CD437, a novel retinoid, causes cell cycle arrest and apoptosis in a number of cancer cells including human breast carcinoma (HBC) by utilizing an undefined retinoic acid receptor/retinoid X receptor-independent mechanism. To delineate mediators of CD437 signaling, we utilized a random antisense-dependent functional knockout genetic approach. We identified a cDNA that encodes ~130-kDa HBC cell perinuclear protein (termed CARP-1). Treatments with CD437 or chemotherapeutic agent adriamycin, as well as serum deprivation of HBC cells, stimulate CARP-1 expression. Reduced levels of CARP-1 result in inhibition of apoptosis by CD437 or adriamycin, whereas increased expression of CARP-1 causes elevated levels of cyclin-dependent kinase inhibitor p21\(^{WAF1/CIP1}\) and apoptosis. CARP-1 interacts with 14-3-3 protein as well as causes reduced expression of cell cycle regulatory genes including c-Myc and cyclin B1. Loss of c-Myc sensitizes cells to apoptosis by CARP-1, whereas expression of c-Myc or 14-3-3 inhibits CARP-1-dependent apoptosis. Thus, apoptosis induction by CARP-1 involves sequestration of 14-3-3 and CARP-1-mediated altered expression of multiple cell cycle regulatory genes. Identification of CARP-1 as a key mediator of signaling by CD437 or adriamycin allows for delineation of pathways that, in turn, may prove beneficial for design and targeting of novel antitumor agents.

The natural and synthetic derivatives of vitamin A, collectively known as retinoids, are involved in many biological processes including development, cellular proliferation, and differentiation (1, 2). Retinoids exert their actions through modulation of gene expression (3). Ligand-bound receptors heterodimerize and bind to specific DNA consensus sequences called RAR\(^3\) elements or RXR elements located in the regulatory regions to modulate ligand-dependent transcription of the target gene (3). Unfortunately, the therapeutic anti-cancer efficacy of both natural and synthetic retinoids, which function through this classical pathway, has thus far been limited to the treatment of acute promyelocytic leukemia, CTCL, AIDS-related Kaposi’s sarcoma, and premalignant processes (4, 5).

We recently described the abilities of a novel retinoid [3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) to induce growth arrest and apoptosis in a wide variety of malignant cell types, including breast, prostate cancer, and leukemia by an RAR/RXR-independent mechanism (6–8). In addition, [3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid has been reported to inhibit the growth of human cancer xenografts in nude mice (9). The mechanism(s) by which CD437 triggers cell cycle arrest and apoptosis is as yet not completely defined. On the basis of our research, CD437 was found to enhance expression of the cyclin-dependent kinase inhibitor (CDKI) p21\(^{WAF1/CIP1}\) and DNA damage-inducible GADD45 genes (10, 11) through a unique mechanism in human breast carcinoma (HBC) cells. We also found that CD437 potently induced c-Jun N-terminal kinase/stress-activated protein kinase and that induction occurred upstream of caspase activation and apoptosis (12). Inactivation of c-Jun, a downstream effector of activated c-Jun N-terminal kinase/stress-activated protein kinase, was also found to impair CD437-dependent apoptosis but not the expression of the CDKI p21\(^{WAF1/CIP1}\) in A549 lung cancer cells (13).

The roles of apoptosis mediators have been ascertained by expressing dominant negative peptides directed to specific cellular proteins involved in apoptotic signaling cascades. This powerful approach allows us to inactivate a known mediator, but not novel mediators that may either be key signaling molecules or co-operate with known mediators. Given that CD437 utilizes unique pathways for growth arrest and apoptosis, we hypothesized that novel mediators of CD437-dependent growth arrest and apoptosis existed. We then utilized a functional knockout genetic approach by overexpressing antisense cDNAs (14) to obtain novel molecules involved in CD437-dependent apoptotic signal cascades. The antisense knockout approach involves random inactivation of genes via a cDNA library cloned in an antisense orientation in an expression vector plasmid (14, 15). This approach is based on the premise that transcription PCR; FBs, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; oligos, oligonucleotides; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGFR, epidermal growth factor receptor; PARP, poly(ADP-ribose) polymerase; CDKI, cyclin-dependent kinase inhibitor; CMV, cytomegalovirus.
cific inactivation or knockout of a growth-inhibitory and/or apoptosis-promoting gene would result in a growth advantage to the transfected cells in a specific restrictive environment. This growth advantage constitutes a powerful forward selection that is helpful in isolating the desired inactivation event from random inactivation. Although nascent protein synthesis may be unnecessary for CD437-dependent apoptosis in certain cell types (such as leukemia), stable overexpression of a particular antisense RNA should over time deplete the target protein, thereby causing a partial or complete block in the CD437-dependent apoptotic signal. This methodology allows, in principle, the identification of important genes, the products of which may be modulated at the transcriptional, post-transcriptional, translational, or post-translational level during the CD437-dependent cell cycle arrest or apoptosis. Thus, if a set of novel mediators of growth arrest and/or apoptosis is activated in the presence of CD437, their specific inactivation via overexpression of a transfected antisense cDNA library should lead to cellular resistance to CD437 apoptosis.

The functional knockout genetic approach yielded several CD437-resistant MDA-MB-468 HBC sublines. Plasmids expressing antisense cDNAs were rescued, and one of the plasmids (AS-6) harbored a cDNA insert that belongs to a novel ~130-kDa protein (referred to as cell cycle and apoptosis regulatory protein (CARP)-1). CARP-1 expression in HBC cells is regulated by serum as well as by agents such as CD437 and adriamycin. This report details the role(s) of CARP-1 in regulating cell cycle and apoptosis pathways.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, Ham’s F-12 medium, and fetal bovine serum (FBS) were purchased from Invitrogen. The reagent kit for probe labeling using [32P]dATP (3000 Ci/mmol) was purchased from DuPont, and fetal bovine serum (FBS) were purchased from Invitrogen. Gentamycin, neomycin (G418), and hygromycin were purchased from Myer Squibb Co., adriamycin from Sigma, and cisplatin from Ameri-...
phoresed on 9–12% SDS-polyacrylamide gels essentially as described before (10, 18, 22). The filters containing protein extracts were independently probed with indicated antibodies per the manufacturer’s guidelines and the protocols described before (10).

**Flow Cytometric Analysis**—Flow cytometric analysis of DNA content was performed to assess the cell cycle distribution as described previously (23). Normally growing parental wild type, the vector plasmid-transfected, c-Myc antisense-transfected sublines, or clone 1.6-transfected HBC sublines were independently stained for DNA content using propidium iodide. After staining, cells were analyzed on a BD Biosciences FACScan cytometer (San Jose, CA). The data were analyzed using the multicycle program from Phoenix Flow Systems (San Diego, CA).

**Gene Regulation by CARP-1**—To study the downstream targets of CARP-1, Atlas Human 1.2 array membranes (Clontech) with ~1200 genes were utilized in conjuction with radiolabeled total RNAs from vector pcDNA3/Hygro subline 1 or clone 1.6 subline 9. Total RNAs (3.0 µg) from each subline were separately labeled with [32P]dATP and hybridized with the array membranes essentially following the manufacturer’s guidelines. The membranes were washed and autoradiographed for 2 weeks, and the hybridization signals were quantitated utilizing the manufacturer’s suggested program.

**Retroviral Expression of CARP-1**—Retroviral gene transfer and expression system (Clontech, Palo Alto, CA) was used to generate retroviruses expressing CARP-1. First, the entire CARP-1-myc-His-tagged cDNA insert of plasmid pLNCX2 (1,554 bases) was excised with BamHI and ligated into BgIII and StuI cut retroviral vector plasmid pLNCX2 to obtain plasmid clone 16.1. Next, the plasmid clone 16.1 or pLAPSIN (retroviral vector expressing alkaline phosphatase) were independently transfected into the RetroPack PT67 packaging cells (Clontech), followed by selection of PT67 sublines in the presence of neomycin (500 µg/ml). Multiple, independent sublines or pooled populations expressing either myc-His-tagged CARP-1 or alkaline phosphatase were obtained. The expression of myc-His-tagged CARP-1 in each subline was determined by Western immunoblot using anti-His (C-terminal) monoclonal antibody (Invitrogen). Two independent PT-67 sublines expressing the highest levels of myc-His-1 CARP-1 (lines 16.4 and 16.6) and one subline expressing alkaline phosphatase were selected for determination of viral titers essentially per the suggested protocols.

**Immunolocalization of CARP-1**—CARP-1 antibodies c1 and c2 were generated (Sigma Genosys, The Woodlands, TX) by immunizing rabbits with keyhole limpet hemocyanin-conjugated peptides containing epitopes RERERRERR (amino acids 320–328) and EDDKEEERIKRQEE (amino acids 698–712), respectively, of the putative sequences deduced from the CARP-1 cDNA. Localization of CARP-1 in parental HBC cells or HBC subline expressing CARP-1-myc-His fusion protein was studied by immunocytochemical analyses (22) by utilizing α anti-CARP-1 (1:2000 dilution) polyclonal antibody or anti-His-tag (27E8) monoclonal antibody (Cell Signaling, Beverly, MA), respectively. In addition, α anti-CARP-1 polyclonal antibody (1:1000 dilution) in conjunction with mouse monoclonal antibody for proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA) was utilized to study expression of CARP-1 and PCNA proteins in a human breast cancer bioppy specimen. Dual labeling of CARP-1 and PCNA was carried out as above followed by confocal microscopic analysis to determine expression of CARP-1 and PCNA proteins. The antibody-stained sections were then photographed under different magnifications utilizing Zeiss microscope with attached 35-mm camera for recording the photomicrographs.

**RESULTS**

**Isolation of CARP-1 cDNA by Functional Gene Knockout**—CD437 is a novel retinoid that utilizes a retinoid receptor-independent mechanism(s) to elicit anti-proliferative and apoptotic effects in the HBC cells (6–8). We hypothesized that novel mediators of CD437 signaling exist, and their specific inactivation via overexpression of the transfected antisense cDNA library should lead to resistance to apoptosis by CD437. To accomplish inactivation of rare proteins and those encoded by low abundance mRNAs, poly(A)+ RNAs were used as template for generating a cDNA library such that mRNA species that are present at ~20 copies per cell are included in the library (14). RNAs were prepared from MDA-MB-468 HBC cells treated with CD437 over a range of time points to allow cloning of molecules involved in early, middle, and late signaling events triggered by CD437. CD437 at a concentration of 1 μM was found previously (6, 10) to be optimal in eliciting cell cycle arrest as well as apoptosis. After cDNA library was transfected into HBC cells, stable sublines were selected in the combined presence of hygromycin and CD437 as described (11). Of the several HBC colonies isolated and propagated to obtain plasmid DNAs, one harbored the recombinant plasmid AS-6 having an ~0.15-kb insert (positions 1760–1900; Fig. 1). A composite restriction map was created from the human and mouse partial cDNAs in the GenBank™ that were homologous
to the insert of the AS-6 plasmid, followed by cloning of the full-length cDNA that encodes a novel 130-kDa protein CARP-1 (Fig. 1).

A protein data base search yielded very high homology scores for two putative motifs in the CARP-1 peptide. A sequence of 31 amino acids (from positions 637 to 667) showed 88% alignment with a putative DNA-binding (bihelical) motif predicted to be involved in chromosomal organization. A 41-amino acid sequence (positions 151–191) showed 76% alignment with a putative cold-shock protein domain that function as an RNA-binding domain. The partial mouse cDNA-encoded peptides also contain DNA-binding (bihelical) and cold-shock protein domains that are identical to those present in the human CARP-1 protein. The plasmid expressing myc-His-tagged wild-type CARP-1 having putative cold-shock and DNA-binding motifs was then generated as detailed under “Experimental Procedures” (clone 6.1, Fig. 1).

Furthermore, the CARP-1 5′-untranslated region subfragment having part of the protein encoding ORF was RT-PCR-amplified using oligos AS-6.23 and AS-6.25 (Table I) and total RNA from MDA-MB-468 HBC cells. The PCR product was subcloned into pBSK vector plasmid, and multiple independent recombinant plasmids were sequenced to confirm the presence of a translation termination codon located 67 nucleotides upstream and in-frame of the CARP-1 ATG (not shown). Thus, CARP-1 peptide encoded by the plasmid clone 6.1 (see Fig. 1) possesses the complete N terminus of the CARP-1 protein.

HBC Cells Expressing Reduced CARP-1 Show Increased Cell Viability and Reduced Apoptosis When Treated with CD437 or Adriamycin—Whether CARP-1 plays a role in apoptosis signaling in the presence of CD437 or other chemotherapeutic drugs was investigated next. The parental MDA-MB-468 HBC cells were treated with different drugs over a range of times, and their viabilities were assessed by MTT assays. Cell viabilities of 20% or less were observed following 72 h of treatment with either adriamycin, etoposide, or cisplatin, while <20% cell viability was observed after 24 h of treatment with taxol (not shown). The reduction in viability of HBC cells treated with adriamycin, etoposide, or cisplatin was due to apoptosis as evidenced by fragmented nuclei following acridine orange staining (not shown).

We found that 1 μM CD437 induced apoptosis in HBC cells within 48–72 h. Whether CARP-1 played a role in CD437-dependent apoptosis was investigated in four independent sublines transfected with vector plasmid pCDNA3/Hygro or 15 independent sublines transfected with clone 1.6. Each subline was separately treated with 1 μM CD437 for 72 h. CD437 treatment of vector-transfected sublines resulted in >80% loss of cell viability, whereas several clone 1.6 transfectants only showed a 20–30% loss (Fig. 2A). The variability in viability of CD437-treated clone 1.6 transfectants could be due to the varying copy number or nuclear integration site(s) of the plasmid

1.6 that, in turn, influenced the levels of antisense RNA and subsequently CARP-1 protein. The resistance to apoptosis by CD437 observed in multiple, independent sublines transfected with clone 1.6 suggested a role for antisense-mediated reduced levels of CARP-1 in this process.

The CD437-resistant HBC sublines transfected with clone 1.6 (see Fig. 2A) were then utilized to investigate whether expression of CARP-1 antisense contributed to chemotherapeutic drug resistance. Independent sublines transfected with either vector (sublines 1–3) or clone 1.6 (sublines 8–11 and 13; see Fig. 2A) were treated with taxol for 24 h or adriamycin, etoposide, or cisplatin for 72 h. Treatment with cisplatin or taxol resulted in reduced viability of the treated sublines transfected either with the vector or clone 1.6 (not shown). However, all clone 1.6-transfected sublines had significantly increased viability when compared with vector alone transfectants after treatment with etoposide or adriamycin (Fig. 2B).

Whether differences in the loss of viabilities of vector or clone 1.6-transfected sublines treated with either CD437 or adriamycin was a result of apoptosis was ascertained by utilizing cell death detection ELISA kit (Roche Applied Science) as described under “Experimental Procedures.” Treatment with CD437 or adriamycin resulted in the levels of apoptosis in the wild-type or vector subline 1 cells that were approximately three times that observed for the clone 1.6-transfected subline 9 or 10 (Fig. 3). Thus, the data suggest that increased viabilities of CD437- or adriamycin-treated clone 1.6 sublines 9 or 10 (Fig. 2) reflect alterations in the cell numbers due to reduced apoptosis.

Expression of CARP-1 Antisense Results in Reduced Levels of CARP-1 Protein—Western immunoblot analysis of clone 1.6 expressing HBC sublines 8–11 and 13 (see Fig. 2A) using α1 anti-CARP-1 polyclonal antibodies showed ~50% reduced levels of CARP-1 when compared with the vector plasmid-transfected HBC subline (Fig. 4, A and B). Moreover, clone 1.6 sublines 1 and 4 that displayed reduced viabilities following treatment with CD437 (see Fig. 2A) did not have reduced CARP-1 when compared with clone 1.6 sublines 9 and 10 (Fig. 4, C and D). Taken together, the data in Figs. 2–4 suggest that reduced expression of CARP-1 contributes to increased cell viability as well as lower apoptosis of HBC cells further implicating CARP-1 in apoptosis signaling pathways utilized by CD437, adriamycin, or etoposide but not by taxol or cisplatin. The chemotherapeutic agents adriamycin, etoposide, or cisplatin are known to cause DNA damage leading to apoptosis in HBC cells. Because CARP-1 contains a predicted motif for DNA (bihelical) binding, it remains to be determined whether CARP-1 binding to DNA induced by CD437, adriamycin, or etoposide leads to DNA damage followed by apoptosis.

CD437, Adriamycin, or Serum Regulate CARP-1 Expression in HBC Cells—Next we studied whether growth arrest and/or apoptosis signaling pathways target CARP-1 expression. HBC cells were treated with either 1 μM CD437 or 34 μM adriamycin

### Table I

| Oligo ID | Oligo sequence (5’ to 3’) | Position (relative to ATG) |
|---------|--------------------------|---------------------------|
| AS-6.1  | GCGGGATCCGGCATCAGGTCGAATTTG | Sense; −5 to +14 |
| AS-6.8  | TATGCGTCTTAAATTGAATG | Sense; 583 to 602 |
| AS-6.9  | CATTCCATTTAAACAGGCAAT | Sense; 1349 to 1368 |
| AS-6.10 | GCTATGCGTCCGCTTATGTA | Antisense; 583 to 602 |
| AS-6.11 | TACTAGGGCTAGCCATCAGC | Antisense; 1349 to 1368 |
| AS-6.12 | GAAAGTGAGCTCTAGTTC | Sense; 1944 to 1963 |
| AS-6.13 | GAACTAAGAGCTCGACTTTC | Antisense; 1944 to 1963 |
| AS-6.14 | CAAAAGGATATCATGACAGAT | Sense; 2684 to 2703 |
| AS-6.15 | ATCTGGTGATATCTCTTTTG | Antisense; 2684 to 2703 |
| AS-6.16 | GGCCTGCTAGTACCTGACCATCCTCTCTT | Antisense; 3429 to 3449 |
| AS-6.23 | CGACTTCTTTAAAAACACGCTGCTCTTGAGGC | Antisense; 427 to 449 |
| AS-6.25 | CTCGGATCCGCTGACGGTGTTGAGG | Sense; −97 to −78 |
for various times followed by Western immunoblotting for CARP-1 expression utilizing 1 anti-CARP-1 polyclonal antibody. Treatment with CD437 (Fig. 5A) or adriamycin (Fig. 5B) resulted in a time-dependent increase in CARP-1 expression. Although both the agents caused increased expression of CARP-1, the existence of specific and significant differences in

**Fig. 2.** HBC sublines expressing CARP-1 antisense show increased viability following treatment with CD437 or chemotherapy drugs adriamycin and etoposide. A, HBC sublines transfected either with vector plasmid pcDNA3/Hygro (columns 1–4) or plasmid clone 1.6 (columns 1–15) were either not treated (−) or treated with 1 μM CD437 for 72 h (+). B, HBC sublines transfected with vector plasmid pcDNA3/Hygro (columns 1–3) or plasmid clone 1.6 (columns 8–11 and 13) were either untreated (−) or treated independently with indicated doses of chemotherapeutic agents (+). Cell viability was determined as described under “Experimental Procedures.” The columns represent means of three independent experiments; bars, S.E.
the signaling events induced by CD437 or adriamycin is supported by the fact that CD437 resulted in increased expression of CARP-1 as early as 6 h, while adriamycin treatment resulted in elevated CARP-1 levels at 12 h or later time points (Fig. 5). To further elucidate mechanism(s) of CARP-1 regulation, CARP-1 expression was investigated in parental MDA-MB-468 HBC cells that were either cultured in the media lacking FBS or in FBS-supplemented media following serum starvation as noted under “Experimental Procedures.” Serum deprivation resulted in elevated levels of CARP-1, whereas addition of...
serum resulted in a time-dependent decrease in CARP-1 expression (Fig. 5C). The data in Fig. 5 demonstrate that CARP-1 expression is regulated by growth factors as well as agents like CD437 and adriamycin. Because CD437 treatment of HBC cells results in G1/G0 cell cycle arrest (6), the increased levels of CARP-1 either in the presence of CD437 or in the absence of serum may support a role for CARP-1 in the progression of the cell cycle.

CARP-1 Is a Perinuclear Protein and Is Expressed in Different Epithelial Cancer Cell Types—To study CARP-1 localization, HBC cells were transfected with plasmid clone 6.1, followed by selection of multiple, independent sublines expressing myc-His-tagged CARP-1 (Fig. 6A). Immunocytochemical localization of CARP-1 was investigated as described under “Experimental Procedures.” As shown in Fig. 6B, CARP-1 is present in the perinuclear compartment. Perinuclear localization of CARP-1 was further corroborated by confocal microscopic analysis of a breast cancer biopsy specimen following dual labeling utilizing antibodies for CARP-1 (α1 antibody) and PCNA. Expression of PCNA (labeled with green fluorescent marker) is exclusive to the nuclear compartment, whereas sporadic expression of CARP-1 (labeled with red fluorescent marker) is noted in the surrounding perinuclear regions of the cells (Fig. 6B). In the absence of yellow color (observed where the red and green colors overlap) fluorescence in the confocal micrograph in either the nuclear or perinuclear compartments together with the data from immunocytochemical analyses strongly suggest that CARP-1 is a perinuclear protein. In addition, CARP-1 is expressed ubiquitously in various cancer cell lines of human breast, colon, and prostate and pancreatic and leukemia origins as indicated by RT-PCR amplification of CARP-1 and ribosomal phosphoprotein 36B4 (24) cDNAs, as well as Western immunoblots (not shown).

CARP-1 Regulates Expression of Key Cell Cycle Regulatory Proteins—To elucidate the CARP-1 role in signaling pathways regulating cellular growth and/or apoptosis, we investigated the CARP-1 target genes by gene array technology as detailed under “Experimental Procedures.” A subset of genes showing alterations in their expression in clone 1.6 subline 9 when compared with their expression in the vector pcDNA3/Hygro subline 1 are listed in Table II. Because clone 1.6-transfected subline 9 expresses 50% reduced levels of CARP-1 (see Fig. 4), it is noteworthy that several genes associated with cell cycle progression and/or cell proliferation such as c-myc, cyclin B1, DNA topoisomerase IIα, p21WAF1/CIP1, and histone deacetylase (HDAC) 3C were significantly up-regulated (Table II). On the other hand, the array data did not show altered expression of genes such as proliferating cell nuclear antigen (PCNA) and stress-activated protein kinase 4 (p38α) in clone 1.6-transfected subline 9 (not shown). Together, the data suggest that CARP-1 targets expression of specific genes including those that are regulators of cell cycle progression and/or cell proliferation.

Whether reduced levels of CARP-1 result in altered expression of the proteins corresponding to the above noted genes was studied by Western immunoblotting. Furthermore, because CD437 treatment induces CARP-1 expression in HBC cells (Fig. 5), we hypothesized that CD437 treatment will also alter expression of above CARP-1 target genes in HBC cells. Expression of oncogene c-myc as well as CDKI p21WAF1/CIP1 proteins was analyzed in HBC sublines expressing reduced CARP-1 (Fig. 4) as well as CD437-treated or untreated parental MDA-MB-468 HBC cells. Indeed, reduced expression of CARP-1 results in increased levels of c-Myc (Fig. 7A) and reduced levels of CDKI p21WAF1/CIP1 (Fig. 7C) in multiple independent sublines reduced CARP-1. CD437 treatment of parental MDA-MB-468 HBC cells, on the other hand, resulted in reduced expression of c-Myc (Fig. 7B) and elevated expression of p21WAF1/CIP1 protein (Fig. 7D). CD437 treatment has been shown to cause elevated expression of p21WAF1/CIP1 mRNA in HBC cells (10), and together with the data in Fig. 7, C and D, strongly suggest that CARP-1 targets p21WAF1/CIP1 expression in the presence of CD437. This conclusion is further supported by the observation where clone 1.6 sublines expressing reduced CARP-1 did not elicit enhanced p21WAF1/CIP1 expression following treatment with CD437. CD437 treatment, on the other hand, resulted in increased levels of p21WAF1/CIP1 in clone 1.6 sublines that did not express reduced CARP-1 (Fig. 7E). Thus, CD437-dependent signaling involves increased expression of CARP-1 that, in turn, down-regulates c-Myc and up-regulates CDKI p21WAF1/CIP1 proteins leading to cell cycle arrest. This property of CARP-1 may then serve as one of the contributing factor(s) in inducing apoptosis in the presence of CD437.

In addition to regulation of c-Myc and p21WAF1/CIP1 by CARP-1, expression of cyclin B1, p21Rac1, HDAC3, and DNA topoisomerase IIα in multiple HBC sublines expressing clone 1.6 was investigated by Western immunoblotting. As expected, reduced levels of CARP-1 results in increased expression of cyclin B1, p21Rac1, HDAC3, and DNA topoisomerase IIα (Fig. 7F), suggesting that CARP-1 not only targets c-Myc and p21WAF1/CIP1 expression, but it also interferes with expression of other genes involved in the pathways regulating cell proliferation and/or cell cycle progression. Because chemotherapeuti-
tic agents such as adriamycin and etoposide are known to target DNA topoisomerases, the increased expression of DNA topoisomerase IIα in clone 1.6-expressing sublines may also be a contributing factor in their resistance to apoptosis in the presence of etoposide or adriamycin.

Because depletion of CARP-1 results in elevated expression of genes that are positive regulators of cell cycle and or cell growth, we wished to determine whether there were alterations in any of the phases of the cell cycle in the case of the sublines expressing reduced levels of CARP-1 that may consequently have resulted in their increased viabilities and reduced apoptosis in the presence of etoposide or adriamycin.

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Expression of CARP-1 Alters Cellular Levels of CARP-1 Target Genes and Induces Apoptosis—Because decreased levels of CARP-1 resulted in reduced expression of CDKI p21\textsuperscript{WAF1/CIP1}, and elevated levels of c-Myc and DNA topoisomerase IIα, we investigated whether overexpression of CARP-1 will result in altered levels of the above genes and induction of apoptosis. To this end, we utilized retroviral expression system to generate CARP-1-expressing retroviruses. Interestingly, multiple transfection attempts yielded a significantly lower number of sublines expressing retroviruses encoding CARP-1-myrc-His fusion protein when compared with transfections with alkaline phosphatase encoding the retroviral vector (not shown). Among a total of 11 PT-67 sublines, CARP-1 expression was noted to be highest in 3 sublines (lines 16.4, 16.5, and 16.6; Fig. 8A). PT-67 sublines 16.4, 16.6 expressing retroviruses encoding CARP-1-myrc-His fusion protein, and PT-67 sublines 1 and 2 expressing retroviruses encoding alkaline phosphatase were selected to determine viral titers per manufacturer’s suggested protocols. Both the PT-67 sublines 1 and 2 yielded viral titers of \(10^4\) colony-forming units/ml, whereas both the PT-67 sublines 16.4 and 16.6 yielded viral titers of \(10^5\) colony-forming units/ml. It is likely that the lower titers of the viruses derived from PT-67 sublines 16.4 and 16.6 were because of apoptosis caused by overexpression of CARP-1 following viral transduction of the target cells (see below). Together with the observation that transfections of retroviral vector encoding CARP-1-myrc-His fu-
Novel Regulator of Cell Cycle and Apoptosis

**TABLE II**

| Gene name                          | Accession No. | -Fold change |
|------------------------------------|---------------|-------------|
| c-Myc oncogene                     | V00568        | 1.8 ↑       |
| c-Myc-hind binding protein MM-1    | D89667        | 2.7 ↓       |
| γ-Interferon-inducible protein; IP 30| J02909        | 3.3 ↓       |
| G2/mitotic-specific cyclin B1      | L125753       | 4.2 ↑       |
| Cell division protein kinase 5 (CDK5) | X66364        | 2.5 ↑       |
| Insulin-like growth factor binding protein 2 (IGFBP2) | M35410 | 2.0 ↑ |
| Protein kinase DYRK4                | Y09305        | 4.5 ↑       |
| Cell cycle protein p38–2G4 homolog; HG4–1 | U59435 | 2.6 ↑ |
| c-Jun N-terminal kinase 2 (JNK2)   | L31951        | 3.2 ↑       |
| Cyclin-dependent kinase regulatory subunit 1 (CKS1) | X54941 | 2.6 ↑ |
| Shb proto-oncogene                  | X75342        | 2.4 ↑       |
| G1 to S phase transition protein 1 homolog | X17644 | 3.45 ↑ |
| Active breakpoint cluster related protein | U01147 | 3.8 ↑ |
| CDC7-related kinase                | AF015592      | 3.4 ↑       |
| DNA topoisomerase IIα              | J04088        | 10 ↑        |
| ADP/ATP carrier protein            | J02683        | 2.55 ↑      |
| Jun activation domain binding protein | U65928       | 2.4 ↑       |
| Dual specificity mitogen-activated kinase 2 (MAPKK2)-ERK activity | L11285 | 2.6 ↑ |
| Protein Phosphatase 2C γ           | Y13936        | 2.22 ↑      |
| α2-Macroglobulin receptor-associated protein precursor | M63395 | 3.0 ↑ |
| B-cell receptor-associated protein (hBAP) | U72511 | 2.57 ↑ |
| Nuclear tyrosine phosphatase PBL-1 | U48926        | 2.1 ↑       |
| STAT-induced STAT inhibitor 3      | AB004904      | 2.4 ↑       |
| Ras-related C3 botulin toxin substrate 1p21 rac1 | M29870; M31467 | 2.5 ↑ |
| DNA ligase 1                       | M36067        | 2.35 ↑      |
| Tumor necrosis factor type 1 receptor associated protein (TRAP1) | U12595 | 2.44 ↑ |
| Sentrin: ubiquitin-like protein SMITC | U36117       | 2.72 ↑      |
| Ornithine decarboxylase            | X16277        | 3.66 ↑      |
| Fas-activated sarine/threonine (FAST)* kinase | X86779 | 2.3 ↑ |
| Nonhistone chromosomal protein HMG17 | M12623       | 4.5 ↑       |
| DNA cytosine-5-methyltransferase   | X63692        | 3.3 ↑       |
| Histone deacetylase 3C             | AF005482      | 2.56 ↑      |
| TNF-α-converting enzyme            | U69111        | 2.37 ↑      |
| Thymidylylate synthase (TS)        | X02308        | 2.0 ↑       |
| Purine nucleoside phosphorylase (PNP) | X09737       | 2.9 ↑       |
| Vimentin                           | X56134        | 9.0 ↑       |
| L-Lactate dehydrogenase H subunit  | Y09177        | 14.6 ↑      |
| Ribonucleoside-diphosphate reductase M2 subunit | X59618 | 2.16 ↑ |
| Inosine-5′-monophosphate dehydrogenase 2 | L33842 | 2.65 ↑ |
| Cytokeratin 2E                     | L199061; S43646 | 5.0 ↓ |
| Elongation factor 1α (EF1 α)       | M27364        | 3.6 ↓       |

Suggestion protein resulted in isolation of greatly reduced numbers of sublines when compared with their alkaline phosphatase-transfected counterparts would suggest that CARP-1 expression beyond a threshold is detrimental to cell survival.

Next, parental MDA-MB-468 HBC cells were cultured in the presence of 5 ml of supernatants derived from either parental non-transfected PT-67 cells, PT-67 pLAPSN subline 1, PT-67 subline 16.4, or subline 16.6 for a period of 72 h, followed by determination of apoptosis. Transduction of HBC cells with retroviruses expressing CARP-1, myc-His fusion protein resulted in apoptosis 4–5 times higher than those transduced with supernatants derived from either untransfected PT-67 cells or PT-67 pLAPSN subline 1 (Fig. 5B). That transduction of HBC cells with retroviruses from PT-67 sublines 16.4 or 16.6 indeed resulted in apoptosis of HBC cells was corroborated by the presence of reduced levels of procaspase 3 noted in Western immunoblots of the respective lysates (Fig. 8C). The data in Fig. 8D also underscore the fact that altered expression of the genes noted in HBC sublines expressing reduced CARP-1 (Fig. 7) was independent of the effects of position and/or site(s) of integration of plasmid clone 1.6.

**CARP-1 Interacts with 14-3-3 Protein**—To further define mechanisms of CARP-1-dependent apoptosis, we utilized HBC cells as well as PT-67 cells expressing myc-His-tagged CARP-1 (Figs. 6 and 8) in conjunction with immunoprecipitation experiments. Because CARP-1 is a perinuclear protein, and in light of the fact that CARP-1 overexpression triggers apoptosis, we hypothesized that CARP-1 interaction with protein(s) located at the plasma membrane or in the perinuclear compartment plays an important role in apoptosis induction. HBC cell lysates were incubated with either anti-epidermal growth factor receptor (EGFR) antibody or anti-14-3-3 antibody, followed by determination of apoptosis. CARP-1 presence is noted in the lanes containing immunoprecipitates derived from 14-3-3 antibody, whereas anti-EGFR antibodies failed to immunoprecipitate CARP-1.

**Depletion of c-Myc Sensitizes Cells to Apoptosis by CARP-1, whereas Expression of c-Myc or 14-3-3 Interferes with CARP-1 Apoptosis**—Whether c-Myc or 14-3-3 proteins play roles in CARP-1-mediated apoptosis was investigated as follows. Independent HBC sublines expressing −50% reduced levels of c-Myc (Fig. 10, A and B) were generated by transfecting c-Myc antisense cDNA as described under “Experimental Proce-
As noted in Fig. 8, treatment of wild-type HBC cells with retroviruses expressing myc-His-tagged CARP-1 resulted in apoptosis over a period of 72 h. Transduction of wild-type or the vector-transfected HBC subline with retroviruses expressing myc-His-tagged CARP-1 over a period of 24 h resulted in marginally elevated apoptosis when compared with apoptosis noted in cells transduced with retroviruses expressing alkaline phosphatase (Fig. 10C). On the other hand, transduction of sublines harboring reduced c-Myc with retroviruses expressing myc-His-tagged CARP-1 protein over a period of 24 h resulted in apoptosis 2.5-fold higher than that noted in cells transduced with retroviruses expressing alkaline phosphatase (Fig. 10C). Thus, data in Fig. 10 underscore the fact that c-Myc and 14-3-3 proteins play important roles in CARP-1-dependent apoptosis.

DISCUSSION

CD437 is a novel, RARγ-selective adamantyl retinoid that causes cell cycle arrest and apoptosis in a number of cell types. CD437 displays poor binding and trans-activation of RARβ and RARα, and no binding to RXXRs (25). CD437 also causes apoptosis of human leukemia cell line HL-60R that does not express any functional RARs (7), indicating that CD437 functions independently of direct interactions with retinoid receptors. Although CD437-mediated growth arrest and apoptosis has been
shown to involve elevated expression of CDKI p21WAF1/CIP1, GADD45, activation of caspases, and release of mitochondrial cytochrome c (6–13), the mechanism(s) involved remain to be fully delineated. We undertook a functional gene knockout approach to further elucidate signaling pathway(s) utilized by CD437. This approach has been used previously (14, 15, 19) to identify genes involved in apoptosis signaling in the presence of interferons and/or retinoic acid. By transfecting episomal plasmids containing antisense cDNAs derived from the cDNA library generated from untreated as well as CD437-treated HBC cells, we obtained multiple independent sublines that exhibited resistance to the combined presence of hygromycin (an antibiotic resistance marker expressed by the vector plasmid) and CD437 (not shown). One subline harbored a recombinant plasmid, termed AS-6, which contained an insert that belongs to an mRNA of ~3.8 kb size. This mRNA encodes a novel ~130-kDa protein termed CARP-1. CARP-1 is an important target of apoptosis signaling induced by agents like CD437 or adriamycin. CARP-1 interacts with cytoplasmic 14-3-3 protein(s) and also plays a role in cell cycle progression. Increased expression of CARP-1 by CD437 results in sequestration of 14-3-3 protein(s) and altered expression of many key cell cycle regulatory proteins that together lead to cell growth inhibition and apoptosis.

CARP-1 serves as a key mediator of apoptosis signaling as supported by data in Fig. 8. Expression of CARP-1, following transduction of HBC cells with retroviruses expressing CARP-1, results in apoptosis and reduced levels of procaspase 3. It is possible that expression of CARP-1 beyond a threshold induces apoptosis, since transfection of plasmids expressing CARP-1 under the control of CMV immediate early promoter (such as plasmid clone 6.1 or 16.1) into HBC, HCT-116 colon cancer cells (not shown), and NIH 3T3-derived PT-67 cells also resulted in their apoptosis. Whether the HBC or the PT-67 sublines (Figs. 6 and 8) express CARP-1 below a threshold that allowed for successful selection and propagation of these sublines or CARP-1 overexpression resulted in their altered genotype that subsequently contributed toward their selection and propagation remains to be determined.

The precise mechanism(s) utilized by CARP-1 to induce apoptosis are yet to be clarified. Whether CARP-1 directly binds 14-3-3 is not clear. Nevertheless, CARP-1 interaction with cy-
toplamic 14-3-3 protein(s) (Fig. 9) suggests that CD437-de-
dependent elevated levels of CARP-1 sequester 14-3-3 protein
and, in turn, may result in inhibition of 14-3-3 binding with the
pro-apoptotic protein Bad. 14-3-3 proteins are a family of small,
evolutionarily conserved proteins that are found in all verte-
brates, invertebrates, plants, and fungi. 14-3-3 proteins display
wide ranging functions. Mammalian 14-3-3 proteins have been
classified as typical (α, β, γ, η, and τ) or atypical (α and ε)

Fig. 9. CARP-1 interacts with 14-3-3. Clone 6.1 HBC sublines 1 and 2 (Fig.
6A) as well as PT-67 subline 16.4 (Fig. 8A) cell lysates were utilized for immunopre-
cipitations (IP) using anti-14-3-3 monoclonal antibody (Neomarkers, Pleasanton,
CA) or anti-EGFR polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY)
as indicated. The indicated immunoprecipitates as well as cell lysates (100 μg)
were analyzed on 10% SDS-PAGE followed by immunoblotting (WB) with anti-CARP-1 (α2) polyclonal anti-
body. The approximate location and size of molecular weight markers is noted on the
right side, whereas location of CARP-1 is noted on the left side of the blot.

![Image of CARP-1 and 14-3-3 interactions](image_url)

Fig. 10. 14-3-3 and c-Myc proteins regulate apoptosis signaling by CARP-1. A, Western immunoblot showing c-Myc protein in indicated
HBC cells. Protein lysate (50 μg) from each subline was analyzed on 10% SDS-PAGE followed by immunoblotting with either anti-c-Myc or
anti-α-tubulin antibodies. B, histogram showing relative expression of c-Myc. The Western blot of A was quantitated by soft laser densitometry.
Columns represent c-Myc levels relative to c-Myc in lysate derived from the wild-type HBC cells, which was arbitrarily defined as 1. C, histogram
showing apoptosis in wild-type MDA-MB-468 HBC cells or their indicated sublines that were treated with noted viral supernatants for 24 h.
Apoptosis was determined as described under “Experimental Procedures.” Columns represent means of three independent experiments; bars, S.E.
D, histogram showing apoptosis in wild-type MDA-MB-468 HBC cells that were transfected with indicated plasmids followed by their treatment
with noted viral supernatants for 48 h. Apoptosis was determined as described under “Experimental Procedures.” Columns represent means of
three independent experiments; bars, S.E.

![Image of c-Myc and apoptosis](image_url)
isoforms based on their distribution and functions. 14-3-3 proteins are involved in signal transduction in the mitogen-activated protein kinase cascade (21, 26). The mitogenic signals activate AKT/PKB kinases that, in turn, phosphorylate the pro-apoptotic protein Bad. The 14-3-3 proteins then bind with phosphorylated Bad and prevent it from inhibiting BclXL, thereby inhibiting apoptosis and promoting cell proliferation. CARP-1 sequestration of 14-3-3s thus likely contributes to apoptosis signaling by promoting Bad inhibition of BclXL followed by release of cytochrome c from mitochondria and subsequent apoptosis. Thus, as summarized in Fig. 11, the data presented in this report suggest that CARP-1-dependent apoptosis utilizes multiple pathways including CARP-1 interaction with 14-3-3 and CARP-1-mediated altered levels of cell cycle regulators such as c-Myc, cyclin B, and CDKI p21WAF1/CIP1. Loss of c-Myc sensitizes cells to CARP-1-mediated apoptosis (Fig. 10C). Overexpression of c-Myc or 14-3-3 proteins, on the other hand, inhibits CARP-1-dependent apoptosis in HBC cells (Fig. 10D) as well as activation of caspase 3 in HBC and monkey kidney COS-7 cells (not shown).

Whether CARP-1 regulation of expression of the above noted cell cycle regulatory genes utilizes 14-3-3-dependent and/or -independent pathways remains to be delineated. CARP-1 regulation of CDKI p21WAF1/CIP1, nonetheless, underscores its growth inhibitory/anti-proliferative property (Figs. 7 and 8). Although reduction p21WAF1/CIP1 mRNA levels was not noted in the array experiment (Table II), it is likely that reduced levels of p21WAF1/CIP1 mRNA in clone L6 subline 9 resulted in signals that were below the threshold of detection set for densitometric quantitation of the autoradiographs. In light of the fact that CD437 regulates CDKI p21WAF1/CIP1 mRNA stability (10), whether CARP-1 regulates stability of p21WAF1/CIP1 mRNA utilizing its RNA-binding cold-shock motif or other novel domain(s) is not known. Alternatively, due to the existence of reciprocal regulation between c-Myc and p21WAF1/CIP1 (27), and given that c-Myc down-regulates p21WAF1/CIP1 (28), it has yet to be determined whether CARP-1 regulation of p21WAF1/CIP1 involves c-Myc. In the absence of direct interactions of CARP-1 with c-Myc or p21WAF1/CIP1, as demonstrated by co-immunoprecipitation and Western immunoblot experiments (not shown), and the fact that CARP-1 is present in the perinuclear region indicate that CARP-1 regulation of c-Myc and p21WAF1/CIP1 may utilize additional factor(s)/mediator(s). Moreover, that CARP-1 may utilize post-transcriptional and/or post-translational mechanisms to regulate levels of its target genes is supported by the fact that CARP-1-dependent alterations in levels of p21 Rac1 and HDAC3 mRNAs (Table II) were quite different when compared with the respective proteins in HBC sublines expressing reduced CARP-1 (Fig. 7F).

That CARP-1 is an important regulator of the cell cycle is supported by the observations that treatment of cells with either CD437 or adriamycin resulted in a time-dependent increase in expression of CARP-1 (Fig. 5). We have shown previously (6, 29) that exposure to CD437 causes G1/G0 and S phase cell cycle arrest in HBC and prostate cancer cells, respectively, followed by their apoptosis. Rapid induction of CARP-1 expression following treatment with CD437 may indicate a critical role for CARP-1 in signaling events regulating the cell cycle. Similar induction of CARP-1 expression was also noted in the case of cells treated with adriamycin, suggesting the existence of overlap among signaling pathways induced by CD437 and adriamycin. CARP-1 involvement in the regulating cell cycle was further highlighted by the experiment where supplementation of serum resulted in a time-dependent decreased expression of CARP-1 (Fig. 5). Whether CD437, adriamycin, or serum components (such as growth factors) regulate CARP-1 expression by transcriptional, post-transcriptional, or post-translational mechanisms remains to be determined.

Our report highlights, for the first time, that CD437 targets pathways converging on topoisomerase IIα (see Figs. 3, 5, 7, and 8). Because topoisomerases are known to regulate cellular DNA topology (30), it is likely that signaling induced by CD437 interferes with the balance between DNA supercoiling forces. In this context, it is interesting to note that HBC sublines expressing CARP-1 antisense do not have similar viability when treated with adriamycin and etoposide. Because both drugs target cellular topoisomerase IIα, the data shown in Fig. 2 suggest that CARP-1 regulation of signaling by adriamycin utilizes both topoisomerase IIα-dependent as well as -independent pathways. Furthermore, it is of note that CD437 treatment of HBC sublines with reduced CARP-1 did not elicit cellular viabilities equal to their non-treated counterparts (Fig. 2), and moreover, that none of the CARP-1 antisense-expressing sublines was totally resistant to CD437-induced apoptosis (Fig. 3) may point to the existence of additional significant signal transducers. This possibility may also be supported by the observation that CD437-dependent alteration in c-Myc levels in parental HBC cells (Fig. 7B) are quite different when compared with the altered levels of c-Myc noted in the HBC sublines expressing reduced CARP-1 (Fig. 7A). CARP-1 protein possesses putative DNA-binding (bihelical) and RNA-binding cold-shock motifs. A number of proteins including poly(ADP-ribose) polymerase (PARP), ATP-dependent DNA helicase II p70 subunit, apurinic endonuclease-redox protein, spliceosome-associated protein 145 (SAP 145), DNA repair protein RAD18, DEAd/HEa (Asp-Glu-Ala-(Asp/His)) box-binding protein 1, and heterogeneous nuclear ribonucleoprotein U possess a similar DNA-binding (bihelical) motif. Accumulating evidence suggests that PARP plays dual roles. In the presence of its substrate NAD and DNA strand breakage, PARP functions as a repair enzyme. In the absence of NAD, PARP promotes activator-dependent transcription by interacting with RNA polymerase II-associated factors (31). PARP also binds transcription enhancer factor 1 and transcription factor AP-2 to increase transcription of muscle-specific genes and AP-2-mediated transcription, respectively (32, 33). PARP has also been shown to up-regulate the E2F-1 gene promoter (34) further corroborating its role as a transcription factor. PARP-dependent silencing of transcription involves poly(ADP-ribosylation) of specific transcription factors, which prevents their binding to the respective DNA consensus sequences and formation of active transcription complexes (35). Whether CARP-1 functions as a DNA-binding transcription factor is unclear. Because CARP-1 is present in the perinuclear compartment (Fig. 6) and also regulates expression of topoisomerase IIα, its function(s) in regulating DNA topology are less likely to entail...
direct interactions with the bihelical nuclear DNA. However, given the perinuclear localization of CARP-1, it remains to be determined whether CARP-1 regulates the expression of gene(s) encoded by the bihelical DNAs of organelles such as mitochondria.

In addition, the proteins like Rho transcription termination factor, Y box factor homolog APY1, ribonuclease R, and exonuclease II proteins possess an RNA-binding cold-shock protein domain similar to that present in CARP-1. In this context, it is noteworthy that reduced expression of CARP-1 results in altered levels of several mRNAs (Table II). Thus, together with the data indicating perinuclear presence of CARP-1 in HBC (Fig. 6) as well as the megakaryocytic cells in the human bone marrow (not shown), it is likely that RNA-binding cold-shock protein domain of CARP-1 is involved in regulating cellular RNA levels. Thus, CARP-1 may regulate levels of target mRNAs using cis-trans interactions involving the RNA-binding cold-shock motif or other novel domain(s). Whether CARP-1 belongs to a family of proteins that bind DNA, RNA, and/or co-factor(s), and whether its interactions with nucleic acid sequences and/or co-factor(s) play a role in growth arrest and/or apoptosis signaling are subjects of our ongoing investigation.

The mechanism(s) by which CARP-1 protein is reduced by overexpression of antisense RNA (Fig. 4) is not clear. Nevertheless, depletion of CARP-1 resulted in interfering with growth arrest and apoptosis signaling and, thereby, allowed for selection of CD437-resistant sublines. Given that CARP-1 targets multiple cell cycle regulatory proteins (Fig. 7), the selection of sublines expressing reduced CARP-1 without significant alterations of chromosomal contents and/or cell cycle distribution may point to the adaptive abilities of the HBC cells expressing CARP-1 antisense. Whether increased expression of c-Myc and/or cyclin B1 in HBC sublines expressing reduced CARP-1 contributes to prolonged survival of cells in the presence of CD437 or adriamycin remains to be determined.

In conclusion, this study reports identification and characterization of a protein involved in signaling pathways utilized by the agents such as a novel retinoid CD437 and chemotherapeutic drug adriamycin. Expression of CARP-1 induces apoptosis, whereas expression of c-Myc or 14-3-3 interferes with CARP-1-mediated apoptosis. Whether CARP-1 functions as a tumor suppressor protein that regulates biological properties of the cancer cells is currently under investigation.

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