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SHP and Sin3A expression are essential for adamantyl-substituted retinoid-related molecule–mediated nuclear factor-κB activation, c-Fos/c-Jun expression, and cellular apoptosis

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Abstract

We previously found that the adamantyl-substituted retinoid-related molecules bind to the small heterodimer partner (SHP) as well as the Sin3A complex. In this report, we delineated the role of SHP and the Sin3A complex in 4-[3′-(1-adamantyl)-4′-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC)–mediated inhibition of cell growth and apoptosis. We examined the effect of loss of SHP and Sin3A expression in a number of cell types on 3-Cl-AHPC–mediated growth inhibition and apoptosis induction, 3-Cl-AHPC–mediated nuclear factor-κB (NF-κB) activation, and 3-Cl-AHPC–mediated increase in c-Fos and c-Jun expression. We found that loss of SHP or Sin3A expression, while blocking 3-Cl-AHPC–mediated apoptosis, had little effect on 3-Cl-AHPC inhibition of cellular proliferation. We have previously shown that 3-Cl-AHPC–mediated NF-κB activation is necessary for apoptosis induction. We have now shown that 3-Cl-AHPC–enhanced c-Fos and c-Jun expression is also essential for maximal 3-Cl-AHPC–mediated apoptosis. 3-Cl-AHPC induction of c-Fos and c-Jun expression as well as NF-κB activation was dependent on SHP protein levels. In turn, SHP levels are regulated by Sin3A because ablation of Sin3A resulted in a decrease in SHP expression. Thus, SHP and Sin3A play an important role in adamantyl-substituted retinoid-related induction of cellular apoptosis. [Mol Cancer Ther 2009;8(6):1625–35]

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Introduction

Apoptosis has been associated with the induction as well as the repression of gene expression. A major mechanism by which gene expression is modulated is through modification of the histone acetylation state. The extent of histone acetylation is primarily regulated through the recruitment of histone acetyltransferases with subsequent gene activation and histone deacetylases (HDAC) with the resultant gene inactivation (1). HDACs are often found associated with large multimolecular repressor complexes such as the Sin3A, the NURD/NED/M12, and the CoREST complexes (1). These are often targeted to specific DNA sequences by the binding of nuclear transcription factors. The Sin3 proteins (A and B) bind a number of docking proteins, SAP30, SAP18, SAP30L, and SAP25, which are responsible for the tethering of HDAC-1 and HDAC-2 as well as other proteins to the Sin3 proteins (2–4). In addition, Sin3A and Sin3B contain four highly conserved paired amphipathic helix domains through which the Sin3 complexes bind specific nuclear transcription factors (2, 5–7). These include the MAD family members p53, E2F-4, p33ING1, MNFβ, Ikaros, MeCP2, and ELK1 as well as the mortality factors MORF4, MRGX, and MRG15 (2). The event(s) that triggers the binding of these nuclear transcription factors to the Sin3A complex and targets the repressor complex to the transcription factor consensus sequences, resulting in subsequent repression of gene transcription rather than its induction, has not been delineated. Once bound to DNA, the Sin3A complex can modify the histone structure through the presence of a number of proteins that are part of the complex, including Swi/Snf chromatin remodeling complex subunits, O-linked *N*-acetylglucosamine transferases, and histone methyltransferases (8–12).

Recently, the nuclear orphan receptor small heterodimer partner (SHP; NROB3) has also been found to be a component of the Sin3A complex (8). SHP is unique as a modulator of gene expression in that it contains a ligand-binding domain but no DNA-binding domain (13). SHP modulation of gene expression is due to its ability to associate with other proteins and it has been found to heterodimerize with a large number of nuclear receptors, including the thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, estrogen receptor, glucocorticoid receptor, and hepatocyte nuclear factor-4 (13). In contrast, SHP has been found to activate

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the NF-κB and Peroxisome Proliferator-Activated Receptor γ (PPARγ) nuclear receptors (14, 15). At least three separate and distinct pathways seem to be used by SHP to silence gene expression. These include (*a*) SHP binding to the nuclear receptor AF-2 coactivator domains, competing for coactivator binding and thus blocking nuclear receptor-mediated transcriptional activity; (*b*) recruitment of corepressors and their associated proteins through direct binding to the SHP repression domain, resulting in silencing of gene transcription; and (*c*) SHP direct interaction with the nuclear receptor and blockade of nuclear receptor DNA binding (13, 16). Several investigators have found that SHP repression of gene expression requires HDAC activity (16). This finding, along with the association of SHP with the Sin3A complex, suggests that the Sin3A complex may play an important role in SHP modulation of gene activity.

The ability of a novel class of compounds, which we have designated adamantyl retinoid–related (ARR) molecules, to induce apoptosis both *in vitro* and *in vivo* in a variety of malignant cells has been well documented (17–23); several of these compounds are now under preclinical or clinical development. These ARRs as typified by 4-[3′-(1-adamantyl)-4′-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC) possess a number of unique features: the presence of a hydrophobic 1-adamantyl group, its adjacent hydrogen-bond donating group, and a distant carboxylic acid hydrogen-bond accepting group are required for robust apoptosis induction (21). Whereas some of the ARRs such as 6-[3′-(1-adamantyl)-4′-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437/AHPN) may bind to certain nuclear retinoic acid receptors and several may activate this receptor, retinoic acid receptor binding and activation do not seem to play any role in their induction of cell death (24–26). We have recently discovered that the ARRs specifically bind to the small heterodimer partner SHP (27). In turn, ARR binding of SHP led to Sin3A/SHP complex formation, resulting in modification of the Sin3A complex members as well enhancement of Sin3A-associated HDAC activity (27). Although SHP binding to the ARRs and the recruitment of SHP/ARR to Sin3A are clear, the role of SHP, if any, in the modification of a Sin3A complex and enhancement of Sin3A-associated HDAC activity remains to be delineated.

In this report, we show that the loss of SHP and Sin3A expression inhibited 3-Cl-AHPC–mediated apoptosis in human breast carcinoma and leukemia cell lines as well as in mouse embryonic cells but had no effect on 3-Cl-AHPC–mediated inhibition of cellular proliferation. 3-Cl-AHPC induction of c-Fos and c-Jun expression as well as NF-κB activation are essential for 3-Cl-AHPC–mediated apoptosis. Loss of SHP or Sin3A expression blocked 3-Cl-AHPC–induced c-Fos and c-Jun expression, NF-κB activation, and 3-Cl-AHPC–enhanced Sin3A-associated HDAC activity.

Materials and Methods

Reagents

3-Cl-AHPC was synthesized as described previously (28, 29). [5,5′-$^3H_2$]AHPN was obtained from Vitrax. DMEM-F12 medium, fetal bovine serum, and Trizol reagent were purchased from Invitrogen. 4-Hydroxytamoxifen and puromycin were obtained from Sigma Life Science. Anti-mSin3A, anti-HSP90, anti–c-Jun, and anti-SHP antibodies were from Santa Cruz Biotechnology. Anti–caspase-3 and anti-cFos antibodies were from Millipore. α-Tubulin antibody was from Oncogene Research Products. The HDAC colorimetric assay kit was from BIOMOL International. The Cell Death Detection ELISA and Caspase-3 activity colorimetric assay kits were obtained from Roche and BioVision, Inc., respectively. Nuclear factor-κB (NF-κB) signaling pathway gene arrays were from Superarray. The c-Fos cDNA plasmid was generously provided by Dr. Paul Chiao (The University of Texas M. D. Anderson Cancer Center, Houston, TX).

Cell Culture

The human leukemia cell line KG-1, breast carcinoma cell line MDA-MB-468, and mouse embryonic fibroblast (MEF) cells were maintained in RPMI 1640 and DMEM-F12 medium as previously described (27). Ablation of Sin3A in MEFs was achieved by adding 4-hydroxytamoxifen for 72 h to MEFs carrying an inducible Cre-recombinase in the Rosa 26-Cre-ER^{T2} (RCM2) as well as one or two conditional alleles for Sin3A (30). MEFs carrying the RCM2 allele and the wild-type allele was used as a control.

Small Hairpin RNA Plasmid Construction

The small hairpin (sh)RNA-SHP, shRNA-Sin3A, shRNA-cJun, and shRNA-cFos expression vectors were constructed by directionally cloning 5′ *Bam*HI and 3′ *Eco*RI overhang nucleotides in a pSIREN-RetroQ vector according to the manufacturer's instructions (Clontech). Sin3A, SHP, cFos, and cJun target sequences were taken from the coding sequence of the PubMed accession numbers NM_015477, L76571, NM_005252, and AY217548, respectively, and synthesized by Integrated DNA Technology, Inc. shRNA regions in the plasmid backbone were confirmed by sequencing. shRNA-SHP and shRNA-Sin3A plasmids were used either to transfect stably adherent cell lines, using the standard calcium phosphate method, or to infect suspension cell lines, after packaging them into the retroviral PT-67 cell line (Clontech). Stable cell lines were selected with puromycin. The sh-vector containing scrambled sequences, GTTATTACTGTTCGATCGC and GCCTTAAGATGACAGCCGAGATCCA, in pSIREN-RetroQ vector was used as a control.

Apoptosis

The SHP knockdown, MDA-MB-468, and KG-1 cells and knockout MEF stable cell lines were treated with 3-Cl-AHPC for 24 h before proliferation and apoptosis were assessed. The mSin3A knockout and wild-type MEF cell lines were treated with 4-hydroxytamoxifen for 72 h for depletion of Sin3A before adding 3-Cl-AHPC for 24 h. MDA-MB-468 and KG-1 cell lines were transiently transduced with retroviral shRNA-Sin3A and then incubated with 3-Cl-AHPC for 48 h. Cell counts were done with a hemocytometer. Cell apoptosis was assessed using acridine orange/ethidium bromide staining method (31) and Cell Death Detection ELISA assay.

Binding Assay

Binding assays were done as we have previously published (27, 29). In the immunoprecipitation binding assays,

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specific primary antibodies (1 μg) were added in 500 μL binding buffer containing nuclear extracts with labeled and nonlabeled ligands and incubated at 4°C for 2 h and binding was assessed (27). Nuclear extracts were prepared using the method as described (27).

Western Blots, RNA Preparation, Northern Blots, and Gene Array

Western blots, RNA preparation, and Northern blots were done as we have previously described (27). Gene arrays were done per manufacturer's instruction (Superarray).

HDAC Assay

Histone deacetylation activation assays were done using the colorimetric HDAC assay kit and according to the manufacturer's instruction (BIOMOL International). Sin3A and SHP were immunoprecipitated from 200 μg of nuclear extracts and added to 1 mmol/L substrate for each HDAC activation assay in 96-well microtiter plates; the HDAC activity was measured at 405 nm in a microtiter plate reader.

Transfection

Transfection in MDA-MB-468 and MEF SHP knockdown and knockout cells for NF-κB activation was done using the calcium phosphate method (28). Cells were treated with 3-Cl-AHPC after 36 h posttransfection and incubated for 24 h and luciferase and β-galactosidase assays were done.

Chromatin Immunoprecipitation Assay

SHP knockdown MDA-MB-468 and control cells were treated with 1 μmol/L 3-Cl-AHPC for 24 h and chromatin immunoprecipitation analysis was done using a described method (27). c-Fos primers were designed from the promoter region exon 1, forward primer 5′-AgAATCCgAAgggAAAggAA; reverse primer, 5′-CTTCTCCTTCAgCAggTTgg-3′.

Quantitative Real-time PCR

Total RNA was prepared from SHP and Sin3A knockout MEF cell lines using TRIzol as recommended by the manufacturer and purified using the Rneasy Mini Kit. For real-time PCR, cDNA was prepared with the First-Strand cDNA synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen) and analyzed in duplicate using the SYBR Green PCR Master Mix (Applied Biosystem) and the ABI Prism 7700 sequence detection system. The oligonucleotide primer set for SHP was forward 5′-TgCCCAgCATACTCAAgAAg-3′ and reverse 5′-GCTCCAgAAggACTCCAgAC-3′. The primer sequences for β-actin were forward 5′-TCCTTCCTgggCATggAg-3′ and reverse 5′-AggAggggCAATgATCTT-3′.

Results

ARR-Mediated Apoptosis but not Growth Inhibition Is Inhibited in SHP and Sin3A Knockdown and Knockout Cells

ARRs initially inhibit the proliferation of cells followed by the induction of apoptosis (17). Previous studies suggested that ARR-mediated growth inhibition and apoptosis seem to involve separate pathways (32, 33). We found that exposure of cells to ARRs resulted in the enhanced association of SHP with the Sin3A complex and modification of the Sin3A complex with the enhanced binding of N-CoR, HDAC-4, and HSP90 and increased Sin3A-associated HDAC activity (27). Therefore, we assessed whether SHP and or Sin3A expression were essential for ARR-mediated growth inhibition as well as induction of apoptosis, ARR binding to nuclear extracts, and ARR modulation of Sin3A-associated HDAC activity in a number of cell types. The effect of knockdown of SHP or Sin3A expression in human MDA-MB-468 breast carcinoma and KG-1 leukemia cell lines and ablation of SHP and Sin3A in MEF cells on these 3-Cl-AHPC–mediated events was examined. shRNA-SHP expression vectors SHP-KD1, SHP-KD2, and SHP-KD3 and Sin3A shRNA expression vectors Sin3A-KD1 and Sin3A-KD2 were stably expressed in the MDA-MB-468 and KG-1 cell lines and clones selected (Supplementary Fig. S1).[3] Cells transfected with the sh-vector only served as controls. Knockdown of SHP in MDA-MB-468 human breast carcinoma cells and KG-1 human leukemia cells resulted in a >50% reduction in 3-Cl-AHPC–mediated apoptosis (Fig. 1A and B). Ablation of SHP levels in MEF cells significantly inhibited 3-Cl-AHPC–mediated apoptosis with 60% apoptosis observed in the controls and 10% apoptosis in the SHP-ablated MEFs as documented by acridine orange staining as well as apoptosis ELISA assay (Fig. 1C; Supplementary Fig. S2A).[3] Reduction in SHP levels had little effect on 3-Cl-AHPC–mediated inhibition of growth (Fig. 1A–C).

We next examined the effect of transiently decreased Sin3A expression on 3-Cl-AHPC–mediated apoptosis and growth inhibition in these cells. Sin3A knockdown in the MDA-MB-468 and KG-1 cells blocked 3-Cl-AHPC–induced apoptosis by 30% to 40% but again had little effect on 3-Cl-AHPC–mediated inhibition of cellular proliferation in a number of the clones examined (Fig. 2A and B). Similarly, by using MEF cells in which one can selectively ablate one or both Sin3A alleles, we found that that knockout of one or both Sin3A alleles inhibited 3-Cl-AHPC–mediated apoptosis by 48% (Fig. 2C).[3] This was further supported when apoptosis was assessed using an apoptosis ELISA assay (Supplementary Fig. S2B). We could not assess the effect of Sin3A ablation on 3-Cl-AHPC–mediated inhibition of proliferation in the MEFs because Sin3A ablation resulted in the cessation of growth in these cells. Thus, decrease of either Sin3A or SHP expression had a significant negative effect on 3-Cl-AHPC–mediated apoptosis but little to no effect on 3-Cl-AHPC inhibition of cellular proliferation in both normal and malignant cells. The MEF cells used in the Sin3A knockout studies displayed increased resistance to 3-Cl-AHPC–mediated apoptosis and inhibition of cellular proliferation when compared with the MEF cells used in the SHP knockout cells (Figs. 1C and 2C). The explanation for this is not clear but is under study.

We investigated the effect of 3-Cl-AHPC on activation of caspase-3 in SHP and Sin3A knockdown MDA-MB-468 and KG-1 cells as well as knockout MEF cells; caspase-3 has an important role in ARR-mediated apoptosis (24). SHP and Sin3A knockdown and knockout significantly inhibited

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://molcanther.aacrjournals.org/).

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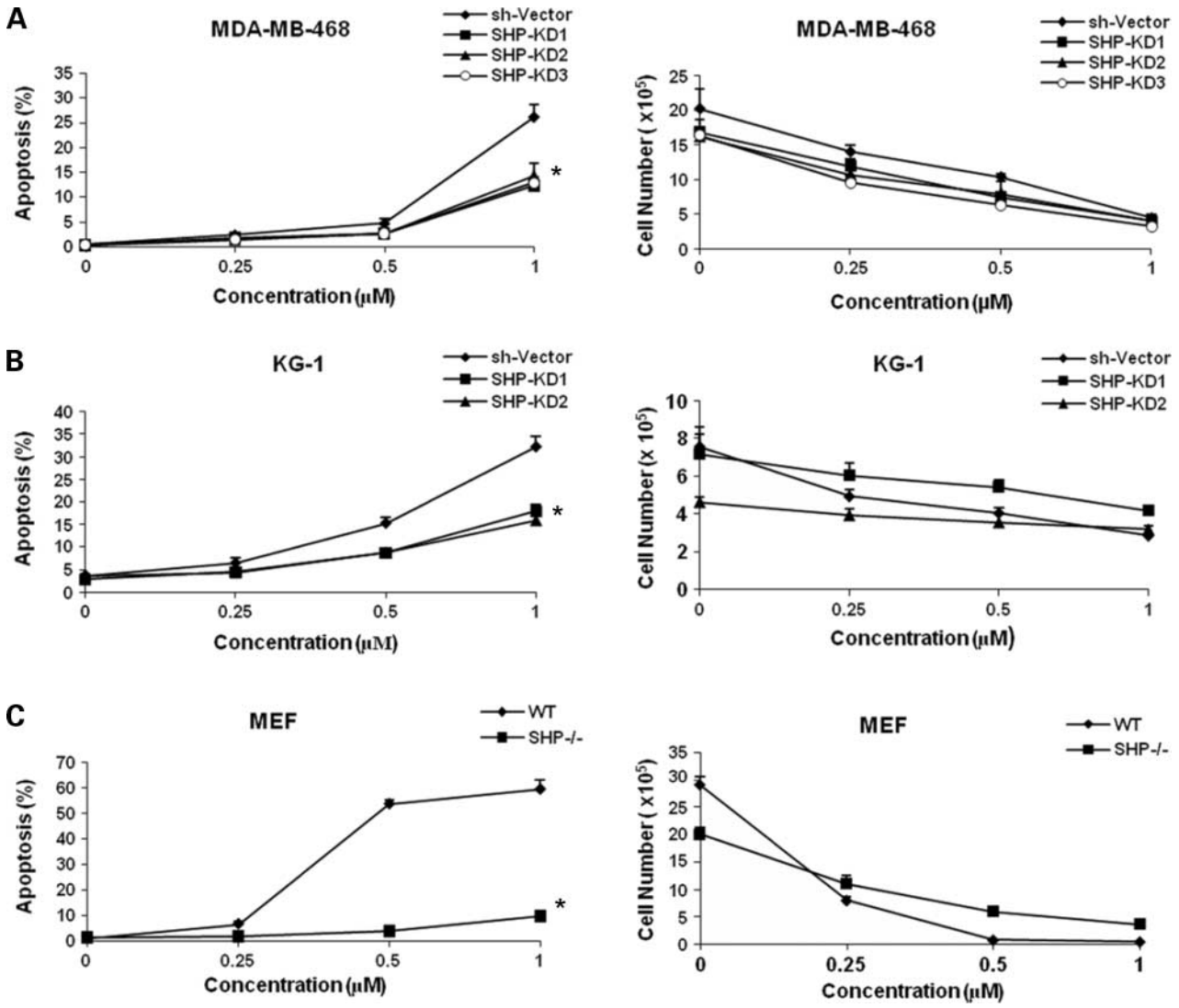

Figure 1. 3-Cl-AHPC–mediated inhibition of proliferation and induction of apoptosis in SHP knockdown MDA-MB-468 and KG-1 cell lines and SHP knockout MEF cells. Cells were grown in the presence of varying concentrations of 3-Cl-AHPC for 48 h. Apoptosis was determined using acridine orange/ethidium bromide staining and cellular proliferation was determined by cell counts as described in Materials and Methods. *Points,* mean of three independent experiments; *error bars,* SD. *, significantly different from SHP wild-type or sh-vector 3-Cl-AHPC–treated cells ($P < 0.05$ as determined by *t* test).

3-Cl-AHPC–mediated caspase-3 activation as well as 3-Cl-AHPC–mediated generation of the active 17 kDa cleaved caspase-3 protein (Supplementary Fig. S3A–C).[3]

[5,5′-$^3H_2$]AHPN Binds to SHP in Nuclear Extracts

We have previously shown that SHP binds [5,5′-$^3H_2$]AHPN with a K_D of 2.88 nmol/L (27). SHP binding of the ARR results in the enhanced binding of ARR-bound SHP to a Sin3A complex (27). We therefore hypothesized that there would be a significant decrease in [5,5′-$^3H_2$]AHPN binding in nuclear extracts in the SHP knockdown cells because it was SHP that was responsible for ARR binding. There was a significant decrease in both SHP and Sin3A complex [5,5′-$^3H_2$]AHPN binding in the nuclear extracts derived from MDA-MB-468 SHP knockdown cells, demonstrating that Sin3A complex [5,5′-$^3H_2$]AHPN binding was dependent on the presence of SHP (Fig. 3A). To assess whether SHP was responsible for the nuclear extract [5,5′-$^3H_2$]AHPN binding in cells, we determined [5,5′-$^3H_2$]AHPN binding in nuclear extracts derived from SHP knockout MEF and Sin3A knockout MEF cells (Fig. 3B). There was a >90% inhibition of [5,5′-$^3H_2$]AHPN binding in nuclear extracts derived from the SHP knockout cells and an ~50% decrease in [5,5′-$^3H_2$]AHPN binding to nuclear extracts derived from Sin3A knockout cells (Fig. 3B). Thus, depletion of SHP resulted in much greater decrease in [5,5′-$^3H_2$]AHPN binding. We noted decreased [5,5′-$^3H_2$]AHPN binding in the

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Sin3A$^{+/+}$ nuclear extracts compared with that seen in nuclear extract in SHP wild-type MEF cells. SHP levels in Sin3A$^{+/+}$ MEF were ~50% decreased compared with those seen in SHP MEF wild-type cells (Supplementary Fig. S4);[3] this is most likely the explanation for the decreased [5,5′-$^3H_2$]AHPN binding in the Sin3A$^{+/+}$ nuclear extract. To further examine why Sin3A knockout inhibited [5,5′-$^3H_2$]AHPN binding to nuclear extracts, we examined SHP levels in the Sin3A MEF knockout cells. We found that Sin3A ablation resulted in a 50% decrease in SHP mRNA and protein levels in Sin3A ablated MEF and Sin3A knockdown MDA-MB-468 cells (Fig. 3C and D). Thus, we believe that the decrease in [5,5′-$^3H_2$]AHPN binding in nuclear extracts derived from cells with decreased Sin3A levels could be attributed to the subsequent decrease in SHP levels in these cells.

Loss of SHP Expression Inhibits 3-Cl-AHPC–Mediated Increase in c-Fos and c-Jun Expression and 3-Cl-AHPC Activation of NF-κB

A number of investigators have shown that exposure to ARRs results in the early induction of c-Jun and c-Fos expression (18, 25, 32). In addition, studies have suggested that AHPN induction of c-Jun and c-Fos may be essential for AHPN-mediated apoptosis (25, 32). Expression of shRNA c-Jun or shRNA c-Fos in MDA-MB-468 cells with

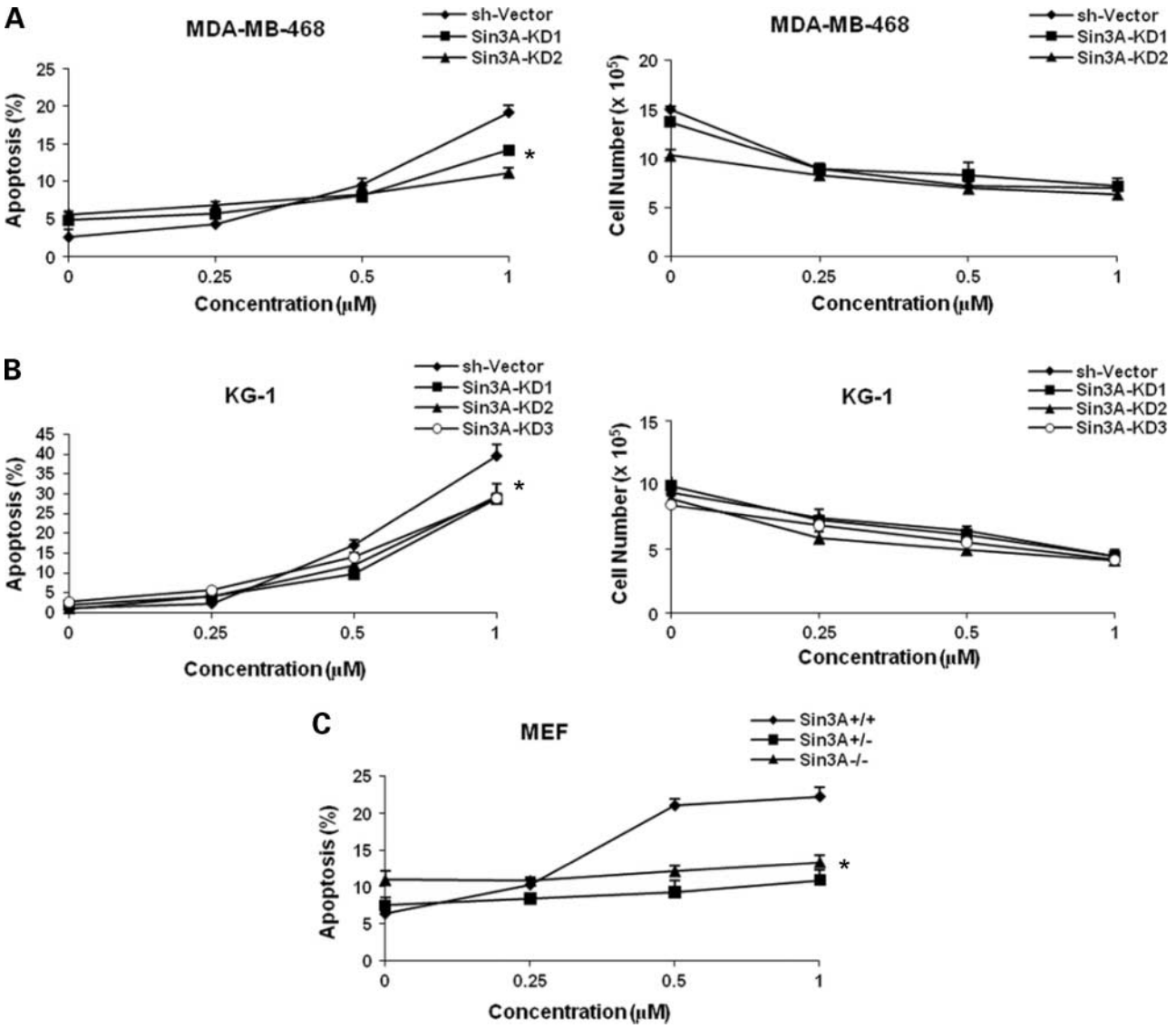

Figure 2. 3-Cl-AHPC–mediated inhibition of proliferation and induction of apoptosis in Sin3A knockdown MDA-MB-468 and KG-1 cell lines and Sin3A knockout MEF cells. Cells were grown in the presence of varying concentrations of 3-Cl-AHPC for 24 h. Apoptosis and cellular proliferation were assessed as described in Fig. 1. *Error bars,* SD. *, significantly different from Sin3A wild-type and sh-vector 3-Cl-AHPC–treated cells ($P < 0.05$ as determined by *t* test).

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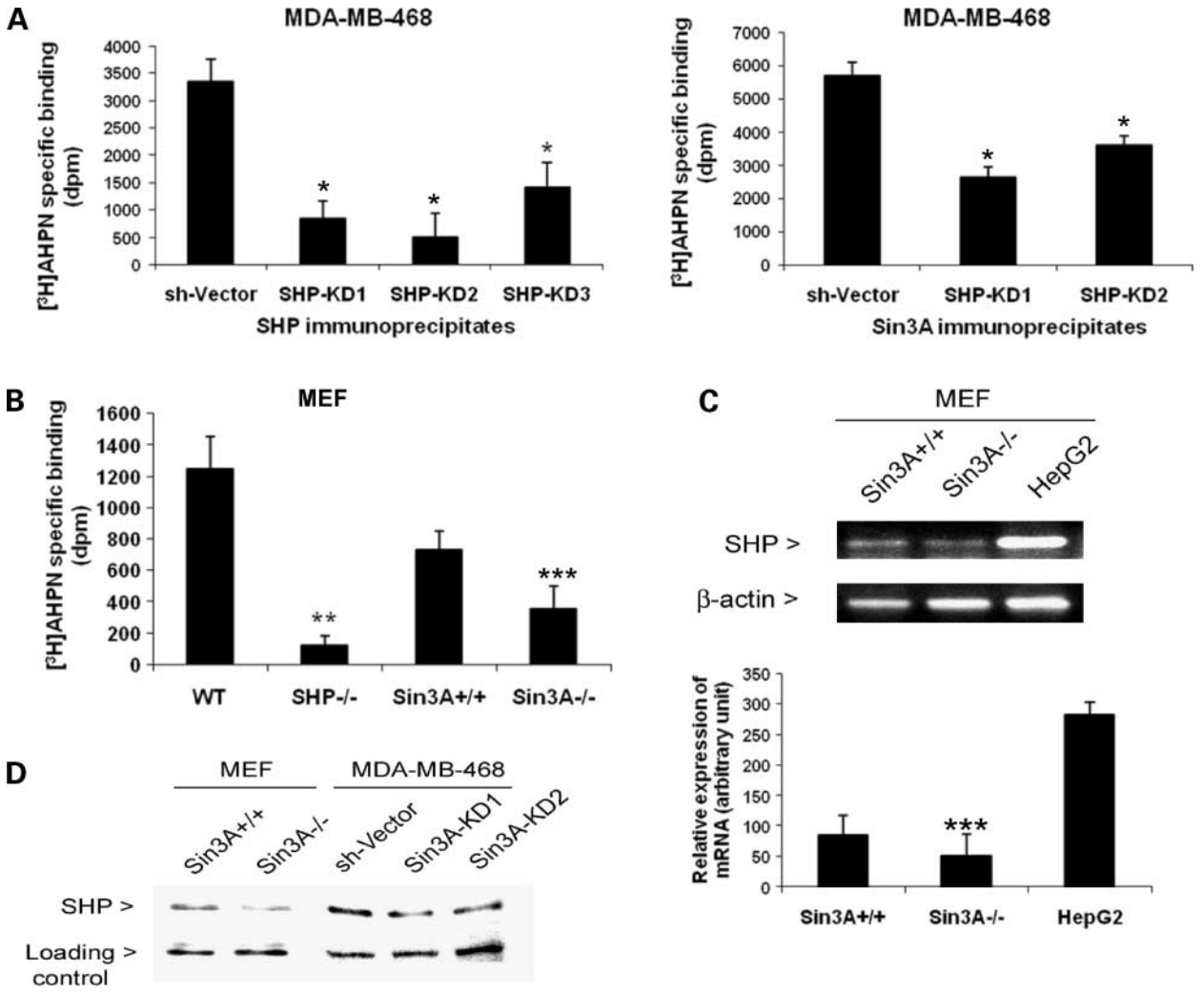

Figure 3. Loss of SHP expression inhibits [5,5′-$^3H_2$]AHPN binding. Inhibition of [5,5′-$^3H_2$] AHPN binding to SHP and Sin3A complex immunoprecipitates in sh-vector control and SHP knockdown cells (**A**) and nuclear extracts derived from MEF SHP and Sin3A knockout cells (**B**). **C,** RT-PCR and quantification of SHP mRNA expression in MEF Sin3A knockout cells. **D,** SHP protein expression in MEF and MDA-MB-468 cells. Cells were grown, nuclear extracts were prepared, and immunoprecipitation and RT-PCR were done as described in Materials and Methods. SHP was quantified by using the ImageJ program. *Columns,* mean of three independent experiments; *error bars,* SD. *, significantly different compared with binding in sh-vector only. **, significantly different from wild-type cells. ***, significantly different from Sin3A+/+ cells ($P < 0.05$ as determined by *t* test).

subsequent decrease of c-Jun or c-Fos expression inhibited 3-Cl-AHPC–mediated apoptosis in these cells, demonstrating a role for c-Jun and c-Fos (Fig. 4A; Supplementary Fig. S5).[3] We found that 3-Cl-AHPC exposure enhanced c-Fos and c-Jun expression and loss of SHP expression completely inhibited 3-Cl-AHPC induction of c-Jun and c-Fos expression in both SHP knockdown MDA-MB-468 and SHP ablated MEF cells (Fig. 4B–D). Sin3A ablation in MEF cells also completely blocked the ARR-mediated increase in c-Fos and c-Jun expression (Fig. 4B). Decrease in SHP levels inhibited ARR induction of c-Fos and c-Jun expression at both the mRNA and protein levels (Fig. 4A–D).

Whereas SHP has been shown to inhibit the induction of gene expression by a number of nuclear receptors, it has been shown to stimulate the transcriptional activity of NF-κB as well as PPARγ (14, 15). We have previously shown that NF-κB is required for ARR-mediated apoptosis (31). Inhibition of IκBα degradation using a dominant-negative IκBα blocked 3-Cl-AHPC apoptosis induction (31). Using NF-κB signaling pathway gene arrays, we found that 3-Cl-AHPC exposure in MDA-MB-468 cells resulted in a 26- and 18-fold increase in c-Fos and c-Jun mRNA levels, respectively (Fig. 4D). Knockdown of SHP expression in these cells inhibited the 3-Cl-AHPC–mediated increase in c-Fos and c-Jun mRNA levels to less than a 1- to 2-fold increases, respectively, in the presence of 3-Cl-AHPC (Fig. 4D). The c-Fos promoter contains a consensus sequence for the NF-κB p65 subunit and c-Fos promoter activity can be enhanced through p65 binding (34).

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Knockout of SHP expression in MEF cells and knockdown of SHP expression in MDA-MB-468 cells resulted in the inhibition of 3-Cl-AHPC–mediated NF-κB activation (Fig. 5A and B); in addition, loss of SHP expression resulted in inhibition of p65 binding to the c-Fos promoter in the presence of 3-Cl-AHPC as shown by chromatin immunoprecipitation assays (Fig. 5C). Inhibition of p65 binding to the c-Fos promoter has been found to reduce c-Fos expression (34). Whether ARR induces c-Fos expression through enhanced p65 binding to the c-Fos promoter is under study. We have previously shown that 3-Cl-AHPC exposure results in the enhanced expression of Sin3A (27). Loss of SHP expression had no effect on ARR-mediated increases in Sin3A levels (Fig. 5D).

SHP Expression and the Binding of the ARR/SHP Complex to Sin3A Are Required for Modification of the Sin3A Complex

We discovered that binding of ARRs to SHP (ARR/SHP) and ARR/SHP to the Sin3A complex have been found to be associated with the enhanced binding of HSP90, TBL-1, GSP-2, and HDAC-4 to the Sin3A complex (27). It is not clear whether the presence of ARR/SHP on the Sin3A complex is responsible for the enhanced binding of HSP90. We therefore examined the effect of reduced SHP expression on HSP90 binding to the Sin3A complex in the presence and absence of 3-Cl-AHPC. Exposure of MDA-MB-468 cells to 3-Cl-AHPC resulted in increased HSP90 levels and increased HSP90 binding to the Sin3A complex (Fig. 6A).

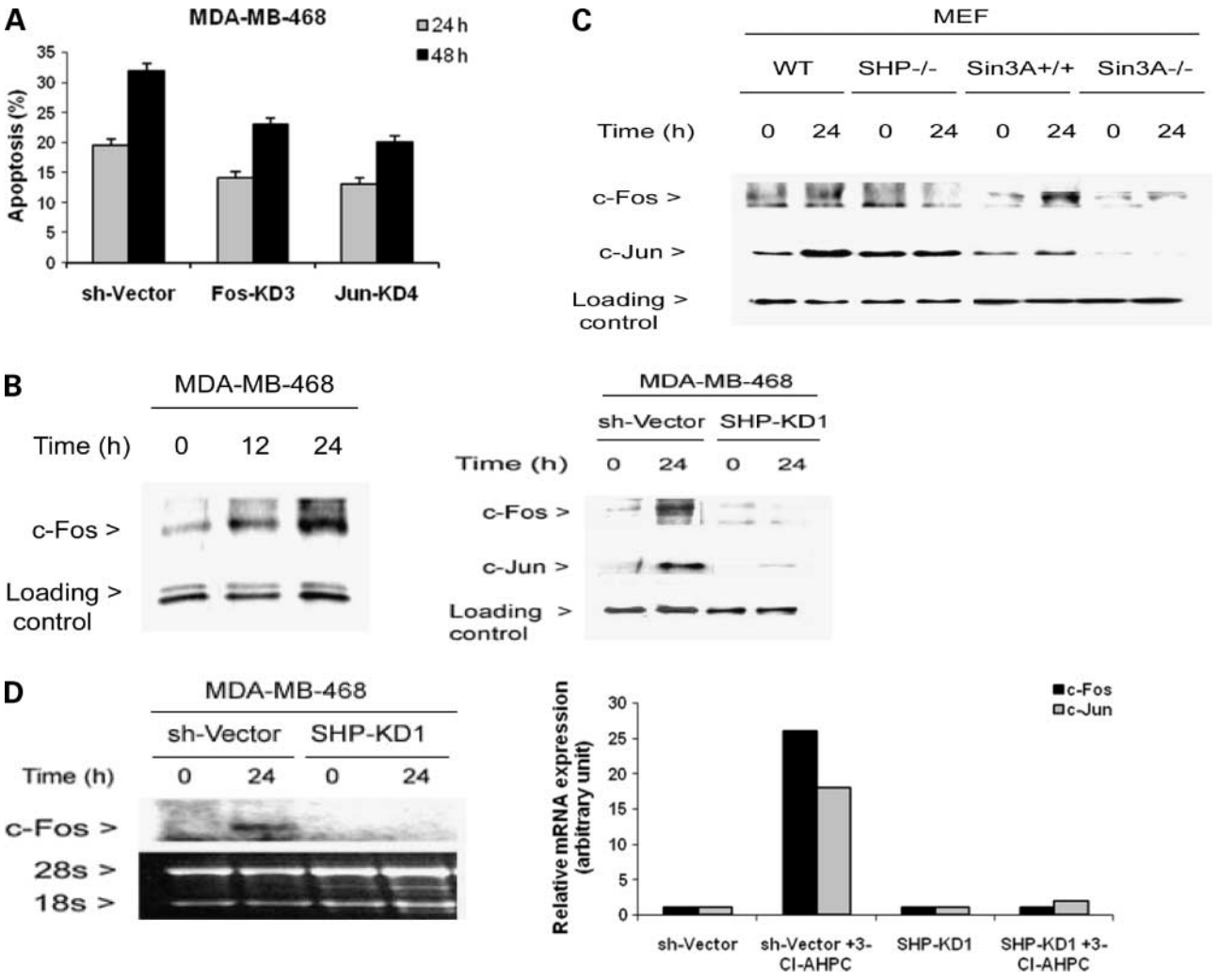

Figure 4. 3-Cl-AHPC–enhanced c-Fos and c-Jun expressions are essential for 3-Cl-AHPC–mediated apoptosis and are regulated through SHP and Sin3A. **A,** decreased c-Jun or c-Fos expression blocks 3-Cl-AHPC–mediated apoptosis in shRNA vector stably transfected MDA-MB-468 cells. **B,** 3-Cl-AHPC exposure enhances c-Fos expression and knockdown of SHP blocks 3-Cl-AHPC–mediated increased c-Fos and c-Jun protein expression in MDA-MB-468 cells. **C,** SHP and Sin3A knockout blocks 3-Cl-AHPC–mediated increase in c-Fos and c-Jun protein levels in MEF cells. **D,** knockdown of SHP blocks 3-Cl-AHPC–mediated increase in c-Fos mRNA and the modulation of c-Fos and c-Jun mRNA expression levels is inhibited in SHP knockdown MDA-MB-468 cells in gene array. Cells were grown and exposed to vehicle or 3-Cl-AHPC (1 μmol/L) for 24 h as described in Materials and Methods. Northern and Western blots were done as described in Materials and Methods. *Columns,* mean of three independent experiments; *error bars,* SD.

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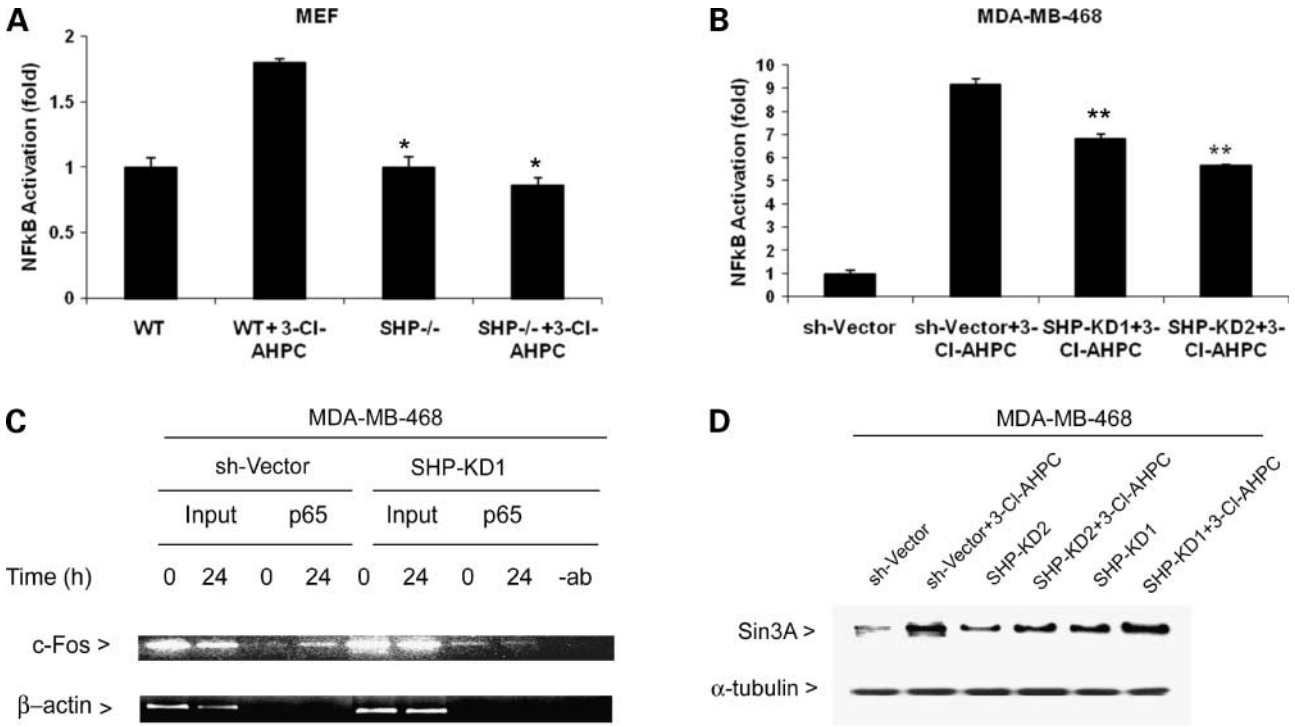

Figure 5. Decreased SHP levels inhibit 3-Cl-AHPC–mediated increase in NF-κB activation but not 3-Cl-AHPC increase in Sin3A expression. SHP ablation in MEF cells (**A**) and knockdown of SHP in MDA-MB-468 cells (**B**) inhibit 3-Cl-AHPC–mediated NF-κB activation. **C,** knockdown of SHP expression in MDA-MB-468 cells inhibits p65 binding to the c-Fos promoter. **D,** loss of SHP expression does not inhibit 3-Cl-AHPC–mediated increase in Sin3A levels. Cells were grown and exposed to vehicle or 3-Cl-AHPC (1 μmol/L) as described in Materials and Methods. Transfection assays, Western blots, and chromatin immunoprecipitation assays were done as described in Materials and Methods. *Columns,* mean of three independent experiments; *error bars,* SDs. *, significantly different from wild-type 3-Cl-AHPC–treated cells. **, significantly different from sh-vector only ($P < 0.05$ as determined by t test).

There was no 3-Cl-AHPC–mediated increase in HSP90β levels; thus, we believe that ARRs specifically increase HSP90α levels and HSP90α association with Sin3A. Loss of SHP expression inhibited the 3-Cl-AHPC–mediated increase in HSP90α expression as well as 3-Cl-AHPC–enhanced association of HSP90α to the Sin3A complex. These results suggested that ARR/SHP association with the Sin3A complex is required for the recruitment of HSP90α to the Sin3A complex (Fig. 6A).

SHP Is Required for ARR-Mediated Increase in Sin3A-Associated HDAC Activity

Exposure of cells to ARRs results in enhanced Sin3A-associated HDAC-1 and HDAC-2 activity but no increase in HDAC-1 and HDAC-2 levels (27). The mechanism by which the ARRs accomplish this is not clear. Whether ARR/SHP binding to Sin3A is required for this increase in HDAC activity remains to be delineated. We found that reduced SHP levels result in a 40% decrease in the 3-Cl-AHPC/SHP–mediated increase in Sin3A-associated HDAC-1 and HDAC-2 activity (Fig. 6B). Treatment of MDA-MB-468 cells with the HSP90 ATPase inhibitor geldanamycin in the presence of ARR/SHP also blocked the 3-Cl-AHPC–mediated increase in Sin3A-associated HDAC-1 and HDAC-2 activity (27). Thus, ARR/SHP recruitment of HSP90α to the Sin3A complex and the associated HSP90α ATPase activity are required for ARR-mediated increase in Sin3A-associated HDAC activity.

Discussion

We have previously shown that ARR-mediated apoptosis requires the expression of SHP and that loss of SHP expression inhibited apoptosis in MDA-MB-468 human breast carcinoma cells (27). We have now shown that 3-Cl-AHPC–mediated apoptosis in the KG-1 human acute leukemia as well as MEF cells requires the presence of SHP. Loss of SHP expression inhibited 3-Cl-AHPC–mediated apoptosis in both cell types. The present study also showed that SHP is responsible for the binding of ARRs to nuclear extracts. The absence of SHP in MEF cells resulted in the loss of [5,5′-$^3H_2$]AHPN binding to nuclear extracts. Furthermore, SHP-bound ARR (SHP/ARR) in turn bound to the Sin3A complex, resulting in increased binding of HSP90α to the Sin3A complex and enhanced Sin3A-associated HDAC activity. Loss of SHP expression blocked the ability of ARRs to bind to the Sin3A complex as well as ARR-mediated increased HSP90α binding to the Sin3A complex and increased Sin3A complex–associated HDAC activity. The mechanism(s) involved by which SHP bound to ARR modulates the Sin3A complex and up-regulates Sin3A-associated HDAC activity remains to be delineated.

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SHP was found to be a coactivator of NF-κB in macrophages and this activation of NF-κB was secondary to SHP binding to the NF-κB p65 subunit with enhanced binding of the p65/p50 complex to the NF-κB consensus sequence (14). We and others have previously shown that ARR activation of NF-κB is essential in ARR-mediated apoptosis (31, 35). Inhibition of NF-κB activation blocked 3-Cl-AHPC–mediated apoptosis (31). In addition, we found that exposure of cells to 3-Cl-AHPC results in the activation of the NF-κB p50 and p65 subunits with increased binding of these subunits to the NF-κB consensus sequence (31). We have now found that inhibition of SHP expression blocks 3-Cl-AHPC–mediated NF-κB activation and apoptosis. In addition, using CHIP assays, we found that decreased SHP levels results in the loss of NF-κB p65 subunit binding to the c-Fos promoter in the presence of 3-Cl-AHPC.

Recent studies have documented that NF-κB can block apoptosis through its enhanced expression of antiapoptotic proteins and function as a tumor promoter (36). Recent investigations have shown that the situation is far more complex and that NF-κB can enhance apoptosis depending on the context in which it is stimulated (36–39). We have previously shown that 3-Cl-AHPC–mediated activation of NF-κB is associated with the down-regulation of a number of antiapoptotic proteins, including XIAP, c-IAP-1, and Bcl-X_L (31). In addition, increased levels of the proapoptotic death receptors DR4 and DR5 as well as Fas were found following 3-Cl-AHPC exposure (31). Thus, 3-Cl-AHPC exposure seems to result in the decreased expression of antiapoptotic proteins and the increased expression of proapoptotic proteins, leading to an environment in which the role of NF-κB seems to be the induction of apoptosis.

A number of investigators have found that ARR exposure results in the enhanced expression of c-Jun and c-Fos (18, 32). In addition, Li et al. (32) reported that the constitutive expression of a dominant-negative inhibitor of c-Jun, c-Jun (Ala63,73), in cells blocked ARR-mediated apoptosis, suggesting that the ARR increase in c-Jun expression plays an important role in ARR-mediated apoptosis. Interestingly, expression of this c-Jun dominant negative had no effect on ARR-mediated inhibition of cellular proliferation (32). We have now determined that inhibition of c-Fos or c-Jun function through the expression of shRNA knockdown vectors blocked 3-Cl-AHPC–mediated apoptosis. The role of c-Jun in ARR-mediated apoptosis remains unclear. We found that the loss of SHP expression inhibited 3-Cl-AHPC increased expression of both c-Jun and c-Fos at both the message and protein levels. Park et al. (40) have recently reported that bile acid–enhanced c-Jun expression in gastric cells occurs through a SHP-mediated mechanism. Elevated c-Fos expression has been found to precede apoptosis in a number of systems and thus may have a proapoptotic function (40, 41). In addition, AHPN-mediated increased c-Fos levels may be essential for AHPN-mediated apoptosis (25). The c-Fos promoter contains a NF-κB consensus sequence (34). In view of the importance of ARR-mediated NF-κB activation in the induction of apoptosis and the role of SHP in enhancing p65 binding to its consensus sequence, we determined whether loss of SHP resulted in decreased p65 binding to the c-Fos promoter. Indeed, we found that decreased SHP expression resulted in decreased p65 binding to the c-Fos promoter in the presence of 3-Cl-AHPC. It has been previously reported that loss of p65 binding to the c-Fos promoter results in the inhibition of transcription (34).

Loss of Sin3A expression also inhibited 3-Cl-AHPC–mediated apoptosis. SHP binding to the Sin3A complex has been previously reported (8). The role of SHP in the Sin3A complex remains unclear. One can speculate that SHP may serve as an additional anchoring device, binding to the Sin3A complex as well as to nuclear proteins and, thus, directing the Sin3A complex to specific promoters resulting in the inhibition of transcription, as is the case for Sin3A/SHP–mediated repression of CYP7A1 transcription (42). We have previously reported that ARR specifically binds to SHP (27). We have now shown that ARR must first bind to SHP, after which this complex binds to the Sin3A complex. The effect of ARR binding on SHP molecular conformation that allows for enhanced SHP binding to the Sin3A complex is under study. Cre-recombinase–mediated deletion of Sin3A

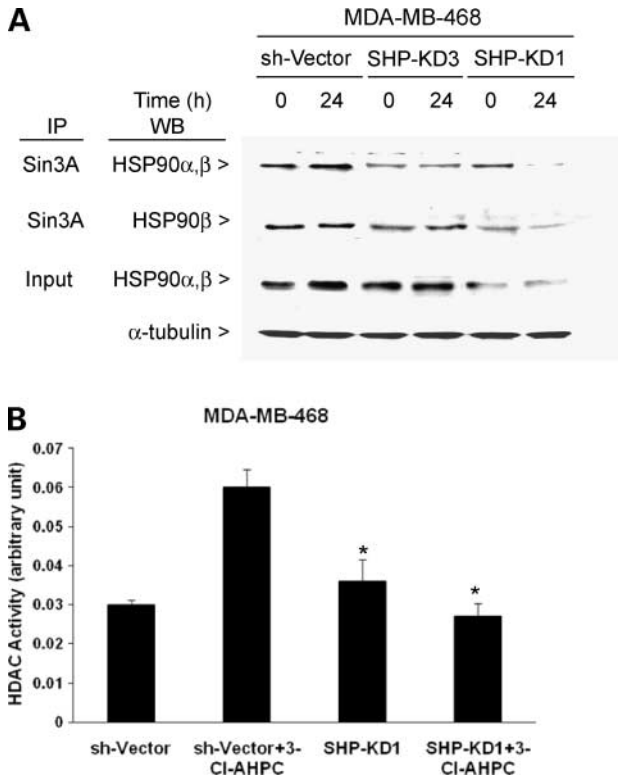

Figure 6. Knockdown of SHP in MDA-MB-468 cells blocks HSP90 binding to Sin3A complex (**A**) and 3-Cl-AHPC–mediated increase in Sin3A-associated HDAC activity (**B**). Cells were exposed to 3-Cl-AHPC (1 μmol/L) for 24 h and Western blots were done and HDAC activity was assessed as described in Materials and Methods. *Columns,* mean of three independent experiments; *error bars,* SD. *, significantly different from sh-vector-only 3-Cl-AHPC–treated cells ($P < 0.05$ as determined by *t* test).

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in the MEF cells and knockdown of Sin3A in KG-1 and MDA-MB-468 cells were found to be associated with decreased SHP levels. This may explain why loss of Sin3A or SHP has similar effects on 3-Cl-AHPC–mediated cellular processes. The importance of Sin3A in the regulation of crucial genes that seem to play vital roles in cell cycle regulation, proliferation, and control of apoptosis is underscored by the fact that permanent Sin3A ablation is lethal to cells (30). A very low expressed protein band (28 kDa) was noted in the MEF SHPKO cells on Western blot; in addition, RT-PCR also identified a band at the SHP location in the MEF SHPKO cells (Supplementary Fig. S4). Whether this band represents SHP or a protein of similar size and base sequence remains to be determined.

The ARRs are a unique class of molecules with a novel mechanism of action. Numerous ARR analogues have been synthesized and the associated efficacy studies have shown that the presence of the 4′-hydroxyl group, the 3′-(1-adamantyl group), and the carboxylic group is essential for the induction of apoptosis by these compounds (43). The ability of these compounds to bind to SHP and modify the biological activity of SHP and the fact that loss of SHP expression markedly inhibits ARR-mediated apoptosis strongly suggest that SHP functions as the receptor through which the ARRs exert their apoptotic activities. In addition, we have previously shown a correlation between ARR binding to SHP and the induction of apoptosis (27). The delineation of the ARR binding pocket in the SHP molecule is under study. Identification of this binding pocket and characterization of those motifs that are responsible for ARR binding to SHP and its subsequent binding to Sin3A as well as its other cellular activities will be essential for the design of more efficacious and more selective ARRs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Correction

Correction: SHP and Sin3A expression are essential for adamantyl-substituted retinoid-related molecule-mediated nuclear factor-κB activation, c-Fos/c-Jun expression, and cellular apoptosis

In this article (Molecular Cancer Ther 2009;8:1625–35), which was published in the June 2009 issue of *Molecular Cancer Therapeutics* (1), one of the authors, Jan-Hermen Dannenberg, was inadvertently omitted. The online article has been changed to reflect this correction and no longer matches the print.

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