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ANALYSIS OF NUCLEAR TRANSPORT SIGNALS OF THE HUMAN APURINIC/APURIMIDINIC ENDONUCLEASE (APE1/REF1)

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To my parents E.B. and Erma Jackson,
with everlasting love and gratitude

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ANALYSIS OF NUCLEAR TRANSPORT SIGNALS OF THE HUMAN APURINIC/APURIMIDINIC ENDONUCLEASE (APE1/REF1)

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The nuclear localization signal (NLS) in human apurinic/apyrimidinic endonuclease (APE1), a key protein in both DNA base excision repair and transcriptional regulation, has not been analyzed in detail. We examined the role of specific residues in nuclear translocation of APE1, using green fluorescent protein (EGFP) fused to APE1 as a reporter. Nuclear localization (NL) of ectopic APE1 was abrogated for the mutant lacking 20 N-terminal aa residues (ND20). Fusion of these 20 residues directed nuclear localization of EGFP. While an APE1 mutant lacking N-terminal 7 residues (ND7 APE1) showed normal nuclear localization, ND7 APE1 with E12A/D13A double mutation resulted in drastic decrease of nuclear localization, indicating that E12 and D13 are critical components of the NLS.

On the other hand, nuclear localization of the full-length APE1 containing the E12A/D13A mutations suggests that the putative NLS and residues 8-13 contribute independently to nuclear import. Nuclear accumulation of the ND7 APE1(E12A/D13A), but not EGFP alone, after treatment with leptomycin B or after oxidative stress suggests the presence of a previously unidentified nuclear export signal in APE1. Together, these

results indicate that the mechanism of nuclear localization of APE1 is complex and regulated via import and export.

In addition increase DNA damage occurs in astronauts during space flight. Humans in space are exposed both to radiation and microgravity. It is clear that the increased exposure to radiation influences DNA damage however it is not clear as to the role that microgravity plays in this increase in damage.

Our investigation on the effects of microgravity on the nuclear translocation of DNA repair enzyme APE1 has revealed that microgravity interferes with the normal trafficking of APE1.

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LIST OF ABBREVIATIONS

APE1, apurinic/apyrimidinic endonuclease 1

AP site, apurinic/apyrimidinic site

EGFP, enhanced green fluorescence protein

Kap, karyopherin

LMB, leptomycin B

NLS, nuclear localization signal

NES, nuclear export signal

PI, propidium iodide

ROS, reactive oxygen species

aa, amino acid

CHAPTER 1: AP ENDONUCLEASE 1

Introduction

DNA continuously suffers the loss of its constituent bases, and thereby, a loss of potentially vital genetic information. Sites of missing bases—termed abasic or apurinic/apyramidinic (AP) sites—form spontaneously, though damage-induced hydrolytic base release, or by enzyme-catalyzed removal of modified or mismatched bases during base excision repair (BER). Apurinic/apyrimidinic (AP) sites are non-coding lesions that are generated via the spontaneous, chemically-induced, or enzyme-catalyzed hydrolysis of the N-glycosyl bond, severing the information-containing purine or pyrimidine base from the deoxyribose sugar of the DNA backbone (9).

Lindahl and Nyberg measured the quantitative release of radiolabeled purine bases from double-stranded DNA as a function of temperature, pH and ionic strength. In a Mg^{2+}-containing buffer, they determined the rate constant for spontaneous in vitro depurination to be $4x\ 10^{-9}\ s^{-1}$ at $70^{o}C$ and physiological pH (7.4). Extrapolating these date to a mammalian cell environment, it was estimated that ~12,000 purines would be lost spontaneously per genome per cell generation (20h), in the absence of the protective effects of chromatin packaging (9).

To add to the burden of spontaneous AP sites, damaging chemicals—e.g. free radicals and alkylating agents—promote base release, often by introducing base modifications that destabilize the N-glycosyl linkage by generating a better leaving group moiety. Atamna et al found that roughly 2 million AP sites are produced in a living

mammalian cell during a 20h period (9). This value presumably reflects AP sites formed by spontaneous decomposition, endogenous chemical-induction and the repair activity of DNA glycosylases. Atamna et al measured the en-vivo steady-state level of abasic sites to be <0.67 per 10^6 nucleotides, which corresponds to <4500 AP sites in a 3 billion nucleotide genome. This number differs dramatically from the steady-state value of 50,000 to 200,000 reported by Nakamura and Swenberg (9). The difference in the estimated AP site levels may also reflect, to some degree, differences in the source of DNA, i.e. the type and age of the target cell. Non-coding AP sites are mutagenic and cytotoxic, and thus, represent a major threat to the integrity and survival of the cell. Their cytotoxicity is perhaps best exemplified by bacterial cells that contain a temperature-sensitive mutation in the gene encoding dUTPase (deoxyuridine triphosphatase) and lack full repair capacity for AP sites (9).

At non-permissive temperature, where dUTPase is inactive, intracellular dUTP levels are elevated, and thus, uracil is incorporated at high frequency (in place of thymine) into chromosomal DNA during replication. Subsequent removal of the uracil bases by uracil DNA glycosylase generates an increased number of AP sites in the genome. When accompanied by a defect in AP site repair, dUTPase-deficient cells are inviable, presumably due to the accumulation of these cytotoxic lesions. Thus, it is presumed that in many instances, DNA (and RNA) polymerases pause and dissociate upon encountering an AP site, leading to the formation of replicative chromosome strand

breaks (or abortive, non-productive transcriptional events), which at high enough frequency result in lethality or cellular dysfunction (9).

APE1

To cope with the large number of mutagenic and cytotoxic abasic lesions in DNA, organisms are equipped with enzymes, termed AP endonucleases, that incise at the first phosphate 5' to the AP site, initiating a repair cascade that involves several proteins of the base excision repair (BER) pathway. AP endonucleases have been divided into two families based on their amino acid sequence identity to either exonuclease III (ExoIII, alternatively called Xth) or EndoIV, the major abasic endonucleases of E. coli. Under normal physiologic conditions, ExoIII comprises ~90% of the total cellular AP endonuclease activity in E. coli. In mammals, the predominant AP endonuclease is APE1 (also called HAP1 or APEX), an enzyme that belongs to the ExoIII family. Based on biochemical projections, APE1 comprises >95% of the total cellular AP site incision activity in human cell extracts (9).

In addition to the AP site incision activity, AP endonucleases possess varying levels of 3'-phosphodiesterase activity for common free radical-induced DNA products, e.g. 3'-phosphates and 3-phosphoglycolates, which require removal prior to repair synthesis or ligation. The bacterial proteins ExoIII and EndoIV, in addition to yeast Apn1, exhibit nearly equal 3'-repair and AP endonuclease activities (9). Conversely, in vitro biochemical and in vivo complementation studies reveal that APE1 exhibits a

relatively poor activity on 3'-blocking termini (>100-fold less efficient than its AP endonuclease activity) (9). Thus, while in vitro reconstitution experiments indicate that APE1 can remove obstructive 3'-termini, including those produced by the AP lyase activity of bifunctional DNA glycosylases, its poor 3'-repair activity suggests alternative repair proteins or processing mechanisms. The APE1 protein has a molecular mass of 35.5kDa (excluding post-translational modifications), a theoretical pI of 8.3 and three consensus nuclear localization signals in the N-terminus. Partial proteolysis studies and crystallography results indicate that the human protein consists of a tight globular nuclease domain, and a flexible, disordered N-terminal region (9).

BER corrects most spontaneous-decomposition, alkylation and oxidative lesions. The finding that APE1 facilitates bonding of PolB. to DNA and stimulates the deoxyribose phosphodiesterase activity of PolB in vitro provided the first evidence that the BER pathway is coordinated. More recently, it has been shown that APE1 promotes the dissociation and in turn the turnover efficiency of the uracil and thymine DNA glycosylases (9) , indicating that the initial step of BER is also coupled. Glycosylases have, thus, been suggested to bind tightly to abasic sites to provide protection to the chemically unstable AP site or prevent mutagenic bypass and/or serve as a beacon for subsequent APE1 binding (and perhaps BER pathway selection) or alternative cellular responses. Interestingly, much of the BER communication appears to occur without tight protein-protein associations. Recent crystallography and biochemical studies have instead suggested that BER coordination is facilitated through strategic bending or

manipulation of the DNA by the initial protein factor, promoting subsequent DNA binding by the downstream enzyme in a "passing the baton" manner. More recent experiments have found that APE1 is inhibited by its incised product, and can in fact form stable complexes with 5'-cleaved AP-DNAs, supporting the "passing the baton" mechanism (9). It is worth pointing out that APE1 undergoes subtle, but significant, conformational changes upon binding AP-DNA, but whether these alterations permit post-complex contacts with proteins of BER remains to be determined (9).

It is worth noting that APE1 was independently purified based on its ability to stimulate the sequence-specific DNA binding activity of Fos-Jun heterodimers (also called AP-1, a complex that functions as an intermediary transcriptional regulator in signal transduction processes) in vitro. Subsequently, it was determined that this binding activation was mediated by reduction of a specific cysteine residue within the DNA binding domain of the target transcription factor. The in vivo significance of such a regulatory mechanism was inferred from the fact that a Jun viral oncoprotein containing a Cys to Ser substitution was insensitive to redox modification, and thus, constitutively active (9). It has since been found that APE1 potentiates the DNA binding activity of several transcription factors, including NF-kB, Myb, members of the ATF/CREB and BSA/Pax families, Egr-1, HIF-1a and p53, through both redox-dependent and – independent mechanisms. The redox states of APE1 itself appears to be modulated by the ubiquitous thioredoxin system (9).

Lastly, APE1 appears to function as part of a multiprotein regulatory complex that binds to a calcium-responsive element (nCaRE) in response to changes in the intracellular calcium homeostasis, and regulates the expression of the parathyroid gene. APE1 similarly negatively regulates its own gene expression in an nCaRE-dependent fashion. compilation of the results suggest that the APE1-mediated genetic responses may be facilitated by a Ca2+-dependent signaling process, as intracellular Ca2+ levels are also influenced by the oxidative status of the cell (9).

Knockout studies indicate an absolute requirement for APE1 in normal embryonic development and animal survival. Specifically, mice lacking both APE1alleles die following blastocyst formation, shortly after implantation, with degeneration for the mutant embryos clearly visible as early a embryonic day E5.5. Heterozygous animals on the other hand display no gross anatomical abnormalities up to 9 months of age, exhibiting normal size, fertility, and no behavioral defects. In a separate study, homozygous null APE1 embryos were found to exhibit successful implantation and nearly normal developmental progression until E7.5, followed by morphogenetic failure and adsorption of embryos by day E9.5. This animal inviability appears to stem from embryonic lineage-specific cell lethality, as these cells, and not the extra-embyonic sections (e.g. the ectoplacental core), were pycnotic at day E8.5. Thus, APE1 may be required for cell viability, and therefore, definitive analysis into the biological roles of the encoded protein may require the construction of conditional-null or site-specifically

altered animals or cell lines—of particular interest will be the specific contributions of the various APE1 activities (9).

While APE1 is generally thought to be a ubiquitous enzyme predominantly localized to the nucleus, several recent studies have discovered striking differences in either APE1 intracellular compartmentalization or expression levels in several cancer types (9). Notably several of these localization and expression differences appear to correlate with specific cancer forms, perhaps providing a means for diagnosing cancer type and/or stage of progression. Moreover, while the reasons for and biological impact of the differential expression is unknown, it has been suggested that cytoplasmic localization observed for APE1 may be related to the oxygen tension of the cell, and thus, may reflect the complex functions of APE/REF-1 (9).

Protein Targeting and Nuclear Import/Export

One of the hallmarks of eukaryotic cells is the containment of DNA in the cell nucleus, where transcription and replication are separated from protein synthesis in the cytoplasm. Transport of molecules between the cytoplasm and nucleus occurs across the nuclear envelope through the nuclear pore complex (NPC), a large protein structure of ~125 megadaltons (MDa) that enables the passage of a variety of complexes, up to 120 kDa, the size of the large ribosomal subunit. In principle, molecules of 60 kDa can diffuse freely across the NPC; however, in reality, the trafficking of several such molecules is tightly controlled (47).

In general, proteins to be imported or exported from the nucleus contain either a nuclear localization signal (NLS) or nuclear export signal (NES), which is recognized by a receptor and carried through the NPC. Once in the correct compartment, the protein cargo is released and the receptor recycled for another round of transport. Ran is the small Ras-like GTPase crucial for maintaining the direction of transport. The asymmetrical distribution of the Ran guanine nucleotide exchange factor (RanGEF) to the nucleus, and the Ran GTPase-activating protein (RanGAP) to the cytoplasm, establishes a gradient of the nucleotide-bound state of Ran. This results in a high RanGTP concentration in the nucleus; the maintenance of this gradient is essential for nuclear transport and cell viability (47).

Members of a growing family of transport receptors carry proteins into and out of the nucleus and bind preferentially to Ran in its GTP-bound form. For protein export, the exportin CRM1 forms a complex with the NES-containing cargo and Ran GTP in the nucleus. Once the complex is translocated into the cytoplasm, the RanGAP promotes hydrolysis of RanGTP to RanGDP, causing the NES-containing cargo to be released. For the nuclear import of many NLS-containing proteins, the adaptor protein, importin a, and this complex translocates across the nuclear envelope through the NPC. Once in the nucleus, RanGTP preferentially binds to importin b, causing the release of the NLS-containing cargo (47).

Many proteins for import are not recognized by the importin-a or –b receptor, but are recognized by other receptors related to importin b. There are at least 21 potential

importin b family members in humans. The full spectrum of cargos for each import receptor is not known, but some might show sufficient specificity to be considered as targets for regulation (47).

DNA damage and Microgravity

Are single cells capable of sensing and responding to alterations in gravity? If so, what are the underlying mechanisms? Are the effects of gravity on cells, tissues, and whole organisms direct (via a gravity sensor), indirect (via related changes in mass transfer and shear rates in the cell microenvironment), or both? These questions provide a philosophical and scientific underpinning for why NASA has invested in gravity-related studies of cells and tissues. It is well established that a stay in space can affect biological systems at a variety of levels, including loss of bone mass, muscle strength, and cardiovascular fitness in astronauts even when they exercise regularly, changes in plant cell growth and metabolism, and in the swimming behavior of aquatic organisms (48).

Humans in space are exposed both to radiation and microgravity. While the adverse effects of the latter on organ function can to a certain extent be overcome by suitable countermeasures and training programs, it is not clear how they influence fundamental physiological processes at the tissue, cellular and subcellular levels. In the context of the present discussion, interactions between radiation damage and microgravity are important. Although radiation levels in space are considerably higher

than on earth they do not reach doses where deterministic effects are expected (with the possible exception of very large solar eruptions). Hence, the main concern is carcinogenesis. The development of a tumor is still poorly understood but it is clear that many stages are involved. The first stage, induction or initiation, can definitely be caused by radiation while its role in promotion and progression is unclear. Cellular repair, tissue reactions, and the immune defense reduce the radiation-related cancer risk under normal circumstances. If microgravity interfered with these processes, the radiation hazard in space would be higher than on earth. Interaction between radiation damage and microgravity is thus clearly not only of fundamental but also of great practical relevance (49).

It is obvious that a space experiment differs grossly from one performed in the home laboratory. This situation requires very careful planning not only regarding experimental procedures but also regarding the logistics of transport and storing. A study undertaken without ground controls, treated exactly like the space samples, will never yield meaningful results. This states a necessary but by no means sufficient condition. A direct comparison in space by using a reference centrifuge is an important improvement but even under these conditions error sources cannot be completely excluded since simulated gravity by centrifugal forces is different from the terrestrial environment. The only answer is "reproducibility," i.e. repeat experiments which are unfortunately neither popular with funding agencies nor with the space agencies who seem sometimes to be more interested in seemingly spectacular findings—even if not respectable—than in

scientific solidity, With regard to radiation safety of astronauts this constitutes not only a scientific but also an ethical issue (49).

It is very difficult to reconstruct the early experimental conditions on Russian spacecraft and the reported synergistic effects cannot be properly evaluated. The two others, chromosomal aberrations in human lymphocytes and DNA double strand break repair have already been mentioned. The first reported synergistic effects could not be confirmed in repeat experiments. This means that the only so far proven case of synergistic interaction is C. morosus {Bucker, 1986 #50}. This is not a cellular system but a developing multicellular organism. Its development is impaired by the action of space particles, which were recorded, and this effect is enforced by microgravity. This makes sense since it is known from studies not involving radiation that microgravity interferes with embryonic development. On the other hand, repair processes are under cellular control and may depend on transcriptional activity. This has so far only been shown for excision repair of UV damage but may also exist for other pathways. Cellular metabolism is under the control of signal transduction systems, which are controlled by environmental parameters, presumably also by gravity. Modifications of cellular repair can hence not be excluded per se. Two groups have recently addressed this question: Horneck et al. in bacteria and human fibroblasts; and the author et al. with the diploid yeast S. cerevisiae. This latter object allows us to tackle the problem in a very straightforward manner. The special strain used, termed rad54-3, is temperature-conditional for the repair of DNA double strand breaks. It is thus possible to keep the

cells at a low temperature in a metabolically inert state during all transportation and handling phases and to start the repair period only in microgravity. A pilot experiment under non-ideal conditions could be flown on IML-1 whose results indicated an impairment of repair. The repeat experiment which was performed under the code-name XRAY on STS-76 and strictly controlled did not confirm this finding. No change in repair was found in the experiment BETARAY on STS-84 (49).

It is clear that APE1 is one the most important DNA repair enzymes and that our understanding of its mechanism of control is still in its infancy. Studies must be performed that focus on characterizing the nature of control of APE1s trafficking. In this report, we aim to characterize the true nuclear localization signal of APE1. In addition, we will analyze the affects of microgravity on the normal trafficking of APE1.

CHAPTER 2: ANALYSIS OF NUCLEAR TRANSPORT SIGNALS IN THE HUMAN APURINIC/APYRIMIDINIC ENDONUCLEASE (APE1/REF1)

Introduction

The integrity of the genome is continuously challenged by endogenous reactive oxygen species (ROS), and exogenously by a variety of toxic reagents, including environmental carcinogens and ionizing radiation (1,2). A variety of DNA damage, including abnormal bases, apurinic/apyrimidinic (AP) sites, and DNA single-strand breaks (SSBs), as well as DNA double-strand breaks (DSBs), is induced by these genotoxic agents (2,3). These lesions, except for DSBs, are repaired primarily via the DNA base excision repair (BER) process (4). For damaged bases, DNA glycosylases hydrolyze the N-glycosylic bonds (5). Subsequent repair requires generation of the 3'-OH primer. In mammalian cells, this reaction is efficiently carried out by AP endonuclease 1 (APE1) (6,7).

Besides its crucial role in BER, APE1 plays an important role in gene regulation. It was independently identified as the redox-enhancing factor, Ref-1, which activates AP-1 (cJun/cFos) and other transcription factors (10-15). Furthermore, APE1 was also discovered as a co-repressor which downregulates the parathyroid hormone gene upon calcium influx (16-18). We have recently found that APE1 is acetylated at Lys6 and Lys7 by the histone acetyltransferase p300, and that this posttranslational modification stimulates its co-repressor activity (19). APE1 was shown to be essential in embryonic development of mice (20-22), because homozygous APE1 knockout mice died at 3.5-5.5

days after implantation (22). Although it is still unclear which function of APE1 is required in embryonic development, it is obvious that nuclear localization is a prerequisite for APE1's *in vivo* functions.

Several studies on subcellular localization of APE1 were reported earlier. We showed that a large fraction of APE1 molecules in human cell lines, HeLa and WI38, were present in the cytoplasm. However, after exposure of these cells to oxidative stress, APE1 was predominantly localized in the nucleus (23). Therefore, the nuclear vs. cytoplasmic distribution of APE1 may be conditional, which is consistent with the variable nuclear/cytoplasmic distribution of this protein observed in various human tissues (24,25). Fan *et al.* recently reported that the majority of APE1/Ref1 molecules were in the cytoplasm, particularly in the perinuclear region, and were associated with the granzyme A complex found in T cells (26). The protein was translocated to the nucleus after proteolysis at residue 31, and then degraded rapidly. However, this regulated nuclear transport may be specific for the cytolytic T cells (26). Using transiently expressed APE1-FLAG fusion polypeptide, Takao *et al.* showed that APE1 was localized exclusively in the nuclei (27). Together, these studies indicate that nuclear transport and accumulation of APE1 is rather complex.

Karyopherin α (Kap α) also known as importin α, recognizes the NLS of target proteins, and plays a crucial role for importing nuclear proteins as an adaptor protein (28). Kap α also interacts with Karyoperin β_1 (Kap β_1 – also known as importin β), and together these proteins form the transportation machinery in which a protein containing a

classical NLS is imported by the Kap α-Kap β1 heterodimer (28). Three categories of NLS have been characterized. One classical type is identified by a four residue pattern (pat 4) consisting of either four basic amino acids (Lys or Arg), or three basic amino acids (Lys or Arg) and a His or Pro. Another classical type, "pat 7", containing seven amino acid residues, begins with a proline, followed by a basic segment of 3 residues (Lys or Arg) (29). The second type of NLS is bipartite, with two basic residues, a 10-residue spacer, and another region consisting of at least 3 basic residues out of the next 5 (30). The third specialized type of NLS is present in yeast protein Mat alpha 2. The Kap α proteins interact with these classical NLS to transport them into nuclei (31).

A putative NLS of the classical pat7 type is found at the N-terminus of APE1, with the segment "PKRGKK" starting at residue number 2 (27). However, its function as NLS was not directly demonstrated. Moreover, while the nuclear localization signal (NLS) directs proteins into the nucleus, nuclear accumulation can also be regulated by inhibiting nuclear export of proteins containing nuclear exporting signals (NES). In order to elucidate the mechanism of nuclear trafficking of APE1 and its regulation, here we report systematic analysis of the N-terminal sequence of APE1 to identify a complex, bipartite NLS in APE1, and the interaction of APE1 with the Kap α2 protein. Furthermore, our results suggest that there is a nuclear export signal in APE1 that responds to leptomycin B, an inhibitor of nuclear export protein CRM1.

Materials and Methods

Cell Culture and Transfections. The mouse BALB/c 3T3 fibroblast line was a generous gift from Dr. M. Tatsuka (Hiroshima University, Japan), and a human colon carcinoma cell line HCT116 was obtained from Dr. Voggelstein (19). These cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and streptomycin/penicillin.

Transfection studies. Cells grown in 35mm dishes were transfected with 0.5μg plasmid DNA, using Lipofectamine 2000 and OptiMEM I (Invitrogen), then incubated at 37˚C in 5% CO_2. After 6 h incubation, the cells were washed and 2 ml of DMEM with 10% FBS solution was added. After incubation for 24 h, the cells were washed with PBS twice, and fixed with paraformaldehyde solution (16% in normal saline) for 30 min at 4ºC, and then rinsed with PBS three times. The cells were permeabilized with Triton X solution for 30 min and incubated with propidium iodide for another 5 min before the cells were rinsed with PBS, and visualized in a confocal microscope (LSM510Meta, Carl Zeiss) at UTMB's Optical Image Core. For live cell analysis, cells spread on glass-coated 35 mm dishes (Mattek) were transfected with DNA as described above, and then examined without fixation. Intensity of EGFP fluorescence in cells was quantified using the digital image generated by the microscope, and with analytical software (MetaMorph). To compare average (area-standardized) intensity of EGFP in nuclei and cytoplasm, the average intensities of the nuclear and total cell areas were measured and the ratios of nuclear to total EGFP intensities were calculated. Fixed cells were used for the

measurement, for which the nuclear area was determined by propidium iodide staining. More than 30 individual cells were analyzed for all the transfection experiments.

Plasmid DNA

The IκBα-EGFP fusion construct was a generous gift from Dr. T.J. Hope (32). The human APE1 cDNA was originally a gift from Dr. S. Seki, and was cloned into various vectors in previous studies (6,33-35). The EGFP gene (pEGFP-N1, Clontech) was inserted into pcDNA 3.1Zeo(+) (Invitrogen) with *Eco*RI and *Not*I. The wild-type (WT) APE1 cDNA was then inserted into this vector using newly created *Bam*HI and *Eco*RI sites at APE1's 5' and 3' ends, respectively. The primers for PCR were: 5' TTG GAT CCA CCA TGC CGA AGC GTG GGA AAA AGG GA 3' and 5' CCG AAT TCG CAG TGC TAG GTA TAG GGT GAT AGG 3'. There are 51 nucleotides between the last codon of APE1 and the initiation codon of EGFP, resulting in a 17 amino acid insertion. Several N-terminal deletions and missense mutations of the APE1 coding sequence were then created in the EGFP fusion vector by PCR cloning, using the WTAPE1-EGFP vector as the template. Table 1 summarizes the N-terminal sequences of those APE1-EGFP fusion proteins. The identity of generated PCR plasmids were confirmed by DNA sequencing at UTMB's Protein Chemistry Core facility. A FLAG epitope tag was introduced at the APE1 C-terminus by inserting a linker containing *Eco*RI and *Xho*I site. The linker replaced the EGFP sequence from the WT APE1-EGFP vector. The linker sequences were: 5' AAT TCT CTG TCG ACT ACA AAG ACG ATG

ACG ACA AGT AAC 3' and 5' TCG AGT TAC TTG TCG TCA TCG TCT TTG TAG TCG ACA GAG 3'.

Oligonucleotides. GFP oligonucleotides were purchased from Invitrogen. The Oligos included: **ND42** (5'-TTT GGA TCC ACC ATG GCC CTG TAT GAG GAC CCC CCA GAT CAC AAA ACC TCA CCC AGT-3'), **ND21** (5'-TTT GGA TCC ACC ATG GAG GCC AAG AAG AGT AAG ACG GCC GCA AAG AAA AAT GAC AAA GAG GCA GCA GGA GAG-3'), **ND7** (5'-TTT GGA TCC ACC ATG GGA GCG GTG GCG GAA GAC GGG GAT GAG CTC AGG ACA-3'), **K6L/K7L** (5'-TTT GGA TCC ACC ATG CCG AAG CGT GGG CTC CTC GGA GCG GTG GCG GAA GAC GGG GAT-3'), **K6R/K7R** (5'-TTT GGA TCC ACC ATG CCG AAG CGT GGG AGG AGG GGA GCG GTG GCG GAA GAC GGG GAT GAT GAG CTC AGG ACA-3'), **L-all** (5'-TTT GGA TCC ACC ATG CCG CTC CGT GGG CTC CTC GGA GCG GTG GCG GAA GAC GGG GAT-3'), **N20** (5'-TTT GGA TCC ACC ATG CCG AAG CGT GGG AAA AAG GGA GCG GTG GCG GAA GAC GGG GAT GAG CTCAGG ACA GAG-3'), **ND7(V10A)** (5'-TTT GGA TCC ACC ATG GGA GCG GCG GCG GAA GAC GGG GAT GAG CTCAGG ACA GAG-3'), **ND7(E12A/D13A)** (5'-TTT GGA TCC ACC ATG GGA GCG GTG GCG GCG GCG GGG GAT GAG CTCAGG ACA GAG-3'), **WT-APE(E12A/D13A)** (5'-TTT GGA TCC ACC ATG CCG AAG CGT GGG AAA AAG GGA GCG GTG GCG GAA GAC GGG GAT GAG CTCAGG ACA GAG-3').

Analysis of Leptomycin B-treated Cells. The cells were transiently transfected with plasmids encoding EGFP fusion proteins, and 24h later treated with 5 nM leptomycin B

(LMB, Sigma), a nuclear export inhibitor, for 2 h. The location of IκB, a control for LMB-inhibited nuclear export (32), was determined along with that of WT APE1 and its mutants. All cells were visualized and differentiated by confocal fluorescence microscopy (LSM510Meta, Carl Zeiss).

Protein purification

APE1, WT and the deletion mutants (ND20 and ND33), were purified as previously described (33). Human Kap α2 protein, tagged N-terminally with a histidine hexamer (36), was purified using Ni-NTA column chromatography as before (33) after expressed in *E.coli* BL21 (Stratagene) harboring the pET-Kap α2 plasmid vector (a generous gift from Dr G. Blobels).

Detection of APE1 protein by His-Kap α2 pull-down assay

Ni-NTA magnetic bead (Qiagen) suspension (50μl) was added to 500 μl of the His-tagged Kap α2 protein (15μg) in microcentrifuge tube. Suspension was incubated on an end-over-end shaker for 30 min to 1hr at room temperature. The tubes were placed in a separator for 1min to remove supernatant. 500μl of interaction buffer (50mM NaH2PO4, 300mM NaCl, 20mM imidazole, .005% Tween 20, pH 8.0) was then added to each tube, mixed, and placed on the magnetic separator for 1min, and then buffer was removed. 500μl of interaction buffer containing APE1 (3 μg) was then added to each tube and incubated on an end-over-end shaker for 1hr at room temperature. After incubation, the suspension was placed on magnetic separator to remove the supernatant, and then

magnetic beads were washed with 500μl of interaction buffer twice. The APE1/Kap α2 mixture was then eluted with 50μl of elution buffer, and the presence of APE1 protein was examined by Western blotting with anti-APE1 antibody.

Far Westeren Analysis WT and truncated APE1 Proteins (10-40 pmol) were separated by 12% SDS-PAGE and transferred overnight to a nitrocellulose membrane. The membrane was washed with cold 1X PBS and treated with 6 M guanidine-HCl in PBS at 4ºC. The proteins were then renatured with successive dilutions of 6M guanidine-HCl in PBS, diluted by 1 mM DTT in PBS at 4ºC (37). After blocking with 5% nonfat dry milk (NFDM) in PBS/0.5% Tween 20 for 1 hr at 4°C, the membrane was incubated with 10 pmol of Kap α2 in 0.5% NFDM/PBS/0.5% Tween 20 containing 1mM DTT and 100mM trimethylamine-N-oxide dihydrate (TMAO) for 3 hr at 4°C (37). Subsequent immunoblot analysis was performed using anti-Kap α2 antibody (Santa Cruz) followed by anti-Goat IgG antibody and detected using ECL (Amersham Biosciences).

APE1-FLAG Immunoprecipitation Cells (HCT116) were transfected with 5 μg of a control vector (pcDNAZeo3.1) or the FLAG tagged WTAPE1 using liptofectamine 2000 (Invitrogen). The cells were then lysed by adding 1ml of lysis buffer (50mM Tris-HCL pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM PMSF, Protease Inhibitors, Roche) to each culture dish and incubating on ice for 30 min. Plates were scraped and cell lysate was placed in a 1.5ml centrifuge tube. Protein concentration of cell lysate was analyzed using the Bradford Assay (Bio-Rad). Then, 60μl of Anti-FLAG M2 Agarose beads (Sigma, cat# A-2220) solution was added to a 1ml centrifuge tube and washed

twice with cold TBS (50mM Tris-HCL pH 7.5, 150mM NaCl). Then 3mg of cell lysate protein normalized to 1ml of TBS was added to tube containing the FLAG beads. The suspension was then incubated at 4°C for 3h while shaking. After incubation the suspension was spun at 10,000 rpm at 4°C and washed 4x with cold TBS removing all the supernatant after the fourth wash. Then 30μl of SDS sample loading buffer was added to the tube and boiled for 5 min. 10 μl of the samples were run in SDS polyacrylamide gel electrophoresis, and then analyzed with Western blotting, using antibodies for APE1, FLAG (anti-FLAG M2-Peroxidase conjugate, Sigma Kapα1 (Santa Cruz), and Kap α2 (Santa Cruz)

Results

Nuclear localization of N-terminal deletion mutants of APE1

We first examined subcellular distribution of WT APE1 fused to EGFP in the fibroblast cell line Balb3T3 (Fig. 1A). N-terminal amino acid (aa) sequences of the APE mutants are listed in Table 1, and the intensities of EGFP in nuclei vs. total intensities were calculated for all APE1-EGFP constructs in this study, and are shown in Table 2. Based on fluorescence, APE1 was localized predominantly in the nuclei. As a control, we used the EGFP expression vector pEGFP-N1. In contrast to APE1-EGFP, the EGFP fluorescence was found to be predominantly in the cytoplasm, although a low level of EGFP was also visible in the nuclei (Fig. 1B). We thus confirmed the previous study indicating that APE1 contains an NLS (27). The obvious candidate for the NLS was the

N-terminal region. We next examined intracellular distribution of ND42 APE1-EGFP, in which the N-terminal 42 aa residues of APE1 were deleted (35). This fusion polypeptide was distributed throughout the cell with a higher level in the cytoplasm compared to nuclei (Fig. 1C, Table 2). The distribution was similar to that of EGFP alone, and thus indicated that the deletion drastically abrogated nuclear targeting. To narrow down the sequence responsible for the nuclear targeting, we transfected the cells with ND21 APE1-EGFP, deleting the N-terminal 21aa residues (Fig. 1D, Table 2). The intracellular distribution of ND21 APE1, as expected, was similar to that of ND42 APE1 and EGFP

Table 1.
APE1-EGFP fusion proteins

EGFP fusion	Amino acid sequence of the N-terminus
WT APE1	^{1}MPKRGKKGAV^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
ND42 APE1	^{1}MPALYEDPP…
ND21 APE1	^{1}MPEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
APE1 (K6R/K7R)	^{1}MPKRG**RR**GAV^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
APE1 (K6L/K7L)	^{1}MPKRG**LL**GAV^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
APE1 (L_{all})	^{1}MP**LL**G**LL**GAV^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
ND7 APE1	^{1}MGAV^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
ND7 APE1 (V10A)	^{1}MGA**A**^{11}AEDGDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
ND7 APE1 (E12A/D13A)	^{1}MGAV^{11}A**AA**GDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
APE1 (E12A/D13A)	^{1}MPKRGKKGAV^{11}A**AA**GDELRTE^{21}PEAKKSKTAA^{31}KKNDKEAAGE^{41}GPALYEDPP…
N20	^{1}MPKRGKKGAV^{11}AEDGDELRTE-**EGFP**

The N-terminal sequences of the APE1-EGFP fusion proteins used in this study are shown. Point mutations introduced in some of the constructs are indicated as bold letters. Note that the first Met, numbered as one in this table, is removed after translation.

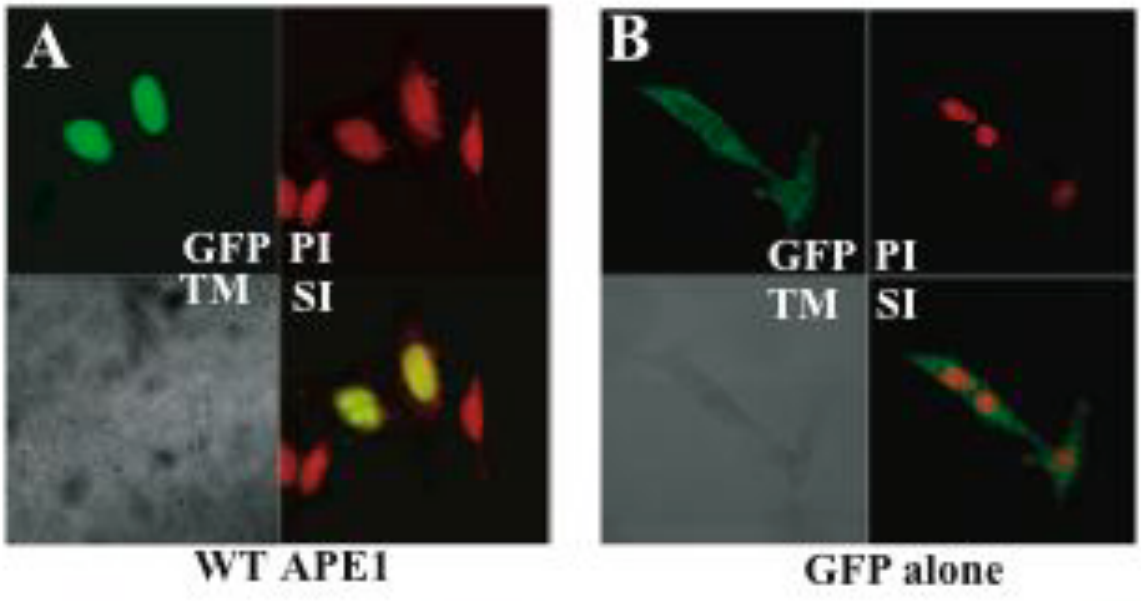


Figure 1, A and B. Intracellular localization of WT and truncated APE1 EGFP fusion proteins in mouse fibroblasts. (A) the WT APE1-EGFP and (B) the GFP vector alone

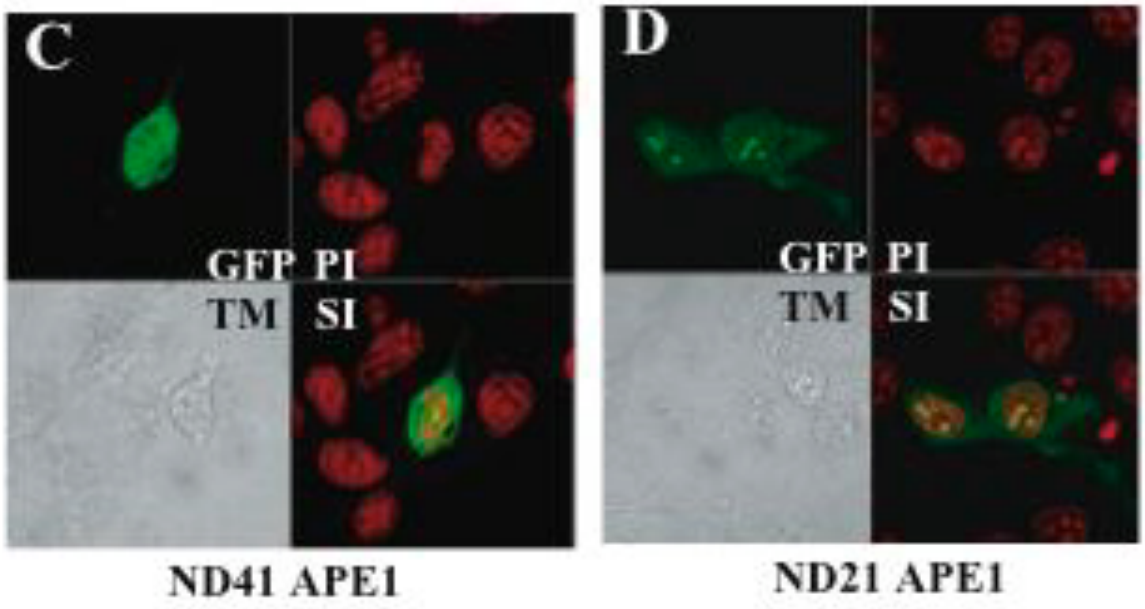


Figure 1, C and D. Intracellular localization of WT and truncated APE1 EGFP fusion proteins in mouse fibroblasts. (C) ND42 APE1-EGFP, and (D) the ND21 APE1-EGFP.

alone. We also examined the subcellular distribution of all the APE1-EGFP fusion proteins in live cells without fixation, and observed the same results as with the fixed

cells (data not shown). We therefore concluded that NLS was located within the first 20 amino acid residues, consistent with the prediction that the first 7 aa residues serve as the NLS (27).

Lack of effect of APE1 acetylation on nuclear localization

Bhakat et al. recently reported that acetylation of APE1 occurs at Lys6 and Lys7 residues (19). Because these residues are components of the putative NLS, it appeared possible that such posttranslational modification regulates nuclear translocation of APE1 by eliminating the basicity of these residues. To test this possibility, Lys6/Lys7 were replaced with Arg, APE1 (K6R/K7R), which is slightly larger than Lys and more basic but cannot be acetylated. No significant difference in nuclear localization was observed between the WT APE1 and the K6R/K7R mutant (Fig 1E, Table 2). We also examined the K6L/K7L mutant, APE1(K6L/K7L). The Leu side chain is more hydrophobic than Lys, but similar in size. This mutant also behaved similarly to the WT and K6R/K7R mutant, being localized predominantly in the nucleus (Fig. 1F, Table 2). Therefore, we concluded that the Lys6/Lys7 residues and their acetylation are not required for the regulation of intracellular localization of APE1.

The putative NLS is dispensable for nuclear localization

Because Lys6/Lys7, basic residues in the putative NLS, was not required for APE1's nuclear import, we sought to identify basic residue(s) in the N-terminal region required for nuclear localization. All basic residues in the putative NLS (^{2}PKRGKK^{7})

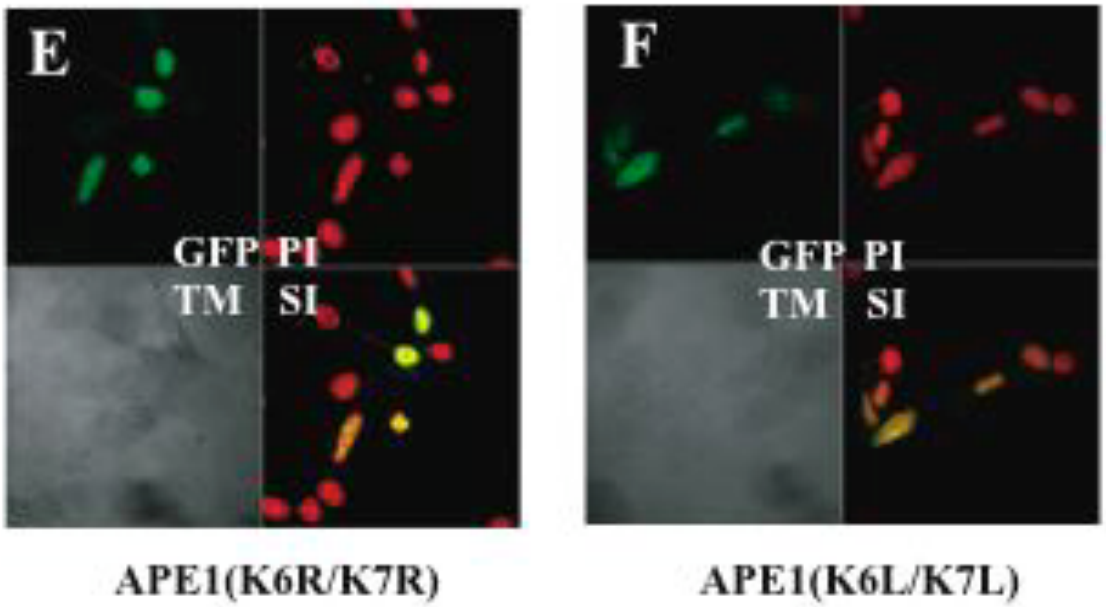


Figure 1, E and F. Effect of mutation of Lys6 and Lys7 on nuclear localization of APE1. (E) APE1(K6R/K7R)-EGFP, and (F) APE1(K6L/K7L)-EGFP.

were simultaneously mutated to Leu. The mutant, K3L/R4L/K6L/K7L (^{2}PLLGLL^{7}), named APE1(L_{all}), was also predominantly localized in the nucleus (Fig. 2A, Table 2).

Although a small fraction of APE1(L_{all}) was found to be cytoplasmic (Table 2), it was evident that the APE1(L_{all}), unlike the ND21 and ND42 APE1, retained a functional NLS. We also examined the intracellular distribution of ND7 APE1-EGFP, in which the putative NLS segment was deleted (Table 1). Its subcellular distribution was almost identical to that of APE1(L_{all})-EGFP (Fig. 2B, Table 2); i.e., the majority of APE1(Lall)-EGFP was translocated in the nucleus. These results indicated that the NLS in APE1 is present within the 20 N-terminal aa residues, but contrary to the prediction, the putative NLS is dispensable.

We tested whether the N-terminal 20 aa residues by themselves could provide the signal for nuclear translocation of EGFP (Fig. 2C). The fusion protein, N-terminal 20-EGFP (N20-GFP), was distributed both in the nucleus and the cytoplasm, although the intensity of EGFP fluorescence in the nucleus was distinctly higher than in the cytosol (Table 2). This result suggests that the first 20 aa residues are enough to translocate APE1 into the nucleus.

Role of E12 and D13 in APE1 nuclear localization

We also examined the subcellular distribution of ND13 APE1 and ND16 APE1 deletion mutants fused to FLAG epitope tag at the C-terminus. Unlike the WT APE1-FLAG, the deletion mutants were primarily present in the cytoplasm (data not shown). The sequence between residues 8 and 13, ^{8}GAVAED^{13}, contains small and nonpolar residues (^{8}GAVA^{11}), followed by two acidic residues (^{12}ED^{13}). We changed V10 to Ala

(V10A) in the ND7 APE1 (Table 1) to increase the hydrophobicity of this region. The intracellular distribution of ND7 APE1(V10A)-EGFP was identical to that of ND7 APE1-EGFP (Fig 2D). Next, the 12E and 13D residues were mutated to A; the double mutant, ND7 APE1(E12A/D13A) abrogated the nuclear localization, resulting in almost identical distribution to that of EGFP alone, or ND21- and ND42-APE1 mutants (Fig. 2E). These results indicate that E12 and D13 play a crucial role in nuclear localization.

Role of the N-terminal residues in nuclear localization

We then introduced the E12A/D13A double mutations in the full-length APE1-EGFP fusion polypeptide. Unlike the E12A/D13A in ND7 APE1, this protein was predominantly nuclear (Fig. 2F), although careful examination revealed some inhibition of nuclear localization compared to the WT-APE1 (Table 2). These results suggest that the N-terminal 7 aa residues can complement the E12A/D13A mutations in promoting nuclear import.

Specific interactions between APE1 and Kap α1 and α2

To understand the mechanism of nuclear import more precisely, we examined interaction between APE1 and the nuclear importins, Kap α1 and α2. FLAG-tagged APE1 was transiently expressed in HCT116 human colon carcinoma cells, and was pulled down by anti-FLAG antibody. Using the corresponding antibodies we were able to detect both karyopherins in the anti-FLAG APE1 enriched extract. In a mock experiment, where FLAG-immunoprecipitation was carried out after transfection with a control vector expressing only FLAG peptide alone, neither karyopherins were detected (Fig. 3).

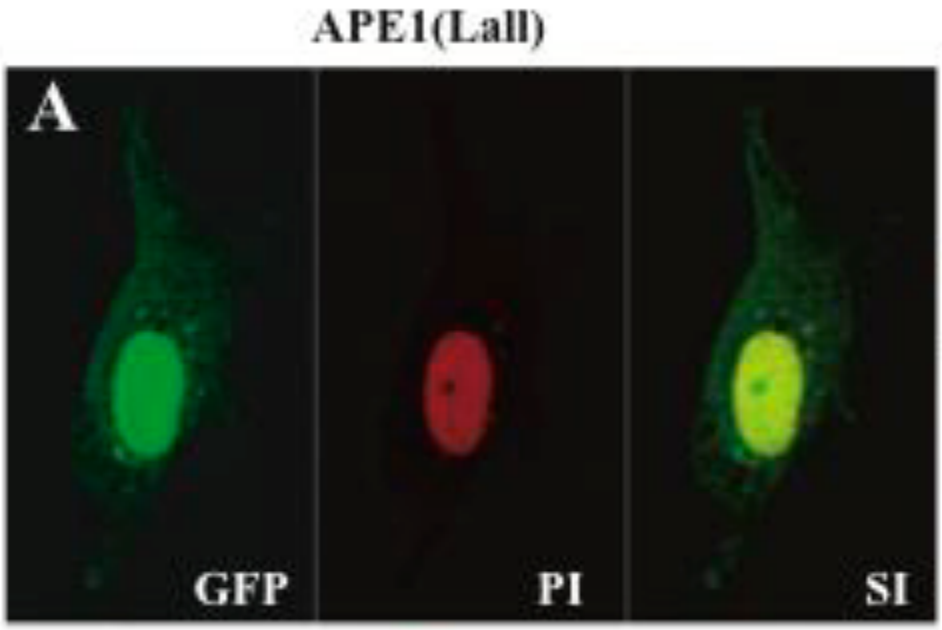


Figure 2, A. Effect of N-terminal mutation in APE1 on the nuclear localization. (A) APE1(L_{all})-EGFP. Cells were fixed and stained with PI.

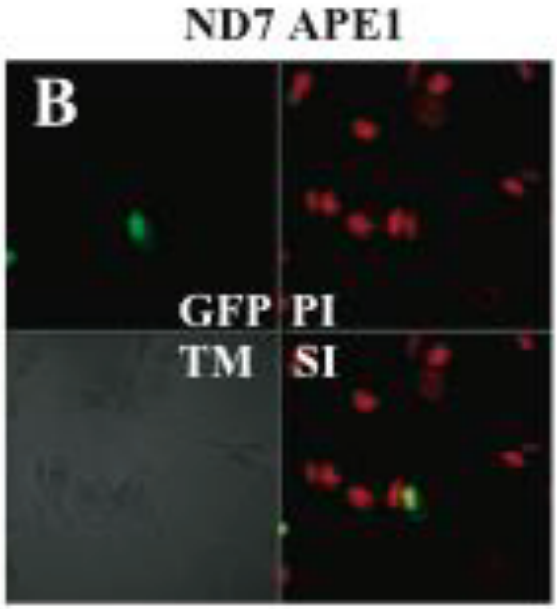


Figure 2, B. Effect of N-terminal mutation in APE1 on the nuclear localization. (B) ND7 APE1-EGFP. Cells were fixed and stained with PI.

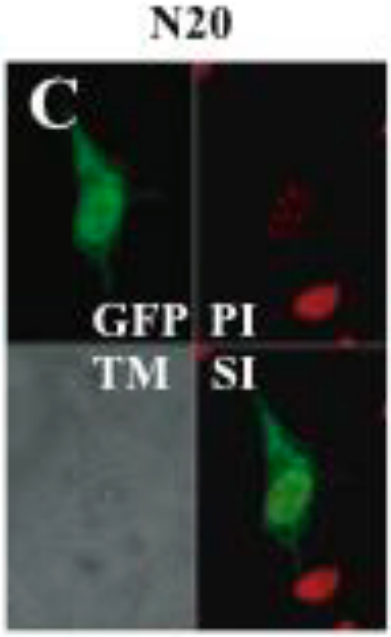
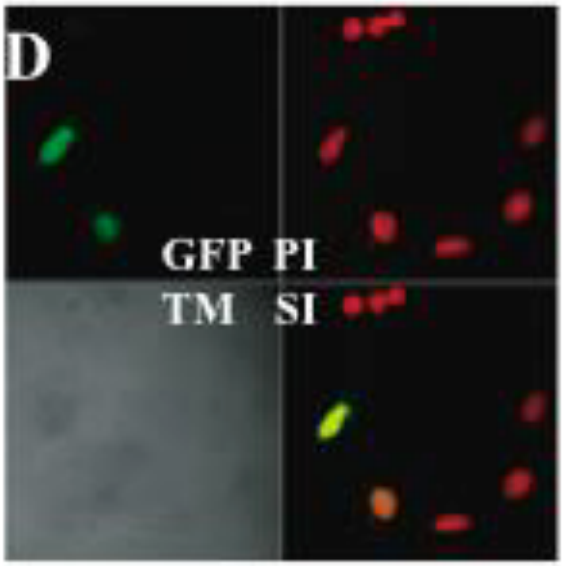


Figure 2, C. Effect of N-terminal mutation in APE1 on the nuclear localization. (C) N20-EGFP. Cells were fixed and stained with PI.

Figure 2, D. Effect of N-terminal mutation in APE1 on the nuclear localization. (D) ND7 APE1(V10A)-EGFP. Cells were fixed and stained with PI.



ND7 APE1(V10A)

Figure 2, E. Effect of N-terminal mutation in APE1 on the nuclear localization. (E) ND7 APE1(E12A/D13A). Cells were fixed and stained with PI.

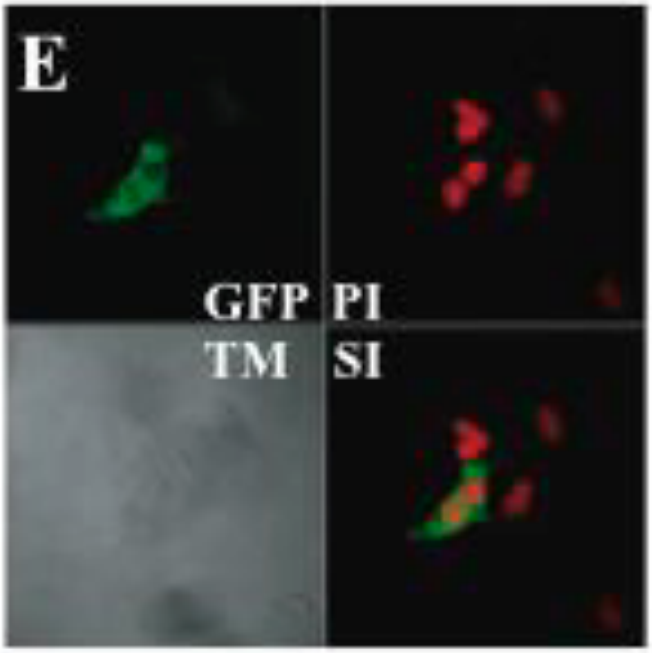
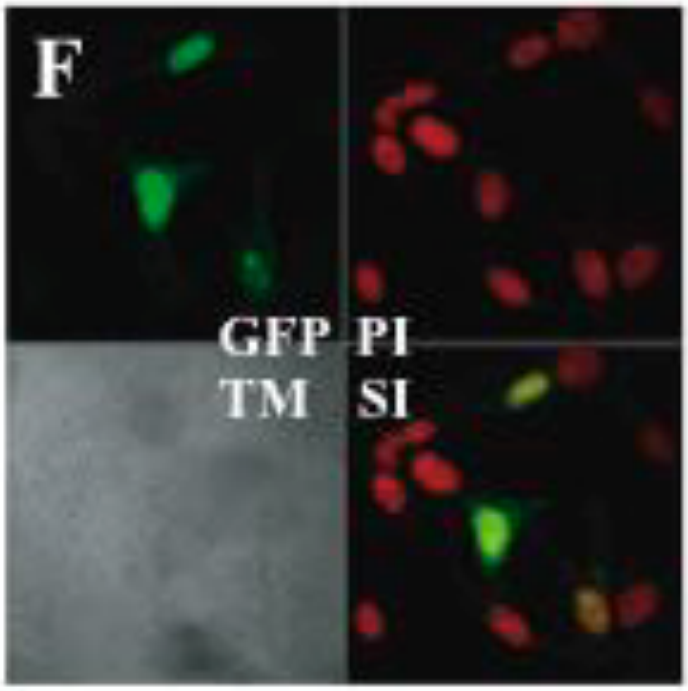


Figure 2, F. Effect of N-terminal mutation in APE1 on the nuclear localization. (F) full-length APE1(E12A/D13A). Cells were fixed and stained with PI.



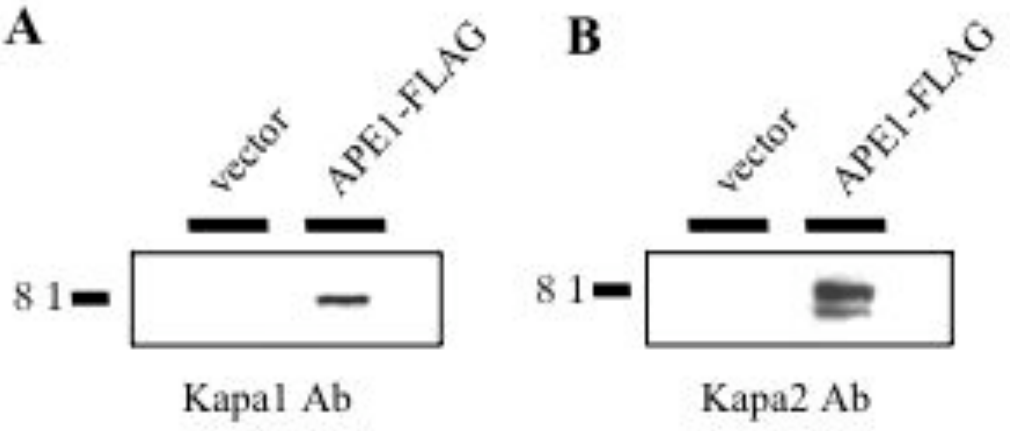


Figure 3, A-B. Interaction of APE1 with Kap α1 □and α2 analyzed by FLAG immunoprecipitation. HCT116 cells were transiently transfected with a vector control or with a plasmid DNA expressing C-terminally FLAG-tagged APE1. The extracts were processed with immunoprecipitation using FLAG antibody to enrich the APE1-FLAG protein, and were analyzed by Western blotting using Kap α1 (A) or Kap α2 (B) antibody.

To analyze the interaction *in vitro*, we purified recombinant human Kap α2 expressed in *E. coli* (35). The interaction between APE1 and Kap α2 was then confirmed by Far-Western analysis (Fig. 4A-C), where Kap α2 clearly interacted with the full-length APE1 as detected by an anti-Kap α2 antibody. However, truncated APE1 proteins, ND21 and ND33, did not show affinity for Kap α2 (Fig. 4A-C). A similar result was obtained when the APE1 protein was pulled down with the his-tagged Kap α2 protein (Fig. 4D). Again, interaction with the ND33 APE1 (N-terminal 33 aa deletion) was not detectable in this experiment, confirming the importance of the N-terminal residues. Although we did not analyze the interaction with Kap α1, we predict a similar mechanism for Kap α1 as for Kap α2, because Kap α2 is able to substitute for Kap α1 in binding to NLS and in proper nuclear export of a target protein (36).

Introduction of mitochondrial translocation signal (MTS) into APE1.

Understanding the transport mechanism for APE1 may enable engineering the APE1 molecule to be localized in a particular organelle. We tested this possibility by replacing the N-terminal 20 aa residues of APE1 with the mitochondrial translocation signal of the human mitochondria-specific Mn^{2+}-superoxide dismutase (MnSOD) gene (38). The FLAG-tagged MTS-APE1 was clearly localized in the mitochondria, with hardly any nuclear fluorescence (Fig. 5A). Mitochondrial localization of the MTS-APE1 was further confirmed by intensity analysis of the Mito-Tracker (mitochondrial) and

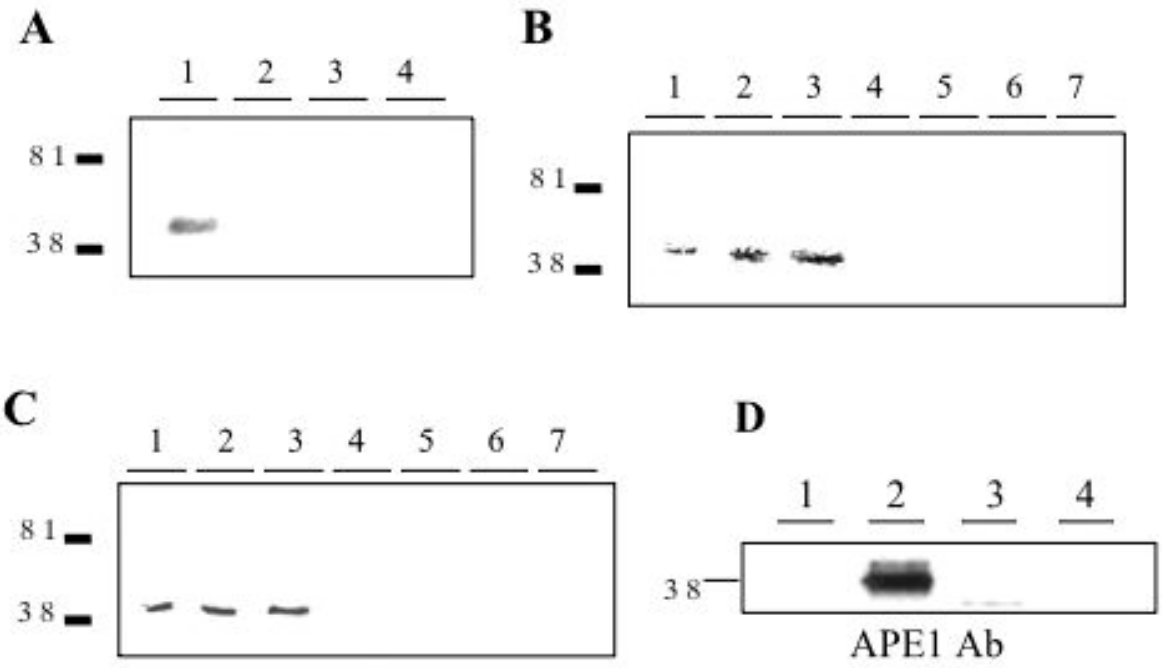


Figure 4, A-D. Analysis of the interaction between APE1 and Kap α2 proteins. (A) 20 pmol of full-length APE1 (lane1), ND33 (lane 2), ND20 (lane3), or BSA (lane 4) were immobilized onto a nitrocellulose membrane, and probed with recombinant Kap α2 protein. Interaction was determined using Kap α2 antibody. (B) Far western with full-length APE1 at 10 pmol (lane 1), 20 pmol (lane2), or 40 pmol (lane 3); and ND20 APE1 at 10 pmol (lane 4), 20 pmol (lane 5), or 40 pmol (lane 6) probed with Kap α2 protein followed by detection of Kap α2 with its antibody (C) Far western with full-length APE1 at10 pmol (lane 1), 20 pmol (lane2), or 40 pmol (lane 3); and ND33 at 10 pmol (lane 4), 20 pmol (lane 5), or 40 pmol (lane 6) probed in the same way as in (B). (D) Interaction between APE1 and Kap α2 analyzed by His-tag pulldown assay. Kap α2 protein (15 μg), N-terminally tagged with histidine hexamer (His-tag Kap α2), was incubated with no protein (lane 1), 3 μg of WT APE1 (lane 2), ND33 APE1 (lane 3), or ND20 APE1 (lane 4) purified through Ni-NTA resin. The eluents were analyzed with anti-APE1 antibody.

rhodamine (FLAG epitope) (Fig. 5B). Therefore, it is possible to control APE1's subcellular distribution by adding a specific localization signal for a subcellular compartment such as mitochondria.

Nuclear accumulation of ND7 APE1 (D12A/E13A) by leptomycin B

Two processes regulate nuclear/cytoplasmic distribution of proteins. One is nuclear import through the NLS, but alternatively, a decrease in nuclear export will result in accumulation of proteins in nuclei (39,40). Since a small fraction of ND7 APE1(E12A/D13A) was still found in nuclei (Table 2), we asked whether the nuclear distribution of APE1 was affected by leptomycin B (LMB), a specific inhibitor of nuclear export (32,41). As a positive control, we monitored the intracellular distribution of IκBα-EGFP, the inhibitor of the transcriptional regulator NF-κB, which is typically cytosolic (32) (Fig. 6A). We observed that IκBα accumulated in the nucleus after 2h incubation with 5 nM LMB, as reported previously (Fig. 6A) (32). The ND7 APE1(E12A/D13A), which showed a marked increase in cytoplasmic distribution under the normal condition, was examined for its distribution after LMB treatment. Surprisingly, the majority of ND7 APE1(E12A/D13A) molecules were accumulated in the nuclei (Fig. 6B). Because there was no effect of LMB on the distribution of EGFP alone (42), this result suggests that APE1 contains an NES.

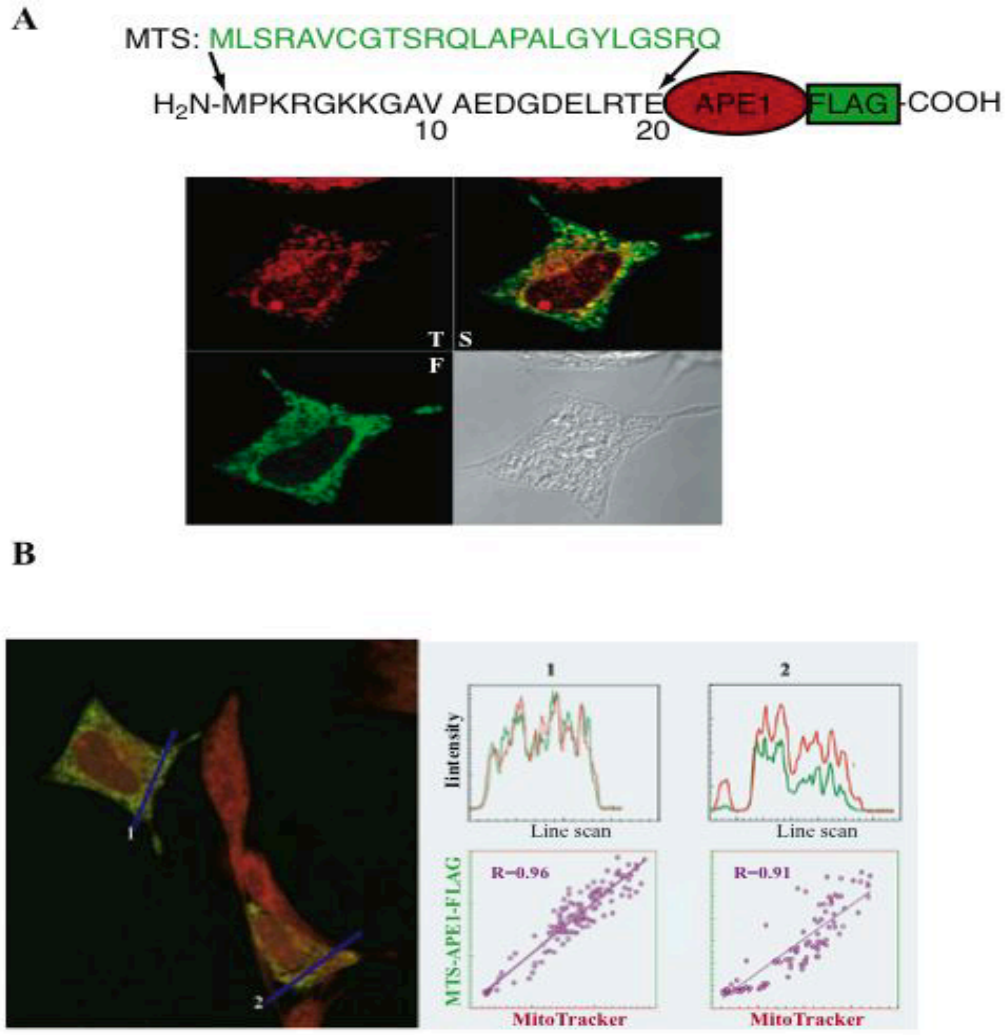


Figure 5, A-B. Localization of APE1 fused to mitochondrial translocation signal (MTS) of the MnSOD. (A) Upper panel: A schematic diagram showing replacement of N1-20 aa residues of APE1 with MnSOD MTS at the N-terminus. The MTS-APE1 is fused with the FLAG peptide at the C-terminus. Lower panel: The BALB/c cells were trasnsfected with cDNA encoding the MTS-APE1-FLAG, and then stained with monoclonal anti-FLAG antibody (M2) and Mitotracker after fixation. (B) Intensities scanned along with the lines (1 and 2) denoted in the left panel, using LSMmeta (Carl Zeiss), are shown at the top plots, and correlation between Mitotracker (red) and FITC (green) are shown in the bottom plots. Arbitrary units are shown in both types of plots.

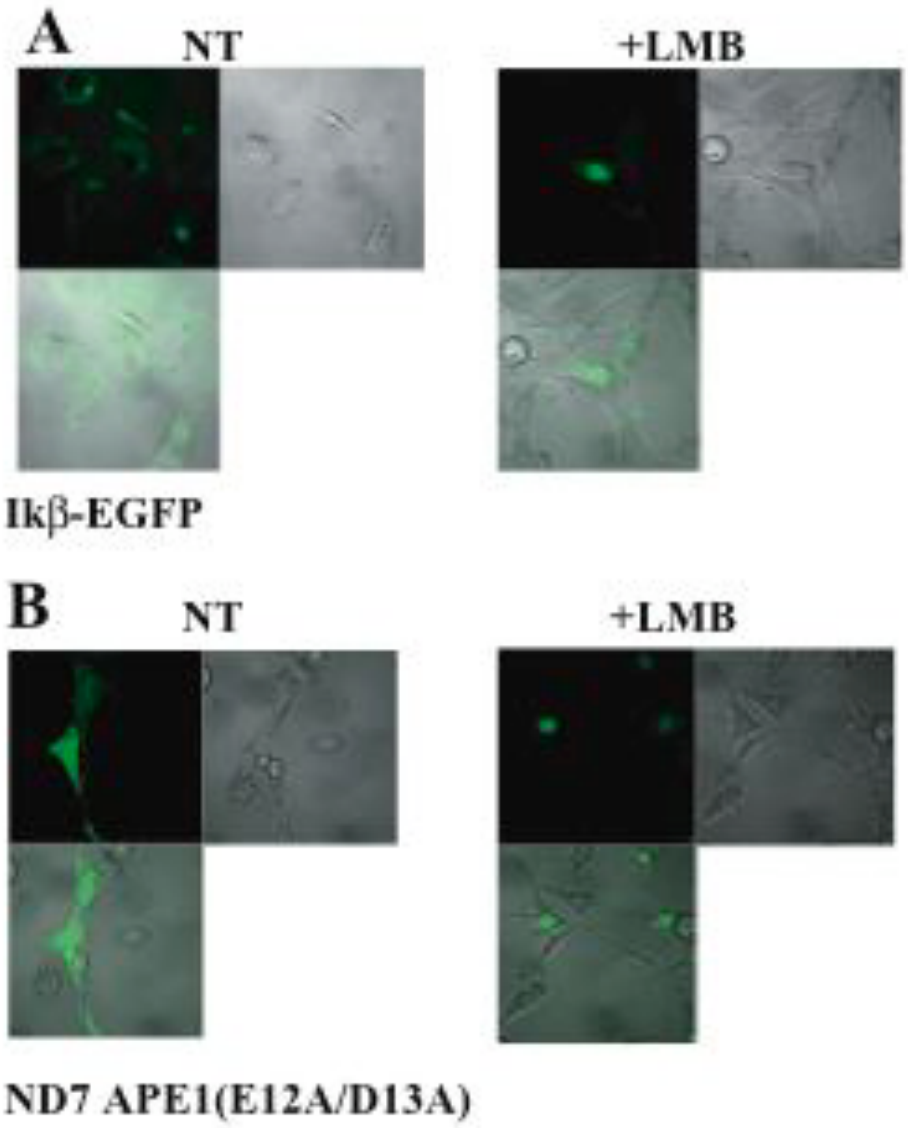


Figure 6, A-B. Effect of leptomycin B on subcellular localization of APE1-EGFP. Transiently transfected cells with (A) IκBα-EGFP, and (B) ND7 APE1(E12A/D13A)-EGFP. Cells were treated with: mock (NT) or 5nM leptomycin (LMB). Live cells were then analyzed in the confocal microscope after 2h.

Table 2.
Nuclear distribution of various EGFP fusion proteins.

EGFP fusion proteins	Nuclear fraction (% of total)
GFP	12.4 ± 3
IκBα-GFP	26.9 ± 4.2
WT APE1-EGFP	97.2 ± 3.6
N20-EGFP	54.9 ± 5.2
ND21 APE1-GFP	40.5 ± 3.3
ND42 APE1-GFP	32.3 ± 2.5
APE1(K6R/K7R)-EGFP	85.6 ± 6.4
APE1(K6L/K7L)-EGFP	87.2 ± 5
APE1(Lall)-EGFP	68.5 ± 8.2
ND7 APE1-EGFP	77.2 ± 6.6
ND7 APE1(V10A)-EGFP	75.3 ± 2.6
ND7 APE1(E12A/D13A)-EGFP	32.8 ± 8.1
Full-length APE1(E12A/D13A)-EGFP	74.5 ± 2.8
ND7-APE1(E12A/D13A)-EGFP+LMB	92.7 ± 2.5

Intensities of whole cells and of nuclei were measured and ratios of nuclear vs. whole cell intensities were calculated from more than 25 individual cells in each experiment.

Discussion

Our first objective in this study was to determine the subcellular localization of the WT APE1. Using BALB/C 3T3, our results were consistent with the earlier report (27), in that APE1 molecules were exclusively present in the nuclei. This pattern was not affected whether the cells were live or fixed, or by the method of fixation nor the type of reporter peptide (EGFP or FLAG). It should be noted that all the APE1-EGFP fusion proteins contain a 17 aa-long polypeptide derived from the vector sequence at the junction between the two gene sequences. We confirmed that the intervening polypeptide did not increase the EGFP's nuclear localization (data not shown). Instead, the sequence appears to decrease nuclear localization of EGFP, because we found more cytoplasmic distribution of the EGFP with the intervening sequence (data now shown) than that of the wild-type EGFP (Fig. 1B). The fact that the WT APE1-EGFP was exclusively in the nuclei indicates that APE1 could overcome the negative effect of the intervening sequence on nuclear import, further supporting the notion that APE1 contains a strong NLS at the N-terminus. This is in striking contrast to the previous observations of the endogenous APE1; a significant level of the endogenous APE1 were found in the cytoplasm when determined by immunocytochemistry with anti-APE1 antibodies (23-25). The ratio between cytoplasmic and nuclear APE1 might vary based on the tissue type, cell line, and growth conditions (24,25). Also importantly, the endogenous APE1 was found to accumulate in the nuclei after ROS generation and during the apoptotic

process (23,26). Therefore, cells seem to use the cytoplasm to store extra APE1, which may be needed in the nuclei under such stressful conditions. While it was not clear why the ectopic APE1 molecules were mostly localized in the nuclei in the BALB/c cells, this cell line provided a convenient tool to identify APE1's NLS, with its high transfection efficiency compared to that of HeLa cells (unpublished observation).

Results with ND42-, ND21-, ND7-, and L_{all}-APE1 compared to EGFP led us to conclude that residues between 8 and 20 are important for proper nuclear localization. Further analysis with ND13- and ND16-FLAG APE1 narrowed the region to the 8th through 13th amino acids.

Small and hydrophobic residues are the main component of residues 8-13 (^{8}GAVAED^{13}), except for the last two acidic residues. Our data suggest that these acidic residues, Glu12 and Asp13, are indeed critical for APE1's nuclear localization. The distribution of E12A/D13A on the ND7 background was essentially identical to that of ND21, and completely different from that of ND7, while a V10A missense mutation in ND7 APE1 did not affect nuclear localization at all. It is unlikely that the dual missense mutation caused significant conformational change, because the N-terminal 6 kDa is known to be a distinct, unstructured domain separated from the 30 kDa domain containing the AP endonuclease activity (34,35,43). Moreover, the double mutation had only a slight effect on the subcellular distribution of full-length APE1, supporting the idea that the mutations did not affect the overall structure of APE1. We thus propose that APE1 has two separate, independent NLS segments. One consists of the N-terminal 7 aa

residues that form a NLS of the classical pat-7 type, and the other is the segment from the 8th to 13th residues revealed in this study. Our data indicate that either segment, but not both, can be mutated without impacting on efficient nuclear localization. The peptide comprising residues 8-13 contains two acidic residues, and lacks the signature of a classical NLS. Interestingly, NLSs without the classical consensus sequences are already known. For example, the NLS of c-Myc and other proteins contain essential acidic amino acid residues (44).

Nuclear importins play a central role in importing target proteins into nuclei. Particularly, importin α (Kap α) can interact with the target proteins by binding to their classical NLS (31). Our observation, that APE1 interacts with Kap α1 and α2, suggests that APE1's nuclear transport is regulated by these importin proteins. The interaction was observed both between recombinant APE1 and Kap α2 proteins (Far-Western and His-tag pull-down assay) and with cell-free extracts (FLAG-tagged APE1-immuno pull-down assay). Although our study did not dissect the two NLS segments for Kap α specificity, it is likely that APE1's classical NLS (^{2}PKRAKK^{7}) is responsible for the Kap α2 interaction, because Kap α proteins are known to interact with classic NLS (31). This idea was supported by our observation that both ND21- and ND33-APE1 failed to interact with the importin protein. It may be interesting to examine how APE1's distribution is affected by the level of cytosolic/nuclear Kap α2, of which nuclear import/export is also regulated (45). The lack of cytoplasmic distribution of transiently transfected APE1 made it difficult to systematically examine the presence of a nuclear

export signal in APE1. We noticed that a small fraction of ND7 APE1(E12A/D13A) was still localized in the nuclei. This suggested that APE1, even without the critical NLS at N-terminus, could be transported into nuclei. The intrinsic property of EGFP, of which small fraction tends to be present in nuclei (Fig. 1B and Table 2) may account to this result. In any case, we tested whether this mutant respond to LMB, a general nuclear export inhibitor (46). We observed that ND7 APE1(E12A/D13A) accumulated in the nuclei. To our knowledge, the present study is the first to show nuclear accumulation of APE1 by LMB. Nearly identical response was observed with IκBα (32), so a similar mechanism may regulate nuclear export of APE1 by the nuclear export protein CRM1 (46).

A CRM1-dependent NES is typically a 10-20 aa long sequence containing a few Leu residues (46). Since APE1's N-terminal 6kDa region is not conserved with the *E. coli* exonuclease III, an APE1 ortholog, it is often speculated that the region may be linked to mammalian specific functions such as nuclear import/export. However, among 37 Leu residues in APE1, there are only two Leu (L17 and L44) in the N-terminal 6kDa peptide. Therefore, it is unlikely that NES is in the N-terminal 6kDa region. By examining 3D structures (35), we found that ^{281}RLDYFLLSHS LLPALCDSKI^{300} near the C-terminus contains 6 Leu residues, of which L291, L292, and L295 are exposed on the surface of the APE1 molecule. These Leu residues are localized at a very short α-helix between two β-sheet structures. Other Leu-rich regions on the surface can be also found: ^{101}ENKLPAELQELPGLSHQYWS^{120}, ^{131}VGLLSRQCPL^{140}, ^{191}AFRKFLKGLA^{200},

^{241}GELLQAVPLA^{250}, and ^{311}PITLYLAL^{318}. In the future, it should be possible to identify the NES by examining Leu mutants in the ND7 APE1(E12A/D13A). Analyzing the interaction of APE1 with CRM1 and identification of APE1's domain for the interaction would also help to further understand the nuclear export mechanism of APE1.

Although APE1 has been known to be present in the cytoplasm (23), it is not clear why in some cells APE1 is localized in both the nuclei and cytoplasm, whereas in BALB/c 3T3 and HeLa transiently expressed APE1 can be detected only in the nuclei. The present study indicates that the distribution of APE1 in the nucleus and cytosol is in a dynamic equilibrium. In HeLa cells, APE1 was translocated into nuclei after the cells were exposed to ROS including H_2O_2 (23). It is possible that decreased nuclear export is responsible for the nuclear accumulation of APE1. Whether APE1 accumulation in nuclei after ROS generation is due to CRM1 inhibition clearly needs more elaborated study. Examination of the activity of the importin and exportin proteins specific to APE1 in each cell type should reveal the complex mechanism to regulate the subcellular distribution of APE1, an essential multifunctional protein.

CHAPTER 3: THE EFFECTS OF MICROGRAVITY ON NUCLEOCTOPLASMIC LOCALIZATION OF HUMAN APURINIC/APURIMIDINIC ENDONUCLEASE 1 (APE1/REF1)

Introduction

The question as to whether microgravity plays a role in the increase of DNA damage to astronauts during spaceflight is ambiguous. The major studies up til now have concluded that microgravity does not play a synergistic role in the increase in DNA damage seen in astronauts. This damage has been attributed to the increased radiation exposure in the space environment. Interestingly, studies which focused on the effects of microgravity on normal development have shown that this environment disrupts normal development in zebrafish.

Recently it has been reported that there is a relationship between the biological effects of space radiation and microgravity. Unfortunately, there is no direct evidence for this phenomenon especially at the molecular level. It has been suggested that microgravity may not have an affect at the stage of DNA rejoining of the damaged DNA and substrate incorporation in the induced-mutation frequency and thus another possible mechanism must be accepted to explain the synergism (1). As one possible mechanism we investigate whether nucleocytoplasmic translocation of DNA repair enzyme is depressed by microgravity. Specifically, DNA repair enzyme AP endonuclease 1 which is involved in base excision repair by recognizing apurinic/apyrimidinic sites (47).

To test the effects of microgravity one must employ the use of the Hydrofocusing Bioreactor (HFB). This apparatus creates simulated microgravity in the earths 1G environment. The hydrodynamic focusing bioreactor (HFB) is a bioreactor system designed for three-dimensional cell culture and tissue-engineering investigations on orbiting spacecraft and in laboratories on Earth. The HFB offers a unique hydrofocusing capability that enables the creation of a low-shear culture culture environment simultaneously with the "herding" of suspended cells, tissue assemblies, and air bubbles.

The HFB, based on the principle of hydrodynamic focusing, provides the capability to control the movement of air bubbles and removes them from the bioreactor without degrading the low-shear culture environment or the suspended three-dimensional tissue assemblies. The HFB also provides unparalleled control over the locations of cells and tissues within its bioreactor vessel during operation and sampling.

We propose that Microgravity effects trafficking of DNA repair proteins which my aid in the increased damage seen during spaceflight. We intend to show that BER enzyme APE1 undergoes a depression of its translocalization to the nucleus in a microgravity environment.

Key Words: APE1, apurinic/apyrimidinic endonuclease 1; AP site, apurinic/apyrimidinic site; EGFP, enhanced green fluorescence protein; NLS, nuclear localization signal; PI, propidium iodide; ROS, reactive oxygen species

Materials and Methods

Cell Culture and Transfections. The mouse BALB/c 3T3 fibroblast line was a generous gift from Dr. M. Tatsuka (Hiroshima University, Japan). These cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and streptomycin/penicillin. The culture materials were purchased from Invitrogen.

Microgravity. Gravitational conditions were accomplished by the Hydrodynamic focusing bioreactor (HFB). A rotating wall bioreactor, which provides a unique hydrofocusing capability, that simultaneously enables a low-shear culture environment and a unique hydrofocusing-based "herding" of suspended cells.

Transfection studies. The cells and cytodex 3 microcarrier beads (sigma) in 35mm dishes were transfected with 0.5ug plasmid DNA, using Lipofectamine 2000 and OPTIMEM I (Invitrogen), then incubated at 37˚C in 5% CO_2. After 6 h incubation, the control cells were washed and 2 ml of DMEM with 10% FBS solution was added. While the sample cells were placed in the bioreactor. After incubation for 24 h, the control and sample cells were washed with PBS twice, and fixed with paraformaldehyde solution (16% in normal saline) for 30 min at 4ºC, and then rinsed with PBS three times. The cells were permeabilized with Triton X solution for 30 min and incubated with propidium iodide (PI) for another 5 min before the cells were rinsed with PBS, and visualized in a confocal microscope (LSM510Meta, Carl Zeiss) at UTMB's Optical Image Core.

Intensity of EGFP fluorescence in cells was quantified using the digital image generated in the microscope, and with analytical software (MetaMorph). To compare average (area-standardized) intensity of EGFP in nuclei and cytoplasm, the average intensities of the nuclear and total cell areas were measured and the ratios of nuclear to total EGFP intensities were calculated.

Plasmid DNA EcoRI The human APE1 cDNA was originally a gift from Dr. S. Seki, and was cloned into various vectors in previous studies (7,9-11). The EGFP gene (pEGFP-N1, Clontech) was inserted into pcDNA 3.1Zeo(+) (Invitrogen) with and NotI. The wild-type (WT) APE1 cDNA was then inserted into this vector using newly created BamHI and EcoRI sites at APE1's 5' and 3' ends, respectively. The primers for PCR were: 5' TTG GAT CCA CCA TGC CGA AGC GTG GGA AAA AGG GA 3' and 5' CCG AAT TCG CAG TGC TAG GTA TAG GGT GAT AGG 3'. There are 51 nucleotides between the last codon of APE1 and the initiation codon of EGFP, resulting in a 17 amino acid insertion.

Results

Analysis of the nuclear localization of APE1 of cells cultured at normal gravity compared to cells cultured in the bioreactor showed a significant difference in the nucleocytoplasmic localization of APE1. Cells were analyzed at 0hr, 2hr, and 24hr time points. Localization of APE1, at the 0hr control and microgravity samples, was the same

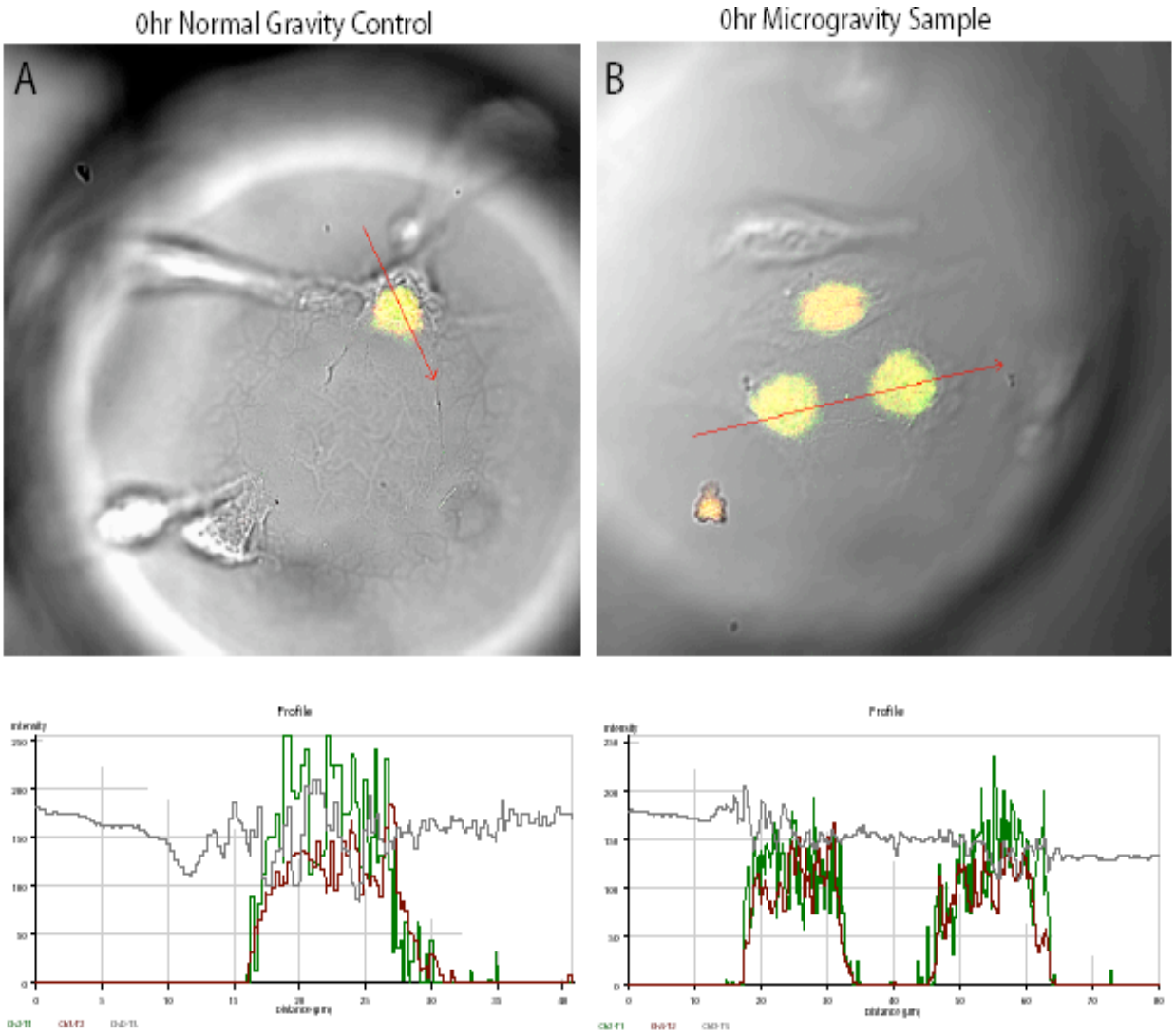


Fig. 1 Confocal analysis of 0hr control and microgravity cell samples with GFP(green)/PI(red) intensity profile analysis. (A) 0hr control cell samples (B) 0hr microgravity cell samples

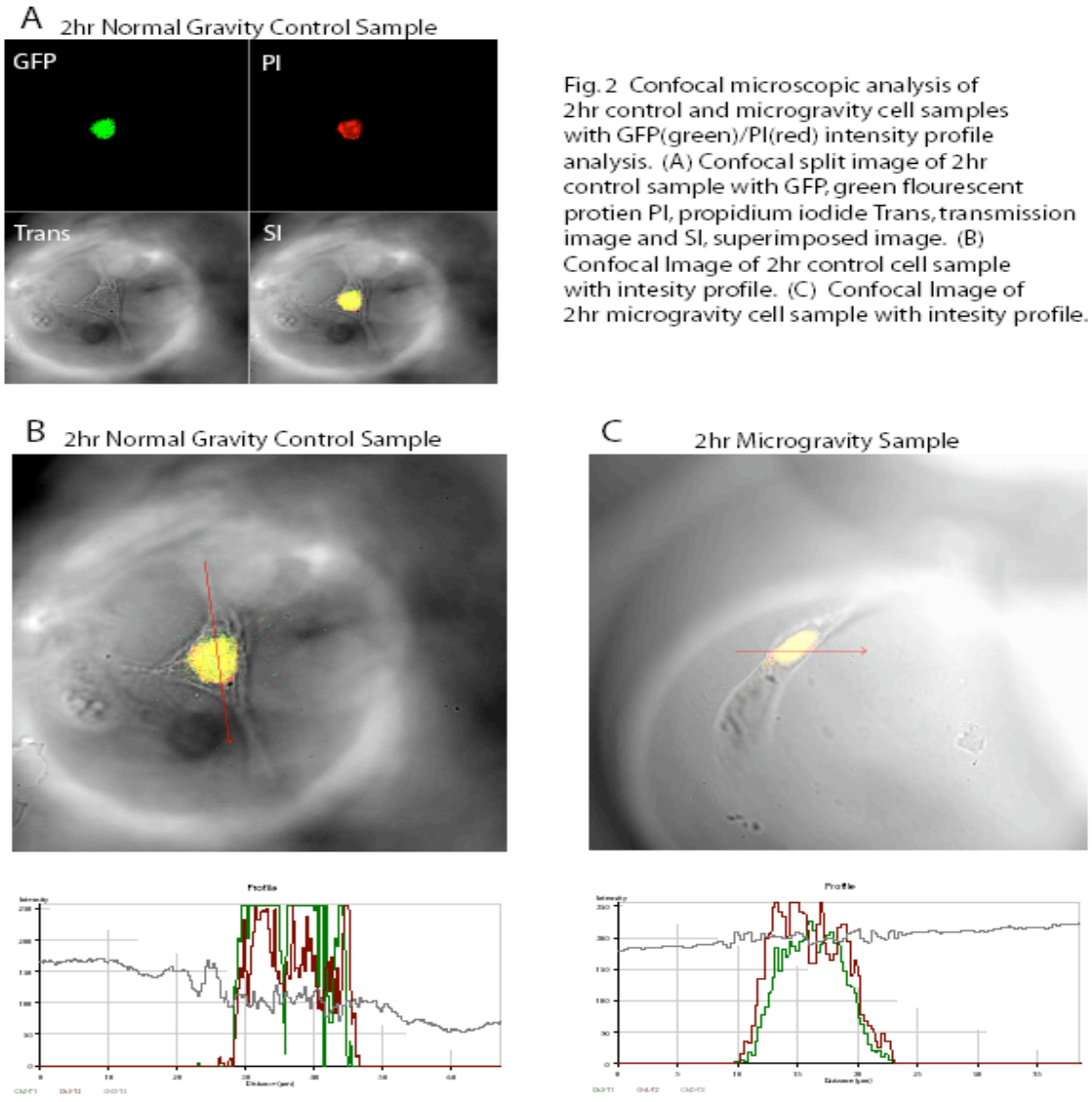
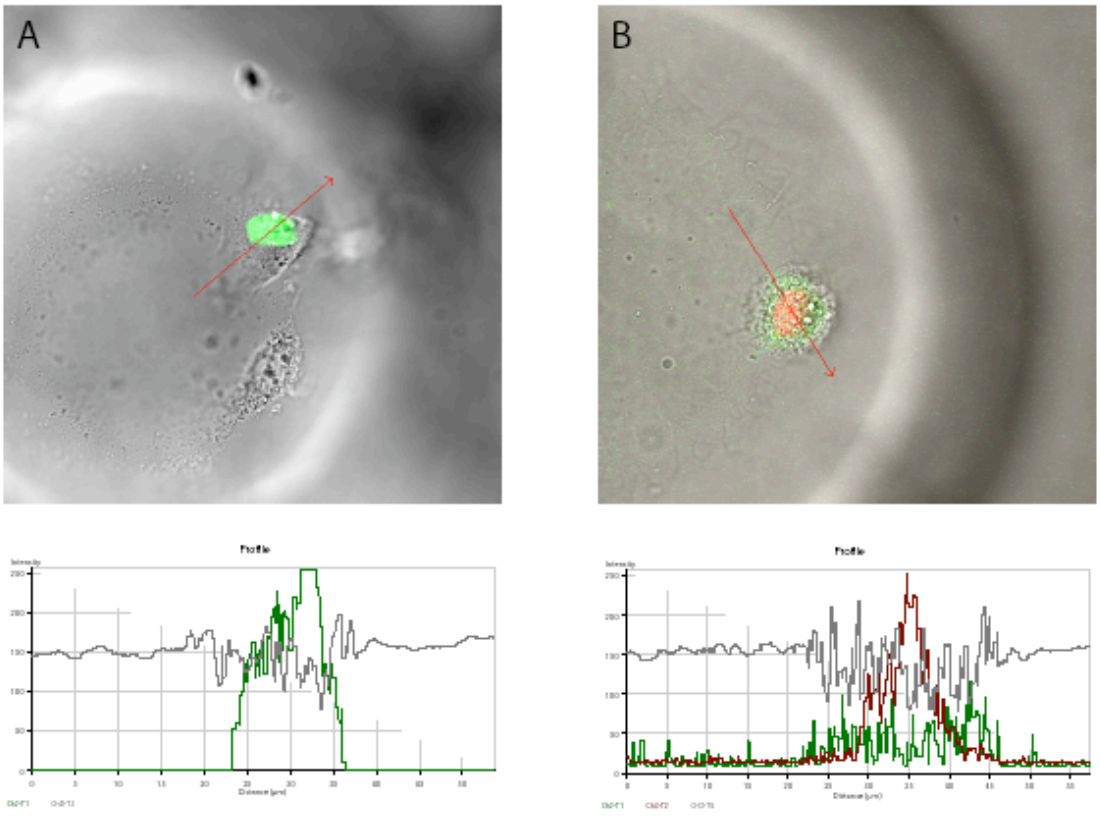


Fig. 2 Confocal microscopic analysis of 2hr control and microgravity cell samples with GFP(green)/PI(red) intensity profile analysis. (A) Confocal split image of 2hr control sample with GFP, green flourescent protien PI, propidium iodide Trans, transmission image and SI, superimposed image. (B) Confocal Image of 2hr control cell sample with intesity profile. (C) Confocal Image of 2hr microgravity cell sample with intesity profile.

in that APE1 localized to the nucleus (Fig. 1). The 2hr samples also showed APE1 nuclear localization (Fig. 2). However, in the 24hr sample, APE1 nuclear localization was disrupted. APE1 was now seen randomly distributed in the cytoplasm and not concentrated in the nucleus as the 0hr and 2hr samples (Fig. 3).

Fig. 3 Confocal micrscopic analysis of 24hr control and microgravity cell sample with intensity proile. (A) 24 hr control cell sample with GFP analysis (B) 24hr microgravity sample with GFP and PI prolie analysis



APE1 percent nuclear localization at 0hr and 2hr was >90% in both the control and microgravity samples. Interestingly, the percent nuclear localization at the 24hr time point was less than 40% (Table 1). This is a significant difference in localization of APE1. These results indicate that under microgravity conditions the % nuclear localization decreased relative to localization at normal gravity. To test whether this depression of APE1 nuclear localization could be reversed the 24hr microgravity samples

were returned to normal gravity and analyzed 24hrs later. In these samples, the percent nuclear localization of APE1 reversed back to >90% (Fig. 4).

Table 1.
Nuclear distribution of EGFP fusion protein

	0 hr	2 hr	24 hr
Control Sample	96.4 ± 2.7	94.1 ± 3.6	94.9 ± 7.5
Microgravity Sample	95.2 ± 4.1	90.8 ± 2.8	38.9 ± 4.8
Reversal	_	_	93.8 ± 2.3

Intensities of whole cells and of nuclei were measured and ratios of nuclear vs. whole cell intensities were calculated from more than 30 individual cells in each experiment.

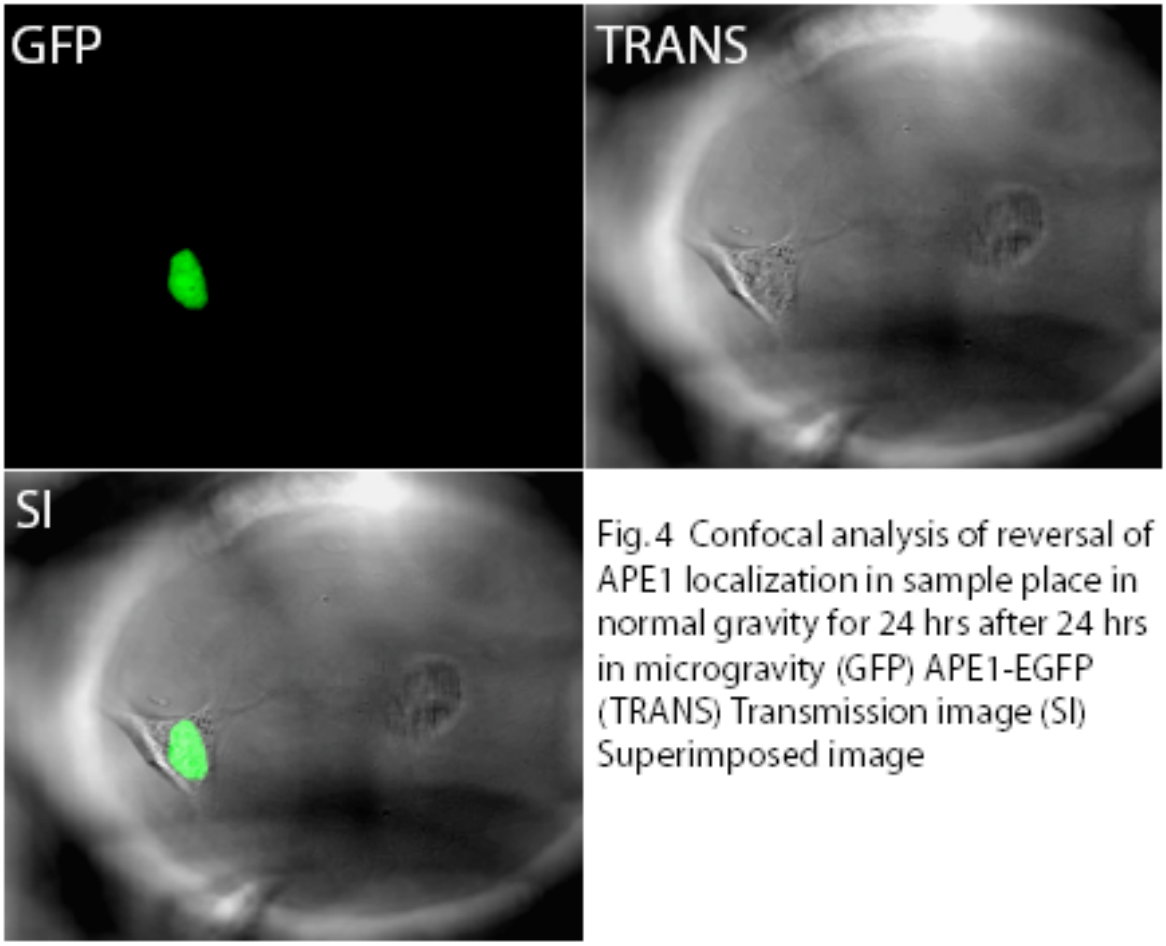


Fig. 4 Confocal analysis of reversal of APE1 localization in sample place in normal gravity for 24 hrs after 24 hrs in microgravity (GFP) APE1-EGFP (TRANS) Transmission image (SI) Superimposed image

Discussion

Understanding the effects of microgravity on cellular mechanisms is crucial to sustaining life in outer space. These results suggest that microgravity depresses nucleocytoplasmic translocation of the DNA repair protein APE1. This indicates that microgravity plays a role in disrupting normal trafficking of proteins that are involved in DNA repair. It is not clear how this disruption arises due to microgravity nor if radiation induced-induced mutations or transformations are also altered.

It is important to note that any statement that indicates an effect found due to "microgravity" be made with the utmost care in light of previous space experimental literature. The problem is that there are so many possible influences on simulated microgravity studies that may have an impact on the final outcome even when all steps are controlled.

Future experiments should try to elucidate the mechanism of this depression of APE1 trafficking from the cytoplasm into the nucleus in a microgravity environment. Specifically, an analysis of how microgravity affects trafficking proteins which are crucial in importing nuclear proteins.

CHAPTER 4: CONCLUSIONS

The nuclear localization signal (NLS) of APE1 was proposed to be that of a classical pat-7 form, consisting of seven amino acids, starting with a proline and containing 3 to 4 basic amino acids within the sequence. This is believed to be the sequence which allows APE1 to recognize and bind to importin proteins which will ferry it into the nucleus. These results show that the putative classical nuclear localization signal for APE1 is not a classical pat-7 form but rather a more complicated form which is composed of the 7 amino acids plus amino acids 12 and 13 in the sequence.

These results are very significant because they indicate that previously accepted dogma of nuclear localization of enzymes with pat7 NLSs does not apply to APE1 which is considered to be in the class of proteins that have a pat7 type of NLS. Studies that utilized this sequence must now be re-examined. Another question is whether this newly discovered sequence can be seen in other proteins which localize to the nucleus which the pat-7 classical NLS.

The interaction between APE1 and its ferrying protein karyopherin-a and karyopherin-b facilitates its nucleocytoplasmic translocation. Regulation of this translocation may be at the level of the adapter protein karyopherin-a because the findings show that two of the six homologs of karyopherin-a, a1 and a2 bind to APE1 to facilitate its binding to the other importin protein karyopherin-b, b then communicates with the nuclear pore complex to allow the entire cargo to enter the nucleus.

The question of synergism between DNA damage and exposure to micrograivity has long been dismissed. It was accepted that microgravity has no significant effect of the occurrence of DNA damage during spaceflight and that the cause of the damage was the only player involved. Now it seems that syngergism does exist in that regardless of the cause of the damage microgravity interrupts the normal programming of the cell and thus makes the DNA predisposed to damage.

Finally these questions and many more must be addressed before our understanding of the mechanistic nature of APE1 is complete. This work has unveiled important findings that add to our understanding of this multifunctional protein. It is only by such detailed investigation that we will be able to understand the functional outcomes of this enzyme. Whether in microgravity or at 1G, damage to the DNA is a problem that must be made less detrimental.

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