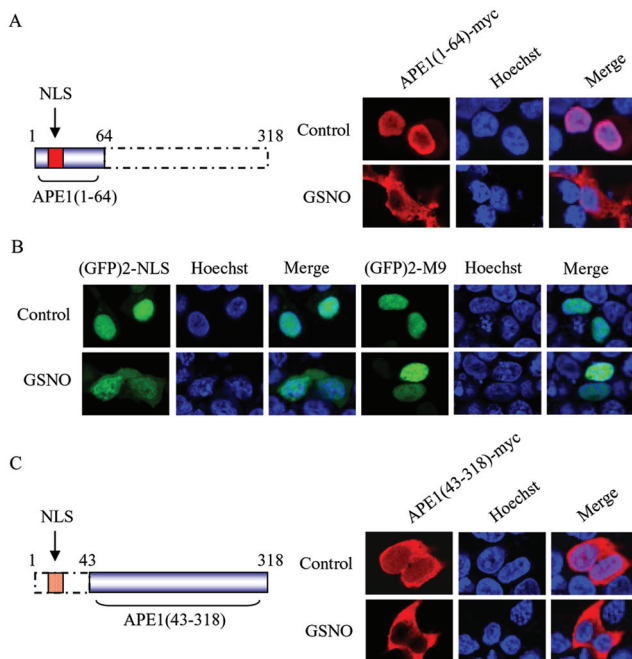
The above experiments clearly indicated that S-nitrosation determined the nuclear export of APE1. However, even if APE1 could be effectively exported into the cytosol by GSNO, it is still likely to be reimported into the nucleus via the classical nuclear import pathway. In fact, we did observe a complete nuclear exclusion of APE1 in response to GSNO stimulation. It has been reported that the classical NLS nuclear import pathway could be impaired by certain stresses such as UV irradiation, heat shock and oxidative stress (47). To address the issue whether nitric oxide was able to suppress the nuclear import of APE1, the subcellular distribution of APE1(1-64), which contains NLS but excludes most of its C-terminal portion, was examined in the presence of GSNO. Immunofluorescence experiment revealed that APE1(1-64) was dominantly localized in the nuclei of untreated cells (Figure 6A). However, once cells were challenged with 1 mM GSNO for 4 h, APE1(1-64) significantly accumulated in the cytoplasm (Figure 6A), suggesting that nitrosative stress may disrupt the nuclear import function of this mutant (Figure 2A). Since nuclear import of APE1 involves a

functional interaction between APE1 NLS and nuclear import receptor importin (21), we next determined whether the impaired nuclear import of APE1(1-64) was due to the defect of importin transport system. Simian virus 40 NLS is an established substrate for the importin receptor; fusion of this NLS to double GFPs could target the latter into the nucleus via importin (36). For this reason, we examined whether the nuclear import of SV40(NLS)-GFP-GFP could be repressed by nitric oxide. As shown in Figure 6B, SV40(NLS)-GFP-GFP was predominantly localized in the nucleus after 40 h transfection. However, when the cells were stimulated with 1 mM GSNO for 4 h, a markedly enhanced cytosolic fluorescence was observed. By comparison, GSNO treatment could hardly affect the nuclear localization of M9-GFP-GFP (Figure 6B), whose nuclear import is independent of the importin pathway (36). Thus, these observations indicated that the nuclear import efficiency of the classical NLS substrates was substantially decreased in the NO-insulted cells.

To exclude the possibility that the defect of importin nuclear import pathway could be totally responsible for GSNO-induced cytosolic translocation of APE1, APE1(43-318), which excludes NLS region but contains the intact APE1 globular structure, was employed. Indirect immunofluorescence showed a diffuse whole-cell distribution for this mutant, with a predominantly cytosolic staining (Figure 6C), indicating that a small proportion of APE1 molecules could diffuse into the nucleus in an NLS-independent manner. Surprisingly, the intra-nuclear APE1(43-318) could be totally exported into cytoplasm in the presence of GSNO (Figure 6C). This result, together with previous observations that the NLS-containing APE1 mutants (2CS, 3CS, 1-305, and  $\Delta$ 64-80) were refractory to GSNO-induced cytosolic translocation (Figure 4B, 5A), and the fact that H<sub>2</sub>O<sub>2</sub>, a functional importin inhibitor (47), cannot promote nuclear export of APE1 (Figure 4A), strongly indicated that repression of nuclear import of APE1 was not sufficient for GSNO-induced nuclear export of APE1. Its contribution may lie in preventing cytosolic APE1 from retransporting into the nucleus.

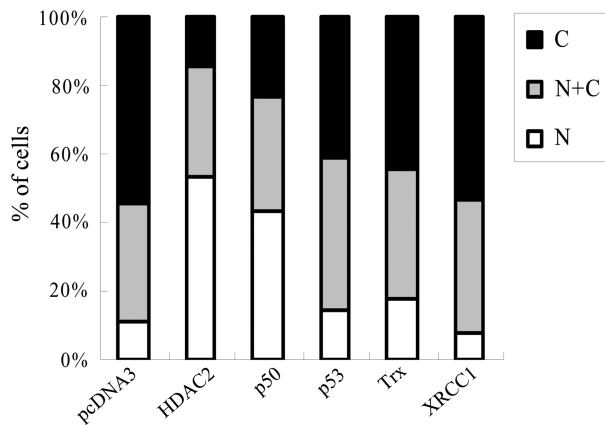
### HDAC2 and p50 antagonized GSNO-induced nuclear export of APE1

The above studies on the APE1 translocation were mainly based on APE1-overexpressed cell systems, in which the expression level of APE1 should be much higher than those of other proteins in cells. Therefore, it is necessary to investigate whether certain proteins could interfere with GSNO-induced APE1 nuclear export at physiologically comparable expression levels. For this consideration, the GSNO-induced APE1 nuclear export was reexamined by cotransfection of HA-APE1 with several established APE1-binding proteins, respectively. It was shown in Figure 7 that, among the tested APE1-binding proteins, only p50 (48) and HDAC2 (13), when overexpressed, respectively, could partially prevent GSNO-induced APE1 nuclear export. Other proteins including p53 (49), thioredoxin (Trx) (3) and XRCC1 (50) had little effect.



**Figure 6.** Nitric-oxide-repressed classical importin-mediated nuclear import pathway. (A) Left: Schematic presentation of the NLS-containing APE1(1-64) mutant. Right: Immunofluorescence images depicting the localization of APE1(1-64)-Myc in HEK293 cells challenged with or without 1 mM GSNO for 4 h. (B) Confocal images depicting the localization of (GFP)2-NLS and (GFP)2-M9 in HEK293 cells treated with GSNO for 4 h. (C) Left: Schematic presentation of the NLS-truncated mutant APE1(43-318)-Myc. Right: Immunofluorescence images depicting the localization of APE1(43-318)-Myc in HEK293 cells challenged with or without 1 mM GSNO for 4 h.





**Figure 7.** Proteins interfering with NO-induced APE1 nuclear export. HEK293 cells were cotransfected with HA-APE1 together with the expression vector encoding HDAC2, p50, p53, Trx, XRCC1, respectively, for 40 h, and then stimulated with 1 mM GSNO for 4 h. The subcellular distribution of HA-APE1 was determined by immunofluorescence.

These data suggested that the nitric-oxide-regulated APE1 nuclear export may be a complicated and cellular-context-dependent process.

## DISCUSSION

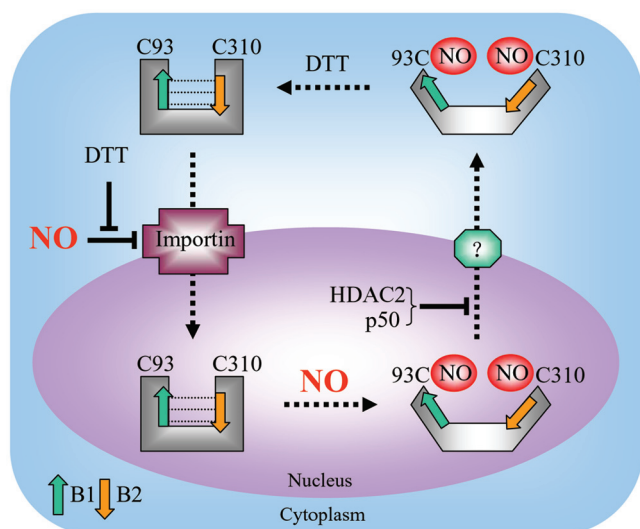
Post-translational protein modification is a crucial mechanism to regulate protein function in eukaryotic cells. Similar to other post-translational modifications, protein S-nitrosation exerts pleiotropic effects on its target proteins, ranging from altered protein stabilization to changed activity or function (26). Recently, Hara et al. reported that S-nitrosation mediated nuclear translocation of GAPDH. In the current investigation, we revealed that S-nitrosation mediated nuclear export of APE1. To our knowledge, this is the first evidence that S-nitrosation modification is able to control protein nuclear export, although other post-translational modifications, including phosphorylation (51), ubiquitination (52), sumoylation (53), acetylation (54), and thiol modification (55) have already been involved in such processes. For APE1, to date, the potential phosphorylation sites, acetylation sites, and granzyme A-cleavage sites have been well established, and the modification of these sites have been known to change APE1 activity (12,13,56). However, the potential S-nitrosation sites of APE1 and the effect of this modification on APE1 have not been reported. Our finding also provided the first evidence that nitrosative stress regulated APE1 by subcellular translocation through S-nitrosation mechanism.

Strikingly, this inducible cytosolic translocation of APE1 is an NO-related phenomenon. Firstly, the nuclear export of APE1 was not sensitive to other oxidative stresses such as  $H_2O_2$  (Figure 4A), which may cause a higher oxidative form of APE1, although the possibility has not been excluded that  $H_2O_2$  may act in a similar manner as NO in the regulation of APE1 trafficking in other unexamined cell types. In addition,

GSNO selectively induced cytosolic translocation of APE1, but not other DNA-repair-related proteins such as XRCC1 (Figure 1B) and thymine DNA glycosylase (data not shown). It should be pointed out that, although the APE1 nuclear export is specific to NO, the endogenous APE1 appears to show different sensitivities to NO-induced nuclear export among different cell types. Of note, the endogenous APE1 in human endothelial cells is strongly refractory to NO-induced nuclear export, although it can still be effectively S-nitrosated under the same circumstances (unpublished data).

Although the precise mechanism by which S-nitrosation controls nuclear export of APE1 is not clear, two anti-parallel beta-strands close to Cys93 and Cys310 were found to be critical for the nitrosation-mediated cytosolic translocation of APE1. Previous study revealed that phosphorylation of MK2 can result in intra-molecular de-masking of an NES and, therefore, mediate its nuclear export (51). Therefore, the conformational change and 'NES' exposure of APE1 caused by S-nitrosation modification can be expected in this study. We demonstrated that one of the beta-strands (B1) was functional, as the exposure of B1-containing sequence through C-terminal truncation could mediate protein cytosolic localization even in the presence of N-terminal NLS (Figure 5A). The direct evidence about whether the C-terminal B2 strand is another NO-responsive "NES" has not been proved. However, either deletion of B2 (Figure 5C) or fusion of GFP to APE1 at the B2 end (unpublished data) strongly repressed NO-stimulated nuclear export, suggesting that a proper conformational change of B2 may contribute to the NO-induced cytosolic translocation of APE1. B1 and B2 may cooperate with each other in mediating APE1 nuclear export. Alternately, they functioned independently, providing that the inter-strand interaction was disrupted as a result of APE1 S-nitrosation (Figure 8). In addition, it should be emphasized that the nuclear export efficiency of APE1 was high, as a 4-h GSNO treatment almost led to a complete nuclear exclusion of APE1. This phenomenon may be attributed to NO-caused impaired nuclear import of APE1 that helped in preventing the already exported APE1 from reimporting into nucleus. Different from the APE1-overexpressed cell system, the nuclear-cytosolic shuttling of APE1 may be interfered by some of its binding proteins at normal physiological conditions. It is possible that the protein-protein interactions can repress export of APE1 from nucleus to cytosol. HDAC2 and p50 proteins may act as such intra-nuclear gatekeepers for their ability to antagonize NO-induced APE1 trafficking (Figure 7). The differential intra-nuclear levels of APE1-binding proteins may partly contribute to the cell-type-specific phenomena in APE1 nuclear export. Therefore, the NO-caused APE1 cytosolic translocation may be a complicated process, simultaneously involved in S-nitrosation of Cys93 and Cys310 leading to "NES" exposure, interaction of APE1 with other nuclear proteins and defect in importin nuclear import pathway.

In summary, we have reported here that nitric oxide controlled the nuclear export of APE1 through S-nitrosation of Cysteines 93 and 310, which described a



**Figure 8.** A model for nuclear-cytosolic translocation of APE1 in response to NO stimulation. APE1 carries an importin-dependent NLS at the N-terminal end (not shown). Two antiparallel beta-strands (B1 and B2) in close proximity to C93 and C310 are masked in the internal structure. In rested cells, APE1 resides in the nucleus due to the existence of NLS. Upon nitrosative stress, S-nitrosation of C93 and C310 contributes to unmasking of the B1 and/or B2 by changing conformation, which may facilitate the nuclear export of APE1 in a CRM1-independent manner (perhaps mediated by an unknown transport protein). At the same time, importin-dependent nuclear import pathway is repressed by NO insult, which may prevent the already exported cytosolic APE1 from re-import into the nucleus. However, once the intracellular environment becomes reductive (e.g. the increased reducing molecules such as DTT), both NO-elicited S-nitrosation of APE1 and NO-caused repression of importin can be abrogated; thus, the inducible nuclear export of APE1 by NO could be prevented or reversed.

novel link between the nitrosative stress and DNA repair/transcriptional regulation system. It has been reported that redox modification of Cys310 of APE1 can repress its AP endonuclease activity (14). Thus, it is reasonable to expect that NO may exert influence on APE1 DNA repair function through nitrosation of this site. Unraveling the detailed mechanism by which S-nitrosation of APE1 controlled its nuclear export (such as whether a vehicle protein is required for the translocation) and revealing whether S-nitrosation of APE1 can affect its other biological functions present an exciting and relevant challenge for future research. Since NO and APE1 are connected to tumorigenesis and neurodegenerative diseases, the present findings will reasonably improve our understanding of how nitrosative stress leads to DNA damaging at the DNA-repair level and have the potential to yield therapeutic strategies to fight these diseases.

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