Mn-SOD serves as the primary cellular defense against oxidative damage by converting superoxide radicals (O$_2^-$) to O$_2$ and H$_2$O$_2$. A unique characteristic of this mitochondrial anti-oxidant enzyme is the conservation from bacteria to man of a rapidly formed product inhibited state. Using site-directed mutagenesis, we have generated an active site mutant (H30N) of human Mn-SOD, which exhibits significantly reduced product inhibition and increased enzymatic efficiency. Overexpression of the H30N enzyme causes anti-proliferative effects in vitro and anti-tumor effects in vivo. Our results provide a teleological basis for the phylogenetically invariant nature of position His-30 and the evolutionary conservation of product inhibition. These data also provide more direct intracellular evidence for the signaling role associated with H$_2$O$_2$.

The chemiosmotic theory (1) describes the mechanism that aerobic organisms utilize to form an electrochemical mitochondrial membrane gradient that is coupled to oxidative phosphorylation. The stepwise reduction of oxygen during respiration generates as a byproduct, reactive oxygen species (ROS), specifically the superoxide radical (O$_2^-$), and ultimately hydrogen peroxide (H$_2$O$_2$), both of which are highly cytotoxic (2). Mitochondria utilize over 90% of the oxygen consumed in eukaryotic aerobic organisms to form an electrochemical mitochondrial network (13) in the active site (Fig. 1).

Mn-SOD is a tetrameric dimer of dimers with one active site manganese atom per 22-kDa monomer (9). We have shown that the coordinated solvent molecule (Wat-1) along with Tyr-34, Gln-143, and His-30 form a hydrogen-bonding network (13) in the active site (Fig. 1A), which may be important for delivery of protons to regenerate the active Mn(III) enzyme. Site-directed mutations at each of these residues have demonstrated their importance in substrate accessibility, overall catalysis, and the redox chemistry of the metal (10–14). Our kinetic results have also established that the wild-type (WT) human enzyme exhibits first-order catalysis within the first 1–5 milliseconds of the reaction followed by a zero-order product inhibition. The catalytic mechanism of Mn-SOD (Reaction 1) is depicted below, where Mn(III)-SOD represents the ground state of the metal-bound enzyme.

These reactions represent an oxidative follows a reductive stage, where two O$_2^-$ are converted to O$_2$ and H$_2$O$_2$, by either the uninhibited or inhibited (Mn(X)-SOD) pathways (15). The product-inhibited state of Mn-SOD has been conserved from bacteria to man with the emergence of the inhibited form appearing 30-fold more rapidly for the human enzyme (11). In addition, in mammals, Mn-SOD has been postulated to function as a tumor suppressor gene (16–19), with its expression and activity being significantly reduced in many cancers (20, 5).
Anti-tumor Effects of a Mutant of Human Mn-SOD

21). Overexpression of Mn-SOD in several human cancer cell lines leads to reversion of the malignant phenotype with a decrease in cell proliferation (16–19), which is thought to be mediated by H2O2, a potential signaling molecule (22–24) and primary product of Mn-SOD catalysis (2).

An inference, therefore, derived from the active site structure, catalytic mechanism, and the signaling properties of H2O2, would be that specific alterations in active site residues may alleviate the inherent inefficiency associated with the product-inhibited complex, thus increasing the anti-proliferative effects through elevated levels of H2O2. To this end, we have generated active site mutants in an attempt to eliminate this rate-limiting portion of the catalytic cycle and have assessed the properties of these more efficient, mutant enzymes through overexpression in cell culture and in an animal model of tumorigenesis. These results have provided further insight into the physiological importance of Mn-SOD activity during oncogenesis as well as the relevance for evolutionary conservation of the product-inhibited complex.

MATERIALS AND METHODS

Reagents—The mammalian expression vectors pcDNA3.1/LacZ, a plasmid containing β-galactosidase; pcDNA3.1/Zeo(+) pcDNA3.1(-); LipofectAMINE Plus; G418 and Zeocin were purchased from Invitrogen Corp (Carlsbad, CA). TNF-α was a gift of Genentech Corp., San Francisco, CA. Cycloheximide (CHX) and catalase were purchased from Sigma-Aldrich Corp. (St. Louis, MO), and WST-1 cell proliferation kit was obtained from Roche Applied Science (Nutley, NJ). The pLPCX retroviral vector was purchased from Clontech (Palo Alto, CA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR).

Plasmid Constructs—An 833-bp human Mn-SOD cDNA fragment was generated by reverse transcription-PCR from mRNA isolated from human renal proximal tubule cells using oligonucleotides containing 5′-HindIII and 3′-EcoRI; (Forward: 5′-CCCAAGCTTGGCGCGCAGCATCGCT-3′ and reverse: 5′-GGGGTACCAATTAGACGCTATACGTTAT-3′) and subcloned into pcDNA3.1 that had been completely digested with NotI and inserted downstream of the cytomegalovirus promoter/enhancer of the mammalian expression vector pcDNA3.1+ that had been completely digested with NotI, and partially digested with BsaI as described previously (25).

Mn-SOD active site mutants were generated using PCR as described previously (14) using the following primer sets: H30N Forward: 5′-CAGCTGCAACATTGGTGGAAAGACCAAGGCGGCTA-3′, H30N Reverse: 5′-TACAGGCTCCTCGTGTGGCTTACGGGATCT-3′; and Q143A Forward: 5′-GCTGCCTCTTCATATATCCGAGTCAC-3′, and Q143A Reverse: 5′-GGTGATCGGGATTTGGACAGGAC-3′ (underlined codon denotes site of mutations). All constructs were verified by DNA sequencing.

HEK 293 Cell Culture, Cell Transfection, and Generation of Stable Cell Lines—Human embryonic kidney cells (HEK293) were cultured as previously described (25) and maintained in growth medium of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected with 1 µg of pcDNA3.1 plasmid containing either wild-type Mn-SOD, the H30N or Q143A mutant using LipofectAMINE Plus (Invitrogen), cultured for 48 h in growth medium and transferred to growth medium containing zeocin (150 µg/ml). Antibiotic-containing medium was replaced every 3 days for approximately 4 weeks. Multiple zeocin-resistant colonies were isolated by clonal selection and grown without antibiotic prior to screening for Mn-SOD or catalase protein expression by immunoblot analysis. Identical stable clones were also generated using selection with G418 to test for the effects of antibiotic selection on the quiescence phenotype, which occurred with cells stably overexpressing the H30N mutant with similar results, as described below.

In transient transfection assays, 1 µg of pcDNA3.1 plasmid containing either wild-type Mn-SOD, H30N, or Q143A mutants and 1 µg of pcDNA3.1/LacZ were co-transfected into 60-mm dishes containing 1.9 x 105 HEK 293 using LipofectAMINE Plus (Invitrogen). Twenty-four hours post-transfection, the cells were split into two 35-mm plates and allowed to recover for an additional 24 h. Cells were washed twice with phosphate-buffered saline and harvested for Northern and immunoblot analyses, as described below.

Enzyme Isolation and Pulse Radiolysis—Wild-type and mutant Mn-SODs (lacking the leader sequence which in eukaryotic cells is excised

![Image](https://example.com/image.png)
FIG. 2. Characterization of HEK293 cells overexpressing vector (pcDNA3.1, Invitrogen), WT, H30N, and Q143A. A, Northern analysis of transiently transfected Mn-SODs. A representative Northern blot analysis is depicted. Arrows indicate the position of the endogenous (4.0 and 1.0 kb) and plasmid-derived Mn-SOD transcripts (1.2 kb). The ~1.2-kb transcript comprises 853 nucleotides of the WT, H30N, or Q143A Mn-SOD cDNAs and 300 nucleotides of pcDNA3.1 and poly(A) sequence. B, TNF-mediated cytotoxicity data of transiently transfected Mn-SOD cDNAs. HEK 293 cells were co-transfected with β-galactosidase reporter plasmid pcDNA3.1/LacZ and pcDNA3.1 alone, or carrying WT, H30N, and Q143A. Transfected cells were split 1:2 and either treated with 50 μg/ml cycloheximide (CHX) for 20 min followed by 100 ng/ml TNF-α for 24 h or not. The numbers of β-galactosidase-positive cells per 10 high power fields were counted blindly. Data are expressed as the quotient of β-galactosidase-positive cells in the control and treated groups. *, p < 0.05 versus pcDNA3.1 alone and Q143A. C, immunoblot analysis of clonal HEK 293 cell populations. Individual clones from HEK 293 cells stably transfected with empty pcDNA3.1 Zeo(+) (vector) or with either WT Mn-SOD (top panel), Q143A (middle panel), or H30N (bottom panel) are shown. The numbers in each panel correspond to the individual clone designation with an additional lane for WT clone #3 for comparison of transgene expression levels. D, flow cytometry of vector, WT, and H30N cells using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). Data are represented by cell number as a function of DCF-DA fluorescence (x axis). E, flow cytometry of the H30N-overexpressing cells following exogenous exposure to purified catalase protein in the culture media.
following targeting of the protein to the mitochondrion) were overexpressed and purified as described previously from *Escherichia coli* (11). Briefly, the bacterial lysates were heat-treated (60 °C) followed by subsequent dialysis and ion exchange chromatography (DE-52 and CM-52). Final protein concentrations were determined by the ratio of total Mn-SOD active sites as determined by the Lowry assay.

Pulse radiolysis experiments were carried out at the Brookhaven National Laboratory utilizing a 2-MeV Van de Graaff accelerator, as described previously (26). Buffered enzyme solutions containing 1 μM enzyme, 2 mM Taps, 30 mM sodium formate, and 50 mM EDTA at pH 8.2 were exposed to the electron beam instantaneously generating 20 μS/m. Thioredoxin dependent and thus activity of the enzymes was followed spectrophotometrically at 260 nm.

**Northern Blot Analysis—**Total RNA was isolated from transfected cells using a modification of the Chomczynski and Sacchi method (7). RNA was quantified and size fractionated on 1% agarose-formaldehyde gels, blotted onto nylon membranes, and hybridized with a 32P-labeled human Mn-SOD cDNA probe. Membranes were then washed and subjected to autoradiography.

**Immunoblot Analysis—**Transfected or retrovirally transduced cells were lysed in buffer containing Triton X-100 (%). Protein concentration was determined by the bicinchoninic assay (Pierce, Rockford, IL), and 20 μg of total protein was electrophoresed on 10% or 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked using 5% nonfat milk and incubated for 1 h with a rabbit polyclonal anti-Mn-SOD (1:2000, Stressgen Bioreagents, Victoria, BC, Canada (25)), mouse monoclonal anti-GFP (1:1000, Chemicon Int., Temecula, CA) or anti-catalase (1:500, Calbiochem, San Diego, CA) antibodies followed by washing and incubation with peroxidase-conjugated goat anti-rabbit or mouse IgG antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA). Labeled protein bands were visualized by chemiluminescence (Pierce).

**Cell Growth and Toxicity Assays—**HEK 293 cells were transiently co-transfected with pcDNA3.1/LacZ and pcDNA3.1 alone, or carrying wild-type Mn-SOD, H30N, or Q143A mutant enzymes. The transfected cells were split 1:2 either treated, or not, with 50 μg/ml CHX for 20 min followed by 100 ng/ml of TNF-α for 24 h. Cell were washed and stained for β-galactosidase. β-Galactosidase-positive cells were blindly counted in 8–10 random 10X high power fields. The data are expressed as the fraction of β-galactosidase positive cells in the control and treated groups.

Cell growth rates and cellular toxicity were also determined using the WST-1 Proliferation Kit (Roche Corp.), where HEK 293 cell lines stably overexpressing mitochondrial targeted catalase as well as wild-type Mn-SOD, H30N, or Q143A enzymes were plated at 4 × 10^4 cells per well in a 96-well plate and grown for 1, 3, 5, 6, and 7 days. 10 μl of the WST-1 reagent was added to each well and for toxicity studies the percent viability was determined by subtracting the ratio of (A690 – A490 in treated cells) to (A690 – A490 control cells) following the addition of WST-1.

To evaluate the effects of protein overexpression in cells that were stably overexpressing mito-catalase (25) and the respective Mn-SOD proteins in the context of a cytotoxic challenge, cells were exposed to CHX/TNF-α cell viability determined by the WST-1 assay. For determination of percent toxicity following TNF and CHX challenge, 4 × 10^4 cells of each stable cell line (replicates of four) were plated 24 h prior to challenge into 96-well plates. Cells were incubated with 50 μg/ml CHX for 20 min followed by the addition of 100 ng/ml TNF. Control and treated cells were incubated with 10 μl of WST-1 reagent and allowed to incubate for 1 h prior to measurement of absorbances at 450 nm and 690 nm as per the manufacturer's instructions. Percent viability was determined by the quotient (A690 – A490 of treated samples/A690 – A490 of control samples) × 100. All growth curves and toxicity measurements represent the means ± S.E. of at least four replicate experiments.

**Measurement of Cellular H2O2 Levels—**HEK cell lines stably expressing wild-type Mn-SOD, H30N, or Q143A enzymes as well as empty vector were exposed to DCF-DA as a probe for ROS as described previously (24). Confirmation of the presence of increased H2O2 levels was determined via quenching with exogenous catalase.

**Retrovector Constructions, Transductions, and Cell Growth—**Wild-type (WT) Mn-SOD and H30N/Mn-SOD mutant cDNAs were subcloned from the pcDNA 3.1 plasmid into a pBMM-IRE-GFP retrovector expression vector (27) and into the puroamycin selectable retrovector expression vector pLPXC (Clontech Corp.). The Mn-SOD cDNAs were excised from the pcDNA 3.1 plasmid using EcoRI and NotI and subcloned into both of these retrovector vectors. Phoenix-packaging cells were used for the production of infectious retroviruses (27). A549 cells were utilized for the retrovector transduction experiments and were cultured in Ham's modified F12K media containing 10% fetal bovine serum.

For the generation of retrovirally transduced A549 cells, Phoenix cells at ~40% confluence were transfected using calcium phosphate with 8 μg of empty vector, wild-type Mn-SOD, H30N Mn-SOD, or GFP (pLPXC only) and incubated at 37 °C (27). The same protocol was used for both the pBMM-IRE-GFP and pLPXC constructs. Eighteen hours post-transfection, the media was changed and the Phoenix cells were moved to 32 °C for 24 h for retrovector generation. Forty-eight hours post-transfection the media from the Phoenix cells was collected and centrifuged to remove live packaging cells. The media containing infectious retrovirus was put on 30–40% confluent A549 cells and placed at 32 °C for 24 h after which fresh media was added, and the cells were moved to 37 °C. For the pBMM-IRE-GFP A549 cells, GFP expression was apparent after 48 h and 80–90% of the cells were GFP-positive as...
FIG. 4. Morphological and anti-proliferative effects of the H30N mutant in retroviral overexpressed A549 cells. A, representative phase contrast images (magnification 15X) of human A549 cells alone (Control) or transduced with an empty retroviral vector (Vector), virus containing the WT Mn-SOD or virus encoding the H30N Mn-SOD (H30N). Cells were maintained in culture following transduction and selection following >21 passages (9 weeks) at which time the morphological differences became evident in the H30N cells. B, retrovirally transduced cells were selected with puromycin for a 2-week period, yielding a cell population that contained over 97% transduced cells based on independent transduction with GFP and FACS analysis. C, 1 × 10⁵ A549 cells were plated in triplicate at each passage following antibiotic selection and allowed to grow over a 5-day period. At this time, cells were counted for each experimental group, and the cell number with standard deviations is depicted in each bar graph for the days post-selection (16, 23, 44, 57, and 64) (passages 5, 8, 14, 19, and 21, respectively). All vector-transduced cells were maintained in puromycin selection media throughout culture except with cells plated for growth studies. D, total cellular protein was isolated from cells at 0, 35, and 64 days post selection and analyzed by immunoblot analysis using a Mn-SOD-specific antibody. This demonstrates that protein levels for both the WT and H30N proteins are maintained over the course of the experiment.
confirmed by flow cytometry. The A549 cells transduced with pLPX vectors were selected at 1 \( \mu g/ml \) puromycin starting at 48 h post-infection, with selection complete at 2 weeks. Independent confirmation of infection efficiency was performed using flow cytometry with the GFP-pLPX cells and demonstrated greater than 98% transduction. Protein was isolated from all groups of transduced cells, and immuno- blot analysis was used to confirm transgene expression.

For evaluation of cell growth, control A549 cells and cells transduced with the empty retroviral vector were compared with retroviral transduced cells overexpressing the wild-type Mn-SOD and H30N proteins. 1 \( \times 10^5 \) cells were plated on 100-mm plates and allowed to grow for 5 days, and cells were then blindly counted in triplicate using a hemacytometer. Cell growth was evaluated at 16, 23, 44, 57, and 64 days post antibiotic selection as described above.

Animal Model of Tumorigenesis—NOD/SCID mice (28) were injected subcutaneously with 2 \( \times 10^5 \) retrovirally transduced A549 cells over-expressing empty pBMN-IRE-EGFP vector (27), wild-type Mn-SOD, or the H30N mutant in pBMN-IRE-EGFP (n = 7 per group). Flow cytometry of GFP fluorescence was performed prior to injection to determine the transduction efficiency, which was between 88 and 92%. Tumor size was determined by measuring the tumor length (L) and width (W) using a Vernier caliper, and the tumor volume (TV) was calculated in cubic millimeters by the formula, TV = L \times W^2/2.

Statistical Analysis—Data are represented as mean \( \pm \) S.E. The \( t \) test was used for comparisons between two groups, and for comparisons involving more than two groups, we used analysis of variance and the Student-Newman-Keuls test. All results are considered significant at \( p < 0.05 \).

RESULTS

Catalytic Efficiency Assessed by Pulse Radiolysis—Our previous studies (11–14) have demonstrated that mutagenesis at position Glu-143 (see Fig. 1A) in the active site leads to changes in the redox chemistry of the manganese atom, converting the metal from Mn(III) to Mn(II) in the ground state dramatically reducing the efficiency of catalysis. This is illustrated in a pulse radiolysis trace of the Q143A mutant (Fig. 1B), where purine mutant enzyme in buffer is placed in a cuvette and bombarded with a collimated electron beam instantaneously producing reproducible levels of \( O_2^- \). Superoxide concentrations are determined based on \( O_2^- \) absorbance at 280 nm. The upper tracing illustrates the slower catalytic efficiency over time of the Q143A mutant relative to the WT enzyme. On the other hand, substitution of asparagine at position His-30 (13), another important residue in the hydrogen bond network of the active site (Fig. 1A), essentially eliminates the product-inhibited phase of catalysis and generates an enzyme that can more efficiently dismutate an equivalent concentration of \( O_2^- \) over the observed time interval relative to the wild-type enzyme (Fig. 1B).

Overexpression of Site-Directed Mutants of Human Mn-SOD—To characterize the cellular and physiological consequences of the more efficient H30N enzyme, stable cell lines (vector, WT, H30N, and Q143A) were established in both HEK293 and A549 cells. Fig. 2A shows a Northern analysis of transiently expressed WT and mutant Mn-SODs in HEK293 cells. The overexpressed message of \( \sim 1.2 \text{ kb} \) is seen relative to the 4.0- and 1.0-kb endogenous mRNA for human Mn-SOD. To evaluate the functional characteristics of these mutant enzymes, we transiently overexpressed the WT, H30N, and Q143A enzymes and tested their effectiveness in a model of TNF-\( \alpha \)-mediated apoptosis (6). Fig. 2B illustrates that cells overexpressing the less efficient Q143A enzyme exhibited cell viability similar to vector transfected cells. In contrast, cells expressing the WT or H30N enzymes showed equivalent cytoprotection with significantly increased viability compared with vector- or Q143A-transfected cells (Fig. 2B).

Fig. 2C illustrates an immunoblot of individual antibiotic-selected HEK 293 clones from cells stably transfected with each of the abovementioned constructs. The presence of the 22-kDa Mn-SOD monomer is indicated in each panel. Protein from WT clone #3 was employed on each immunoblot for a comparison to the mutant protein expression levels. Most significantly, selection of stable, clonal transformants in HEK 293 cells employing mammalian expression vectors with either zeocin or G418 selection caused significant effects on cellular growth. Over the time period necessary for stable selection, overexpression of the H30N enzyme caused significant slowing of cell growth in multiple independent clones and separate transfections, irrespective of the vector or selection regimen. To explain the anti-proliferative effects of this mutant enzyme, we evaluated the level of ROS in the stably transfected cells using DCF-DA (Fig. 2D). These results demonstrate that the H30N stably overexpressing cells produce elevated levels of ROS and that the anti-proliferative effects are most likely a consequence of increased \( H_2O_2 \) production based on the reduction in signal following catalase treatment (Fig. 2E). Other than these limited studies, H30N-overexpressing cells established through stable transfection protocols were essentially refractory to further study due to the potent anti-proliferative effects of this mutant enzyme.

Rescue of H30N-overexpressing Cells by Mitochondrial-targeted Catalase—As additional evidence for the relevance of \( H_2O_2 \), we tested the strategy that overexpression of mitochondrial-targeted catalase (mito-CAT) (24) would effectively provide a sink for the increased mitochondrial \( H_2O_2 \) generated from H30N overexpression and thus detoxify \( H_2O_2 \) sufficiently to regenerate normal cellular growth patterns. We first established cloned cell lines that stably overexpress mito-CAT (25) followed by the development of a number of independent double stable mito-CAT cell lines containing a second integration of either vector, WT, H30N, or Q143A, as shown in Fig. 3A. As predicted, the inclusion of mitochondrial targeted catalase activity abrogated the quiescent phenotype associated with H30N allowing a growth pattern that more closely approximated that of the mito-CAT cells transfected with vector or Q143A (Fig. 3B). In addition, unlike the single stables for H30N, which are essentially quiescent, the ability to sustain growth in a mito-CAT background has allowed for the further characterization of the functional/physiological characteristics of this mutant. As observed with transient transfection (Fig. 2A), mito-CAT cells overexpressing H30N or WT enzyme could equally protect cells from the pro-apoptotic effects of TNF-\( \alpha \) as compared with vector or Q143A (Fig. 3C). These data demonstrate that the H30N enzyme is capable of both protecting against TNF-\( \alpha \)-mediated apoptosis through the dismutation of \( O_2^- \) and causing significant anti-proliferative effects most likely as a result of increased \( H_2O_2 \) production.

Cellular Effects of Retroviral-mediated Overexpression of WT and H30N Mn-SOD—To circumvent the anti-proliferative effects of H30N apparent during the extended time frame necessary for stable selection, we transduced A549 cells, originally derived from a human lung adenocarcinoma, using retroviral-mediated gene targeting. The inclusion of the puromycin resistance gene in these retroviral constructs (pLPX) allowed for the generation of a uniform population of overexpressing cells following antibiotic selection over a shorter period of time relative to the time frame for plasmid-based selection of stable transformants. H30N-overexpressing cells grew slowly, and with time they exhibited an elongated morphology (Fig. 4A). Fig. 4B illustrates the uniformity of the cell population following antibiotic selection as demonstrated by FACS analysis of the GFP-expressing cells with a 97% pure population. As shown in Fig. 4C, we observed that the growth of the H30N cells was different as compared with cells transduced with the retroviral vector alone or expressing the WT Mn-SOD. Equal numbers of cells from each group were plated, and the cell number was quantified following 5 days of growth. H30N cells...
displayed a significantly slower growth rate compared with the vector and WT Mn-SOD cells (Fig. 4C). To verify continued overexpression of Mn-SOD, we performed immunoblot analysis on the cells at 0, 35, and 65 days post-selection. As shown in Fig. 4D, both the WT- and the H30N-overexpressing cells had similar levels of Mn-SOD protein over time thus excluding the possibility that changes in Mn-SOD protein levels were responsible for the phenotypic and growth characteristics of the H30N mutant. The growth studies show marked difference between WT and H30N, whereas in the apoptosis studies (Fig. 2B), the enzymes afforded equal levels of cytoprotection indicating that TNF cytotoxicity has effects other than increasing O$_2^-$ levels. The difference in the results highlights the effect of continued H30N expression in contrast to a transient expression in an apoptosis assay.

Anti-proliferative Effects of H30N Mn-SOD in an Animal Model of Tumorigenesis—The lack of product inhibition, the increased efficiency of catalysis, and the concomitant anti-proliferative effects of the H30N enzyme, provided the impetus for evaluation of this mutant enzyme as a potential for anti-tumor therapy. This assumption is further substantiated by the observation that Mn-SOD activity is generally low in cancer cells (20, 21), suggesting that the oncogenic phenotype may be associated with or a consequence of lowered H$_2$O$_2$ levels. To test the anti-proliferative effects of H30N overexpression in vivo, A549 cells were transduced with a bicistronic retroviral vector (pBMN-Mn-SOD-IRES-eGFP, Fig. 5A) co-expressing both the WT enzyme or H30N with GFP. This vector lacks an antibiotic resistance gene; however, these viral constructs are capable of transduction efficiencies of 80–90% at 48 h as verified by flow cytometry based on GFP fluorescence (Fig. 5B) and protein overexpression (Fig. 5C). 48 h following transduction, 2 x 10$^6$ cells were injected subcutaneously into NOD/SCID (28) mice, and tumor volume was assessed over a period of 27 days. As shown in Fig. 5D, a striking inhibition of tumor growth was observed in the H30N group as compared with the vector or WT animals. These results suggest that mutations that eliminate product inhibition and, thus, the rate-limiting portion of the catalytic mechanism of human Mn-SOD, can cause potent anti-tumor effects, most likely through the increased generation of H$_2$O$_2$.

DISCUSSION

These results offer important insights regarding the rationale for evolutionary conservation of product inhibition associated with Mn-SOD and the importance of Mn-SOD-derived H$_2$O$_2$ generation in the control of cellular growth. As stated, we have previously demonstrated that product inhibition is conserved from bacteria to man, with the human enzyme entering the product-inhibited state 30-fold faster than the analogous bacterial enzyme (11). Additionally, although Mn-SODs are highly conserved enzymes, the residues (most relevantly His-30) in Fig. 1A comprising the hydrogen bond network in the active site are invariant from E. coli or Thermus thermophilus through the mouse, rat, and human enzymes (10). The conservation of product inhibition, on first approximation, could be rationalized based on the highly regulated nature of the Mn-SOD gene, which can be elevated as much as 25-fold following stimulation with pro-inflammatory mediators (5–8). Intuitively, simple increases of overall enzyme concentration could compensate for any perceived shortcomings associated with the product-inhibited phase of catalysis. Based on the present results, it is now apparent that the loss of product inhibition accompanied by the increase in efficiency resulting from substitution at position His-30 would not be an advantageous mutation, but rather one leading to a lethal phenotype. Therefore, teleologically speaking, the phylogenetically invariant nature of position His30 in Mn-SOD results from a trade-off between increased enzymatic efficiency associated with cellular/organisinal lethality versus
evolutionary conservation of product inhibition.

An additional rationale for the observed sequence conservation would be the overall selective advantage associated with a product-inhibited enzyme that more slowly releases a product, H}_2O}_2, which is a potent regulator of cell growth and/or viability (22). Recently, increasing evidence has implicated H}_2O}_2 as a second messenger with the unique effects of stimulating cellular proliferation at 100 nM to 1 μM but causing cell cycle arrest and induction of cell quiescence at concentrations of 200 μM and higher (22, 29–32). Furthermore, as with our data using extracellular catalase exposure (Fig. 2D) or prior overexpression of catalase to overcome the anti-proliferative effects of H30N overexpression (Fig. 3), the slow growth phenotype observed with elevated Mn-SOD activity documented by other investigators has also been rescued by the overexpression of catalase, either in the cytosol or targeted to the mitochondrion (23, 24). It therefore appears that overexpression of the more efficient H30N Mn-SOD must generate H}_2O}_2 levels approaching 200 μM based on its anti-proliferative and anti-tumor effects. Reciprocally, it is also important to recognize that the reduction in tumor size linked to H30N overexpression clearly provides evidence for the critically important signaling role for H}_2O}_2 (22, 29–32) in the control of cell growth.

The conventional strategy underlying structure/function relationships generally targets enzymatic/kinetic mechanism or test structural hypotheses. The present studies are unique in that we have utilized fast kinetic and crystallographic analysis coupled with site-directed mutagenesis as a strategy for altering the inherent character of the enzyme in vitro, ultimately culminating with a potential therapeutic outcome. As previously documented (18–21), overexpression of the wild-type Mn-SOD results in a slow growth phenotype in most tumor cells in vitro coupling with site-directed mutagenesis as a strategy for altering the inherent character of the enzyme in vitro, ultimately culminating with a potential therapeutic outcome. As previously documented (18–21), overexpression of the wild-type Mn-SOD results in a slow growth phenotype in most tumor cells along with reported decreases in tumorigenicity. Molecular targeted modification of enzymatic behavior, namely overcoming Mn-SOD product inhibition as evident in the H30N enzyme, has yielded a more efficient enzyme that consequently generates H}_2O}_2 at levels that significantly retard tumor growth. As further support of this strategy, we have also generated other single and double site mutants of Mn-SOD, which exhibit similar characteristics in vitro (data not shown) and are currently testing their efficacy in vitro tumor models. Equally promising are the prospects of coupling increases in cellular production of O}_2 from conventional radiation and chemotherapy with gene targeting of mutant Mn-SODs lacking product inhibition and exhibiting increased enzymatic efficiency. Combination therapy could possibly enhance the anti-proliferative response through increasing levels of H}_2O}_2 signaling, thus providing a potent avenue for anti-tumor therapy.

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