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

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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-azadeoxycytidine 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 pHA-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 haploinsufficient 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).
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-tetradecanoylphorbol-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-terminal 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 was found to preferentially mediate apoptosis, whereas JNK2 tends to enhance cell proliferation (38–42). In addition, JNK1 deficiency increases TPA-induced AP-1 DNA binding activity (37).

The Rho, Rac, and Cdc42 GTPases coordinate regulation of the actin cytoskeleton and the JNK/mitogen-activated protein kinase (MAPK) pathway. Plenty of SH3 (POSH) is a scaffold protein that links activated Rac1 to the downstream JNK pathway by binding, in a multiprotein complex, JNK1/2, MAPK kinase (MKK) 4/7, and mixed-lineage kinases (MLK; ref. 43). Ectopic expression of POSH leads to activation of the JNK pathway and also nuclear translocation of nuclear factor-κB (44). Recent studies have revealed a separate function of POSH, in which it colocalizes in cells with the protein hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs). The RING domain of POSH specifically regulates the stability of Hrs, but not that of JNK1, via an ubiquitin-proteosomal degradation pathway (45). POSH may be directly relevant to the molecular mechanism of action of HINT1 because recent two-hybrid studies in yeast using POSH revealed an unexpected interaction with HINT1. Therefore, the possible role of POSH with respect to effects of HINT1 on AP-1 activity has been specifically explored in the present study.

With the above findings as a background, in the present study, we have explored the relevance of the Hint1 gene to human colon cancer by examining its level of expression in a series of five human colon cancer cell lines. We found that in SW480 colon cancer cells, HINT1 expression is partially suppressed by promoter methylation and that increased expression of HINT1 inhibits the growth of these cells. We also found that HINT1 inhibits the transcriptional activity of AP-1 and that this seems to be due to binding of HINT1 to the POSH protein, which subsequently inhibits the phosphorylation of c-Jun by JNK2. The latter effects could contribute to the tumor-suppressive activity of the Hint1 gene.

**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 mutant 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 DF10 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 mmol/L hydroquinone and 3 mol/L sodium bisulfite 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′-TTGGGCGCGGGTTTTGTTTGCTTAC-3′ (methylated forward) and 5′-GTGACCGGCACCCCCACTA-3′ (methylated reverse) and 5′-TTGGGTTTTGTTTGGTTTATG-3′ (unmethylated forward) and 5′-GATACAAACACCCACCCACTA-3′ (unmethylated 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, 72°C for 3 min, and a final extension at 72°C for 7 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-Azade (1 μmol/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 a 25 μL reaction mixture containing 12.5 μL of 2× reaction mix, 2 μL (1 μg) template RNA, 0.25 μL sense primer (20 μmol/L), 0.25 μL antisense primer (20 μmol/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′-GAGATGGCAGATGAGATT-3′ (forward) and 5′-TAAACCCAGGAACTGTTCTG-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control; the primer set

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


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included GAPDH-RT 5'-TCGTGGAAAGACTAGTACGCTA-3' (forward) and 5'-GGGATGATTGTTCTGGAGAGC-3' (reverse). Twelve micro liters of each PCR product were analyzed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination, and the intensities of the specific bands were analyzed.

### 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'-CCGGAATTCGATGGCAGG-3' (forward) and 5'-CCGGAATTCCTCCCAAGAAGAGAA-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/His112 to Asn112. The above-described pHA-Hint1-WT plasmid was used as the template. Twelve microliters of each PCR product were then cloned into the retroviral shuttle plasmid pLNCX2 (Clontech) by EcoRI and NotI endonuclease cut sites. Thus retroviral-HA-Hint1-His112/Asn112 plasmid were constructed.

Retrovirus-mediated Hint1 gene transduction. PT67 packaging cells by directly applying the diluted retrovirus as described above. The cells were replated into 100-mm dishes with 1 × 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 analyzed on 2% agarose gels and visualized under UV illumination. All assays were repeated three times, and gave similar results. C, cells were treated with 5-AzadC (1 μmol/L) for 96 h. Total RNA was isolated and semiquantitative RT-PCR was done with specific Hint1 and GAPDH (internal loading control) primers. PCR products were analyzed on 2% agarose gels and visualized under UV illumination. All assays were repeated three times, and gave similar results. D, cells were treated with 5-AzadC as described above, and HINT1 expression levels were detected by Western blot analysis. Actin was used as loading control. No treatment (−); 5-AzadC (+). Expression ratios for Hint1 mRNA were calculated after normalization for GAPDH. All assays were repeated three times and gave similar results. D, cells were treated with 5-AzadC as described above, and HINT1 expression levels were detected by Western blot analysis. Actin was used as loading control. No treatment (−); 5-AzadC (+). Expression ratios for Hint1 protein were calculated after normalization for β-actin. Assays were done in duplicate and gave similar results.
(provided by Lloyd A. Greene, Columbia University, New York, NY). Antimouse and antirabbit IgG (Amersham Biosciences) antibodies were used as the secondary antibodies. Each membrane was developed with an enhanced chemiluminescence system (Amersham Biosciences). The intensities of specific protein bands were quantified with NIH Image software version 1.62, corrected for the intensity of the respective β-actin band, and expressed as a ratio with respect to a control lane.

**Luciferase Reporter Assays**

Cells were plated at 1 x 10^5 per 35-mm-diameter plates 18 h before transfection. The cells were transfected with 500 ng of the pAP-1-luciferase reporter [kindly provided by Dr. 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-Myr 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-Myr (43) separately or together, as indicated, using the Lipofectin reagent. At 48 h after transfection, cells were washed twice with cold PBS and incubated with ice-cold lysis buffer [10 mmol/L Tris (pH 7.4), 1.0% Triton X-100, 0.5% NP40, 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 lysates (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
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 promoter methylation. Indeed, a previous study indicated that expression of HINT1 in H522 cells could be induced by treating the cells with the DNA-demethylating agent 5-AzadC, although these investigators did not actually examine the extent of methylation of the Hint1 promoter in these lung cancer cells (3). Therefore, we treated both H522 and SW480 cells with 1 μmol/L 5-AzadC for 96 h and then examined by RT-PCR and Western blots the levels of expression of HINT1 mRNA and protein, respectively (Fig. 1C). In the H522 cells, treatment with 5-AzadC led to about a 1.7-fold increase in expression of HINT1 mRNA and protein, and in SW480 cells, the increase was ~ 1.3-fold (Fig. 1C). In contrast, similar treatment of HCT15, HCT116, HT29, or Caco2 cells with 5-AzadC did not increase cellular levels of HINT1 mRNA (data not shown), which is consistent with our evidence that the Hint1 promoter is not methylated in these cells (Fig. 1B). Taken together, the studies described in Fig. 1 provide evidence that the relatively low level of expression of HINT1 in SW480 cells is 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 specifically 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).
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\textsubscript{112}/Asn\textsubscript{112} caused \textasciitilde60% 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\textsubscript{112}/Asn\textsubscript{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 \textasciitilde60% inhibition of cell proliferation (Fig. 3A). The same amount of a HA-Hint1-His\textsubscript{112}/Asn\textsubscript{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 JNK2, we used a homologous set of three MEF cell lines that were either WT (+/+), or JNK2\textsuperscript{−/−} to study the relative roles of JNK1 and JNK2 with respect to inhibition of AP-1 activity. AP-1 luciferase reporter assays indicated that the WT-MEF and MEF JNK1\textsuperscript{+/−} cells had relatively high AP-1 activity, and with both cell types, cotransfection with HINT1 markedly inhibited this activity. However, AP-1 activity was very low, both in the absence and in the presence of HINT1 expression in the MEF JNK2\textsuperscript{−/−} cells (Fig. 3C). These results, together with those obtained in Fig. 3B, indicate that JNK2, but not JNK1, plays a critical role in activating the AP-1 transcription factor. These findings are consistent with previously published studies (36, 37).

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 haplosufficient 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 haplosufficient 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. 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 His112/Asn112 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 coordinate regulates 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 activity 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 proteosome 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.

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References


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