Transactivator of transcription–tagged cell cycle and apoptosis regulatory protein-1 peptides suppress the growth of human breast cancer cells *in vitro* and *in vivo*

Liyue Zhang,[1,2] Edi Levi,[1] Pralay Majumder,[5] Yingjie Yu,[1] Amro Aboukameel,[2] Jianhua Du,[1,2] Hu Xu,[1,2] Ramzi Mohammad,[2] James S. Hatfield,[1] Anil Wali,[3,5] Volkan Adsay,[4] Adhip P.N. Majumdar,[1,2,5] and Arun K. Rishi[1,2,5]

[1]Veterans Affairs Medical Center, Departments of [2]Internal Medicine, [3]Surgery, and [4]Pathology, [5]Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

Abstract

Deregulated signaling by the epidermal growth factor receptor family of proteins is encountered in human malignancies including breast cancer. Cell cycle and apoptosis-regulatory protein-1 (CARP-1), a novel, perinuclear phosphoprotein, is a regulator of apoptosis signaling by epidermal growth factor receptors. CARP-1 expression is diminished in human breast cancers, and correlates inversely with human breast cancer grades which could be attributed to increased methylation. The expression of CARP-1, on the other hand, interferes with the ability of human breast cancer cells to invade through the matrigel-coated membranes, to form colonies in the soft agar, and to grow as s.c. tumors in severe combined immunodeficiency (SCID) mice. To test whether CARP-1 is a suppressor of human breast cancer growth, we generated transactivator of transcription (TAT)–tagged CARP-1 peptides. Treatment of human breast cancer cells with affinity purified, TAT-CARP-1 1–198, 197–454, and 896–1150 peptides caused inhibition of human breast cancer cell proliferation and elevated apoptosis. In contrast, TAT-tagged enhanced green fluorescent protein or CARP-1 (1–198$^{Y192/F}$) peptide failed to inhibit cell proliferation or induce apoptosis. Apoptosis by CARP-1 peptides, with the exception of CARP-1 (1–198$^{Y192/F}$), involves the activation of p38 stress-activated protein kinase and caspase-9. Moreover, administration of TAT-CARP-1 (1–198), but not TAT-tagged enhanced green fluorescent protein or TAT-CARP-1 (1–198$^{Y192/F}$), inhibits growth of human breast cancer cell–derived tumor xenografts in SCID mice. We conclude that CARP-1 is a suppressor of human breast cancer growth, and its expression is diminished in tumors, in part, by methylation-dependent silencing. [Mol Cancer Ther 2007;6(5):1661–72]

Introduction

Aberrant regulation of apoptosis, also known as programmed cell death, is a factor in pathogenesis of many disorders including cancer (1), whereas resistance to apoptosis is a characteristic feature of cancer cells (2). Sustained mutations in genes that control cell proliferation and survival are frequently noted in the development and progression of many cancers. For example, in breast carcinoma, several commonly mutated genes noted in patient-derived tumors include the amplification or overexpression of *HER-2/neu, cyclin D1,* and *c-myc* genes in certain tumors, whereas mutations or methylation-dependent silencing of tumor suppressor genes such as *p53, pRb,* or *p16^{INK4a}* which lead to the loss of function/expression of these proteins in other tumors (3–7). In addition to mutations in the oncogenes and tumor suppressor genes, alterations in Ras-signaling pathways are also frequently noted in human breast carcinomas (8).

The balance between proliferation and apoptosis often influences the response of tumors to various anticancer therapies. Signaling pathways that regulate cancer cell proliferation and apoptosis serve as attractive targets for the design and development of specific biological and/or pharmacologic inhibitory molecules with potential as anticancer therapeutics (9). Indeed, several peptides and/or pharmacologic agents that target specific mediators of intracellular signaling to either inhibit proliferation or promote apoptosis are currently used as anticancer agents. Defects in apoptosis signaling in the cancer cells, however, often lead to acquisition of their resistance towards chemotherapeutic agents that function by targeting these pathways (9). Therefore, the elucidation of molecular bases of diverse intrinsic as well as extrinsic apoptosis signaling pathways is necessary to reveal novel targets for inhibition and, in turn, to facilitate the development of strategies to counteract resistance of cancer cells towards conventional anticancer agents. Hence, the search for novel regulators of

Received 10/23/06; revised 2/23/07; accepted 3/2/07.

Grant support: Susan G. Komen Foundation for Breast Cancer Research (A.K. Rishi), the medical research services of the Department of Veterans Affairs (A.K. Rishi, A. Wali, and A.P.N. Majumdar), and by NIH RO1 AG 14343 (A.P.N. Majumdar).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Arun K. Rishi, Room B4334, VA Medical Center, 4646 John R., Detroit, MI 48201. Phone: 313-576-4492; Fax: 313-576-1112. E-mail: Rishia@Karmanos.org

doi:10.1158/1535-7163.MCT-06-0653

apoptosis, and their potential utility in the design and development of efficacious agents that target these molecules, are the subjects of intense investigation.

Transactivator of transcription (TAT) from the HIV protein has an amino-terminal sequence that is essential and sufficient for the translocation of HIV-TAT protein in a receptor-independent fashion (10). This TAT-leading sequence is rich in arginine and is highly basic, and facilitates the internalization of TAT peptide. Subsequent studies have generated heterologous TAT fusions to deliver a wide, size-independent variety of molecules into mammalian cells, including liposomes, iron beads, antisense oligonucleotides, and proteins (11). TAT protein transduction domain fusion peptides or proteins are usually expressed in *Escherichia coli* and purified by affinity chromatography under denaturing conditions. The protein transduction domain serves to facilitate the translocation of the denatured fusion proteins across the plasma membrane, and once inside, the fusion proteins regain their biological and functional properties. Multiple studies have used protein transduction domain fusion proteins in *in vitro* and *in vivo* preclinical models of human disease including cancer, psoriasis, and stroke (11, 12). Thus, the protein transduction domain–based expression of a protein product is complementary to the goals of gene therapy that could, in principle, allow the delivery of tumor suppressor proteins to induce tumor cell apoptosis.

Cell cycle and apoptosis-regulatory protein-1 (CARP-1)/CCAR1 is a novel mediator of apoptosis signaling that was recently identified by using an antisense-dependent functional gene-knockout strategy (13). CARP-1 mRNA is ~3.5 kb in size and encodes a protein of 1,150 amino acids with an approximate molecular mass of 130 kDa (13). Western immunoblot and immunocytochemical analyses using anti–CARP-1 antibodies revealed that CARP-1 is a perinuclear protein that is present in diverse cell types. The human CARP-1 gene is located at the long arm of chromosome 10 (10q21–10q22), and the nucleotide sequences in the Genbank indicate the presence of mouse, rat, dog, chimpanzee, fowl, *Xenopus*, honey bee, and *Caenorhabditis elegans* CARP-1 proteins. CARP-1 regulates apoptosis by diverse agents including chemotherapeutics, adriamycin and etoposide. Moreover, inhibition of epidermal growth factor receptors (EGFR) by EGFR-related protein, a recently reported pan-ErbB inhibitor (14), causes the induction of apoptosis that involves increased CARP-1 expression and tyrosine phosphorylation (15). That CARP-1 is a key and specific regulator of apoptosis induced by these agents was underscored by the observations in which antisense-dependent loss of CARP-1 resulted in the abrogation of apoptosis by adriamycin or EGFR-related protein, but not cisplatin (13, 15). Increased expression of CARP-1 results in the repression of various cell cycle and proliferation-related genes such as *p21Rac1, c-Myc, cyclin B1,* and *topoisomerase IIα,* although causing elevated levels of CDKI $p21^{WAF1/CIP1}$, and activation of caspase-3 and caspase-9 (13, 15). Based on our observations thus far, CARP-1 seems to be a transducer of diverse pathways of cell growth regulation.

In this report, we tested the hypothesis that CARP-1 is a human breast cancer growth suppressor, and its expression and/or function is inhibited in human breast cancers. Stable expression of CARP-1 or TAT-mediated transduction of its peptides suppressed human breast cancer growth, in part, by activating apoptosis. Our investigation further revealed that higher grade breast cancers that are usually poorly differentiated with worsened prognosis had reduced to absent levels of CARP-1, and that the loss of CARP-1 is due, in part, to the increased methylation of its promoter.

Materials and Methods

Materials

DMEM, Ham's F-12 medium, fetal bovine serum, and LipofectAMINE-based transfection kits were purchased from Life Technologies, Inc. Anti–CARP-1 (α1) and (α2) polyclonal antibodies and their affinity purification were as previously described (13). Anti–myc-tag, caspase-9, p38α/β and phospho-p38α/β antibodies were purchased from Cell Signaling, whereas anti–hemagglutinin (HA)-tag antibodies were purchased from Covance. The ProBond purification system for affinity purification of TAT–enhanced green fluorescent protein (eGFP) and TAT-CARP-1 peptides was purchased from Invitrogen, Corp.

Recombinant Plasmid Constructs

The construction of plasmid clone 6.1 expressing myc-His–tagged CARP-1 protein has been previously described (13). Vector plasmid pTAT/HA, and the plasmid pTAT/HA-eGFP for expression of TAT-eGFP have been described elsewhere (16), and were kindly provided by Dr. Steve Dowdy, University of California, San Diego (San Diego, CA). Various recombinant plasmids harboring CARP-1 cDNA fragments were generated by a combination of PCR and standard cloning methodologies (17). CARP-1 cDNAs were subcloned into the vector plasmid pTAT/HA to generate CARP-1 peptides having hemagglutinin and polyhistidine tags, as well as a retroviral TAT transduction domain (10–12, 16) positioned at the amino termini (see Fig. 2A below). A construct encoding His-TAT-HA-CARP-1 ($1–198^{Y192/F}$) was generated by subcloning a PCR-amplified CARP-1 cDNA fragment. This PCR amplification used CARP-1 sense oligonucleotide AS-6.1 (13) and an antisense oligonucleotide–harboring mutation introducing a codon for phenylalanine instead of tyrosine at position 192 of the CARP-1 1–198 peptide. In addition, substitution of Tyr^{42} in CARP-1 ($1–198^{Y192/F}$) was created by a similar PCR-based strategy to obtain a construct for the expression of a His-TAT-HA-CARP-1 ($1–198^{Y42/F;\ Y192/F}$) peptide. All the constructs were sequenced to confirm the validity of the CARP-1 cDNAs.

Cell Lines and Cell Culture

The generation and characterization of MDA-MB-468 human breast cancer sublines expressing pcDNA3 vector or myc-His–tagged wild-type CARP-1 (plasmid clone 6.1) have been previously detailed (13). Routine maintenance and culture of estrogen receptor–negative, p53-negative wild-type MDA-MB-468 human breast cancer and their sublines were carried out essentially as described (13).

Affinity Purification of Peptides

The recombinant plasmids for expression of TAT-CARP-1 or TAT-eGFP proteins were used to transform *E. coli* (BL-21) DE3 strain and independent *E. coli* isolates expressing their respective proteins were obtained. The expression of TAT-tagged CARP-1 peptides and eGFP proteins was induced by isopropyl-L-thio-β-D-galactopyranoside followed by affinity purification of recombinant proteins from bacterial lysates using nickel-charged agarose resin (ProBond resin; Invitrogen, Inc.) using manufacturer-suggested protocols. The eluted denatured proteins were then desalted, dialyzed, followed by their analysis on SDS-PAGE to confirm their purity and stored either at 4°C or −20°C for subsequent use.

Western Blot and Apoptosis Assays

Logarithmically growing cells were treated with TAT-tagged CARP-1 peptides or eGFP for various time periods, and cells were lysed to prepare protein extracts. In all Western blot analyses, protein concentration was standardized among the samples. Briefly, 100-μg proteins were separated on a 10% SDS-PAGE and subsequently electroblotted to supported nitrocellulose membranes. The membranes were blocked overnight with 5% bovine serum albumin or nonfat dried milk in buffer containing 20 mmol/L of Tris-HCl (pH 7.6), 100 mmol/L of NaCl, and 0.01% Tween 20 (TBS-T). The membranes were incubated from 3 h to overnight with p-p38, p38, caspase-9, or anti-actin primary antibodies at the manufacturer-suggested dilutions in TBS-T buffer containing 5% bovine serum albumin at 4°C. After the membranes were washed thrice with TBS-T buffer, they were incubated with a horseradish peroxide–conjugated goat anti-rabbit IgG (1:5,000 dilution) as a second antibody for 1 h at room temperature. Protein band(s) were visualized using an enhanced chemiluminescence detection system.

In addition, cell lysates were prepared from cells transduced with different peptides and used to determine cell growth inhibition and apoptosis levels. The cell growth inhibition was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, whereas apoptosis levels were determined by using either DNA fragmentation–based ELISA kit (Roche Diagnostics) or Live/Dead assay kit (Molecular Probes) essentially following manufacturer-suggested guidelines and our published protocols (13, 15, 17). Apoptosis levels in tumor explants were determined by either immunostaining of the sections with anti–phospho-p38 stress-activated protein kinase (SAPK) antibody following the methodology detailed below or using the terminal deoxynucleotidyl transferase–mediated nick end labeling assay kit (Roche Diagnostics). Briefly, for the terminal deoxynucleotidyl transferase–mediated nick end labeling assay, the sections were incubated in terminal deoxynucleotidyl transferase reaction mixture for 2 h at 40°C to 45°C in a humidified chamber to allow the transfer of biotin-UTP to the strand breaks of the cleaved DNA in the apoptotic cells *in situ* by terminal deoxynucleotidyl transferase. The biotin-labeled cleavage sites were then detected by reaction with horseradish peroxidase–conjugated streptavidin and visualized with 3,3′-diaminobenzidine (Vector Laboratories) staining showing brown color, essentially following vendor-suggested protocols.

Immunolocalization of CARP-1

The deidentified, paraffin-embedded human breast cancer, and benign breast tissue biopsies were obtained from the Pathology Department, Harper Hospital (Detroit, MI) and their use was granted exempt status by the Wayne State University-Human Investigation Committee (Institutional Review Board). The breast cancer array slides were purchased from Cyberdi, Inc. and Petagen Inc. For immunohistochemical staining, an immunoperoxidase method was used with a streptavidin-biotinylated horseradish peroxidase complex (Dako). The tissue biopsies were formalin-fixed and paraffin-embedded, and 5-μm serial sections were generated. The tissue sections or the array slides were deparaffinized and microwaved for 15 min in citrate buffer (0.1 mol/L citrate acid and 0.1 mol/L sodium citrate; pH 6.0) for antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide, and subsequently incubated with 5% horse serum for 10 min to block nonspecific binding. The slides were then incubated at room temperature for 2 h with polyclonal antibodies to CARP-1 (α1), anti–HA-tag, or anti–phospho-p38 antibodies at 1:1,000, 1:250, or 1:500 dilutions, respectively. The avidin-biotin technique was done with matched components (secondary biotinylated antibody and avidin-peroxidase complex) from the DAKO-labeled streptavidin-biotin system according to the manufacturer's suggested protocol. The slides were then reacted with amino ethyl carbazole, counterstained with Harris' hematoxylin and examined by a pathologist. For immunocytochemical analysis, human breast cancer cells transduced with TAT-tagged eGFP or CARP-1 peptides in conjunction with a 1:2,000 dilution of anti–HA-tag monoclonal antibodies were used. The antibody-stained cells were then photographed under different magnifications using a Zeiss microscope with a 35 mm camera attached for recording the photomicrographs. In addition, the antibody-stained slides were photographed separately by confocal microscopy at the confocal core facility of Wayne State University School of Medicine. For quantitative determination of CARP-1 levels in the breast cancer arrays, at least five randomly selected fields on each array spot were examined. Staining scores were determined independently by two individuals according to the following formula to reflect quantitative as well as qualitative intensity: overall tumor staining was reported as the percentage of staining. The percentage staining was multiplied with the intensity score to obtain a single numerical staining score [score = staining intensity (0–3; 0 = negative, 1 = weak, 2 = moderate, and 3 = strong) × % tumor cells staining]. The level of CARP-1 in tumors was considered high when the score was ≥100, whereas tumors with <100 score were considered low expressers.

Methylation-Specific PCR Analysis of Human CARP-1 Promoter

Genomic DNA was extracted from formalin-fixed normal and breast cancer biopsy specimens essentially as described previously (18). Bisulfite modification of DNA to convert all unmethylated cytosines to uracil and then to thymidine during the subsequent PCR step, whereas leaving the methylated cytosines unaffected was done as described by Herman et al. (19). Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer's instructions (Promega) and eluted into 50 μL of water. Modification was completed by the addition of NaOH (0.3 mol/L final concentration) for 5 min at room temperature followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20°C.

The bisulfite-treated DNA was used for PCR amplification of a human CARP-1 promoter fragment. Amplification of a methylated 147 bp product (from positions 98422 to 98569; accession no. AL513534) was carried out using a combination of forward (5′-TTTTTGAGTAGTTGGGATTATAGGC-3′) and reverse primers (5′-CAACACTTTAAAAAACCGAAACGAA-3′). The primers for amplification of the unmethylated 124 bp DNA fragment were 5′-TTGAGTAGTTGGGATTATAGGTG-3′ (forward primer) and 5′-CAAATCACAAAATCAAAAAATCAA-3′ (reverse primer). The forward primer for methylated template had a single mismatch at the 3′-end for methylated template, whereas the reverse primer for the methylated template has two mismatches at the third and fourth positions from the 3′-end. These mismatches were expected to confer sufficient selectivity and specificity for amplification of the methylated template. For PCR amplification, the annealing temperature was 66°C. PCR amplification was done by using 3 ng of treated DNA as the template in a final volume of 50 μL. Reactions were hot-started at 95°C for 5 min before amplification in an Eppendorf thermal cycler for 40 cycles (20 s at 95°C, 20 s at 66°C, and 20 s at 70°C) followed by a final 4 min extension at 72°C. Controls without DNA were done for each set of PCRs. Each PCR product (15 μL) was directly electrophoresed and visualized following ethidium bromide staining of 2% agarose gels.

Human Breast Cancer Cell Clonogenic and Invasion Assays

Parental MDA-MB-468 human breast cancer cells or their sublines (vector subline 1, clone 6.1 sublines 1, 2, and 3; ref. 13) were used in soft agar clonogenic assays (18). Briefly, a 1 mL suspension containing 500 cells was sandwiched between 0.6% and 0.3% agarose in DMEM-5% fetal bovine serum. Plates were incubated at 37°C for 14 days, followed by counting of colonies from multiple, randomly selected fields from each plate. The transformed properties of CARP-1-myc-His expressing human breast cancer cells were studied by using the cell invasion assay kit (Chemicon International, Inc.) following the manufacturer-suggested guidelines. The assay was done in a 24-well tissue culture plate with cell culture inserts in each well. The cell culture insert contained an 8 μm pore size polycarbonate membrane, over which a thin layer of extracellular matrix was dried. A cell suspension containing 1×10^6 cells/mL in serum-free DMEM/F-12 medium was applied on the membrane inside the insert, whereas 0.5 mL of DMEM/F-12 medium with 5% fetal bovine serum was added to each well in the space outside of the insert. The cells were incubated at 37°C for 72 h, followed by staining of the invasive cells on the lower surface of the membrane of the insert. The invasive, stained cells on the membranes were photographed (data not shown). In addition, multiple random fields of the invasive, stained cells from each well were independently counted.

Establishment of Tumors in SCID Mice

Three-week-old, female, ICR SCID mice were obtained from Taconic Laboratories. After a period of adaptation, two to three mice were s.c. injected on each flank with $\sim 10^6$ human breast cancer cells. When tumors developed, mice were killed; tumors were dissected, cut into small fragments, and subsequently transplanted s.c. into similarly conditioned animals ($n = 6$ for each group) by using a 12-gauge trocar. Mice were checked thrice a week for tumor development. Once palpable tumors developed (usually <100 mg in size) by day 7 of xenograft implantation, groups of six mice were removed randomly for the efficacy trial using affinity-purified TAT-tagged proteins. The proteins were injected intratumorally at a dose of 25 μg/tumor/d for 5 consecutive days, and the tumor growth monitored for an additional 20 days. The tumor measurements were carried out at multiple time points during the course of treatments and observation periods. Mice were observed for changes in weight and side effects followed by measurement of tumors thrice per week. All studies involving mice were done under Wayne State University Animal Investigation Committee–approved protocols (nos. A 09-22-04 and A 10-06-03). Tumor weights in SCID mice were calculated as previously published (20, 21). Tumor weight (mg) = $(A \times B^2) / 2$, where A and B are the tumor length and width (in mm), respectively.

Results

CARP-1 Expression Inhibits Growth of Human Breast Cancer Cell–Derived Tumor Xenografts

Enhanced activation and expression of cell surface EGFR is an indicator of worsened prognosis in human breast cancers, and correlates inversely with the expression of estradiol receptor-α (22). In the absence of estradiol receptor-α, EGFRs and their downstream signaling mediators have emerged as targets for therapeutic intervention strategies in breast cancers (23). Our recent findings showing CARP-1 regulation by growth factor signaling (13), and elevated CARP-1 expression and tyrosine phosphorylation following inhibition of EGFRs by agents such as Iressa or EGFR-related protein in the human breast cancer cell model (15) suggested a potential inverse correlation between functions of EGFRs and CARP-1. Moreover, ectopic expression of CARP-1 results in the

Table 1. CARP-1 expression in human breast cancers (N = 100)

Tumor histologic grade	CARP-1 expression	
	Low	High
Low	16%	27%
High	47%	10%

NOTE: CARP-1 expression inversely correlates with the tumor histologic grade (Fisher's exact P = 0.00000378).

apoptosis of human breast cancer cells. Because aberrant activation and expression of EGFRs, particularly EGFR and Her-2/neu, is associated with poor differentiation and thus a prognostic factor in breast carcinoma, we speculated that CARP-1 expression is diminished or lost in poorly differentiated breast cancers. We investigated this postulation by first determining the levels of CARP-1 in breast cancers. Multiple primary breast cancer array slides were used for immunolabeling of CARP-1. Breast cancers were graded according to the modified Scarf-Bloom Richardson grading system (24) and assigned into categories of low (moderate to higher differentiation) or high grades (poorly differentiated) by staff pathologist at the Detroit VA Medical Center. The cells lining the benign ducts of the breast tissue show abundant CARP-1 expression, whereas its expression was higher in low-grade breast tumor when compared with the higher-grade tumor (data not shown). Analysis of the array data further revealed that 82% (47 out of 57) of high-grade breast cancers expressed low to absent levels of CARP-1, whereas ~62% (27 out of 43) of the low-grade tumors showed high levels of CARP-1 (Table 1).

Whether CARP-1 expression alters the biological properties of the breast cancer cells and is a factor in their inability to grow as tumors when xenografted in SCID mice was investigated next. Parental MDA-MB-468 human breast cancer cells and their sublines stably expressing vector plasmid or plasmid 6.1 that encodes myc-His–tagged wild-type CARP-1 (Fig. 1A) that were generated and characterized in our previous study (13), were used initially in soft agar clonogenic and cell invasion assays. Human breast cancer cells expressing myc-His–tagged CARP-1 formed 50% fewer colonies in soft agar when compared with the parental cells or vector subline 1 cells (Fig. 1B). Expression of CARP-1 also interfered with the ability of the human breast cancer cells to invade as suggested by 40% to 60% reduction in the number of stained cells that were able to migrate across the Matrigel-coated membranes when compared with their wild-type or vector-transfected counterparts (Fig. 1C). Next, we investigated the antitumor properties of human breast cancer sublines overexpressing CARP-1 by determining their growth as tumors following xenografting in SCID mice. Human breast cancer cells started to develop tumors after 14 days of implantation (which is typical for s.c. injections of cancer cells). However, both human breast cancer sublines expressing myc-His–tagged CARP-1 formed reduced-sized tumors

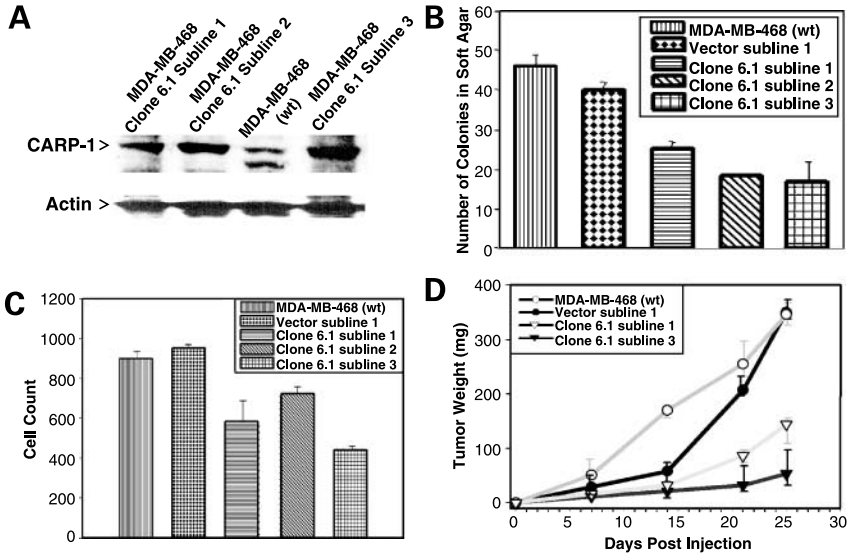

Figure 1. CARP-1 expression alters the biological properties of human breast cancer cells. **A**, Western immunoblot of wild-type human breast cancer cells and their sublines that were transfected with plasmid clone 6.1 for the expression of CARP-1-myc-His fusion protein. Analysis of proteins by SDS-PAGE and their transfer to nitrocellulose membranes were carried out essentially as in Materials and Methods. The membrane was probed with anti-CARP-1 (α1) antibodies followed by hybridization with anti-actin antibody to assess loading. **B**, histogram of relative number of colonies of indicated cell types growing in soft agar. *Columns,* means of three independent experiments; *bars,* SE. **C**, each of the indicated cell types were separately seeded in chambers with Matrigel-coated membranes, followed by staining of the cells migrating through membranes. Histogram, average cell counts of multiple, independent fields having stained human breast cancer cells in two independent experiments. **D**, histogram of human breast cancer cell – derived tumor weights (in mg) in SCID mice. *Points,* average of tumor weights from two independent experiments; *bars,* SE.

when compared with the parental or the vector subline 1 cells (Fig. 1D). The data in Fig. 1 suggests that expression of CARP-1 alters the biological properties of human breast cancer cells. Taken together with an inverse correlation of CARP-1 levels with the breast cancer grades (Table 1), these data indicate a potential tumor suppressor property for CARP-1.

TAT-Tagged CARP-1 Peptides Transduce Human Breast Cancer Cells

Our recent mutagenesis analyses revealed that CARP-1 possesses multiple, nonoverlapping apoptosis-inducing subdomains. CARP-1 amino acids 1–198 harbor one such apoptosis-inducing subdomain, and regulate apoptosis signaling by EGFRs *in vitro* (15). Because EGFR signaling pathways are often deregulated in breast cancer, we hypothesized that apoptosis-promoting peptide(s) of CARP-1 will suppress human breast cancer growth, and could be potential anticancer agents alone or in conjunction with currently used therapies. To test this hypothesis, we first generated various recombinant plasmids for the expression of eGFP and CARP-1 peptides in *E. coli*, and subsequently affinity-purified these proteins. Because CARP-1 is a cytoplasmic/perinuclear protein, each of the peptides were generated as a fusion of the TAT domain positioned at its NH_2-terminal. The fusion peptides bearing TAT protein transduction domain domains are known to translocate across the plasma membrane, and have been useful in treating preclinical models of cancer and cerebral ischemia (11, 12). On this basis, we specifically generated His-TAT-HA–tagged eGFP as well as CARP-1 peptides from positions 1–198, 197–454, and 896–1150 of the wild-type CARP-1 protein (Fig. 2A), as detailed in Materials and Methods. Additional CARP-1 peptides having substitutions of tyrosines at positions 192 and/or 40 [His-TAT-HA-CARP-1 ($1–198^{Y192/F}$), His-TAT-HA-CARP-1 ($1–198^{Y40/F, Y192/F}$)] were also generated and subsequently purified (Fig. 2A). The purified peptides were then analyzed on SDS-PAGE, followed by Coomassie staining of the gels. His-TAT-HA-eGFP, CARP-1 (1–198), and CARP-1 ($1–198^{Y192/F}$) proteins displayed high levels of purity with minimal background proteins (Fig. 2B). However, in the case of His-TAT-HA-CARP-1 (197–454) and (896–1150) peptides, significant quantities of smaller-sized peptides copurified with each preparation (Fig. 2B). Subsequent Western immunoblotting using anti–HA-tag antibodies (data not shown) confirmed the identity of each of the purified peptides (*arrow* in Fig. 2B). Interestingly, the anti–HA-tag antibody also stained multiple, smaller-sized peptides present in the preparations of His-TAT-HA-CARP-1 (197–454) and His-TAT-HA-eGFP proteins, suggesting that these peptides are likely derivatives of these proteins. The reasons for the presence of smaller derivatives of His-TAT-HA-eGFP or His-TAT-HA-CARP-1 (197–454) proteins, and their biochemical properties as well as biological function remain to be determined.

Next, parental MDA-MB-468 human breast cancer cells were incubated with various TAT-tagged affinity-purified, denatured peptides to show their transport across the plasma membrane. Human breast cancer cells were separately incubated with their respective peptides, and their intracellular presence was determined by immunocytochemical labeling using anti–HA-tag antibodies. Consistent with the previous studies showing the transport of various TAT fusion proteins across the plasma membrane, our data revealed the translocation of TAT-tagged eGFP or CARP-1 (1–198) proteins across the plasma membrane, resulting in their predominant localization in the cytoplasmic/perinuclear region (Fig. 2C). Incubation of human breast cancer cells with TAT-tagged CARP-1 (197–454), (896–1150), ($1–198^{Y192/F}$), or ($1–198^{Y40/F, Y192/F}$) also resulted in the translocation of these proteins across the plasma membrane with their predominant presence in the cytoplasmic/perinuclear region (data not shown).

TAT-Tagged CARP-1 Peptides Inhibit Human Breast Cancer Cell Growth, In Part, by Inducing Apoptosis

Because CARP-1–mediated inhibition of cell growth involves the induction of apoptosis (13, 15), we further investigated whether and to what extent TAT-tagged CARP-1 peptides suppress human breast cancer cell growth and the mechanisms involved in this process. Human breast cancer cells were separately treated with each of the peptides, and the number of live/viable cells were determined in the first instance. TAT-tagged CARP-1 ($1–198^{Y192/F}$)–treated cells displayed a marginal, statistically insignificant, loss of their viability when compared with TAT-tagged eGFP-treated human breast cancer cells (Fig. 3A). Treatments with TAT-tagged CARP-1 peptides 1–198, 197–454, or 896–1150, on the other hand, resulted in significant loss of cell viability when compared with their TAT-tagged eGFP-treated counterparts (Fig. 3A). Inhibition of human breast cancer cell growth by TAT-tagged CARP-1 peptides 1–198, 197–454, or 896–1150 was due, in part, to enhanced apoptosis, when compared with the apoptosis noted in the cells treated with either TAT-tagged eGFP or CARP-1 ($1–198^{Y192/F}$) proteins (Fig. 3B). In addition, Western immunoblot analysis revealed enhanced activation (phosphorylation) of p38 SAPK (Fig. 3C) and activation of caspase-9 (Fig. 3D) in cells that were treated with TAT-tagged CARP-1 peptides 1–198, 197–454, or 896–1150 when compared with their untreated counterparts. Moreover, treatments with TAT-tagged CARP-1 ($1–198^{Y192/F}$) or eGFP failed to induce p38 and caspase-9 activation (Fig. 3C and D). The data in Fig. 3 strongly suggests that TAT-tagged peptides that have nonoverlapping CARP-1 sequences, with the exception of CARP-1 ($1–198^{Y192/F}$), inhibit human breast cancer cell viability, cause activation of p38 SAPK and caspase-9, and induce apoptosis. These observations are consistent with our previous findings showing cell growth inhibition by retrovirally encoded CARP-1 mutants, whereas the retroviral expression of CARP-1 ($1–198^{Y192/F}$) failed to inhibit cell growth. Together, these data corroborate our hypothesis that CARP-1 harbors distinct apoptosis-promoting subdomains, and that Tyr^{192} regulates apoptosis induction by CARP-1 (15).

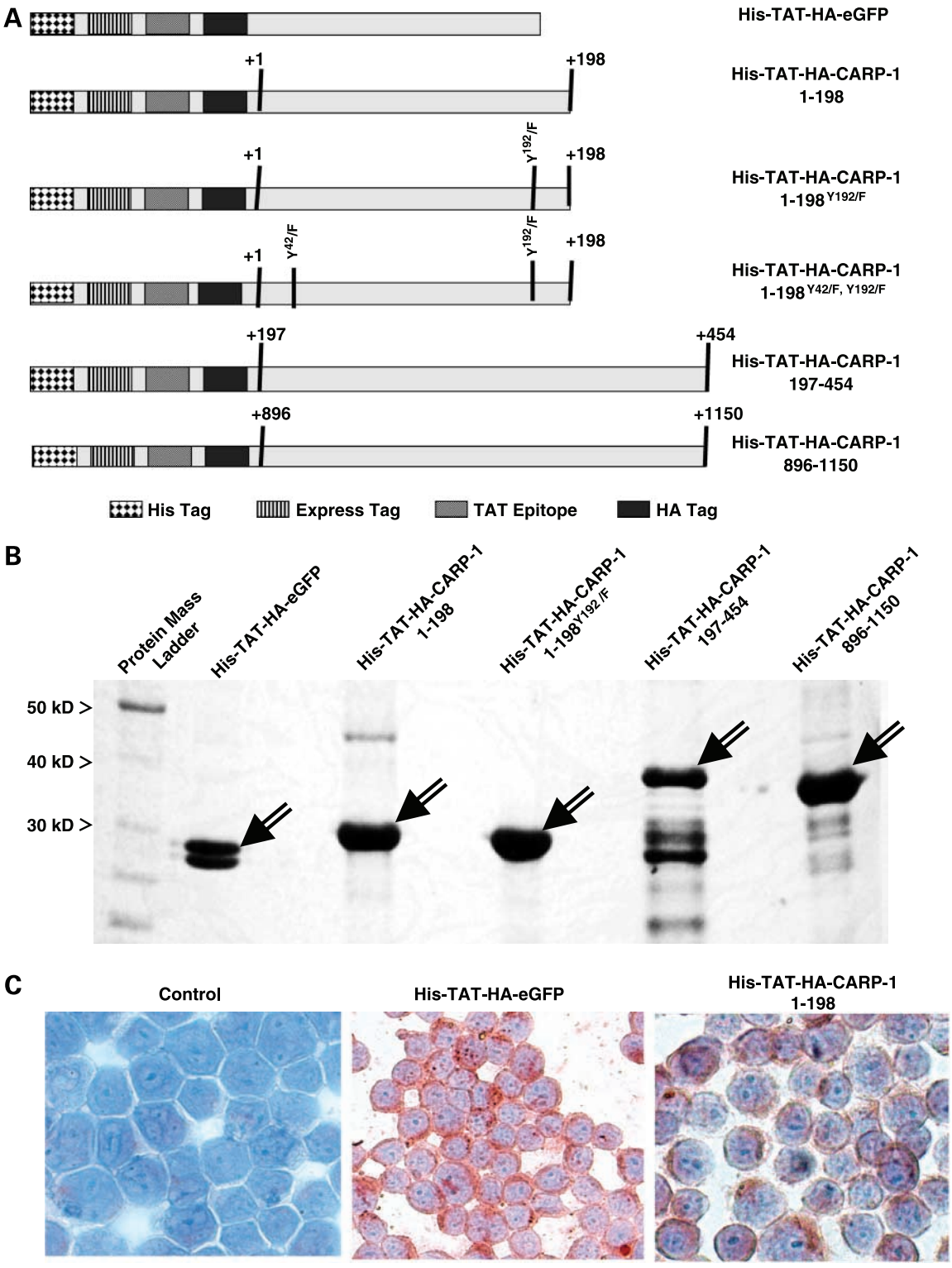

Figure 2. Human breast cancer cell transduction by TAT-tagged proteins. **A**, diagrammatic map of eGFP and various CARP-1 peptides (patent pending) having noted epitopes positioned at their amino termini. *Above the respective columns,* CARP-1 amino acid numbers; *right,* construct names. **B**, Coomassie blue–stained SDS-polyacrylamide gel having affinity-purified TAT-tagged peptides. *Arrows,* purified protein/peptide; *left,* molecular mass (in kDa) of the select protein ladder. **C**, human breast cancer cells were incubated with 50 μg/mL of affinity-purified respective peptides for a period of 24 h. Cells were stained with anti–HA-tag antibodies as in Materials and Methods. *Dark brown,* presence of the transduced peptides in the cytoplasmic/perinuclear region. The vehicle-treated viable cells were spun down and immunostained similarly to their treated counterparts (*Control*).

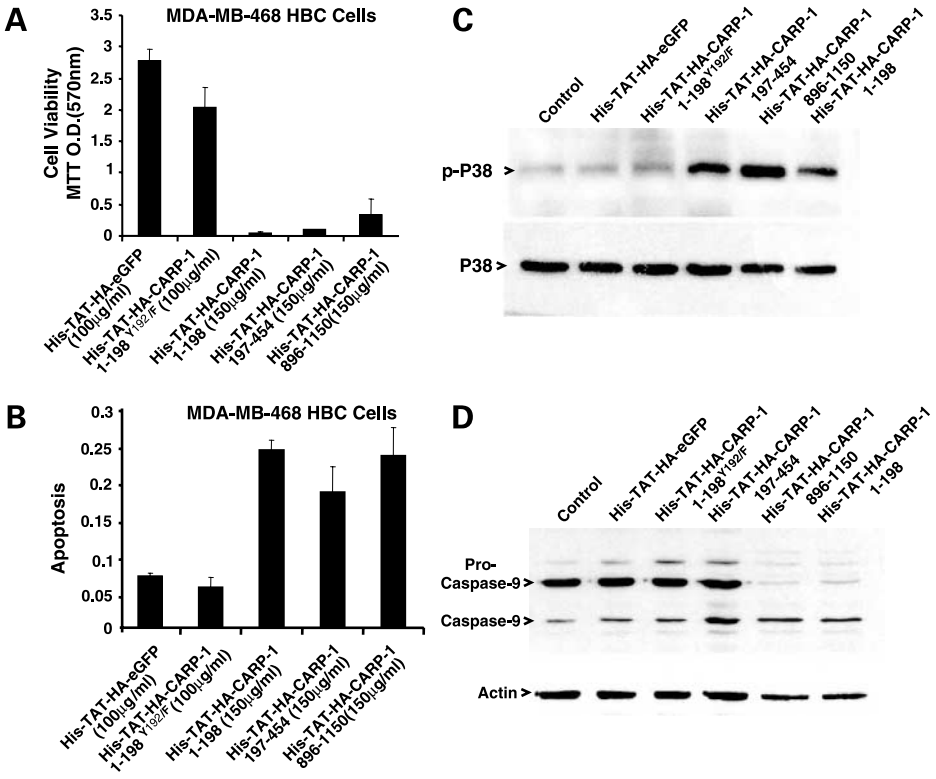

Figure 3. Cell growth inhibition and apoptosis induction by CARP-1 peptides involves the activation of p38α/β SAPK and caspase-9. Human breast cancer cells were incubated with the noted doses of each of the indicated peptide for 48 h. Cell lysates were prepared and used for determination of cell viability (**A**) or apoptosis (**B**) by conducting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or ELISA-based DNA fragmentation assays, respectively, as in Materials and Methods. *Columns,* means of three independent experiments; *bars,* SE. **C** and **D,** protein lysates from each sample were analyzed on SDS-PAGE followed by immunoblotting with anti-p38 and caspase-9 antibodies, respectively, as detailed in Materials and Methods. Presence of phospho-p38 (*p-P38*), p38, pro-caspase-9, and caspase-9 proteins (*left*). As a control for sample loading, the membrane was reprobed with anti-actin antibodies (*Caspase-9*).

His-TAT-HA-CARP-1 (1–198) Inhibits the Growth of Human Breast Cancer Cell–Derived Xenografts in SCID Mice

Our studies thus far suggest a growth-suppressor property for CARP-1, and prompted us to further hypothesize that affinity-purified TAT-tagged CARP-1 peptides will inhibit the growth of xenografted human breast cancer cells in a preclinical mouse model. To test this possibility, we conducted efficacy trials by intratumoral administration of TAT-tagged eGFP, CARP-1 (1–198), or CARP-1 ($1–198^{Y192/F}$) proteins. Groups of SCID mice bearing human breast cancer cell–derived tumor xenografts were administered with purified His-TAT-HA-eGFP, His-TAT-HA-CARP-1 (1–198), or His-TAT-HA-CARP-1 ($1–198^{Y192/F}$) proteins essentially following procedures detailed in Materials and Methods. We found that administration of His-TAT-HA-CARP-1 (1–198) caused a significant (~40%) reduction in tumor burden when compared with the tumor weights of His-TAT-HA-eGFP or His-TAT-HA-CARP-1 ($1–198^{Y192/F}$)–treated animals (Fig. 4). Immunohistochemical analyses of the xenografted tumor biopsies from the untreated as well as treated animals revealed that although the animals administered with His-TAT-HA-CARP-1 (1–198), His-TAT-HA-eGFP or His-TAT-HA-CARP-1 ($1–198^{Y192/F}$) had similar levels of the respective proteins, only those treated with His-TAT-HA-CARP-1 (1–198) had elevated levels of phosphorylated p38 mitogen-activated protein kinase and apoptosis (Fig. 5). Consistent with our observations in the *in vitro* model (15), the data in Figs. 4 and 5 suggest that human breast cancer growth suppression by CARP-1 (1–198) protein, *in vivo*, involves the activation of p38 SAPK and apoptosis, whereas substitution of Tyr^{192} to phenylalanine interferes with its function.

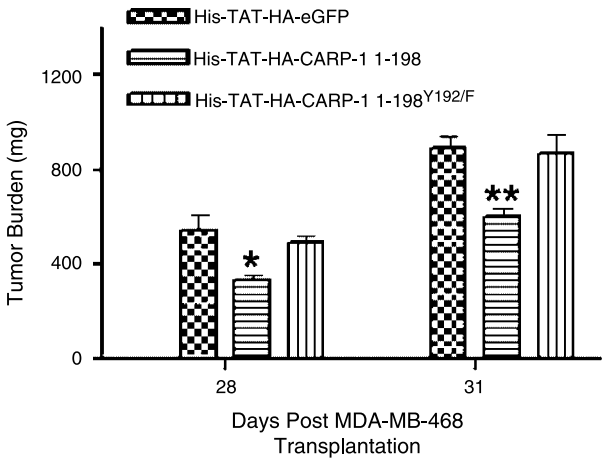

Figure 4. His-TAT-HA-CARP-1 (1–198) protein inhibits the growth of human breast cancer cell–derived tumor xenografts in SCID mice. The efficacy studies were carried out as in Materials and Methods, data analyzed essentially as described previously (20, 21). *Columns,* mean tumor weight; *bars,* SD; *, $P = 0.0015$; **, $P = 0.003$ compared with the corresponding His-TAT-HA-eGFP–treated tumors.

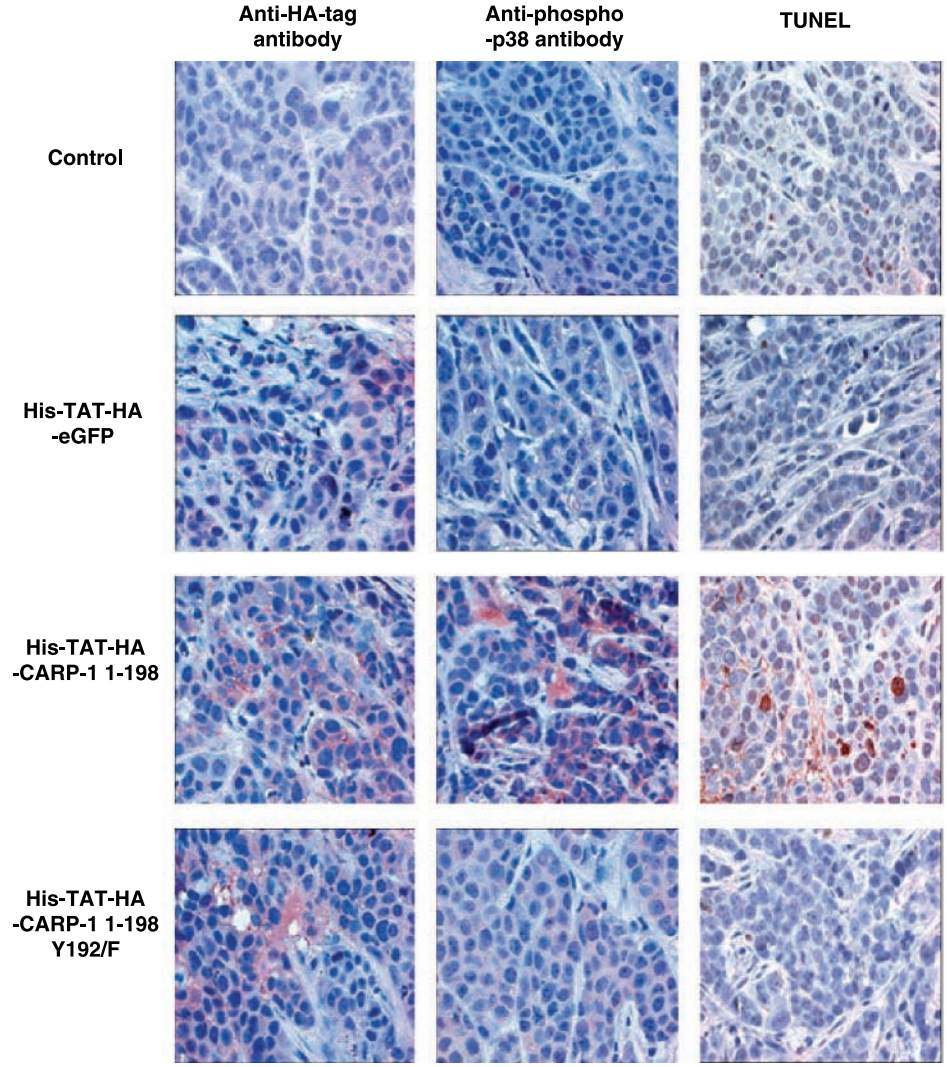

Figure 5. Treatments of human breast cancer cell – derived tumor xenografts with His-TAT-HA-CARP-1 (1 – 198) results in p38 mitogen-activated protein kinase activation and apoptosis. The mice were either untreated (*Control*) or treated with different affinity-purified peptides (*left*) as described in the legend to Fig. 4. The formalin-fixed tumor xenograft biopsies were paraffin-embedded, processed, and subsequently subjected to immunohistochemical staining as detailed in Materials and Methods. Representative photomicrograph showing intratumoral presence (*brown*) of respective peptides is indicated by staining with anti-HA-Tag antibodies (*left*). Representative photomicrographs are also presented showing apoptosis in xenografted tumors following their staining with anti – phospho-p38 antibodies (*middle*), or using terminal deoxynucleotidyl transferase – mediated nick end labeling assay (*right*).

Decreased Expression of CARP-1 in Breast Cancer is Associated with Enhanced Methylation of CARP-1 Promoter

Our immunohistochemical analyses of human breast cancer arrays suggested decreased levels of CARP-1 protein in the high-grade, poorly differentiated tumors (Table 1). We speculated that diminished levels of CARP-1 in poorly differentiated breast cancers could be due to the repression of CARP-1 transcription. This speculation is based, in part, on the database search[6] that suggested the presence of a potential methylation island (CpG sequences) in the promoter of the human CARP-1 gene that is located on chromosome 10 (accession no. AL513534; data not shown). Because aberrant methylation is thought to transcriptionally silence expression of several tumor suppressors, and is suspected to begin early in tumor progression (25, 26), we determined whether hypermethylation of the CARP-1 promoter also occurs in breast cancers. Methylation-specific PCR amplification of a genomic DNA subfragment that is contained within the predicted methylation islands was carried out as detailed in Materials and Methods using the primers as depicted in Fig. 6A, and the 147 bp methylated DNA PCR product was sequenced to confirm with CARP-1 promoter amplification. CARP-1 promoter was hypermethylated in four out of five poorly differentiated breast cancers when compared with the benign tissues as reflected by the intensities of the amplified 147 bp product (Fig. 6B). Subsequent immunohistochemical staining revealed reduced levels of CARP-1 in a breast cancer specimen when compared with its levels in the benign, well-differentiated specimen (Fig. 6C), suggesting the involvement of promoter methylation in regulating CARP-1 expression in breast cancer.

[6] http://www.uscnorris.com/cpgislands2/cpg.aspx

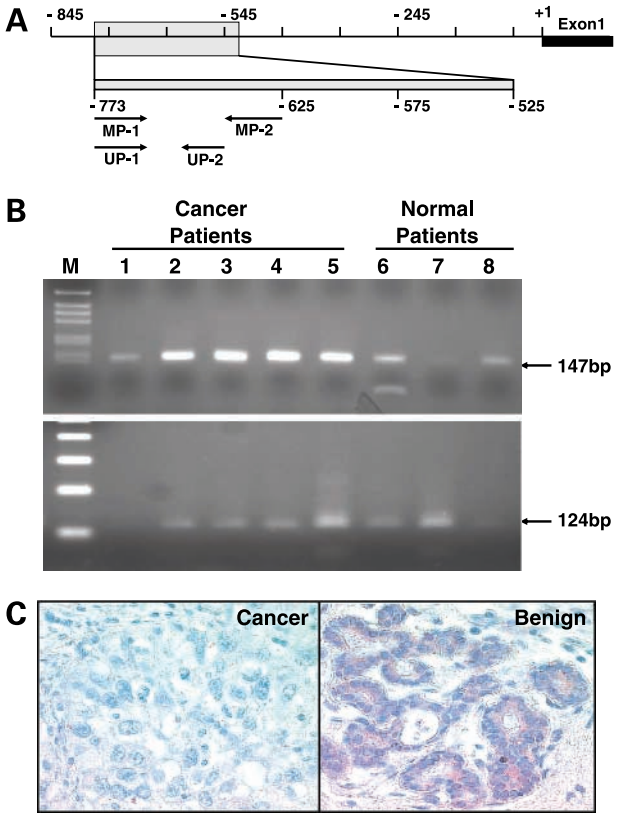

Figure 6. Methylation-specific PCR amplification of human CARP-1 promoter. **A**, diagrammatic overview of the methylation-specific PCR primers and the CARP-1 gene region targeted for amplification. *+1,* putative transcription start site in the untranslated exon upstream of CARP-1 ATG in Genbank accession no. AL513534. *MP* and *UP,* methylated DNA primer and unmethylated DNA primers, respectively. **B**, ethidium bromide–stained agarose gels of methylated (*top*) and unmethylated (*bottom*) DNA fragments (*arrows, right*) that was PCR-amplified from bisulfite-treated genomic DNAs as described in Materials and Methods. *M,* 1 kb (*top*) and 1 kb plus (*bottom*) DNA size ladder. **C,** paraffin-embedded tissue from specimen in (*A, lanes 2* and *8*) were stained for the presence of CARP-1 using anti–CARP-1 (α1) polyclonal antibodies as detailed in Materials and Methods. Representative photomicrographs showing the presence of CARP-1 (*dark brown*) are presented.

Discussion

EGFR family of receptor tyrosine kinases are overexpressed in 60% of breast cancers and often correlate with estrogen receptor negativity (27). These factors are associated with the late stage and aggressive forms of breast cancers. Therefore, interference with EGFRs activation, and/or blockage of downstream signaling mediators by pharmacologic or biochemical means continues to be an attractive therapeutic strategy.

CARP-1 is a recently identified perinuclear/cytoplasmic regulator of apoptosis signaling by a variety of pathways including EGFRs. Deprivation of serum growth factors stimulates CARP-1 expression, whereas loss/depletion of CARP-1 results in resistance to apoptosis by EGFR inhibitors (13, 15). In this study, we tested our hypothesis that CARP-1 is a potential tumor suppressor, and that its expression would inhibit breast cancer growth. Indeed, diminished expression of CARP-1 was noted in poorly differentiated breast cancers when compared with their moderately differentiated counterparts as well as benign breast tissues. Moreover, stable overexpression of CARP-1 in human breast cancer cells interferes with their ability to form tumors when xenografted in SCID mice. Because tumor suppressor molecules are either mutated or repressed/inactivated in a variety of cancers, including breast carcinoma, the observation of an inverse correlation between breast cancer grades and CARP-1 expression and its inhibition of human breast cancer cell–derived tumors suggests a potential tumor suppressor role for CARP-1. This is consistent with the notion that loss of one and/or multiple tumor suppressors is associated with aggressive cancers.

CARP-1 possesses multiple, nonoverlapping apoptosis-promoting domains, and retroviral transduction of wild-type CARP-1 or proteins harboring its apoptosis-inducing subdomains results in growth inhibition of human breast cancer cells that involves the induction of apoptosis (15). These findings, together with our current observation showing the reduced growth of xenografts of human breast cancer cells that expressed elevated levels of CARP-1, further suggests that expression CARP-1 protein or its mutant(s) harboring apoptosis-inducing subdomain(s) are effective inhibitors of breast cancer with potential therapeutic value. In a proof-of-principle approach, we first generated different, affinity-purified CARP-1 peptides and used them to investigate human breast cancer cell growth inhibition *in vitro* and *in vivo*. Incubation of human breast cancer cells with different, denatured, TAT-bearing nonoverlapping CARP-1 peptides, as well as eGFP, resulted in their accumulation in the cytoplasm. Furthermore, all the CARP-1 peptides, with the exception of eGFP and CARP-1 ($1–198^{Y192/F}$), caused the inhibition of cell proliferation and induction of apoptosis, suggesting that these peptides are able to regain their renatured conformations, and consequently, their functions following entry into the cells. On the other hand, the cells that were transduced with eGFP, whereas showing no alterations in their viability or apoptotic variables, displayed green fluorescence (data not shown). These findings not only show that denatured CARP-1 peptides bearing distinct apoptosis-inducing subdomains, like their retrovirally transduced counterparts (15), are sufficient in causing human breast cancer cell growth inhibition, but also underscore their abilities to activate intrinsic apoptosis signaling. Our current studies have further revealed that CARP-1 (1–198) and CARP-1 (896–1150) peptides are also effective in inhibiting the growth of diffuse large B cell lymphomas *in vitro* as well as *in vivo*.[7] Human breast cancer growth inhibition by TAT-CARP-1 (1–198) peptide is unlikely to be a stress response

[7] Manuscript in preparation.

because the presence of eGFP or CARP-1 ($1–198^{Y192/F}$) failed to activate p38 mitogen-activated protein kinase/SAPK and caspase-9. The anti–breast cancer properties of TAT-CARP-1 (1–198) involve the activation of intrinsic apoptosis, which is further supported by the fact that although eGFP, CARP-1 (1–198), or CARP-1 ($1–198^{Y192/F}$) proteins were present in the xenografted tumors, only CARP-1 (1–198) caused a significant inhibition of growth as well as elevated phosphorylation of p38 mitogen-activated protein kinase/SAPK and apoptosis.

That TAT-CARP-1 peptide–induced apoptosis was further examined by conducting immunologic staining of TAT-CARP-1 (1–198)–transduced human breast cancer cells as well as TAT-CARP-1 (1–198)–treated xenografted tumor biopsies. Immunocytochemical labeling was carried out to detect TAT-CARP-1 (1–198) peptide using anti-HA or anti-His tag antibodies, and apoptosis by terminal deoxynucleotidyl transferase–mediated nick end labeling or activated caspase-3 levels. Both methods independently showed apoptosis. However, staining with anti-His or hemagglutinin antibodies showed weak/diffused signals (data not shown). Although the precise reason for this is unclear, it is likely that activation of caspase-3 leads to cleavage of HA-tag from CARP-1 peptides resulting in the loss of hemagglutinin immunoreactivity. This inference is consistent with a recent report showing the loss of hemagglutinin immunoreactivity in the HeLa and Jurkat cells undergoing apoptosis (28). It was shown that HA-tag of SNAP-25 fusion protein was cleaved by activated caspase-3/7, and this phenomenon resulted in a total loss of hemagglutinin immunoreactivity in the cells undergoing apoptosis (28). Because CARP-1 peptides bear a cassette of His-TAT-HA epitopes at their NH_2 termini (Fig. 2A), it is likely that caspase-3–dependent cleavage of HA-tag results in the degradation/loss of these epitopes. Nevertheless, our ELISA-based DNA fragmentation assay data (Fig. 3B) clearly show apoptosis by TAT-tagged CARP-1 peptides.

The cellular uptake of denatured TAT-fusion proteins is thought to involve lipid raft–dependent macropinocytosis, a nonselective form of endocytosis (11, 29). Although the injected peptides were predominantly present in the xenografted human breast cancer cells, it remains to be determined whether these proteins also accumulate inside and inhibit various host tissue cells. Although the detailed toxicology studies remain to be carried out, our ongoing studies have thus far revealed that SCID mice are able to tolerate His-TAT-HA-CARP-1 (1–198) doses of up to 100 mg/kg when administered by tail vein injection without any visible signs of toxicity, weight loss, or diarrhea (data not shown). Together with the immunohistologic analyses of the breast cancer and benign tissue biopsies, as well as various mouse tissues (data not shown), showing the abundant presence of CARP-1 in well-differentiated regions, these observations indicate that the His-TAT-HA-CARP-1 (1–198) protein is likely well tolerated by SCID mice, and the xenografted human breast cancer cells are the targets of its growth-inhibitory effects.

Diminished expression of CARP-1 in poorly differentiated breast cancers involved methylation-dependent silencing of the CARP-1 gene (Fig. 6). One of the major roles of DNA methylation in mammals is thought to be in the control of gene regulation. This is because methylation within promoters and/or enhancers of genes, particularly tumor suppressors or oncogenes, results in the altering of their expression and consequent function. Methylation-induced suppression is thought to occur either by the blocking of transcription factor binding and/or by formation of an inactive chromatin state. However, it is still unclear whether methylation directly elicits gene inactivation or is a consequence of gene silencing (30). Because the CARP-1 promoter has a predicted methylation island (CpG sequences) that is hypermethylated in breast cancers (Fig. 6A), reduction in CARP-1 protein in the poorly differentiated breast cancers may, in part, be due to decreased transcription of CARP-1. The predicted methylation island of CARP-1 promoter encompasses predicted consensus sites for transcription factors such as E2F and c-Rel. The extent of binding of these and other transcription factors to the cognate consensus elements in the CARP-1 promoter is modulated by methylation, and contributes to silencing of CARP-1 transcription in poorly differentiated breast cancers, remains to be determined.

In summary, this report shows that CARP-1 expression is repressed in breast cancers. Although loss of CARP-1 is, in part, due to methylation-dependent transcriptional silencing, expression of CARP-1 or its apoptosis-promoting peptides causes the inhibition of human breast cancer cell growth both *in vitro* and *in vivo*. His-TAT-HA-CARP-1 (1–198) protein possesses anti–breast cancer properties, and Tyr^{192} regulates tumor suppressor function of CARP-1 *in vivo*.

References

1. Reed JC, Doctor KS, Godzik A. The domains of apoptosis: a genomics perspective. Sci STKE 2004;239:1 – 29.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100: 57 – 70.
3. Ozbun MA, Butel JS. Tumor suppressor p53 mutations and breast cancer: a critical analysis. Adv Cancer Res 1995;66:71 – 141.
4. Brenner AJ, Paladugu A, Wang H, et al. Preferential loss of expression of p16(INK4a) rather than p19(ARF) in breast cancer. Clin Cancer Res 1996;2:1993 – 8.
5. Gillett C, Fantl V, Smith R, et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994;54:1812 – 7.
6. Slamon DJ, Godolphin W, Jones LA, et al. Studies of HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244: 707 – 12.
7. Escot C, Theillet C, Lidereau R, et al. Genetic alteration of c-myc protooncogene (MYC) in human primary breast carcinomas. Proc Natl Acad Sci U S A 1996;83:4834 – 8.
8. Clark GJ, Der CJ. Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res Treat 1995;35: 133 – 44.
9. Johnstone RW, Ruefli AA, Lowe SW. A link between cancer genetics and chemotherapy. Cell 2002;108:153 – 64.
10. Vives E, Brodin P, Lebleu B. A truncated HIV Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 1997;272:16010 – 7.

11. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 2004;10:310 – 5.

12. Snyder EL, Saenz CC, Denicourt C, et al. Enhanced targeting and killing of tumor cells expressing the CXC chemokine receptor 4 by transducible anticancer peptides. Cancer Res 2005;65:10646 – 50.

13. Rishi AK, Zhang L, Boyanapalli M, et al. Identification and characterization of a cell-cycle and apoptosis regulatory protein (CARP)-1 as a novel mediator of apoptosis signaling by retinoid CD437. J Biol Chem 2003; 278:33422 – 35.

14. Xu H, Yu Y, Marciniak DJ, et al. EGFR-related protein (ERRP) inhibits multiple members of EGFR family in colon and breast cancer cells. Mol Cancer Ther 2005;4:435 – 42.

15. Rishi AK, Zhang L, Yu Y, et al. Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor. J Biol Chem 2006;281:13188 – 98.

16. Wadia JS, Dowdy SF. Modulation of cellular function by TAT mediated transduction of full length proteins. Curr Protein Pept Sci 2003;4:97 – 104.

17. Ausubel FM, Brent R, Kingston RE, et al., editors. Current protocols in molecular biology. vol. 2. 1990.

18. Nagothu KK, Rishi AK, Jaszewski R, et al. Folic acid mediated inhibition of serum-induced activation of EGFR promoter in colon cancer cells. Am J Physiol Gastrointest Liver Physiol 2004;287:G541 – 6.

19. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821 – 6.

20. Mohammad RM, Al-Katib A, Pettit GR, Sensenbrenner LL. Successful treatment of human Waldenstrom's macroglobulinemia with combination biological and chemotherapy agents. Cancer Res 1994;54: 165 – 8.

21. Mohammad RM, Aboukameel A, Nabha S, et al. Rituximab, cyclophosphamide, dexamethasone (RCD) regimen induces cure in WSU-WM xenograft model and a partial remission in previously treated Waldenstrom's macroglobulinemia patient. J Drug Target 2002;10: 405 – 10.

22. Keen JC, Davidson NE. The biology of breast carcinoma. Cancer 2003;97:825 – 33.

23. Rusch V, Mendelsohn J, Dmitrovsky E. The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors. Cytokine Growth Factor Rev 1996;7:133 – 41.

24. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. A study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 1957;11:359 – 77.

25. Lin SY, Yeh KT, Chen WT, et al. Promoter CpG methylation of tumor suppressor genes in colorectal cancer and its relationship to clinical features. Oncol Rep 2004;11:341 – 8.

26. Baylin SB, Esteller M, Rountree MR, et al. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687 – 92.

27. Harris JR, Lippman ME, Veronesi U, Willett W. Breast cancer (3). N Engl J Med 1992;327:473 – 80.

28. Schembri L, Dalibart R, Tomasello F, et al. The HA tag is cleaved and loses immunoreactivity during apoptosis. Nat Methods 2007;4:107 – 8.

29. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature 2003;422:37 – 44.

30. Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002;21:5380 – 7.