Hint1 Inhibits Growth and Activator Protein-1 Activity in Human Colon Cancer Cells

Lin Wang,1,5 Yujing Zhang,1 Haiyang Li,1 Zhiheng Xu,2,4 Regina M. Santella,1 and I. Bernard Weinstein1

1Herbert Irving Comprehensive Cancer Center, Departments of Pathology and Medicine, and Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, New York and 2Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Kumming Medical College, Kumming, China

Abstract

There is accumulating evidence that histidine triad (HIT) nucleotide-binding protein 1 (HINT1), a member of the evolutionary highly conserved HIT protein superfamily, is a novel tumor suppressor. However, the mechanism of action of HINT1 with respect to tumor suppression is not known. In the present study, we found that a series of human colon cancer cell lines displayed various levels of expression of HINT1, with a very low level in SW480 cells. This cell line also displayed partial methylation of the promoter region of the HINT1 gene, and treatment of these cells with 5-azadeoxycitidine increased expression of Hint1 mRNA and protein. Therefore, the decreased expression of HINT1 in SW480 cells seems to be due to epigenetic silencing. Increased expression of HINT1 in these cells, using a retrovirus vector (pLNCX2) that encodes either wild-type (WT) Hint1 or a point mutant (His112/Asn112) of Hint1, inhibited the proliferation of SW480 cells. Because of the important role of the activator protein-1 (AP-1) transcription factor in cancer cells, we examined possible effects of Hint1 on AP-1 transcription factor activity in SW480 cells transfected with an AP-1-luciferase reporter. We found that cotransfection with a pH2-Hint1 plasmid DNA significantly inhibited this activity. Studies with inhibitors indicated that AP-1 activity in SW480 cells requires the activity of c-Jun NH2-terminal kinase (JNK) 2 and not JNK1. Cotransfection with the Hint1 plasmid DNA also inhibited AP-1-luciferase reporter activity in WT mouse embryo fibroblast (MEF) studies, and studies with JNK1 deleted or JNK2 deleted MEFs confirmed the essential role for JNK2, but not JNK1, in mediating AP-1 activity. Recent studies indicate that the protein plenty of SH3 (POSH) provides a scaffold that enhances JNK activity. We found that cotransfection of a plasmid DNA encoding POSH stimulated the phosphorylation of c-Jun and also AP-1 reporter activity, and cotransfection with Hint1 inhibited both of these activities. Furthermore, coimmunoprecipitation studies provided evidence that HINT1 forms an in vivo complex with POSH and JNK. These results suggest that HINT1 inhibits AP-1 activity by binding to a POSH-JNK2 complex, thus inhibiting the phosphorylation of c-Jun. This effect could contribute to the tumor suppressor activity of HINT1. [Cancer Res 2007;67(10):4700–8]

Introduction

There is accumulating evidence that the histidine triad (HIT) nucleotide-binding protein 1 (HINT1) is a novel tumor suppressor (1–3). This protein belongs to the evolutionary conserved HIT protein superfamily, which consists of at least three subfamilies: HINT1, Fhit, and GALT (4). The well-known tumor suppressor gene Fhit is mutated or transcriptionally silenced in a variety of human carcinoma (5–8) and overexpression of the Fhit protein can induce growth inhibition in tumor cell lines (9–11). In previous studies, we developed Hint1 deleted mice and found that they have a marked increase in susceptibility to chemical carcinogen-induced gastric tumors (1), mammary tumors (2), and ovarian tumors (2). In addition, with aging, Hint1 deleted mice displayed an increase in the occurrence of a variety of spontaneous tumors (2). Our studies in mice also provided evidence that the Hint1 gene may be haplosufficient with respect to its function as a tumor suppressor. Other investigators reported recently that the Hint1 gene is transcriptionally silenced in some human non–small cell lung cancer (NSCLC) cell lines and that increased expression of HINT1 inhibits growth of the NSCLC cell lines H522 and H538 (3). In the present study, and in unpublished studies, we have obtained similar results in other types of human cancer cell lines, thus providing evidence that the Hint1 gene may function as a tumor suppressor in certain types of human cancers. However, the precise mechanism by which this gene functions as a tumor suppressor is not known.

The Hint1 gene was originally discovered in our laboratory in studies using the yeast two-hybrid system to identify proteins that interact with the regulatory domain of protein kinase Cβ (12). Since then, other investigators have obtained evidence that the HINT1 protein can also interact with other proteins, which include the product of the ATDC gene (13), which seems to play a role in cellular responses to ionizing radiation; cyclin-dependent kinase 7 (14), which functions in growth control and transcriptional regulation; the human mu-opioid receptor, which is a G-protein–coupled receptor that mediates analgesia, euphoria, and other important central and peripheral neurologic functions (15); the transcription factor microphthalmia also called MITF (16), which plays an important role in mast cell and melanocyte growth; the ubiquitously expressed transcription factor USF2 (17); and Pontin and Reptin (18), which complex with β-catenin and thereby regulate the β-catenin/TCF4 signaling pathway. Indeed, the latter study indicated that overexpression of HINT1 significantly inhibited both β-catenin/TCF4 and cyclin D1 transcription factor activity in SW480 colon cancer cells. These investigators also reported that the HINT1 protein is also associated with the Tip60/Pontin/Reptin complex, which plays a role in transcription (19).

Requests for reprints: I Bernard Weinstein, Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, HHSC-1509, New York, NY 10032-2704. Phone: 212-305-6921; Fax: 212-305-6889; E-mail: lbw1@columbia.edu.

©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-4645
Thus, the HINT1 protein seems to play an inhibitory role in several pathways that control gene transcription. Therefore, in the present study, we examined possible effects of HINT1 on the activator protein-1 (AP-1) transcription factor complex because AP-1 plays an important role in enhancing the transcription of several genes that stimulate cell proliferation (20–22). The AP-1 transcription factor was first described as a 12-O-tetradecanoyl-phorbol-13-acetate (TPA)–activated transcription factor (23). The AP-1 dimeric complex contains members of the c-Jun, c-fos, Fra, and ATF protein families (24–26). AP-1 activity is often increased in cancer cells and exerts its oncogenic effects by regulating genes involved in cell proliferation, angiogenesis, and tumor invasion (27–30). Inhibition of AP-1 activity is associated with cell growth inhibition in several types of cancer cells (31–33). The c-Jun protein can form homodimers or heterodimers with c-fos or Fra. These dimers have a high affinity for the TGACTCA DNA target sequence and thereby regulate AP-1 activity in the promoter regions of several genes (20–22). The c-Jun protein has important regulatory phosphorylation sites at Ser63 and Ser73, which are near the NH2 terminus of this protein (30). The phosphorylation of these amino acids by c-Jun NH2-terminus kinase (JNK) stimulates the transcriptional activation of c-Jun. Among the three JNK subfamilies, JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is mainly expressed in the central nervous system. Both JNK1 and JNK2 seem to share the same substrates, including activating transcription factor 2 and c-Jun, and were, therefore, assumed to complement each other and mediate the same or similar biological functions (34–37). However, JNK1 deficiency increases TPA-induced AP-1 activity (38–42). In addition, JNK1 is preferentially expressed in cells expressing Hint1-RT 5CyC[-tetradecanoyl-dehydrogenase (GAPDH) was used as a loading control; the primer set

Materials and Methods

Chemicals and Agents

5-Azadeoxycytidine (5-Azadc) was purchased from Sigma, the JNK inhibitor SP600125 was from Calbiochem, and protein G Plus-Agarose was purchased from Santa Cruz Biotechnology.

Cell Lines and Cell Culture

The SW-480, Caco2, HCT116, HT29, SW837, and HCT15 human colorectal cancer cell lines, the NCI-H522 NSCLC cell line, the human embryonic kidney (HEK) 293 cells, and the mouse fibroblast NIH3T3 cell line were obtained from American Type Culture Collection. The cultures of JNK wild-type (WT) mouse embryonic fibroblast (MEF) cells (JNK−/−) and cultures of the JNK mutated MEF JNK1−/− cells and MEF JNK2−/− cells were established from JNK mutant mice with the respective genotypes and were generously provided by Dr. Zigang Dong (University of Minnesota, Austin, MN; ref. 34). All of the cell lines, but not NCI-H522, were maintained in DF1 medium containing DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). NCI-H522 NSCLC cells were maintained in RPMI 1640 (Invitrogen) and 10% FBS. Cells were incubated in a 100% humidified incubator at 37°C with 5% CO2.

Methylation-Specific PCR

Whole-cell DNA was isolated from cultured cells using QiAamp DNA Mini kit (Qiagen, Inc.). A DNA bisulfite modification kit (Chemicon Co.) was used to modify genomic DNA as described before (46). Briefly, 40 ng of genomic DNA were denatured with 0.2 mol/L NaOH and then 10 mol/L hydroquinone and 3 mol/L sodium bisulfate were added and this mixture was incubated at 50°C for 16 h. The modified DNA was purified using Wizard DNA purification kit (Promega Corp.). The specific PCR primer sets for detection of methylated CpG dinucleotides within a CpG island in the promoter region of the Hint1 gene were as follows: 5'-TTGGGGCCGCGTTGGGTTTTTACAGCC-3' (methylated forward) and 5'-CTAACGAGCCGACCCCACTA-3' (methylated reverse) and 5'-TTGGGGCCGCGTTGGGTTTTTACAGCCCACC-3' (methylated forward) and 5'-CTAACGACCCGACCCCCACCCACA-3' (methylated reverse). Each PCR mixture system contained 10× PCR buffer (Qiagen), deoxynucleotide triphosphates (1.25 mmol/L), primers (0.6 mmol/L), 1 unit HotStart Taq (Qiagen), and bisulfite-modified DNA (100 ng). The PCRs were cycled in a thermal controller (Programmable Thermal Controller, MJ Research, Inc.) under the following conditions: preheat at 94°C for 3 min, 94°C for 30 s, 62°C for 30 s, and 72°C for a final extension at 72°C for 5 min. Methylation-specific PCR (MSP) products were analyzed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

5-Azade Treatment and Semiquantitative Reverse Transcription-PCR

Cells were treated with solvent or with 5-Azadc (1 mmol/L) for 96 h. Total RNA was extracted with a single-step method using the Trizol reagent (Life Technologies). Reverse transcription-PCR (RT-PCR) was conducted using a SuperScript One-Step RT-PCR system (Life Technologies) in a total volume of 25 μL reaction mixture containing 12.5 μL of 2× reaction mix, 2 μL (1 μg) template RNA, 0.25 μL sense primer (20 mmol/L), 0.25 μL antisense primer (20 mmol/L), 0.4 μL RT/Platinum Taq mix, and 9.6 μL distilled water. Initially, cDNA was generated at 50°C for 30 min. The PCR was then conducted for 28 cycles in a thermal controller (Programmable Thermal Controller); each amplification cycle consisted of 0.5 min at 94°C for denaturing, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension. The sequences of PCR primers were as follows: Hint1-RT 5'-GGATTTCGAGAGTCCTAAACGAGGGA-3' (forward) and 5'-TAAACCAGAGGGAAGAACATT-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control; the primer set

4 Z. Xu, et al., unpublished data.

www.aacrjournals.org

4701


Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2007 American Association for Cancer Research.
using the Lipofectin agents (Invitrogen). Fresh DMEM/10% FBS medium was replenished 18 h after transfection, and the retrovirus-containing medium was harvested 48 h later. Virus titers were determined by assays for plaque-forming units. NIH3T3 cells (1 × 10^5 per well for six-well plates) were infected with the retroviral vectors by directly applying the diluted viruses into the growth medium at different multiplicities of infection (MOI).

**Plasmid Construction and Gene Transduction**

Full-length human Hint1 cDNA was amplified from HCT116 cells by a one-step RT-PCR procedure as described above, using the primer sets 5′-CCGGAATTCCC-TAAAGAAGAGAA-3′ (forward) and 5′-TGAGATGGCAGATCCGGAATTCCC-3′ (reverse). The PCR products were then cloned into the EcoRI-CIP-treated plasmid pHANE, which contained the ATG start codon and the NH2-terminal HA epitope tag (YPYDVPDYA; ref. 47). To carry out site-directed mutagenesis of the Hint1 gene, we used the QuickChange Site-Directed Mutagenesis kit (Stratagene) to mutate Hint1/HINT1 dIII and NotI endonuclease cut sites. Thus retroviral-HA-Hint1-His112/Asn112 plasmid was constructed. The proper sequences of the above mentioned constructs were confirmed by DNA sequencing.

Retrovirus mediated Hint1 gene transduction. PT67 packaging cells were plated 18 h before transfection. Then, the cells were transfected with the pLNCX2 retroviral shuttle plasmid encoding the indicated Hint1, by using the Lipofectin agents (Invitrogen). Fresh DMEM/10% FBS medium was added and the retrovirus containing medium was harvested 48 h later. Virus titers were determined by assays for plaque-forming units. NIH3T3 cells (1 × 10^5 per well for six-well plates) were infected with the retroviral vectors by directly applying the diluted viruses into the growth medium at different multiplicities of infection (MOI).

**Cell Proliferation Assays**

**Colonization formation assay.** SW480 cells were infected with the indicated retrovirus as described above. The cells were replated into 100-mm dishes at 10^5 per dish, and the cells were cultured in the presence of 0.8 mg/mL G418 for 3 weeks. The colonies were then fixed with formalin, stained with Giemsa, and counted. All assays were done in triplicate, and the results were expressed as "percentage of control" (i.e., the number of colonies obtained compared with the vector control retrovirus).

**Growth curves.** SW480 cells were cultured at 1 × 10^5 in 60-mm dishes. The cells were infected with the indicated retroviruses and counted every day for 1 week. The number of viable cells per well was determined at the indicated times, in triplicate assays.

**Protein Extraction and Western Blot Assays**

Cells were cultured and treated with the various conditions as described above. Protein extracts were then prepared as described previously (29). The proteins were separated by SDS-PAGE with 7.5% to 12.5% polyacrylamide gels and then blotted with the indicated antibody. The primary antibodies included HINT1 antisera (12), anti-HA-Tag antibody (Sigma), anti-FLAG-Tag antibody (Sigma), anti-Myc-Tag antibody (Cell Signaling), anti-β-actin antibody (Sigma), anti-c-Jun antibody (Cell Signaling), anti-phosphorylated c-Jun (p-c-Jun) antibody (Ser63; Cell Signaling), and anti-POSH antibody.
Overexpression of HINT1 inhibits growth of SW480 cells. As reviewed in Introduction, there is increasing evidence that HINT1 is a novel tumor suppressor gene (1–3). In view of the prevalence of human colon cancer, it was therefore of interest to examine the levels of HINT1 in various colon cancer cell lines (Fig. 1A). As shown in Table 1, the expression of HINT1 was markedly lower in several colon cancer cell lines than in the H1299 lung adenocarcinoma cell line. Analysis of the levels of HINT1 in 10 colon cancer cell lines and two HNSCLC cell lines, which was reported previously to display a very low level of this protein (3), showed that the colon cancer cell lines displayed about a 1.5- to 2-fold higher level of HINT1 than the SW480 cells. Similar results were obtained in a repeat study.

The expression of specific tumor suppressor genes is often inhibited in cancer cells via methylation of specific cytidine residues in the corresponding promoter regions of these genes and also via other modifications that alter chromatin structure (46, 50, 51). Therefore, we examined the state of methylation of a CpG-rich sequence in the promoter region of the HINT1 gene using bisulfite-treated DNA samples obtained from the above series of cell lines and using sets of methylation-specific primers as described in Materials and Methods. No DNA methylation was detected in the samples from the SW480, HCT15, HCT116, HT29, and H522 cell lines and using sets of methylation-specific primers as described in Materials and Methods. No DNA methylation was detected in the samples from the above series of colon cancer cell lines. Methylation was detected in the samples from the H522 cell line, which was reported previously to display a very low level of this protein (3). Among the five colon cancer cell lines, SW480 cells displayed the lowest level of HINT1, and this level was somewhat higher than that in the H522 cells. The other colon cancer cell lines displayed about a 2-fold higher level of HINT1 than the SW480 cells. Similar results were obtained in a repeat study.

The expression of specific tumor suppressor genes is often inhibited in cancer cells via methylation of specific cytidine residues in the corresponding promoter regions of these genes and also via other modifications that alter chromatin structure (46, 50, 51). Therefore, we examined the state of methylation of a CpG-rich sequence in the promoter region of the HINT1 gene using bisulfite-treated DNA samples obtained from the above series of cell lines and using sets of methylation-specific primers as described in Materials and Methods. No DNA methylation was detected in the samples from the HCT15, HCT116, HT29, and

 Luciferase Reporter Assays

Cells were plated at 1 x 10^3 per 35-mm-diameter plates 18 h before transfection. Then, the cells were transfected with 500 ng of the pAP1-luciferase reporter plasmid (kindly provided by Drs. J.H. Pierce, National Cancer Institute (NCI), Bethesda, MD; ref. 48) and 200 ng of a cytomegalovirus (CMV)-β-galactosidase reporter plasmid, with or without cotransfection with the indicated plasmids (see figure legends), which included pCMV-JNK2-Myc and pCMV-POSH-FLAG (kindly provided by Lloyd A. Greene; ref. 43) and pcDNA3-JNK1-KR (49), using the Lipofectin reagent. At 36 h after transfection, cell extracts were prepared, and each sample was assayed, in triplicate, using a luciferase assay system (Promega). Luciferase activities were normalized to β-galactosidase activities to correct for differences in transfection efficiency. A difference in reporter activities between the experimental groups was assigned the value of “percentage.” All statements of significance are P < 0.05.

Immunoprecipitation

Cells were plated into 10-cm-diameter plates 18 h before transfection. Then, the cells were transiently transfected with pHA-Hint1, pCMV-POSH-FLAG, and pCMV-JNK2-Myc (43) separately or together, as indicated, using the Lipofectin reagent. At 48 h after transfection, cell extracts were washed twice with cold PBS and incubated with ice-cold lyses buffer [10 mmol/L Tris (pH 7.4), 1.0% Triton X-100, 0.5% NP-40, 150 mmol/L NaCl, 20 mmol/L NaF, 0.2 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L phenylmethylsulfonyl fluoride] for 15 min at 4°C. Immunoprecipitation from precleared cell lysesates (200 μg protein) was done with 4 μg of an anti-HA M2 affinity gel (Sigma) or an anti-FLAG M2 affinity gel (Sigma), which contained anti-HA-Tag monoclonal antibody (mAb) or anti-FLAG-Tag mAb covalently prebound to protein G-Agarose, or in other assays, we used an anti-Myc-Tag mAb together with the protein G Plus-Agarose, with in all cases an incubation time of 18 h at 4°C. The immunocomplexes were washed with cold cell lyses buffer for five times and then boiled for 5 min. The samples were then analyzed by Western blot using the indicated antibody.

Statistical Methods

Results of the experimental studies are reported as mean ± SD. Differences were analyzed by Student’s t test. P < 0.05 was regarded as significant.

Results

Expression of HINT1 in colon cancer cells. As reviewed in Introduction, there is increasing evidence that HINT1 is a novel tumor suppressor gene (1–3). In view of the prevalence of human colon cancer, it was therefore of interest to examine the levels of expression of the HINT1 protein in a series of five human colon cancer cell lines (Fig. 1A). For comparison, we included the H522 HNSCLC cell line, which was reported previously to display a very low level of this protein (3). Among the five colon cancer cell lines, SW480 cells displayed the lowest level of HINT1, and this level was somewhat higher than that in the H522 cells. The other colon cancer cell lines displayed about a 1.5- to 2-fold higher level of HINT1 than the SW480 cells. Similar results were obtained in a repeat study.

The expression of specific tumor suppressor genes is often inhibited in cancer cells via methylation of specific cytidine residues in the corresponding promoter regions of these genes and also via other modifications that alter chromatin structure (46, 50, 51). Therefore, we examined the state of methylation of a CpG-rich sequence in the promoter region of the HINT1 gene using bisulfite-treated DNA samples obtained from the above series of cell lines and using sets of methylation-specific primers as described in Materials and Methods. No DNA methylation was detected in the samples from the HCT15, HCT116, HT29, and

Figure 2. Overexpression of HINT1 inhibits growth of SW480 cells. A, the retrovirus shuttle plasmid pLNCX2 encoding HA-Hint1-WT or HA-Hint1-His112/Asn112 or an empty control plasmid was transfected into the P267 packaging cell line. After incubation for 48 h, the retrovirus-containing medium was harvested. SW480 cells were then infected with the indicated retroviruses for 24 h at a MOI of 800 and the cells were then incubated in fresh medium for 72 h. A, total cell lysates were prepared and Western blots were done with an anti-HA antibody. B, colony formation assay. The infected cells were grown in the presence of the selection agent G418 for 3 wks. The colonies were then fixed, stained with Giemsa solution, and counted. Top, representative plates; bottom, percentage inhibition of colony formation. Columns, mean of triplicate assays; bars, SD. *, P < 0.01, significant inhibition. C, growth curves. Cells were cultured in 24-well plates, infected with the above described retrovirus-containing medium, and the number of cells in replicate wells was counted every day for the subsequent 8 d. Points, mean of triplicate assays; bars, SD. Similar results were obtained in a repeat assay.
Caco2 cell lines (Fig. 1B), which is consistent with their relatively high levels of expression of the HINT1 protein (Fig. 1A). However, both methylated and unmethylated bands were detected in the H522 and SW480 DNA samples (Fig. 1B). The above results suggest that the relatively low levels of HINT1 in the H522 and SW480 cells may be due, at least in part, to partial methylation of the promoter region of this gene.

The treatment of SW480 cells with 5-AzadC markedly inhibited their growth (data not shown), but because of possible nonspecific cytotoxic effects of 5-AzadC, this could not be specially attributed to the growth-inhibitory effects of HINT1. However, studies described below (Fig. 2) indicate that overexpression of HINT1 in SW480 cells is sufficient to inhibit their growth, and this was also shown previously with H522 cells (3).

Overexpression of HINT1 inhibits growth of SW480 colon cancer cells. To facilitate our subsequent studies, we developed retrovirus expression vectors that encode either the HA-tagged WT Hint1 cDNA sequence (Hint1-WT) or a HA-tagged mutant Hint1 sequence, in which histidine residue 112 was replaced with asparagine (Hint1/His112/Asn112). We thought that this mutant would be of interest because this substitution might disrupt the HIT motif that is characteristic of HINT1 and related members of the HIT superfamily of proteins (52, 53). Infection of SW480 cells with either of these vectors caused approximately similar overexpression of the related proteins (Fig. 2A). The infected
cells were then analyzed for their colony-forming efficiency. They were plated at relatively low density and grown in G418 selection medium for 3 weeks. The cells were then stained with Giemsa and the number of colonies were counted in triplicate plates. The overexpression of either WT-HINT1 or HINT1-His
\textsuperscript{112}/Asn
\textsuperscript{112} caused \~60% inhibition of colony formation (P < 0.05) when compared with the control pLNCX2 vector-infected cells (Fig. 2B). We also carried out cell proliferation assays over a period of 8 days and found that both the Hint1-WT and HINT1-His
\textsuperscript{112}/Asn
\textsuperscript{112}–infected cells displayed a 50% inhibition of cell proliferation when compared with the vector-control-infected cells (Fig. 2C).

**Hint1 inhibits JNK2-mediated AP-1 transcription factor activity.** As discussed in Introduction, there is emerging evidence that Hint1 is a tumor suppressor gene and that it might exert its suppressive activity, at least in part, by inhibiting the function of specific transcription factors. Because of the important role of the AP-1 transcription factor in enhancing the expression of genes that stimulate cell proliferation (see Introduction), we examined possible effects of the HINT1 protein on this factor using transient transfection reporter assays. When SW480 cells were transfected with the AP-1-luciferase reporter, they displayed high luciferase activity. Cotransfection with increasing concentrations of a pHA-Hint1-WT plasmid produced a concentration-dependent inhibition of this activity. Two micrograms of this plasmid DNA caused \~60% inhibition (Fig. 3A). The same amount of a HA-Hint1-His
\textsuperscript{112}/Asn
\textsuperscript{112} mutant plasmid caused a similar inhibition. Controls included an empty pHANE plasmid and all assays were normalized for CMV-\beta-galactosidase reporter activity.

JNKs can phosphorylate the c-Jun component of the AP-1 transcription factor, thereby activating its functions (22). Indeed, when we treated SW480 cells with the compound SP600125, which inhibits the kinase activity of both JNK1 and JNK2 (54), there was discovered that HINT1 binds to POSH.\textsuperscript{6} Therefore, we examined a possible role for POSH in mediating the inhibition of AP-1 activity (43). In recent yeast two-hybrid assays, it was found that Hint1 binds to POSH.\textsuperscript{6} Therefore, we examined a possible role for POSH in mediating the inhibition of AP-1 activity.

**The proteins POSH and JNK seem to be the target of HINT1 with respect to inhibition of AP-1 activity.** As reviewed in Introduction, the POSH protein is a scaffold that interacts JNKs and upstream signaling molecules, thereby enhancing the activation of JNK, subsequent phosphorylation of c-Jun, and activation of JNK activity (43). In recent yeast two-hybrid assays, it was discovered that Hint1 binds to POSH.\textsuperscript{6} Therefore, we examined a possible role for POSH in mediating the inhibition of AP-1 activity.

![Figure 4](image-url)  
**Figure 4.** The proteins POSH and JNK play a role in HINT1 inhibition of AP-1 activity. The AP-1-luciferase and \beta-galactosidase plasmid DNAs were cotransfected into SW480 (A) or HEK293 (B) cells as described in Fig. 3. The cells were also cotransfected with pCMV-POSH or pHA-Hint1 plasmid DNAs. Luciferase activity was assayed at 36 h after transfection and normalized for \beta-galactosidase activity. All assays were done in triplicate. HEK293 (C) and SW480 (D) cells were transiently transfected with pCMV-POSH and/or pHA-Hint1 plasmid DNAs. Whole-cell lysates were prepared and analyzed by Western blots to detect the expression levels of p-c-Jun (Ser\textsuperscript{63}) and total c-Jun, using the respective antibodies. Relative levels of p-c-Jun were determined by densitometry. A repeat assay gave similar results.
by HINT1. Indeed, in transient transfection reporter assays using the AP-1-luciferase reporter in SW480 cells, we found that transfection with a POSH plasmid stimulated AP-1 activity, whereas cotransfection with a Hint1 plasmid largely abrogated this stimulation by POSH (Fig. 4A). Very similar results were obtained when these assays were done in HEK293 cells (Fig. 4B).

As mentioned above, phosphorylation of c-Jun by JNK is a major rate-limiting event in the activation of AP-1. Therefore, in parallel with the studies shown in Fig. 4A and B, we also collected cell extracts from SW480 cells that were transfected with the various plasmids and analyzed by Western blot cellular levels of c-Jun and p-Jun using the respective antibodies. Figure 4C indicates that in the control cells (Fig. 4C, lane 1) and in the cells transfected with Hint1, there was only a very low level of p-Jun. Transfection with POSH markedly increased the level of p-Jun (Fig. 4C, lane 3), whereas cotransfection with POSH and Hint1 abrogated the simulation obtained with POSH alone (Fig. 4C, lane 4). In contrast, the total level of the c-Jun protein was similar in all four lanes. Similar results were obtained when this experiment was repeated with the HEK293 cells (Fig. 4D).

In view of the above results, it was of interest to determine whether HINT1 binds to POSH in intact cells. Therefore, HEK293 cells were transfected with expression vectors encoding FLAG-tagged POSH and HA-tagged HINT1, either alone or in combination, and cell extracts were prepared. When proteins in these extracts were immunoprecipitated with an anti-FLAG antibody and analyzed by Western blots using an anti-HA antibody, we observed a distinct HINT1 protein band only in the extract from cells transfected with both the POSH-FLAG and the Hint1-HA plasmids (Fig. 5A, lane 3). In a reciprocal experiment when the cells extracts were immunoprecipitated with an anti-HA antibody and the Western blots were probed with an anti-FLAG antibody, we only observed the POSH protein in the extract of cells that had been cotransfected with both the Hint1 and the POSH plasmids (Fig. 5B, lane 3). Similar coimmunoprecipitation results were obtained when the same studies were done with SW480 cells (data not shown).

In additional studies, cotransfection studies with Myc-tagged JNK2 and HA-tagged Hint1 plasmid DNAs, we obtained evidence that in HEK293 cells, HINT1 also forms an immunocomplex with JNK2 (Fig. 5B). However, similar assays failed to show that HINT1 forms an immunocomplex with c-Jun (data not shown). Taken together, these results provide evidence that the HINT1 protein can bind to a POSH-JNK2 complex in vivo. This may explain its ability to inhibit the phosphorylation of c-Jun by JNK2 (Fig. 3C) and thereby cause inhibition of AP-1 activity.
Discussion

In previous studies with genetically engineered mice, we obtained evidence that Hint1 is a novel haploinsufficient tumor suppressor gene (2). However, its precise mechanism of action and relevance to various human cancers is not known. In the present study, we found a low level of expression of the HINT1 protein in the SW480 cell line when compared with four other human colon cancer cell lines and obtained evidence that this is due to methylation of the promoter region of the HINT1 gene in SW480 cells (Fig. 1). We obtained similar findings in a subset of human hepatoma and prostate cancer cell lines. Other investigators found decreased expression of HINT1 in a subset of human NSCLC cell lines, which also seemed to be due to promoter methylation based on studies using 5-AzadC, although this study did not actually determine the state of methylation of the promoter region of HINT1 (3). These decreases in expression of HINT1 in a variety of cancer cell lines may be functionally significant, although these cells retain detectable levels of the HINT1 protein because of our evidence that Hint1 is haploinsufficient with respect to tumor suppression (2). Further studies are required to determine whether epigenetic silencing of HINT1 expression, or inactivating mutations in this gene, occur in a significant fraction of specific types of primary human cancer.

In the present study, we showed that increased expression of HINT1 in SW480 cells inhibited their growth (Fig. 2) and we obtained similar results with MCF-7 human breast cancer cells (4). Other investigators found that increased expression of HINT1 also inhibits the growth of H522 and H538 NSCLC human lung cancer cells (3). Furthermore, transfection of Hint1 in SW480 or MCF-7 cells induced apoptosis and this was associated with increased expression of p53 and Bax and decreased expression of Bcl-2 (19). Taken together, these findings strongly suggest the potential role of Hint1 as a tumor suppressor in a variety of human cancers. The HINT1 protein contains a canonical HIT motif (i.e., His-X-His-X-His-X-X), in which X is a hydrophobic amino acid (4), but the relevance of this motif to the tumor-suppressive effect of HINT1 is not known. Therefore, we generated a point mutant form of Hint1 with a histidine to asparagine substitution at position 112. This mutant also inhibited growth of SW480 cells (Fig. 2) and H522 cells (data not shown) and, as discussed below, also inhibited AP-1 activity (Fig. 3A and C). Further studies are required to assess the possible roles of other components of the HIT motif in these assay systems.

As discussed in Introduction, there is evidence that HINT1 can inhibit the activities of specific transcription factors. Thus, in mast cells, HINT1 inhibits the activities of the bHLH transcription factors MITF and USF2 by binding directly to these proteins (16, 17), and in SW480 cells, HINT1 inhibits β-catenin/TCF4 activity by binding to the associated proteins Pontin and Reptin (18). The latter study also found that because of this effect, HINT1 also inhibits the transcription of cyclin D1 and expression of the cyclin D1 protein (18).

In the present study, we focused on possible effects of HINT1 on the activity of the AP-1 transcription factor because of its important role in cell proliferation and also in tumor promotion and progression in various type of cancer (21, 27, 29, 55). Indeed, we found that in transient transfection assays, SW480 cells displayed high transcriptional activity of an AP-1-luciferase reporter and this activity was inhibited in a dose-dependent manner by cotransfection with plasmid DNAs encoding either WT HINT1 or the His<sup>112</sup>/Asn<sup>112</sup> mutant HINT1 protein (Fig. 3A). HINT1 inhibition of AP-1 activity was also seen in MEFs (Fig. 3D) and HEK293 cells (Fig. 4B). Thus, this inhibitory effect of HINT1 was highly reproducible and not confined to SW480 cells, but for reasons that are not apparent, it was only in the range of 50% to 70%. Further studies with JNK inhibitors and with MEFs deleted in either JNK1 or JNK2 indicated that the AP-1 activity in our cell systems was dependent on JNK2 but not on JNK1 (Fig. 3B–D). This finding is consistent with previous studies on the role of JNK2 in AP-1 activity, presumably by phosphorylation of the c-Jun component of AP-1 (36). The Rho, Rac, and Cdc42 GTPases coordinately regulate organization of the actin cytoskeleton and also the JNK/MAPK signaling pathway. The protein POSH acts as a scaffold that binds activated Rac1, MKK4/7, MLKs, and JNK1/2, thus enhancing JNK activation and downstream signaling events (43–45). During the course of the present study, three of our colleagues (Z. Xu, A. Sproul, and L.A. Greene) discovered that Hint1 binds to the POSH protein in the yeast two-hybrid system, and we found that POSH is expressed in SW480 cells and other cancer cell lines. Therefore, we then focused on this protein. We found that transfection with POSH enhanced AP-1 activity in SW480 cells and that this stimulation was inhibited by cotransfection with Hint1 (Fig. 4A). We also found that POSH increased cellular levels of the phosphorylated form of c-Jun and stimulated AP-1 transcription factor activity in HEK293 cells, and both of these effects were inhibited by cotransfection with Hint1 (Fig. 4B and C). Coimmunoprecipitation studies provided evidence that in intact cells Hint1 binds to both POSH and JNK, presumably as a complex containing both proteins (Fig. 5A and B). Taken together, these results suggest that Hint1 inhibits activation of the AP-1 transcription factor by binding to a POSH-JNK2 complex, thereby inhibiting the ability of JNK2 to phosphorylate the c-Jun component of AP-1. Because of the results obtained in the yeast two-hybrid system, we assume that Hint1 binds directly to POSH but the molecular details of this interaction and the possible role of the HIT motif in the Hint1 protein remain to be determined. In addition to its role in JNK signaling, POSH also plays a separate role via its RING domain as an E3 ubiquitin ligase that mediates proteosomal degradation of the protein Hrs. This kinase plays an essential role in multivesicular body biogenesis. Indeed, POSH localizes to early endosomes (45). Further studies are required to determine whether Hint1 also modulates this function of POSH.

The present study indicating that Hint1 inhibits the activity of the AP-1 transcription factor, coupled with previous evidence indicating that it also inhibits the activities of the transcription factors β-catenin (18), MITF (16), and USF2 (17), suggests that a major cellular function of Hint1 is to modulate gene transcription. Presumably, this explains its potential role as a tumor suppressor. Obviously, the possible clinical relevance of impairments in the expression and function of Hint1 with respect to specific human cancers remains to be determined.

Acknowledgments

Received 12/18/2006 accepted 3/2/2007.

Grant support: Entertainment Industry Foundation-National Colorectal Cancer Research Alliance, the T.J. Martell Foundation, and the National Foundation for Cancer Research (L.B. Weinstein); National Natural Science Foundation of China grant 30660184 (L. Wang); and NCI grants ES09089 (R.M. Santella).

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.

We thank Dr. Zigang Dong for providing the JNK WT and knockout MEFs, Dr. Audrey Minden (Columbia University) for providing the POSH plasmid, and Lloyd A. Greene and Andrew Sproul (Columbia University) for providing the POSH constructs and antibody and their available advice.
References


3. Yuan BZ, Jefferson AM, Popescu NC, Reynolds SH. FRA3B expression is frequently increased in multiple malignancies and in normal tissues and mimics alterations previously described in tumours. Oncogene.


Hint1 Inhibits Growth and Activator Protein-1 Activity in Human Colon Cancer Cells

Lin Wang, Yujing Zhang, Haiyang Li, et al.