J. Biochem. **133**, 713–718 (2003)
DOI: 10.1093/jb/mvg091

HCC-Associated Protein HCAP1, a Variant of GEMIN4, Interacts with Zinc-Finger Proteins

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Received January 23, 2003; accepted March 3, 2003

The gene *HCAP1* (HCC-associated Protein 1), one variant of *GEMIN4*, has been mapped in a minimum LOH region on chromosome 17p13.3 and encodes a 1047-amino acid protein. Function predictions based on the amino acid sequence of protein HCAP1 revealed it to contain one helix-loop-helix motif and one leucine zipper domain. Using yeast two-hybrid screening, five zinc-finger proteins were identified as HCAP1-interacting proteins. Among them, NDP52 (nuclear dot protein 52) appeared most frequently in positive clones and was the most strongly interacting protein. Then, the interaction between HCAP1 and NDP52 was confirmed by GST pull-down assay and a coimmunoprecipitation experiment. Moreover, an immunofluorescent staining assay indicated that NDP52 colocalizes with HCAP1 in the cytoplasm. By deletion analysis, the leucine zipper domain of HCAP1 and the zinc finger domain of NDP52 were identified as important regions responsible for the interaction.

Key words: coimmunoprecipitation, co-localization, HCAP1, NDP52, yeast two-hybrid.

Abbreviations: ARS2, similar to arsenate resistance protein; GST, Glutathione-S transferase; HCC, human hepatocellular carcinoma; HCAP1, HCC-associated protein 1; IPTG, isopropyl-beta-D-thiogalactopyranoside; LOH, loss of heterozygosity; MITF, microphthalmia-associated transcription factor; NDP52, nuclear dot protein 52; SNP, single nucleotide polymorphism; SMN, survival of Motor Neurons; OPNG, *o*-nitrophenyl-β-D-galactopyranoside; STAT, signal transducers and activators of transcription; 3AT, 3-amino-1,2,4-triazole.

Previous studies of LOH in HCC have shown that chromosome 17p13 is one of the most frequently affected chromosomal regions (*1–2*). In China, genome-wide screening in HCC further revealed a high incidence of genomic imbalance at 17p13 (65%) (*3*). The LOH region was further minimized to a 1.5 Mb region on 17p13.3 (*4*). After sequencing the PAC579 clone of the region, with screening of a normal human liver cDNA library and RACE approaches, five novel genes were identified within this region, including *HCAP1, HCCS1, C17orf25, HC90* and *CT120* (*5–9*).

The cDNA sequence of *HCAP1* (originally named HC56, GenBank accession number: AF177341) comprises 4752bp and encodes a 1047-amino acid protein (*5*). Its sequence is identical to the last 4752bp of *GEMIN4* cDNA (GenBank accession number: P57678), which encodes a 1058-amino acid protein (*10*). The protein GEMIN4 is one component of the SMN complex, which functions as a factor essential for the efficient assembly of Sm proteins on U snRNAs and likely protects cells from illicit, and potentially deleterious, nonspecific binding of Sm proteins to RNAs (*11*). In our previous studies, *HCAP1* was identified as a variant of *GEMIN4* with a different splicing form and different starting code. Two isoforms are expressed differently in different tissues. Northern blot revealed that *HCAP1* is expressed as a 5Kb transcript (*5*). By means of western blot assays with antiserum against HCAP1, a 118kD protein was found in different cell lines (*12*). From SNP screening within a collection of 140 normal controls and 22 HCC patients, we found a high incidence of SNPs in the coding region of *HCAP1*. There are two kinds of haplotypes, *HCAP1*-N and *HCAP1*-M, which contain common alleles and minor alleles at 5 SNP sites, respectively. Transfection of these two haplotypes into liver cancer cell lines could result in a remarkable reduction in the number of formed colonies and a suppression of cell growth, although the inhibitory effects of *HCAP1*-N were stronger than those of *HCAP1*-M (*13*). Function predictions based on the amino acid sequence of protein HCAP1 with EXPASY programs revealed it to contain a myc-like Helix-loop-Helix motif (256–264 aa) and a leucine zipper domain (703–724 aa).

Considering the close relation of HCAP1 with HCC and its structural features, we searched for HCAP1-interacting proteins by yeast two-hybrid screening and confirmed their presence in cells, which will deepen our understanding of the function of HCAP1.

MATERIALS AND METHODS

PCR Primer Sequences—Primer sequences used in this study are summarized in Table 1.

Plasmid Construction—The full coding region of *HCAP1* was previously isolated from a human liver

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Table 1. **Sequence of primers used in this work.**

Gene	No. of primer	Orientation	Sequence*
HCAP1	1	R	5′-GTCGACTATTCTAGGAGGCTTC-3′
	2	F	5′-ACTAGTGGCCATGGCAATGGAAGC-3′
	3	R	5′-GTCGACCGCCATGGCCGTCATCCAG-3′
	4	F	5′-ACTAGTCTCTAGAGTCTGGATGAAGATCCT-3′
	5	R	5′-GTCGACCACTCTAGAGGCAAACGCGTGT-3′
	6	F	5′-ACTAGTTCAGAAGCTGCTCATCTTCTG-3′
NDP52	N-1	F	5′-ACTAGTCTGCTGTTGCTCCAAGGTGTGATCA-3′
	N-2	F	5′-ACTAGTTCCATAGGCAAGTCCTGG-3′
Gal4 AD	4522	R	5′-TATAACGCGTTTGGAATCACT-3′

*Nucleotide sequences underlined are the restriction sites.

cDNA library and subcloned into pCMV-script vector (pCMV-script-HCAP1) (*5*). The ORF of *HCAP1* was amplified with primer 1 and primer 6 using pCMV-script-HCAP1 as the template, and the amplified 3153bp fragment was inserted into the "bait" vector, pDB-Leu (Invitrogen, Carlsbad, CA). The full ORF sequence of *HCAP1* was inserted into the *SalI*/*NotI* sites of the pGEX4T-2 (Amersham Pharmacia biotech, Buckinghamshire, UK). The full ORF sequence of *NDP52* was constructed into pcDNA3.1 (Invitrogen) under the T7 promoter. To construct eukaryotic expression plasmids, the full ORF sequences of *HCAP1* and *NDP52* were inserted into eukaryotic expression vectors pCMV-Myc and pCMV-HA (Clontech, Palo Alto, CA). The deletions of *HCAP1* and *NDP52* were accomplished by PCR using different primers pairs as listed in Table 1. These deleted *HCAP1* fragments were subcloned into the pDB-Leu vector. The deleted fragments of *NDP52* were subcloned into *salI* and *speI* sites of pPC86 vector (Invitrogen).

Yeast Two-Hybrid Analysis—The yeast two-hybrid system used in our experiment was the ProQust™ (Invitrogen). A human skeleton-muscle cDNA library and a human liver cDNA library were used for screening. In each library, more than 3×10^6 cDNA colonies were screened. After the positive colonies had grown out on leucine-, tryptophan-, histidine-lacking, 3AT (Sigma, St. Louis, MO)-added plates, the prey plasmids were isolated and their cDNA inserts were sequenced. Homology algorithm comparisons were performed using the Blast algorithm through the NCBI web site (www.ncbi.nlm.nih.gov/BLAST).

We quantitatively estimated the expression level of reporter gene *LacZ* with the β-Gal activity according to the manufacturer's instructions. The β-Gal activity was assayed using ONPG (Sigma) as a substitute. Control strains C and D were used in the experiments.

In Vitro Binding Assays—The GST fusion protein was expressed by inducing 0.04 mM IPTG in *E. coli* strain BL-21. Then the expressed products were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia biotech). The plasmid pcDNA3.1-NDP52 was used in the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) and the products with biotinylated-lysines. The purified GST-HCAP1 fusion protein or an equivalent amount GST bound to 20 μl glutathione-Sepharose 4B beads was incubated with 5 μl of the *in vitro* translated and biotinylated protein NDP52 in 200 μl binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM PMSF). After incubation for 2 h at 4°C, the resin was washed 3 times with 200 μl binding buffer and twice with 200 μl buffer H (20 mM HEPES, 50 mM KCl, 20% glycerol, 0.1% Nonidet P-40, and 0.007% β-mercaptoethanol). Then, the pellets were boiled in 20 μl 1 × SDS sample loading buffer, subjected to 12% SDS-PAGE, transferred to a PVDF Plus membrane using the BioRad Mini protein transfer system, and detected with a Transcend™ Colorimetric Translation detection System (Promega).

Co-Immunoprecipitation—For immunoprecipitation experiments, COS-7 cells were transfected independently with pCMV-Myc-HCAP1 or pCMV-Myc-NDP52, or cotransfected with pCMV-Myc-HCAP1 and pCMV-Myc-NDP52. Forty-eight hours after transfection, cells were collected in 0.6 ml of lysis buffer (Roch. IN) for 15 min at room temperature. The lysate supernatant was incubated with protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antiserum against HCAP1 for 4 h at 4°C. The pellets were washed 3 times with lysis buffer. The precipitated proteins were eluted from the beads with protein loading buffer and separated on 10% SDS-PAGE, routinely transferred to PVDF plus membranes, blocked and detected with mouse monoclonal antibody (McAb) c-myc (diluted 1:2,000) (Invitrogen). Bands were visualized by the enhanced chemoluminescence system (Pierce, Rockford, IL).

Immunofluorescent Staining—COS-7 cells were cultured on glass cover slides in a 6-well plate, and cotransfected as indicated with plasmids pCMV-HA-HCAP1 and pCMV-Myc-NDP52. Forty-eight hours later, the cells were washed twice in cold PBS, fixed at 4°C with freshly prepared 4% paraformaldehyde (pH 7.4) for 30 min, permeabilized with 0.2% TritonX-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 30 min. Samples were reacted with a rabbit polyclonal antibody against HCAP1 and McAb c-myc as a primary antibody diluted 1:200 in PBS for 1 h at room temperature. Cells were then rinsed with PBS and stained with FITC-conjugated anti-mouse IgG (Sigma) and Alexa 594-conjugated goat anti-rabbit IgG (Molecular probes, Eugene, OR) diluted 1:500 for 25 min at room temperature. The cells were rinsed three times with 0.5% Tween-20 in PBS. Fluorescent image analyses were performed on an Axioskop 2 universal microscope with an ISIS system (Carl Zeiss, Germany).

Deletion Assay of HCAP1 and NDP52—The deleted fragments of *HCAP1* were cloned into the pDB-Leu vector, and cotransformed with pPC86-NDP52 into MAV203 cells. The deleted *NDP52* fragments were cloned into the pPC86 vector and cotransformed with pDB-Leu-HCAP1.

The RING finger domain and LIM-like domain of the protein NDP52:
392 SIKKCPICKADDICDHTLEQQQMQPLCFNCPICDKIFPATEKQIFEDHVFCHSL 446
The cysteine-rich domain in C-terminal of the protein similar to sprouty homologue 1:
181CEQCGKCKCGECTAPRTLPSCLACNRQCLCSAESMVEYGTCMCLVKGIFYHCSNDDEG
DSYSDNPCSCSQSHCCSRYLCMGAMSLFLPCLLCYPPA 276
The predicted IBR domain of the protein similar to sprouty homologue 1:
91 GCLKLCRRCYDWIHRPGCRCKNSNTVYCKLESCPSRGQGKPSRHTSHLGHAVLPSNA
RGPILSRSTSTGSAASSGSNSSASSEQGLLGRSPPTRPVPGHRSERAIRTQPKQLIVDDLKGSL
KEDLTQHKFICEQCGKCKCGECTAPRTLPSC 201
The Zinc finger-ZZ domain of the protein sequestosome1:
122 HPNVICDGCNGPVVGTRYKCSVCPDYDLCSVCEGKGLHRGHTK 165
The Zinc finger-MIZ domain the protein inhibitor of activated STAT3:
314 VSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCDKKAPY 366
The ZnF-U1 domain of the protein similar to arsenate resistance protein ARS2:
263 DKWLCPLSGKKFKGPEFVRKHIFNKHAEKIEEV 296

Fig. 1. **Domain analysis of HCAP1-interacting proteins.** The results of function predictions based on the amino acid sequences of HCAP1-interacting proteins. Their zinc finger domains and cysteine-rich domains are showed. The protein NDP52 contains a RING finger domain and a LIM-like domain; the protein similar to sprouty homologue 1 contains a cysteine-rich C-terminal and a IBR domain; Sequestosome 1 contains a Zinc finger-ZZ domain, the protein inhibitor of activated STAT3 contains a Zinc finger-MIZ domain; and the protein similar to arsenate resistance protein ARS2 contains a ZnF-U1 domain. The cysteine and histidine residues in the amino acid sequences are underlined.

The expressions of reporter genes *His3* and *LacZ* of transformed clones were detected as described above.

RESULTS

Yeast Two–Hybrid—Using *HCAP1* as the "bait" to screen a human skeletal muscle cDNA library and a human liver cDNA library with a yeast two-hybrid system, we obtained 65 positive colonies. The cDNAs of the positive clones were sequenced and the sequences were used as queries to search the GenBank database. Homology searches showed these clones encoded seven different proteins. Compared with the controls in the yeast two-hybrid system, five proteins interacted with HCAP1 at a stronger level. These included NDP52 (NP_005822), a protein similar to sprouty homologue 1 (XP_036349), Sequestosome 1 (NP_011018), a protein inhibitor of activated STAT3 (BAA78533), and a protein similar to arsenate resistance protein ARS2 (AAH00082). Among them, NDP52 appeared most frequently and interacted most strongly with HCAP1. NDP52 was chose for further studies.

Function predictions based on the amino acid sequences with SMART (hppt;//smart.embl-heidelberg.de/) programs revealed the frequent presence of zinc-finger domains and cysteine-rich domains in these proteins. NDP52 contains a RING finger domain C3HC4 (395–434 aa) and a LIM-like domain C2H2 (420–444 aa); the protein similar to sprouty homolog 1 contains a cysteine-rich C-terminal and an IBR domain (91–201 aa) (the IBR domain has also been called the C6HC domain and DRIL, for double RING finger linked domain); Sequestosome 1 contains a Zinc finger-ZZ domain (121–164 aa); the protein inhibitor of activated STAT3 contains a Zinc finger-MIZ (314–366 aa); the protein similar to arsenate resistance protein ARS2 contains a ZnF-U1 domain (236–263 aa) (Fig. 1).

HCAP1 Can Interact with NDP52 In Vitro and In Vivo—The expression of the fusion protein GST-HCAP1 is shown in Fig. 2A as already well purified.

From Fig. 2B, it was clear that the fusion protein GST-HCAP1 bound to glutathione-Sepharose 4B beads could bind NDP52; glutathione-Sepharose 4B beads coupled to GST alone did not cause NDP52 to bind to the beads. This means that the interaction between NDP52 and HCAP1 occurs not only in yeast cells, but also *in vitro*.

The results of the co-immunoprecipitation experiments are shown in Fig. 3. Two bands in lane1 indicate that both Myc-HCAP1 and Myc-NDP52 are co-expressed in cotransfected COS7 cells. Using the rabbit antiserum against HCAP1, the precipitated proteins were also found to contain two components, Myc-NDP52 and Myc-HCAP1 (lane 2). This result reveals that HCAP1 can also interact with NDP52 in mammalian cells.

Co-Localization of HCAP1 and NDP52 in Cells—Immunofluorescent staining of COS7 cells shown that HCAP1 localizes in the cytoplasm and nuclear fractions (Fig. 4A) and NDP52 is exclusively localized in the cytoplasm (Fig. 4B). The results of granular staining clearly

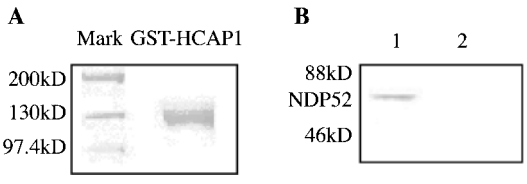

Fig. 2. **HCAP1 interacts with NDP52 *in vitro*.** (A) The expressed GST fusion HCAP1 protein in *E. coli* strain BL-21 and purified with glutathione-sepharose 4B beads. (B) NDP52 specifically binds to HCAP1 *in vitro*. Lane 1, NDP52 specifically binds to GST-HCAP1 *in vitro*; lane 2, NDP52 does not bind to GST alone as a control.

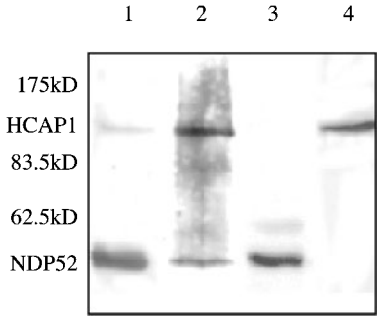

Fig. 3. **Interaction of HCAP1 with NDP52 *in vivo*.** Western blot was performed with McAb c-myc. Lane 1, Myc-HCAP1 and Myc-NDP52 co-expressed in COS7 cell; lane 2, the two proteins were co-immunoprecipitated with rabbit anti-HCAP1 antibody; lane 3, Myc-NDP52 expressed in COS7 cells; lane 4, Myc-HCAP1 expressed in COS7 cells.

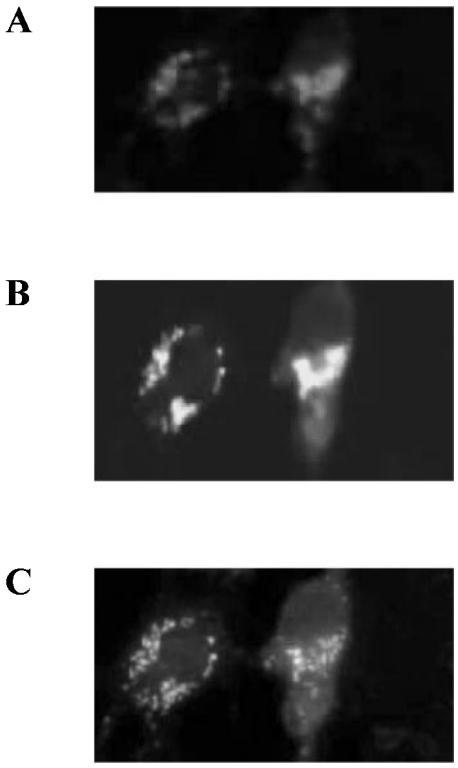

Fig. 4. **Subcellular localization of HCAP1 and NDP52 in COS7 cells.** (A) Cytoplasm and nuclear localization of HCAP1 (red). Immunofluorescent staining with an antibody against HCAP1 in COS7 cells. (B) Cytoplasm localization of NDP52 (green). Immunofluorescent staining with McAb c-myc-tag of Myc-NDP52 in COS7 cells. (C) Overlay of A and B. Colocalization (yellow) of HCAP1 and NDP52 in cytoplasm.

showed that both HCAP1 and NDP52 colocalize in the cytoplasm. (Fig. 4C).

Deletion Assay of HCAP1 and NDP52—The results showed in Fig. 5 reveal that the important region for the interaction of HCAP1 with NDP52 is located at the C-terminus. Neither HCAP1-2 nor HCAP1-3 could interact with NDP52, but HCAP1-23 including both portions could (Fig. 5, A and C). This suggests that both the leucine zipper domain and the proximal region are required. The C-terminus of NDP52 is critical for the interaction with HCAP1. Compared with NDP52, the deletion fragment NDP52-1 lacks only 26 amino acids at the C-terminus. NDP52-1 could interact with HCAP1, but the interaction is much weaken. Furthermore, the NDP52-2, fragment without the Ring and LIM domains, almost completely failed to interact with HCAP1 (Fig. 5, B and C).

DISCUSSION

The gene *HCAP1* was mapped to 17p13.3, a high frequency LOH region in HCC. It is expressed ubiquitously in all examined tissues (*5*). The results of SNP screening revealed a high incidence of SNPs in the coding region of *HCAP1*. Two kinds of haplotypes, *HCAP1*-N and *HCAP1*-M, were transfected into HCC cells and found to remarkably reduce formation and suppress cell growth (*13*)**.** The full-length *HCAP1* cDNA encodes a 1047-amino acid protein, which localizes in the cytoplasm and nucleus (*12*). The protein HCAP1 contains a HLH motif and a leucine zipper domain (*5*), two structures usually conserved in RNA-interacting proteins, DNA-interacting proteins, or proteins involved in protein–protein interaction (*14*).

Through yeast two-hybrid screening, we found several different functional proteins could interact with HCAP1. Among them, NDP52, a nuclear dot protein, not only was most frequently found, but also interacted strongly with HCAP1. The interaction between NDP52 and HCAP1 was confirmed by GST pull-down assay *in vitro*, co-immunoprecipitation in COS7 cells, and colocalization in the cytoplasm. These results show that the interaction between HCAP1 and NDP52 exists in mammalian cells.

The protein NDP52 contains a basic region leucine zipper domain (BRLZ, 185–239 aa), a RING finger domain

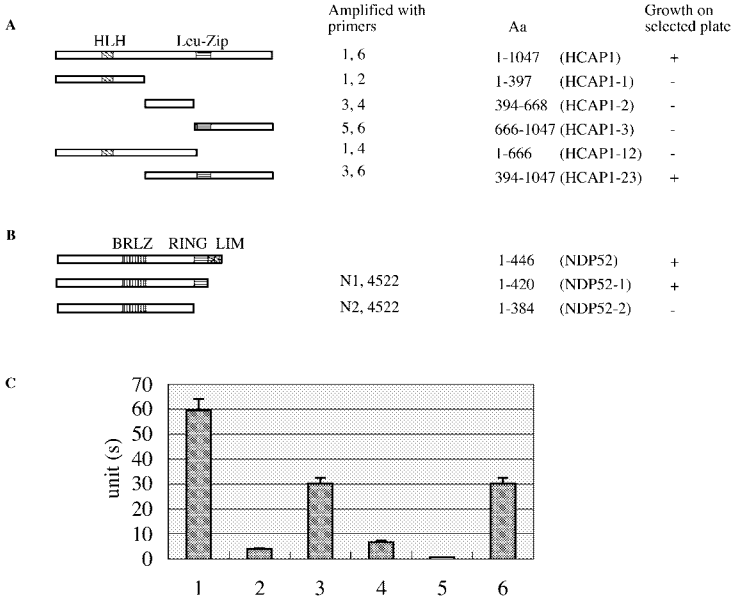

Fig. 5. **Deletion assay to identify the regions of HCAP1 and NDP52 responsible for interaction.** (A) The sequential deletion of HCAP1. (B) The sequential deletion of NDP52. (C) Quantitative assays for β-Gal. Quantitative assays were performed in MAV203 cells containing two different plasmids. 1, pPC97-Fos and pPC86-Jun (Control D); 2, pPC97-dDP and pPC86-dE2F (Control C); 3, pDB-leu-HCAP1 and pPC86-NDP52; 4, pDB-leu-HACP1 and pPC86-NDP52-1; 5, pDB-leu-HACP1 and pPC86-NDP52-2; 6, pDB-leu-HCAP1-23 and pPC86-NDP52 (pDB-leu is derived from pPC97).

C3HC4 (395–434 aa), and a LIM-like domain C2H2 (420–444 aa) (*15*). BRLZ domains are usually present in DNA-binding transcription factors that contain a basic region mediating sequence-specific DNA-binding followed by a leucine zipper required for dimerization (*16*). The LIM-like domain C2H2 and RING finger domain C3HC4 have the ability to bind to both RNA and DNA, and also are involved in protein interactions (*17*, *18*). The results of the deletion assay suggest that the important region for the interaction of NDP52 with HCAP1 is the cysteine-rich C-terminus containing the RING finger domain C3HC4 and the LIM-like domain C2H2. The homodimer of NDP52 was identified by the yeast two hybrid screening method (*15*). Other nuclear dot proteins, such as PML and Sp100 proteins, have been shown to play important roles in the development of acute promyelocytic leukemia and primary biliary cirrhosis (*15*, *19*). Recently, in the differential display analysis of cell line BTL-26, which is derived from the calf type sporadic bovine lymphosarcoma, the bovine epithelial cell line CKT-1 and healthy bovine T thymocytes, a bovine nuclear domain protein homologous to human NDP52, were found to express BTL-26 differently. Bovine NDP52 shows 83.1% homology to human NDP52 at the amino acid level and 85.5% homology at the nucleotide level (*20*). NDP52 was a relatively conserved protein in different species. It is reasonable to expect that the NDP52 may play an important role in cancer development.

The deletion assay identified the region of HCAP1 responsible for the interaction. The larger C-terminus of HCAP1 (amino acids 394–1047) could interact with NDP52 (Fig. 4), and both the leucine zipper domain and proximal region are required to this interaction. Besides interacting with NDP52, this region of HCAP1 is also responsible for binding with other zinc finger proteins as described above (data not shown).

Other interaction proteins play rather different roles: the protein similar to sprouty homolog 1 protein has been reported to inhibit the Ras/Raf/MAP kinase pathway (*21*, *22*); Sequestosome 1 is the phosphotyrosine independent ligand for the SH2 domain of p56-lck and also a component of intracytoplasmic hyaline bodies in hepatocellular carcinoma (HCC) cells (*23*); the protein inhibitor of activated STAT3 was identified as an inhibitor of activated STAT3 and binds to MITF, a key DNA-binding protein, in rat basophilic leukemia cells and mouse melanocytes (*24–26*). We have predicted that HCAP1 probably is a multifunction protein that binds all of them. Most of the amino acid sequence of GEMIN4 is similar to HCAP1, with a potient leucine zipper domain, and also might interact with zinc finger proteins.

These interactions of a leucine zipper protein and a zinc finger protein may play important roles in cells through binding to DNA or RNA. These interactions are also involved in other important complexes, such as the interaction of MITF with a basic helix-loop-helix leucine zipper domain and myc-associated zinc-finger protein-related factor (MAZR) for transcription (*27*). In further studies, it is necessary to clarify the function of the interactions of HCAP1 and zinc finger proteins in cells.

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