A novel protein overexpressed in hepatoma accelerates export of NF-κB from the nucleus and inhibits p53-dependent apoptosis

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Summary

NF-κB is a transcription factor that can protect from or contribute to apoptosis. Here we report identification of HSCO that binds to NF-κB and inhibits apoptosis. HSCO mRNA was overexpressed in 20 of 30 hepatocellular carcinomas analyzed. Overexpression of HSCO inhibited caspase 9 activation and apoptosis induced by DNA damaging agents, while it augmented apoptosis induced by TNFα. Like IκBα, HSCO inhibited NF-κB activity and abrogated p53-induced apoptosis. However, the underlying mechanism was different. HSCO is a nuclear-cytoplasmic shuttling protein, bound to RelA NF-κB, and HSCO sequestered it in the cytoplasm by accelerating its export from the nucleus. These results suggest that overexpression of HSCO suppresses p53-induced apoptosis by preventing nuclear localization of NF-κB during signaling and thus contributes to hepatocarcinogenesis.

Introduction

One of the most frequent carcinomas throughout the world is hepatocellular carcinoma (HCC) (Murray and Lopez, 1997; Hamilton and Aaltonen, 2000). HCC is rapidly fatal, with a life expectancy of about 6 months from time of diagnosis. The disease is more prevalent in parts of Africa and Asia than in North and South America and Europe, with a strong etiological association with viral hepatitis, hemochromatosis, known carcinogens, and toxins. In areas where exposure to aflatoxin B_1 is prevalent, mutation of the p53 gene, one of the most commonly mutated genes identified in various types of human cancers (Vogelstein et al., 2000), is observed in 50%–67% of HCCs (Tannapfel and Wittekind, 2002). In areas such as Japan, Europe, and USA, however, p53 mutation is not frequent, especially in low-grade or low-stage HCCs (Konishi et al., 1993). To date, many genes have been shown to be altered in HCCs, but the molecular mechanisms of hepatocarcinogenesis have not been fully elucidated (Feitelson et al., 2002).

The vertebrate NF-κB/Rel family of transcription factors includes five members: RelA (p65), RelB, c-Rel, NF-κB1(p105)/p50, and NF-κB2(p100)/p52 (Baldwin, 1996; Pahl, 1999). These can form most combinations of homodimers or heterodimers, which have distinct affinities for DNA target sites. The most abundant forms are RelA-p50 heterodimers and RelA-RelA homodimers. The NF-κB/Rel family plays major roles in the inducible expression of a large number of genes involved in inflammation, host defense, cell survival, and proliferation. NF-κB is one of the key proteins that modulates the apoptotic response (Barkett and Gilmore, 1999; Karin and Lin, 2002). The *rela* knockout mouse dies in utero as a result of massive liver apoptosis (Beg et al., 1995). Fibroblasts established from *rela* knockout mice show increased sensitivity to tumor necrosis factor α (TNFα)-induced apoptosis, and RelA expression reverses their sensitivity to TNFα in these cells (Beg and Baltimore, 1996). The accumulating data have suggested a role for NF-κB in the prevention of apoptosis. However, under some circumstances, activation of NF-κB is required for apoptosis (Barkett and Gilmore, 1999). For example, Dengue virus-infected HepG2 hepatocytes undergo apoptosis in vitro, and treatment of these cells with NF-κB decoys inhibits the virally induced cell death (Marianneau et al., 1997). Ryan et al. (2000) have shown that NF-κB activation is required for p53-mediated apoptosis and that *rela*$^{-/-}$ mouse embryonic fibroblasts are resistant to p53-induced cell death. Depending on a variety of circumstances,

SIGNIFICANCE

Although there are many modalities of treatment for hepatocellular carcinoma (HCC), the prognosis is unsatisfactory. We identified a novel phylogenetically conserved protein, HSCO, that is overexpressed in a substantial number of human HCCs and so is likely to have relevance to clinical cancers. A function for HSCO was identified in the cytoplasmic sequestration of RelA NF-κB, resulting in the inhibition of p53-mediated apoptosis. Thus, HSCO is a promising target for HCC treatment, especially in combination with DNA-damaging agents. The present study will contribute to the development of therapeutic strategies to repair the apoptotic p53 response in cancers.

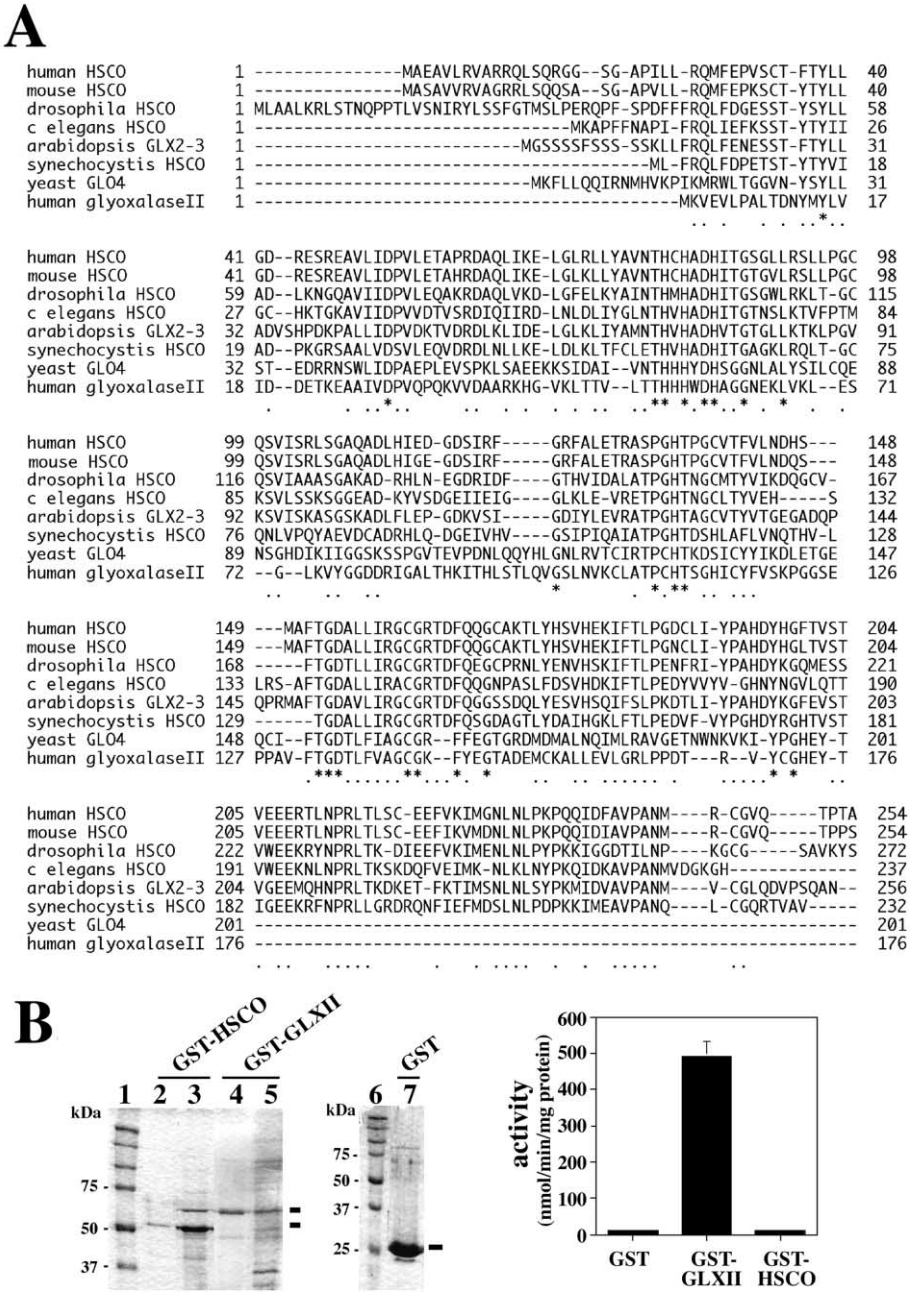

Figure 1. Identification of HSCO and its relation to glyoxalase II

A: Deduced amino acid sequence of human HSCO and its alignment with those of human glyoxalase II (accession number X90999), putative HSCOs in the mouse, *Drosophila* (AE003826), *C. elegans* (Z68493), *Arabidopsis* (U74610), *Synechocystis* (D90909), and yeast GLO4 (CAA99230). Identical and conserved amino acid residues are indicated by asterisks and dots, respectively. The human and mouse sequence data are available from GenBank/EMBL/DDBJ under accession number D83198 and AB049623, respectively. **B:** No glyoxalase II activity detected in HSCO. Left: GST-HSCO, GST-human glyoxalase II (GLXII), and GST alone were purified from bacterial lysates with glutathione beads. Parts of the samples were separated by SDS-PAGE and stained with silver. Lanes 2, 4, and 7: purified samples; lanes 3 and 5: lysates; lanes 1 and 6: molecular weight markers. Right: Glyoxalase II activity in purified proteins.

including the cell type and the inducing agent, NF-κB complexes seem to have pro- or antiapoptic effects.

To identify genes involved in hepatocarcinogenesis and find targets for therapeutic agents, we have constructed subtracted cDNA libraries enriched for the genes overexpressed in HCCs, and previously identified gankyrin (Higashitsuji et al., 2000). Gankyrin is an oncogenic ankyrin-repeat protein, was overexpressed in all examined HCCs, and accelerates the degradation of the retinoblastoma gene product (Rb). Here we report the identification and characterization of HSCO. HSCO bound to NF-κB, accelerated its export from the nucleus, and inhibited p53-mediated apoptosis.

Results and Discussion

Identification of HSCO, a novel glyoxalase II-related protein overexpressed in HCC cells

One of the clones overexpressed in HCC encoded a novel protein, which we named HSCO (Hepatoma Subtracted-cDNA library Clone One). The amino acid sequence of HSCO showed similarity to that of human glyoxalase II, but was more similar to those of putative HSCO homologs in the mouse, *Drosophila*, *C. elegans*, *Arabidopsis*, *Synechocystis*, and yeast GLO4 (Figure 1A). In *Arabidopsis thaliana*, five isozymes are known for glyoxalase II, and HSCO showed strongest homology to GLX2-3 in amino acid sequence. HSCO contained a metallo-β-lactamase-

like region that encompasses between 35 and 196 in amino acid sequence. This metallo-β-lactamase-like region is conserved within the related proteins, and a consensus sequence of HXHXD(X)iH(X)jC(X)kH (in single-letter code for amino acids where X indicates any amino acid and i = 55–74, j = 18–24, and k = 37–41) conserved throughout this family is believed to be the Zn(II) ligands (Daiyasu et al., 2001; Zang et al., 2001). The N-terminal and C-terminal extensions have no homology to known proteins.

In biological systems, methylglyoxal is detoxified to S-D-lactoylglutathione by glyoxalase I with reduced glutathione as a cofactor, and S-D-lactoylglutathione is converted to D-lactic acid by glyoxalase II with simultaneous regeneration of glutathione (Zang et al., 2001). Glyoxalase I is selectively overexpressed in some human tumor cells and can function as a resistance factor to antitumor agent-induced apoptosis (Sakamoto et al., 2000). The target(s) of glyoxalase I responsible for induction of apoptosis has not been identified. Because of a suggested key role of glyoxalase system to detoxify cytotoxic methylglyoxal in tumor cells and a structural similarity of HSCO to glyoxalase II, we analyzed the glyoxalase II activity of HSCO in vitro. As shown in Figure 1B, the control GST-glyoxalase II fusion protein showed the expected enzymatic activity. However, HSCO did not show any activity, indicating that HSCO is a phylogenetically conserved protein related to but distinct from glyoxalase II. Possible enzymatic activity of HSCO and its substrates are yet to be determined.

The mRNA level of HSCO was increased between 155% and 1198% (mean, 332%) in 20 of 30 HCCs studied (Figure 2A and data not shown). As shown in Figure 2B, HSCO encoded protein of about 27 kDa in size, and its expression was increased in HCCs compared with those in noncancerous liver tissues. Immunohistochemical analysis revealed that HSCO protein localized mainly in the cytoplasm of tumor cells (Figure 2C). We next examined the relation between the overexpression of HSCO and the clinicopathological findings such as virus infection, α-fetoprotein, histologic grade of HCC, portal involvement, number of tumors, and clinical stage, but no significant association was identified (data not shown).

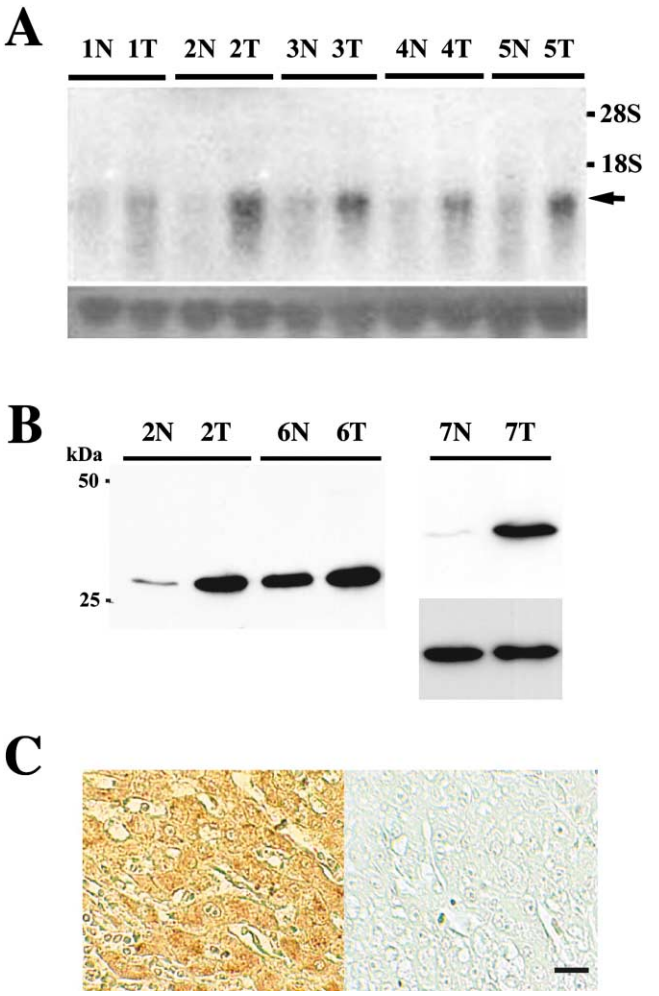

Figure 2. Increased expression of HSCO in hepatocellular carcinoma (HCC) cells

A: Increased expression of HSCO mRNA in HCCs. Total RNA from five pairs of HCCs (1T to 5T) and noncancerous liver tissues (1N to 5N) were analyzed by Northern blotting using human HSCO (top) or 18S rRNA (bottom) cDNA as a probe. An arrow indicates the mobility of HSCO mRNA. **B:** Expression of HSCO protein in HCCs. Cell lysates from three pairs of HCCs (2T, 6T, 7T) and noncancerous liver tissues (2N, 6N, 7N) were analyzed by Western blotting using an anti-HSCO antibody (top) or anti-actin antibody (bottom). **C:** Expression of HSCO protein in tumor cells. Immunohistochemical staining of HCC thin sections was performed using anti-HSCO antibody (left) or normal rabbit IgG (right). The bound antibody was visualized using diaminobenzidine as a substrate. The bar equals 50 μm.

Resistance to p53-dependent apoptosis, but sensitization to TNFα-induced apoptosis conferred by HSCO

To elucidate the role(s) played by HSCO in hepatocarcinogenesis, we stably overexpressed HSCO in mouse NIH/3T3 fibroblasts to the level almost equivalent to those observed in HCCs (Figure 3A). No changes in cellular morphology, population doubling times, or anchorage-dependent growth appeared in the clones analyzed (data not shown).

Using these stable transfectants, we explored an involvement of HSCO in apoptosis. Antitumor agents and TNFα were used to induce p53-dependent and p53-independent apoptoses, respectively (Evan and Vousden, 2001; Johnstone et al., 2002). The NIH/3T3 cells overexpressing HSCO were more resistant to adriamycin and etoposide compared with vector-transfected control cells (Figure 3B). The p53 protein levels were comparable between these cells (data not shown). The activity of caspase 9 induced by etoposide in HSCO-overexpressing cells was 21% ± 3% of that induced in control cells (n = 3, each), consistent with a notion that HSCO conferred resistance to p53-dependent apoptosis. By contrast, HSCO sensitized the cells to apoptosis induced by TNFα (Figure 3B). Several studies have demonstrated that NF-κB plays a critical role in the modification of cellular sensitivity to TNF-mediated killing (Liu et al., 1996; Beg and Baltimore, 1996). TNF sensitivity is also shown to be regulated by oncogenes and viral infection (Perez and White, 2000; You et al., 2002). To exclude a possibility that the observed findings were due to clonal differences of transfectants rather than overexpression of HSCO, we generated the HeLa Tet-On-HSCO cells in which HA-tagged HSCO is expressed at a level equivalent to those observed in HCCs only in the presence of doxycycline (Dox) (Figure 3C). Similar to the findings in NIH/3T3 transfectants, HSCO conferred resistance

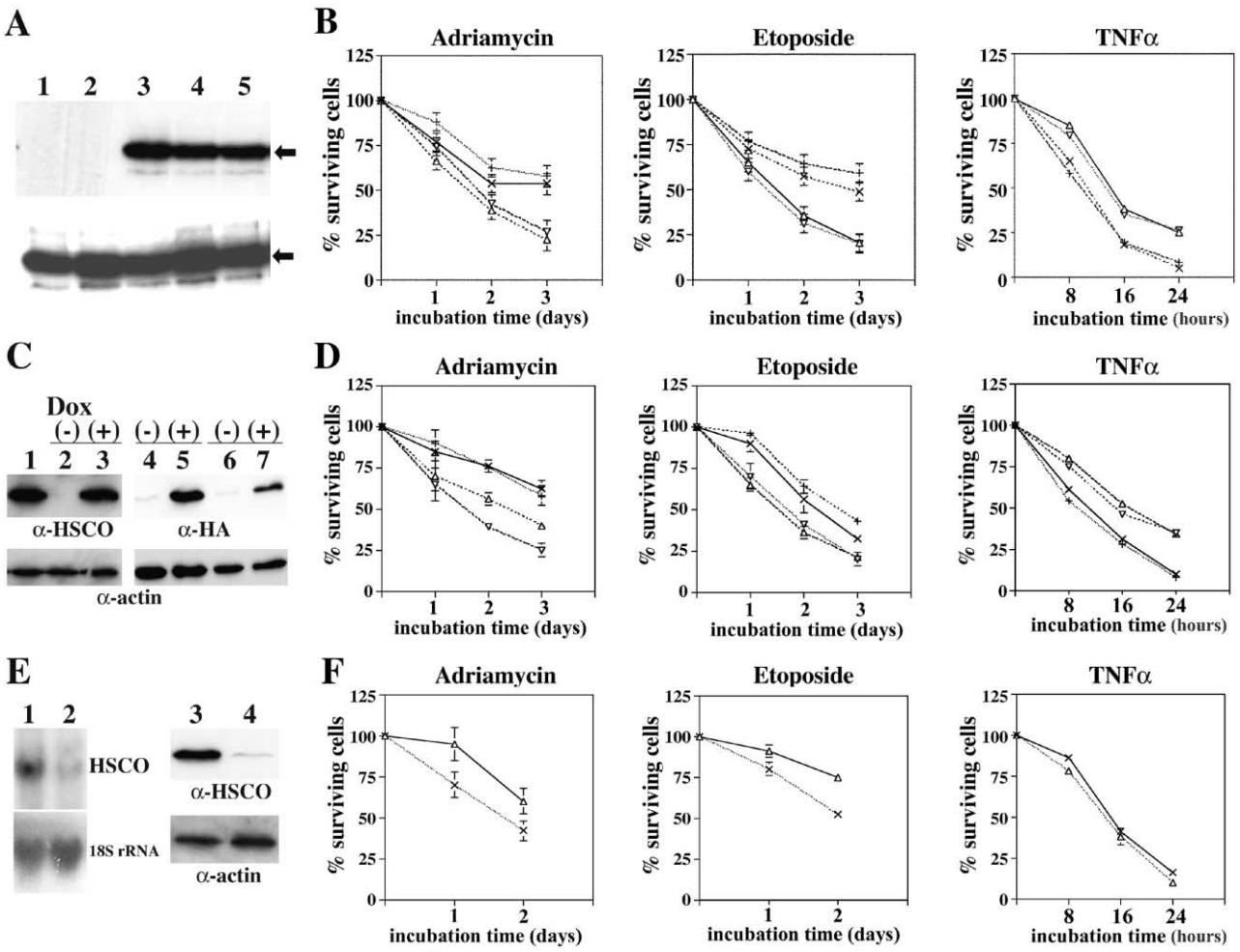

Figure 3. Differential effects of HSCO on apoptosis induced by antitumor agents and TNFα

A and B: Effects of stably overexpressed HSCO on apoptosis in NIH/3T3 transfectants. HSCO protein levels in two clones each of NIH/3T3 transfectants stably overexpressing HSCO (lanes 3 and 4) and vector-transfected control (lanes 1 and 2) and HCC (lane 5) were analyzed by Western blotting using anti-HSCO antibody (**A**). Two clones each of NIH/3T3 trnsfectants overexpressing HSCO (+, ×) and control (△,▽) were exposed to adriamycin (left), etoposide (middle), or TNFα with cycloheximide (right), and the surviving cell numbers were estimated by MTT assay (**B**). **C and D:** Effects of inducibly expressed HSCO on apoptosis in HeLa cells. **C:** HSCO protein levels in HCC (lane 1) and two HeLa Tet-On-HSCO clones (lanes 2 to 5 are from one clone) cultured with (+) or without (−) doxycycline (Dox) were analyzed by Western blotting using anti-HSCO, anti-HA, or anti-actin antibody as indicated. **D:** HeLa Tet-On-HSCO clones cultured in the presence (+, ×) or absence (△,▽) of Dox were assayed as in **B**. **E and F:** Effects of reduced HSCO level on apoptosis in HeLa cells. **E:** HSCO mRNA and protein levels in HeLa cells transfected with pSUPER-HSCOwt (lanes 2 and 4) or pSUPER-HSCOmut (lanes 1 and 3) that contain sequences derived from wild-type or mutant HSCO cDNA, respectively, were analyzed by Northern (lanes 1 and 2) and Western (lanes 3 and 4) blottings using cDNA probes and antibodies as indicated. **F:** HeLa cells cultured in the presence of pSUPER-HSCOwt (×) or pSUPER-HSCOmut (△) were assayed as in **B**.

to apoptosis induced by adriamycin and etoposide, while it sensitized the cells to apoptosis induced by TNFα (Figure 3D).

Recently, efficient and specific downregulation of gene expression has been achieved by using small interfering RNAs (siRNAs) in mammalian cells (Brummelkamp et al., 2002). To address the questions of how general the effect of HSCO on apoptotic sensitivity is and how relevant it is to HCCs that overexpress HSCO, we inhibited the expression of HSCO by siRNAs and assessed the changes in sensitivity to apoptosis. As shown in Figure 3E, siRNAs successfully decreased the level of endogenous HSCO protein in HeLa cells. These cells were more sensitive to apoptosis induced by adriamycin and etoposide compared with control HeLa cells, while no effect was observed on sensitivity to TNFα (Figure 3F). Essentially similar results were obtained with human HCC cell lines, Huh-7 and PLC/PRF/5, using siRNAs or oligoDNAs antisense to HSCO transcripts (data not shown). The reason why the decrease in the level of HSCO did not result in a decrease in sensitivity to TNFα is presently unknown, but one possibility may be that during establishment and/or maintenance of cancer cell lines, those that have lost the increase in TNFα sensitivity had advantage and predominated the culture.

Recently, NF-κB has been shown to be essential for p53-induced apoptosis (Ryan et al., 2000). To assess the effects of HSCO on this NF-κB-dependent apoptosis, we engineered Saos-2 cells, a tumor cell line that does not express endogenous

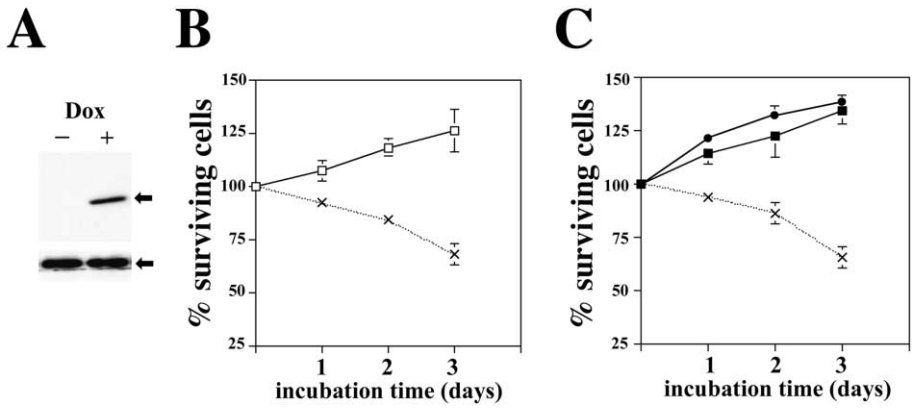

Figure 4. Effect of HSCO on NF-κB-mediated apoptosis in Saos-2 cells

A: Expression of p53 in Saos-2 Tet-On-p53 cells in the presence (+), but not absence (−), of doxycycline (Dox) detected by Western blot analysis using anti-p53 (top) and anti-actin (bottom) antibodies. **B:** Apoptosis of Saos-2 Tet-On-p53 cells induced in the presence (×), but not absence (□), of Dox. **C:** Abrogation of p53-induced apoptosis by HSCO. Saos-2 Tet-On-p53 cells were transfected with plasmid encoding HSCO (●), SRIκBα (■), or empty vector (×) and cultured in the presence of Dox. The numbers of surviving cells were counted as indicated. Data represent the average of triplicates ± S.D. Three independently established Saos-2 Tet-On-p53 cell clones gave similar results, and representative results are shown.

p53, to inducibly express wild-type p53 (Figure 4A). As reported by Ryan et al. (2000), induction of p53 in these cells led to apoptosis (Figure 4B), which was abrogated by expression of super-repressor IκBα (SRIκBα), which completely inhibits NF-κB activity (Figure 4C; Brockman et al., 1995). HSCO abrogated the apoptosis induced by expression of p53 in these cells as efficiently as SRIκBα (Figure 4C). Western blot analysis showed that expression of SRIκBα or HSCO had no effect on the level of p53 protein (data not shown). Taken together, these results demonstrated that HSCO confers resistance to antitumor agent-induced, p53-dependent apoptosis, but sensitizes to TNFα-induced apoptosis in HCC and other cell lines, and suggest that HSCO has a suppressive effect on NF-κB activity. They also suggest that HSCO may be related to resistance of HCC to chemotherapy.

Suppressive effect of HSCO on NF-κB activity and its interaction with RelA/p65 but not IkBα

We, therefore, examined the effect of HSCO on the transactivation activity of NF-κB. After serum withdrawal, luciferase activity was induced in 293 cells transfected with an NF-κB-dependent luciferase reporter construct (Figure 5A). HSCO dose-dependently suppressed this transactivation. HSCO suppressed the NF-κB activities induced by MEKK and RelA/p65 subunit of NF-κB as well (Figures 5B and 5C). These effects were specific to NF-κB because HSCO did not affect AP-1 activity nor E2F-1 activity (Figure 5D and data not shown). Although HSCO has a structural similarity to glyoxalase II (Figure 1), expression of glyoxalase II had no effect on the transactivation activity of NF-κB (Figure 5E). We further confirmed the suppressive effect of HSCO on NF-κB activity by decreasing its endogenous level. In HeLa cells, the HSCO protein levels were decreased by transfection with either pSUPER plasmid that generates siRNAs or oligoDNAs antisense to HSCO transcripts (Figure 3E and data not shown). As shown in Figure 5F, when HSCO level was decreased, the etoposide-induced NF-κB activity was increased in them.

The activity of NF-κB is known to be regulated by IκBα (Karin and Lin, 2002). Activation of NF-κB occurs after the dissociation of the cytoplasmic NF-κB-IκBα complex, which is preceded by the phosphorylation of IκBα. Human papilloma virus oncoprotein E7 targets the IκB kinase complex and impairs IκBα phophorylation and degradation, resulting in attenuation of NF-κB activation (Spitkovsky et al., 2002). The suppressive effects of HSCO on NF-κB activation, however, did not seem to be mediated by IκBα, because HSCO did not affect its amount or phosphorylation status after stimulation with TNFα or etoposide (Figure 6A). By immunoprecipitation analysis, no complex formation was demonstrated between HSCO and IκBα (data not shown).

As HSCO showed an activity similar to SRIκBα (Figure 4C), we suspected that HSCO might bind to NF-κB. To investigate this possibility, we used the HeLa Tet-On-HSCO cells in which HSCO is expressed in the presence of Dox (Figure 3C). After stimulation with TNFα or etoposide and only in the presence of Dox, HA-HSCO was coimmunoprecipitated with endogenous RelA by anti-RelA antibody (Figure 6B, top). We did not observe the coimmunoprecipitation with anti-c-Rel nor anti-p50 antibody (data not shown). In a reciprocal experiment, we detected RelA, but not c-Rel or p50, in the immune complex precipitated by anti-HA antibody (Figure 6B, bottom, and data not shown). We obtained similar results using the 293 cells expressing the exogenous RelA and HA-HSCO (data not shown). The interaction between endogenous HSCO and RelA was also observed in HeLa cells after stimulation with etoposide (Figure 6C). These results demonstrated that the overexpressed endogenous HSCO and RelA specifically interacts in human tumor cells.

The interaction was further analyzed by the in vitro protein binding assay. We prepared recombinant wild-type HSCO protein fused to glutathione-S-transferase (GST). The GST-HSCO fusion protein pulled down the in vitro translated full-length RelA (Figure 6D). No interaction of RelA was observed with the control GST protein. Analysis of various mutant RelA proteins demonstrated that the HSCO-interacting domain resided in residues 1 to 300 of RelA (Figure 6D), indicating that the nuclear localization signal (NLS) of RelA is not involved in this interaction.

Decreased activity and amount of nuclear NF-κB in HSCO-overexpressing cells caused by its accelerated export from the nucleus

To clarify the mechanisms by which HSCO inhibits the activity of NF-κB, we analyzed the effect of HSCO on NF-κB DNA binding activity by the electrophoretic mobility shift assay (EMSA) (Figure 7A). Treatment of 293 cells with etoposide induced an elevation of NF-κB DNA binding activity in the nuclear extracts. Cotransfection of HSCO cDNA in expression vector

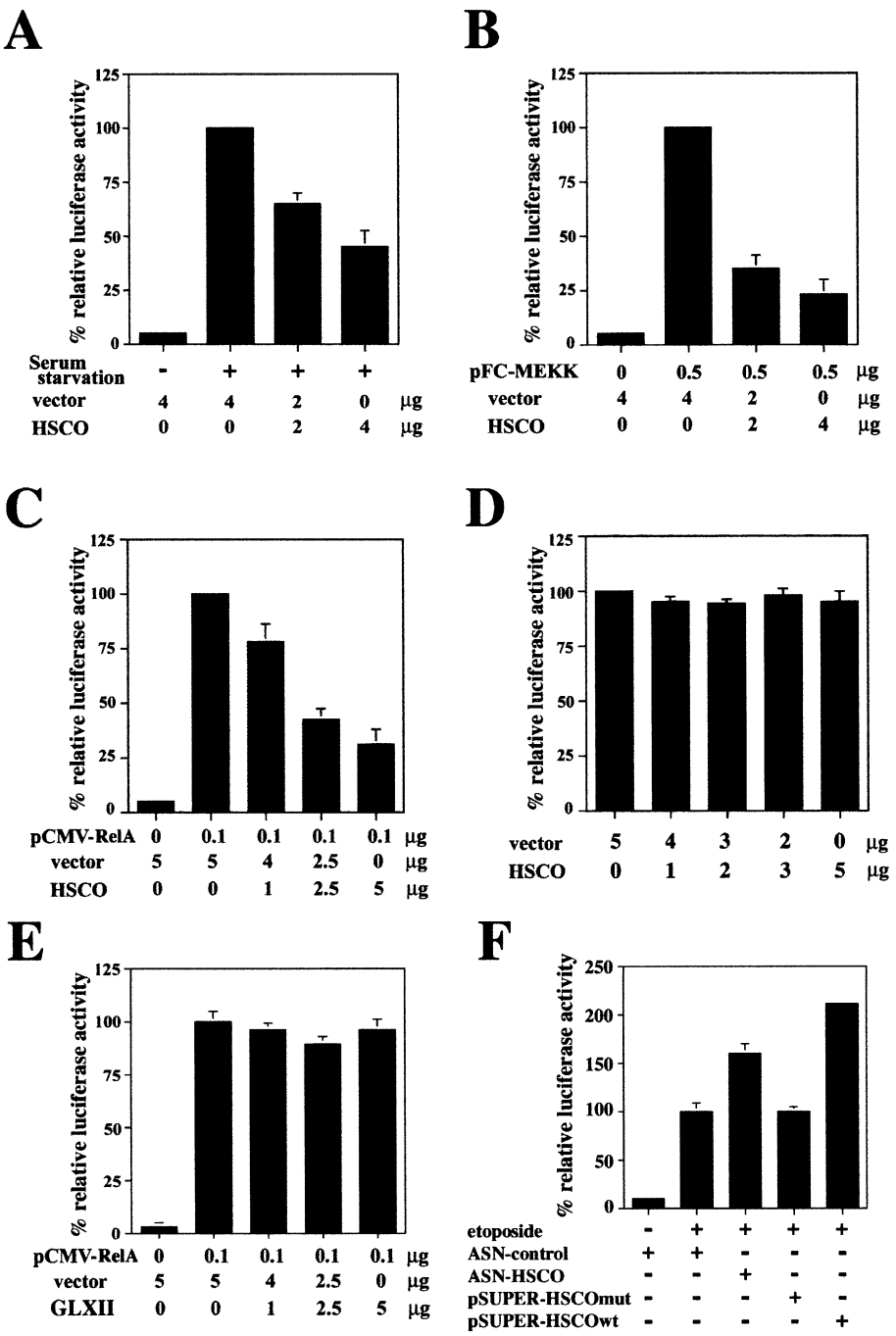

Figure 5. Inhibitory effect of HSCO on NF-κB activity

A: Effect on NF-κB activity induced by serum starvation. 293 cells were cotransfected with NF-κB-responsive luciferase reporter and plasmids expressing HA-tagged HSCO (HSCO) and HA alone (vector) as indicated. Cells were subjected to 32 hr of serum deprivation, and then luciferase activity was determined. **B and C:** Effects on NF-κB activity induced by overexpression of MEKK or RelA. 293 cells were transfected with plasmids expressing MEKK (pFC-MEKK) or RelA (pCMV-RelA) together with NF-κB-responsive reporter and plasmids expressing HA-HSCO as indicated. Luciferase activities were determined 48 hr after transfection. **D:** No effect of HSCO on AP-1 activity. 293 cells were cotransfected with AP-1-responsive luciferase reporter and plasmids expressing HA-HSCO as indicated **E:** No effect of human glyoxalase II on NF-κB activity. 293 cells were cotransfected with pCMV-RelA and NF-κB-responsive reporter together with plasmids expressing HA-glyoxalase II (GLXII) and HA alone (vector) as indicated. Luciferase activities were determined 48 hr after transfection. **F:** Increased NF-κB activity induced by decreased HSCO expression. HeLa cells were cotransfected with the reporter plasmid and either oligoDNAs (ASN-control and ASN-HSCO, control and antisense to HSCO, respectively) or pSUPER constructs (pSUPER-HSCOmut and pSUPER-HSCOwt, siRNA-nonproducing and siRNA-producing constructs, respectively), then treated with etoposide and analyzed as above. The values are expressed as percentage to those in vector-transfected controls. Data represent the average of triplicates ± S.D.

dose-dependently decreased the amount of nuclear NF-κB specifically bound to the DNA probe. The specificity of the band was confirmed by its disappearance with 100-fold excess of unlabeled wild-type κB oligonucleotides but not with those mutated in the κB binding site. The supershift assay using anti-RelA, anti-p50, or anti-cRel antibody indicated that the induced protein-DNA complex mainly composed of RelA-RelA homodimers. These results indicated that the DNA binding activity and/or the amount of nuclear NF-κB is decreased by overexpression of HSCO.

We therefore examined the effect of HSCO on the DNA binding activity of NF-κB in vitro (Figure 7B). When radiolabeled κB sites and in vitro translated RelA were incubated with increasing amounts of GST-IκBα, the amount of bound NF-κB was decreased. By contrast, GST-HSCO up to 10 times molar excess to RelA had no effect on the complex formation between RelA and κB sites. However, when RelA and GST-HSCO were first incubated for 30 min and then radiolabeled κB sites were added, HSCO dose-dependently decreased the amount of RelA-RelA homodimer bound to the κB sites (Figure 7C). GST-glyoxalase II showed no such effect. These results demonstrated that HSCO binds to RelA and partially prevents it from binding to the κB sites, but this effect is weak compared with that of IκBα, and HSCO cannot dissociate RelA from the bound

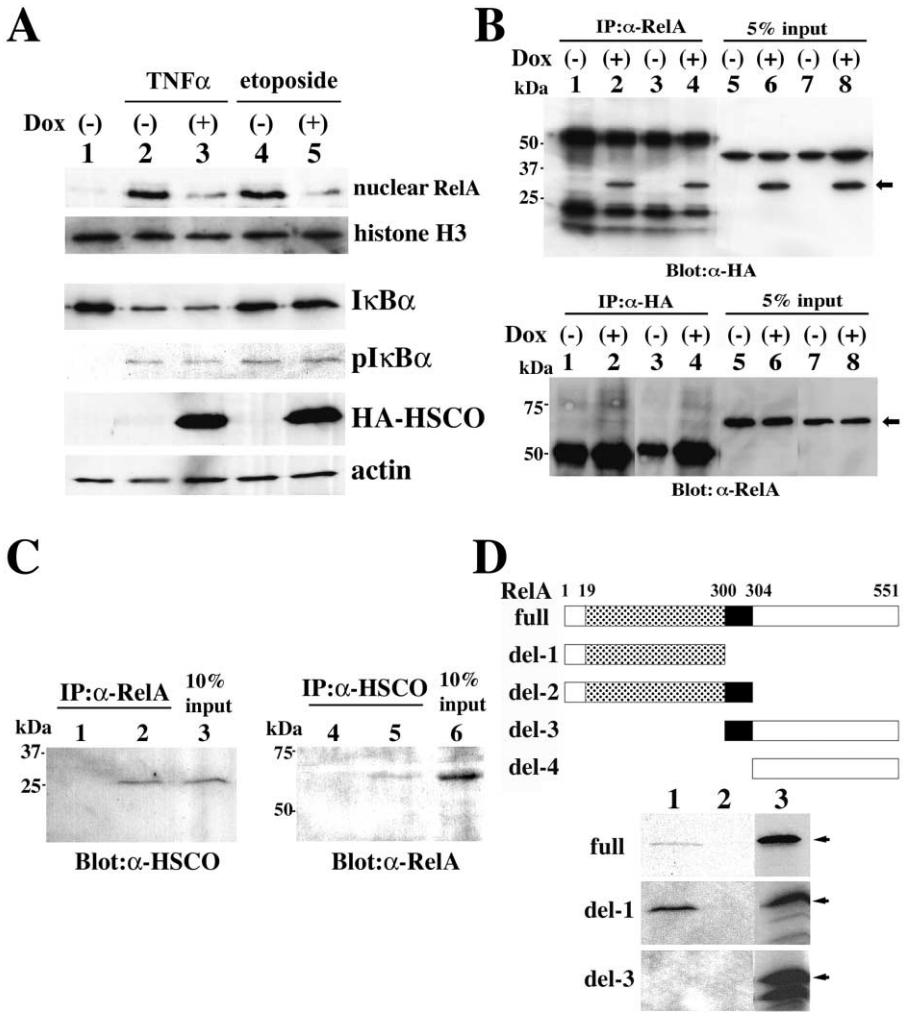

Figure 6. Interaction of HSCO with RelA in vitro and in vivo

A: No effect of HSCO on the amount and phosphorylation of IκBα protein. HeLa Tet-On-HSCO cells were cultured in the presence (+) or absence (−) of doxycycline (Dox) as indicated. They were then treated with vehicle alone (lane 1), TNFα (lanes 2 and 3), or etoposide (lanes 4 and 5). Nuclear extracts (top two gels) or whole cell lysates (bottom four gels) were prepared and analyzed by Western blotting using anti-RelA, anti-histone H3, anti-IκBα, anti-phospho-IκBα (Ser32/36), anti-HA, and anti-actin antibodies as indicated. **B:** Interaction of inducibly overexpressed HSCO with RelA in vivo. HeLa Tet-On-HSCO cells were cultured in the presence (+) or absence (−) of Dox as indicated. They were then treated with TNFα (lanes 1, 2, 5, and 6) or etoposide (lanes 3, 4, 7, and 8), and lysates were prepared from them. 5% of the input lysates (lanes 5 to 8) and one-quarter of immune complexes precipitated by anti-RelA antibody (top, lanes 1 to 4) or anti-HA antibody (bottom, lanes 1 to 4) were analyzed by Western blotting using anti-HA or anti-RelA antibody as indicated. Arrows indicate mobilities of the HA-HSCO (top) and endogenous RelA (bottom). **C:** Interaction of endogenous HSCO with endogenous RelA in vivo. HeLa cells were cultured in the presence (lanes 2, 3, 5, and 6) or absence (lanes 1 and 4) of etoposide, and lysates were prepared from them. One-quarter of immune complexes precipitated by anti-RelA antibody (lanes 1 and 2) or anti-HSCO antibody (lanes 4 and 5), and 10% of the input lysates (lanes 3 and 6) were analyzed by Western blotting using anti-HSCO or anti-RelA antibody as indicated. **D:** Interaction of HSCO with RelA in vitro. Top: Schematic representation of RelA (full) and its mutants (del-1 to -4). The numbers above the bar indicate the amino acid residue numbers in wild-type RelA. Black boxes, nuclear localization signal; shaded boxes, Rel-homology domain. Bottom: Interaction of HSCO with the amino terminus of RelA. ^{35}S-labeled wild-type RelA (full) or its deletion mutants (del-1, del-3, and other data not shown) were incubated with glutathione beads containing GST-HSCO fusion protein (lanes 1) or GST alone (lanes 2). Specifically bound proteins were resolved by SDS-PAGE and autoradiography. One-tenth of the input were run in parallel (lanes 3). Arrows indicate mobilities of the expected recombinant proteins.

κB sites. We next examined the amount of RelA in the nucleus of cells overexpressing HSCO. As shown in Figure 7D, expression of HSCO dose-dependently decreased the amount of nuclear NF-κB. Recently, c-*myc* has been shown to sensitize cells to TNF-mediated apoptosis by interfering with RelA transactivation but not nuclear translocation of NF-κB (You et al., 2002). By contrast, HSCO sensitized cells to TNF-mediated apoptosis by interfering with nuclear accumulation of RelA.

Immunohistochemical analysis suggested that HSCO localized mainly in the cytoplasm of HCC cells (Figure 2C). As shown in Figure 8A, HSCO was diffusely present in the cytoplasm and nucleus in HeLa cells. In the presence of leptomycin B, a nuclear export inhibitor that disrupts the interaction between CRM1/exportin 1 and nuclear export signal (Kudo et al., 1999), HSCO accumulated in the nucleus, indicating that HSCO is a nuclear-cytoplasmic shuttling protein. In the amino acid sequence of HSCO, there are several regions rich in leucine and possibly acting as the nuclear export signal (Figure 1A). We made five different double mutants, i.e., 24A/25A, 68A/70A, 70A/72L, 215A/217A, and 229A/231A, of HSCO and expressed each in COS7, HeLa, and 293 cells, but did not observe accumulation of the mutant proteins in the nucleus (Hiroaki Higashitsuji and J.F., unpublished). Thus, the identity of the nuclear export signal is presently unknown.

When 293 cells were transfected with plasmids expressing RelA, RelA accumulated in the nucleus (Figure 8B, top). After cotransfection with HSCO expressing plasmid, however, RelA colocalized with HSCO and was sequestered in the cytoplasm (Figure 8B, middle). This is consistent with the above finding that expression of HSCO dose-dependently decreased the amount of nuclear NF-κB (Figure 7D). In the presence of leptomycin B, both HSCO and RelA mainly localized in the nucleus (Figure 8B, bottom), indicating that HSCO does not inhibit nuclear translocation of RelA, but stimulates its export from the nucleus. The effect of HSCO on RelA was specific because it showed no effect on the localization of a nuclear-cytoplasmic shuttling protein CIRP (Nishiyama et al., 1998) (data not shown). By treating HeLa Tet-On HSCO cells with TNFα, we also observed that HSCO caused the cytoplasmic accumulation of endogenous RelA during signaling (Figure 8C). To assess

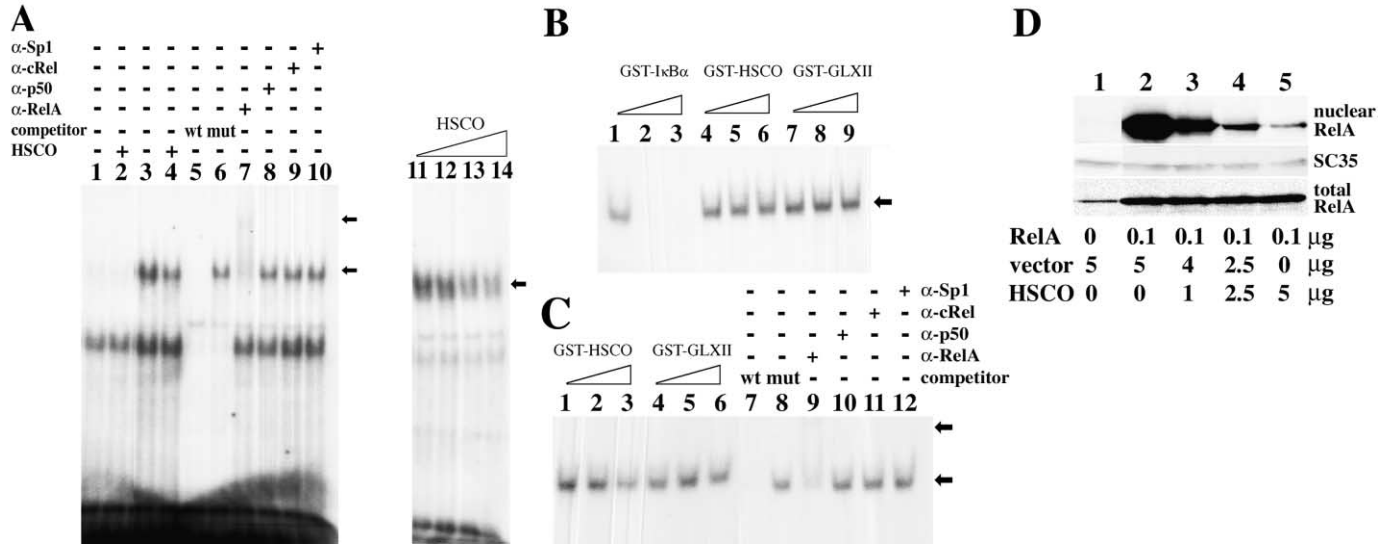

Figure 7. Decreased activity and amount of nuclear NF-κB in cells overexpressing HSCO

A: Inhibition of etoposide-induced nuclear NF-κB DNA binding activity by HSCO in vivo. Equal amounts (10 μg) of nuclear extracts were subjected to electrophoretic mobility shift assays (EMSAs) with a radiolabeled consensus kB site oligoDNAs. 293 cells were transfected with plasmids expressing HA alone or HA-tagged HSCO as indicated and were treated with etoposide (lanes 3 to 14), and nuclear lysates were prepared from them. For competition analysis, a 100-fold excess of unlabeled wild-type (lane 5) or mutant (lane 6) kB site oligoDNAs were added to the reaction mixture. For supershift assays, nuclear extracts were incubated with 1 μg of antibody as indicated (lanes 7 to 10). Mobilities of the NF-kB specific complex and supershifted complex are indicated by arrows. Comparability of the various nuclear extracts was verified by EMSA with a radiolabeled Sp1 probe (data not shown). **B:** Dissociation of RelA from bound DNA induced by IκBα but not by HSCO in vitro. Radiolabeled κB probe was preincubated with RelA, and then increasing amounts (molar ratio to RelA, 1:0, 1:2, and 1:10) of GST-IκBα, GST-HSCO, or GST-glyoxalase II (GLXII) were added to the reaction mixtures as indicated. They were analyzed after further incubation. **C:** Inhibition of RelA binding to DNA by preincubation with HSCO. In vitro EMSA was done as described for **B**, except that RelA and GST-HSCO or GST-GLXII were preincubated for 30 min at 25°C and then mixed with radiolabeled κB site probe. In some reactions, nonradiolabeled wild-type or mutant κB competitors or antibodies were added as indicated. **D:** Decreased amount of nuclear RelA induced by HSCO. 293 cells were cotransfected with plasmids expressing RelA, HSCO, or none (vector) as indicated. The nuclear extracts (top two gels) and whole cell extracts (bottom) were analyzed by Western blotting using anti-RelA antibody (top and bottom) or anti-SC35 antibody (middle).

whether the overexpressed endogenous HSCO observed in HCC cells functions to sequester endogenous RelA as in transfected cells, we treated a human HCC cell line, Huh-7, that overexpresses HSCO with TNFα or etoposide and then analyzed the localization of RelA. RelA was not translocated to the nucleus of Huh-7 cells after stimulation (data not shown). Similar results were obtained when Huh-7 cells were transfected with pSUPER vector that does not produce siRNA and treated with etoposide (Figure 8D, top). However, when expression of HSCO was suppressed with siRNA, RelA accumulated in the nucleus (Figure 8D, bottom), indicating that overexpressed endogenous HSCO sequestered RelA in the cytoplasm in human HCC cells.

The traditional model postulates that, in unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form bound to an inhibitory IκB protein (Foo and Nolan, 1999; Ghosh et al., 1998; Cyert, 2001). IκB binds to and hides the NLS of RelA. Upon stimulation, IκB is rapidly phosphorylated, ubiquitinated, and subsequently degraded by the proteasome. This releases NF-κB, allowing it to translocate to the nucleus, where it can induce the expression of a diverse array of genes. We found that HSCO did not affect IκBα, but did affect NF-κB activity. We found that HSCO binds to RelA subunit of NF-κB and causes its localization in the cytoplasm. However, the NLS of RelA was not involved in the binding. By using leptomycin B, we showed that the accelerated export from, rather than decreased translocation into, the nucleus is the cause of the observed cytoplasmic accumulation of RelA. We demonstrated that HSCO is a nuclear-cytoplsmic shuttling protein. Recently, IκBα has also been shown to shuttle between nucleus and cytoplasm (Arenzana-Seisdedos et al., 1997; Johnson et al., 1999; Tam et al., 2000; Carlotti et al., 2000). Chen et al. (2001) has further demonstrated that RelA is subject to inducible acetylation in the nucleus, and the acetylated form interacts weakly, if at all, with IκBα. It is postulated that after acting as a transcription factor, acetylated RelA is deacetylated, binds to IκBα, and is exported from the nucleus. Since HSCO is mainly bound to the nonacetylated form of RelA (Hiroaki Higashitsuji and J.F., unpublished) and HSCO did not dissociate RelA once it bound to DNA, HSCO probably binds RelA in the nucleus before it is acetylated and exports it to the cytoplasm through a CRM-1-dependent pathway, resulting in inhibition of NF-κB activity.

Several oncoproteins such as human papilloma virus E6 and E7 (Spitkovsky et al., 2002) and c-Myc (You et al., 2002) have been shown to inhibit NF-κB activity by various mechanisms. In the present study we have demonstrated that overexpressed HSCO shows antiapoptotic activity by inhibiting NF-κB activity. Although NF-κB in most situations shows antiapoptotic activities, it also has proapoptotic activities (Barkett and Gilmore, 1999; Ryan et al., 2000). Furthermore, Gapuzan et al. (2002) has recently shown that RelA has tumor-suppressing activity. We have also demonstrated that the expression of HSCO was increased in 67% of HCCs examined and that the overexpressed endogenous HSCO confers resistance to p53-dependent apoptosis induced by adriamycin and etoposide in HCC

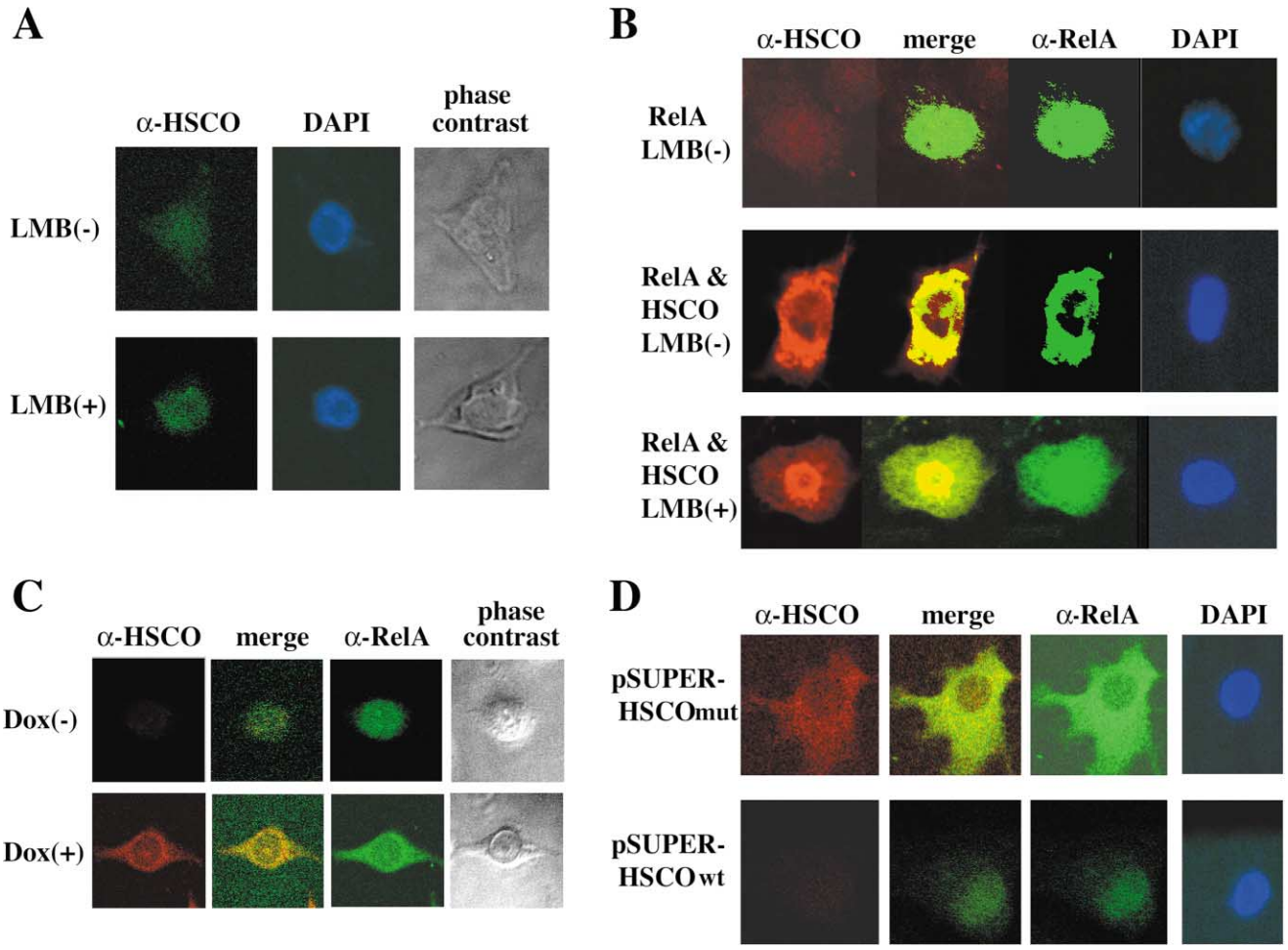

Figure 8. HSCO-induced cytoplasmic sequestration of NF-κB due to accelerated export from the nucleus

A: Nuclear-cytoplasmic shuttling of HSCO. HeLa cells were cultured in the presence (+) or absence (−) of leptomycin B (LMB) and analyzed by confocal microscopy after staining with rabbit anti-HSCO antibody and FITC-conjugated secondary antibody. Blue signals indicate localization of nucleus stained with DAPI. **B:** Cytoplasmic sequestration of exogenous RelA by overexpression of HSCO. COS-7 cells were transfected with RelA cDNA alone (top) or in combination with HSCO cDNA (middle and bottom). They were then cultured in the presence (+) or absence (−) of LMB and analyzed by confocal microscopy after staining with anti-HSCO antibody, anti-RelA antibody, respective secondary antibody, and DAPI. Red, green, and blue signals indicate the localization of HSCO, RelA, and the nucleus, respectively. Yellow signals (merge) indicate colocalization of HSCO and RelA. **C:** Cytoplasmic sequestration of endogenous RelA in cells overexpressing HSCO. HeLa Tet-On HSCO cells were cultured in the presence (+) or absence (−) of doxycycline (Dox), treated with TNFα, and then analyzed by confocal microscopy after staining with anti-HSCO and anti-RelA antibodies as described in **B**. **D:** Cytoplasmic sequestration of endogenous RelA in HCC cells and its correction by siRNA. Huh-7 cells overexpressing HSCO were transfected with pSUPER-HSCOwt that produces siRNA specific to HSCO or pSUPER-HSCOmut that does not produce siRNA. They were stimulated with etoposide and then analyzed by confocal microscopy after staining with anti-HSCO and anti-RelA antibodies and DAPI as described in **B**.

and uterine cancer cell lines. HCC has been regarded as a tumor quite resistant to chemotherapeutic agents (Treiber, 2001). In one study, of the 21 patients treated with etoposide, only 1 showed a partial response. Monochemotherapy has yielded unsatisfactory results with response rates of around 20% but survival is often not improved. Polychemotherapies may have somewhat higher response rates, but with substantial side effects. The most common antiapoptotic lesion that is detected in cancers is inactivation of the p53 tumor-suppressor pathway (Vousden and Lu, 2002). Mutations within the TP53 gene (which encodes p53) itself are found in about half of all cancers, but often at a rather late stage in malignant development. At presentation, most cancers have lost p53 function by one mechanism or another, and in HCCs, especially those at early stages, many are expected to retain wild-type p53 (Konishi et al., 1993) with increased expression of HSCO suppressing, at least partially, its proapoptotic activity. By inhibiting HSCO and repairing the apoptotic p53 response in these HCCs, we may be able to design effective therapeutic interventions. Further studies on the regulation and function of HSCO will contribute to the understanding of molecular mechanism of hepatocarcinogenesis and development of therapeutic strategies against HCCs.

Experimental procedures

Cell culture

NIH/3T3, COS-7, 293, Saos-2, HeLa, Huh-7, and PLC/PRF/5 cells and their derivatives were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of

5% CO_2 in air. For induction of apoptosis, cells were treated with etoposide (100 μg/ml), adriamycin (0.5 μg/ml), or TNFα (50 ng/ml) + cycloheximide (10 μg/ml). In serum deprivation experiment, 293 cells were cultured in medium without fetal bovine serum for 32 hr. The plasmid expressing SRIκBα was a kind gift from Dr. D.W. Ballard, Nashville, TN. Transfection and isolation of stable clones were performed as described (Higashitsuji et al., 2000). 293 and NIH/3T3 cell clones stably expressing HSCO were obtained by transfecting the cells with pMKit-Neo-HSCO plasmids containing HSCO cDNA. As a control, vector alone was transfected. Saos-2 Tet-On-p53 cells were made by cotransfection of Saos-2 cells with pTet-On plasmid and pTRE2 plasmid (Clontech, Tokyo, Japan) containing p53 cDNA, followed by selection of clones inducibly overexpressing p53. Similarly, 293 Tet-On-HSCO cells and HeLa Tet-On-HSCO cells were made by transfecting 293 cells and HeLa cells, respectively, with pTet-On plasmid and pTRE2 plasmid containing HA-tagged HSCO cDNA. Numbers of viable cells were counted by the trypan blue dye exclusion method using a hemocytometer under a microscope or estimated by the MTT assay.

For production of siRNA in the cells, we made the pSUPER vector containing the polymerase-III H1-RNA gene promoter and the gene-specific insert that specifies a 19 nt sequence derived from the target transcript, separated by a short spacer (9 nt long) from the reverse complement of the same 19 nt sequence as described by Brummelkamp et al. (2002). pSUPER-HSCOwt contained the 19 nt derived from the HSCO cDNA (nt number 393 to 411, GenBank accession number D83198) as the target sequnce. As a control, we made pSUPER-HSCOmut containing mutations at HSCO cDNA nt numbers 406 (A to G) and 408 (A to C). The sequence of the oligo DNA antisense to HSCO mRNA was derived from the cDNA nt number 55 to 74. Scrambled 20-mer oligoDNA served as a control.

cDNA cloning and analysis of gene expression

Pairs of primary HCCs and noncancerous liver tissues were obtained from patients undergoing surgery at Kyoto University Hospital. Informed consent was obtained from all patients. cDNA subtraction, cDNA cloning, and nucleotide sequence analysis have been described (Higashitsuji et al., 2000). Immunohistochemistry and Western and Northern blot analyses were performed as described (Danno et al., 2000). An anti-HSCO polyclonal antibody was produced by immunizing rabbits with a carboxy-terminal oligopeptide (NMRCGVQTPTA). For fractionation experiments, cytoplasmic extracts were prepared by lysis of cells in hypotonic buffer containing protease inhibitors and a mixture of phosphatase inhibitors. Nuclear extracts were prepared by plasma membrane permeabilization in hypotonic buffer containing 0.5 mM DTT, 10 mM HEPES (pH 8.0), 10 mM KCl, and 1.5 mM $MgCl_2$. Nuclei were then lysed in hypertonic buffer containing 20 mM HEPES (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, and protease inhibitors. Cytoplasmic and nuclear extracts were analyzed by Western blotting using rabbit anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-histone H3 (Santa Cruz), goat anti-p65/RelA (Santa Cruz), anti-actin (Santa Cruz), mouse anti-SC35 (Sigma Aldrich Japan, Tokyo, Japan), anti-phopho-IκBα (Ser32/36) (Cell Signaling Tech., Beverly, MA), and mouse anti-HA (Roche Diagnostics, Tokyo, Japan) antibodies. Caspase-9 activity was measured by the Caspase calorimetric protease assay kit (MBL, Nagoya, Japan) following the manufacturer's protocol.

Analysis of protein-protein interactions

HeLa Tet-On-HSCO cells were cultured in the presence or absence of Dox. Endogenous RelA NF-κB activity was induced by incubating these cells with TNFα (50 ng/ml) for 1 hr or etoposide (50 μg/ml) for 4 hr. Then cell lysates were prepared, and immunoprecipitation and Western blot analysis were performed using goat or rabbit anti-RelA; rabbit anti-cRel, anti-p50, anti-IκBα, and anti-HSCO; and mouse anti-RelA and anti-HA antibodies as described (Higashitsuji et al., 2000). To analyze interaction between endogenous HSCO and endogenous RelA, HeLa cells were treated with etoposide, and cell lysates were immunoprecipitated using rabbit anti-HSCO or goat anti-RelA antibody immobilized to the protein G support (Seize X Mammalian Immunoprecipitation Kit, Pierce, Rockford, IL), followed by Western blot analysis. Interaction of exogenous RelA with HSCO was examined by cotransfecting 293 cells with plasmid pCMV-RelA (Yang et al., 1999), kindly provided by Dr. T. Okamoto, Nagoya, Japan, and HA-tagged HSCO cDNA or HA cDNA in pCMV4 expression vector. Then, immunoprecipitation and Western blot analysis were performed as above. For the GST pull-down assay, HSCO cDNA was cloned into the expression vector pGEX-4T (Amersham Pharmacia Biotechnology, Tokyo, Japan) and expressed as GST-HSCO fusion protein. ^{35}S-labeled RelA and its mutant proteins were prepared by using a TNT Trascription/Translation System (Promega, Madison, Wisconsin) and assayed as described (Higashitsuji et al., 2000).

Electrophoretic mobility shift assay (EMSA)

293 cells were transfected with plasmids, and 24 hr later the nuclear extracts were prepared as described by Schreiber et al. (1989). Binding reactions were performed by adding 10 μg of the nuclear extracts to a mixture containing 0.1 ng of ^{32}P-labeled, double-stranded κB site oligoDNAs (wild-type κB site, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; mutant κB site with G to C substitution in the Rel DNA binding domain, 5′-AGTTGAGGCGACTTTCCCAGGC-3′) in 20 μl of binding buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 5% glycerol) containing 10 μg of bovine serum albumin and 2 μg of poly(dI-dC):poly(dI-dC). For supershift experiments, 1 μg of antibody directed against RelA, p50, c-Rel, or Sp-1 (Santa Cruz) was added to aliquots of extract and incubated for 30 min on ice before addition of the reaction mixture. Competition reaction mixtures contained a 100-fold molar excess of nonradioactive κB sites.

For in vitro EMSA, human RelA protein was produced in vitro by using a TNT Transcription/Translation System (Promega) as above. Human IκBα, HSCO, and glyoxalase II cDNAs were introduced into pGEX4T vector series (Amersham Pharmacia), and each GST fusion protein was purified according to the manufacturer protocol. The nonradiolabeled consensus and mutant κB oligoDNAs were used in competition assays. Antibodies were added to an EMSA reaction mixture in a supershift assay as above. A dissociation assay was performed by preincubating RelA with radiolabeled κB probe for 15 min at 25°C, followed by addition of 1:2 or 1:10 of GST-IκBα, GST-HSCO, or GST-glyoxalase II. The mixtures were incubated for 15 min at 25°C. Another in vitro RelA EMSA study was performed by preincubating RelA with GST-IκBα, GST-HSCO, or GST-glyoxalase II for 30 min at 25°C, and then κB probe was added. The mixtures were further incubated for 15 min at 25°C. Free and bound DNAs were separated by electrophoresis in a nondenaturing 4% polyacrylamide gel. Gels were dried and exposed to film at −80°C with an intensifying screen.

Assay for glyoxalase II activity

GST-glyoxalase II fusion protein and GST-HSCO fusion protein were prepared as described above using plasmids containing full-length human glyoxalase II cDNA and HSCO cDNA, respectively. Glyoxalase II activity was assayed by measuring the initial rate of hydrolysis of S-D-lactoylglutathione to GSH and D-lactic acid as described by Di Ilio et al. (1995). The assay solution contained 0.5 mM Tris-HCl buffer (pH 7.5), 300 μM of S-D-lactoylglutathione, and appropriate amounts of purified proteins. Glyoxalase II from bovine liver (Sigma) and GST alone served as a positive and negative control, respectively.

Reporter gene assay

Luciferase reporter vectors containing NF-κB binding sites (pNF-κB-Luc) or AP-1 binding sites (pAP-1-Luc) in the promoter were purchased from Stratagene (La Jolla, CA). The luciferase reporter vector containing E2F-1 binding sites was provided by Dr. A. Harel-Bellan, Centre National de la Recherche Scientifique, France. The reporter plasmid and pRL-TK (Promega) were cotransfected with increasing amounts of HSCO cDNA in expression vector pCMV4-3HA with or without pFC-MEKK, pCMV-RelA, or pCMV-GLXII expressing human glyoxalase II, into 293 cells by the calcium phosphate method. After 48 hr, cells were lysed and luciferase activity was measured by a Dual luciferase assay system (Promega) following the manufacturer's protocol. Effects of HSCO on endogenous RelA activity were also analyzed using 293 cells cotransfected with the reporter plasmid, pRL-TK, and increasing amounts of HSCO cDNA as above and then cultured without serum. To analyze the effects of decreasing the HSCO protein level, HeLa cells were cotransfected with the reporter plasmid, pRL-TK, and either oligoDNAs or pSUPER constructs, then treated with etoposide and analyzed as above.

Immunofluorescence staining

Cells were replated on chamber slides after transfection and cultured in the presence or absence of leptomycin B (2 ng/ml) kindly provided by Dr. M. Yoshida, Tokyo, Japan. For some experiments, Dox, TNFα, etoposide, or

vehicle alone were added to the culture with or without transfection. The cells were fixed with PBS containing 4% paraformaldehyde for 30 min, then rendered permeable with PBS containing 0.2% Triton X-100 for 30 min at room temperature. After blocking nonspecific antibody binding sites with bovine serum albumin, the cells were incubated with a rabbit anti-HSCO and/or a mouse anti-RelA antibodies. Bound antibodies were detected with a goat anti-rabbit secondary antibody conjugated to TRITC or FITC (Dako Japan, Kyoto, Japan) and a sheep anti-mouse secondary antibody conjugated to FITC (BD PharMingen, San Diego, CA) and viewed under a confocal microscope (Olympus, Tokyo, Japan). Chromosomal DNA was visualized by staining with blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Inc., Eugene, OR).

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