A Novel Cytoplasmic Protein with RNA-binding Motifs Is an Autoantigen in Human Hepatocellular Carcinoma

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Summary

In hepatocellular carcinoma (HCC), autoantibodies to intracellular antigens are detected in 30–40% of patients. Patients with chronic hepatitis or liver cirrhosis develop HCC, and when this occurs, some patients exhibit autoantibodies of new specificities. It has been suggested that these novel autoantibody responses may be immune system reactions to proteins involved in transformation-associated cellular events. One HCC serum shown to contain antibodies to unidentified cellular antigens was used to immunoscreen a cDNA expression library, and a full length cDNA clone was isolated with an open reading frame encoding 556 amino acids with a predicted molecular mass of 62 kD. The 62-kD protein contained two types of RNA-binding motifs, the consensus sequence RNA–binding domain (CS-RBD) and four hnRNP K homology (KH) domains. This protein, provisionally called p62, has close identity (66–70%) to three other proteins at the amino acid sequence level, and all four proteins may belong to a family having CS-RBD in the NH_2-terminal region and four KH domains in the mid-to-COOH–terminal region. The homologous proteins are: KH domain–containing protein overexpressed in cancer (Koc); zipcode binding protein, a protein which binds to a conserved nucleotide element in chicken β-actin mRNA (ZBP1); and a protein which binds to a promoter cis element in *Xenopus laevis* TFIIIA gene (B3). p62 protein is cytoplasmic in location, and autoantibodies were found in 21% of a cohort of HCC patients. Patients with chronic hepatitis and liver cirrhosis, conditions which are frequent precursors to HCC, were negative for these autoantibodies, suggesting that the immune response might be related to cellular events leading to transformation. However, the possible involvement of p62 autoantigen as a factor in the transformation process remains to be elucidated.

Key words: hepatocellular carcinoma • cloning • tumor antigen • autoantibody • RNA-binding motif

Autoantibodies against intracellular antigens are commonly found in a number of systemic autoimmune diseases (1, 2) and have been used to screen cDNA expression libraries to isolate cDNA clones encoding target autoantigens. Such studies have revealed that many autoantigens are components of subcellular particles involved in important cell functions such as DNA replication (3, 4), DNA transcription (5–7), RNA processing (8), and cell division (9–11). Studies of autoimmune diseases such as systemic lupus erythematosus have shown that the autoantibody responses in the majority of if not all patients are targeted at multiple components present within subcellular particles. It has been well documented that autoantibodies such as anti-Sm in systemic lupus erythematosus may target several proteins in such particles, including the A, B/B′, C, D, and 70-kD proteins, all of which are components of small nuclear ribonucleoprotein complexes (8). These and other observations have led to the hypothesis that an antigen-driven mechanism underlies the production of these autoantibodies (12–14) and that certain intracellular particles or their components become immunogenic because of dysregulation of their function or alterations in molecular structure or localization, leading to the provocation of an immune response (1, 12).

Autoantibodies have been described in cancer patients, including patients with leukemia, malignant melanoma, lung, breast, gastrointestinal, gynecological, nasopharyngeal, and prostate cancer, paraneoplastic neurological syndromes,

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hepatocellular carcinomas, and a variety of other neoplasms (15–30). On the premise that identification of the autoantigens might provide some information regarding intracellular molecules possibly engaged in the transformation process, this laboratory has focused on hepatocellular carcinoma (HCC)[1] as the subject of investigation. This interest is related to the observation that in certain patients with HCC, novel autoantibody responses appeared coincident with or sometimes immediately before the clinical detection of HCC (23, 24). These observations were made possible by the availability of serial samples of stored sera from patients with chronic hepatitis and liver cirrhosis, two precursor conditions to HCC; HCC eventually occurs in ~30–40% of these patients. One previously identified nuclear antigen, HCC1, is a nuclear protein with structural motifs found in the serine–arginine family of alternative splicing factors (25). Another antigen targeted by autoantibodies is a DNA-binding nuclear antigen called SG2NA, a protein highly expressed in the S and G2 phases of the cell cycle (26). In this study, an HCC serum that contained autoantibodies to a 62-kD cellular protein was used to immunoscreen a T24 λzap cDNA expression library. A novel cytoplasmic autoantigen named p62 was identified. p62 is a putative RNA-binding protein, and antibodies to p62 were detected in 21% of a cohort of HCC patients from Henan Province, People's Republic of China.

Materials and Methods

Sera and Antibodies. HCC sera were obtained from 95 subjects included in an epidemiological study previously described and were from Henan Province, People's Republic of China (31). Sera from 77 patients with liver diseases (26 asymptomatic HBsAg carriers, 31 patients with acute hepatitis, and 20 patients with chronic hepatitis and liver cirrhosis), and 30 normal human sera, all from the same province, were available for these studies. All of the above sera came from the Sanitary and Anti-Epidemic Station (Henan Province). 40 normal human sera from the San Diego, CA area were also included as controls. Human prototype sera containing autoantibodies to previously identified intracellular antigens were from patients with systemic autoimmune diseases (2) and obtained from the serum bank of the Autoimmune Diseases Center of The Scripps Research Institute (La Jolla, CA).

Cell Culture and Cell Extracts. MOLT-4 (human acute lymphoblastic leukemia), T24 (human transitional cell bladder carcinoma), HEp-2 (human epidermoid laryngeal carcinoma), HeLa (human epitheloid cervical carcinoma), HepG2 (human hepatocellular carcinoma), A549 (human lung carcinoma), and 3T3 (mouse fibroblast) cell lines were obtained from the American Type Culture Collection and cultured following the specific protocol for each cell line. Cells grown in monolayers were solubilized directly in Laemmli's sample buffer containing protease inhibitors (Boehringer Mannheim). Solubilized lysates were briefly sonicated before electrophoresis on SDS–polyacrylamide gels.

Immunofluorescence Studies. Initial identification of autoantibodies in sera was performed using methanol- and acetone-fixed commercial HEp-2 cell slides (Bion Enterprises, Ltd.). The findings were usually confirmed in other experiments using T24, HepG2, and 3T3 cells that were grown on coverslips, fixed for 5 min at −20°C in 100% methanol, and permeabilized for 3 min at −20°C in 100% acetone. As a second antibody, FITC-conjugated goat anti–human IgG (Caltag Laboratories) was applied. A titer of >1:40 dilution was interpreted as positive.

Western Blotting. Western blotting was performed essentially as described by Chan and Pollard (32). Cell extracts were electrophoresed on SDS-PAGE and transferred to nitrocellulose paper. After preblocking with PBS containing 0.5% Tween-20 and 5% nonfat milk for 30 min at room temperature, the nitrocellulose strips were incubated for 60 min at room temperature with a 1:100 dilution of serum. As secondary antibody, horseradish peroxidase–conjugated goat anti–human IgG (Caltag Laboratories) was applied (1:2,000 dilution). The detection of immunoreactive bands was performed with an ECL kit (Amersham Corp.) according to the manufacturer's instructions and followed by autoradiography.

T24 and HeLa Cell Labeling and Immunoprecipitation. T24 and HeLa cells were cultured and radiolabeled with [^{35}S]methionine. For preparation of T24 and HeLa cell extracts, cells were collected by centrifugation, combined with two times packed cell volume buffer A (10 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP-40), and held on ice for 10 min to allow cell lysis. The supernatant obtained by centrifugation at 10,000 *g* for 10 min at 4°C was used as antigen preparation in immunoprecipitation studies. Before immunoprecipitation, labeled cell extracts were precleared by adding 100 μl 10% protein A–Sepharose stock/ml extract, mixed for 5 min on ice, and centrifuged to collect supernatant. Typically, 100 μl 10% protein A–Sepharose, 500 μl buffer B (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.5% NP-40; 0.5% deoxycholic acid; 0.1% SDS; and 0.02% sodium azide) containing BSA at 10 μl (stock: 10 mg/ml), 40 μl labeled cell extract, 10 μl serum, and 10 μl protease inhibitor (Boehringer Mannheim) was added to a standard immunoprecipitation reaction. After incubation for 1 h, the immunoprecipitated beads were washed five times with 1 ml buffer B. Finally, the beads were eluted with an equal volume of 2× Laemmli's sample buffer and analyzed in SDS-PAGE followed by autoradiography.

cDNA Cloning, 5′ Rapid Amplification of cDNA Ends (RACE) and Sequence Analysis. HCC serum YZ was diluted 1:100 and used for screening a T24 λzap cDNA expression library. Before screening of the cDNA library, the serum was extensively absorbed against bacteria infected with wild-type λzap phage (33). The preabsorbed serum was used to immunoscreen 3.0×10^5 recombinant plaques using ^{125}I-labeled goat anti–human IgG as the secondary detecting reagent. Screening was carried out on duplicate filters, and one double-positive clone, JY1, was isolated and subcloned in vivo into pBK-CMV plasmid using ExAssist helper phage (Stratagene Inc.) as recommended in the manufacturer's instructions. The clone JY1 was amplified, purified, and used for sequence analysis. cDNA insert was analyzed by restriction mapping and sequencing. The clone JY1 was a partial sequence, and RACE methodology was used to obtain overlapping 5′ clones using the Marathon-Ready cDNA from human colorectal adenocarcinoma SW480 cell line (Clontech). Nucleotide sequence was determined in both strands using a semiautomated sequencer from Applied Biosystems (model 373). Oligonucleotide primers were synthesized with a DNA synthesizer (Applied Biosystems; model 394). DNA and protein sequences were analyzed by the Genetics Computer Group Sequence

[1]*Abbreviations used in this paper:* CS-RBD, consensus sequence RNA–binding domain; HCC, hepatocellular carcinoma; KH, K homology; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

Analysis Software Package for UNIX computers (version 7.4; 34). Alignment of protein sequences was achieved with a Multiple Alignment Program (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html; reference 35).

Reverse Transcription (RT)-PCR and Confirmation of p62 Open Reading Frame (ORF). The ORF of p62 was reamplified and confirmed by RT-PCR using T24 cell mRNA as template. One set of sense and antisense primers was designed, and their positions with respect to the p62 full length cDNA are indicated (see Fig. 2 A): rt3 sense, 5′-TTGAATTCGCCATGGTGAACAAG-CTTTACATCGGGAACC-3′ and rt4 antisense, 5′-TTTATGT-CGACGGTGTTGGAAGGGCTACATT-3′, incorporating an EcoRI and SalI site, respectively. RT-PCR was performed using the one-tube method as described by Pfeffer et al. (36). In brief, 1 μl T24 mRNA (0.5 μg/μl), 10 μM primer (1 μl each), 1.25 U Taq polymerase (GIBCO BRL), 100 U SuperScript II RNase H–reverse transcriptase (GIBCO BRL), 20 U RNase inhibitor (Promega Corp.), 0.25 μl of 10 μM dNTPs, and 2.5 μl 10× PCR buffer containing 500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; and 0.1% gelatin were added to a final total volume of 25 μl, and the PCR steps were programmed using a thermocycler (Eppendorf). The reactions were performed at 50°C for 1 h and followed by 30 cycles at 57°C for 10 s, 72°C for 2 min, and 94°C for 10 s. RT-PCR products were analyzed by agarose gel electrophoresis.

Purification of Recombinant Protein. For increased expression and purification of recombinant protein, p62 cDNA derived from RT-PCR was subcloned into the EcoRI and SalI sites of pET28a vector, which provides the NH_2-terminal fusion protein with 6× histidine and T7 epitope tags. The recombinant protein was overexpressed in *Escherichia coli* BL21 (DE3) and purified using nickel column chromatography. The protocol used for the high-level expression and purification of 6× histidine–tagged proteins was performed as described (Qiagen, Inc.). Elution buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 4.5) was used to elute the recombinant protein.

In Vitro Transcription and Translation. The p62 cDNA was transcribed and translated in vitro using TnT-coupled reticulocyte lysate system (Promega Corp.) in the presence of [^{35}S]methionine (ICN) as described (Promega Biotec). Labeled products were used as substrates for immunoprecipitation analysis.

Affinity Purification of Antibodies. Recombinant protein was electrophoresed on 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were cut into strips and the recombinant protein bands confirmed by Western blotting. Nitrocellulose strips were incubated with diluted serum at 1:100, and unbound antibodies were removed by washing with PBS containing 0.5% Tween-20 before elution of bound antibodies with 0.5 ml elution buffer (200 mM KH_2PO_4, 150 mM NaCl, and 0.1% BSA, pH 2.5). Affinity-purified antibodies were immediately neutralized by the addition of 1 M Tris-HCl, pH 8.7. The antibodies were concentrated with Centricon-30 microconcentrators (Amicon Corp.), and different dilutions (1:5, 1:25, and 1:50) were used for immunofluorescence assay and Western blotting analysis.

Rabbit Immunization. Four female New Zealand White rabbits were immunized by subcutaneous injections of 0.5 mg of p62 recombinant protein in complete Freund's adjuvant. Rabbits were boosted two times with 0.5 mg p62 recombinant protein in incomplete Freund's adjuvant at 1-mo intervals, and blood was collected 10 d after the last booster injection.

Northern Blotting. Nylon membranes blotted with poly A^+ RNA isolated from multiple human tissues and several human cancer cell lines were obtained from Clontech. An antisense riboprobe was generated from a 480-bp fragment corresponding to the NH_2-terminal domain of p62 (see Fig. 2 A) and labeled with [α-^{32}P]UTP (Clontech) as described. In brief, the membranes were hybridized with ^{32}P-labeled p62 riboprobe for 2 h at 74°C, washed in 2× SSC and 0.1% SDS at 74°C for 20 min and in 0.1× SSC and 0.1% SDS at 65°C for 20 min, and exposed to x-ray film for 4 h at −70°C. A 2.0-kb human β-actin cDNA provided by Clontech was used as control probe.

ELISA. Standard protocol for ELISA was used as described by Rubin (37). Purified p62 recombinant proteins were diluted in PBS to a final concentration of 1 μg/ml for coating Immulon 2 microtiter plates (Dynatech Laboratories). Human sera diluted 1:100 were incubated in the antigen-coated wells. Horseradish peroxidase–conjugated goat anti–human IgG (Caltag Laboratories) and the substrate 2.2′-azinobis (3-ethylbenzthiazoline sulfonic acid; Boehringer Mannheim) were used as detecting reagents. Each sample was tested in duplicate, and the average OD at 490 nm was used for data analysis. The cutoff value designating positive reaction was the mean OD of normal sera + 3 SD.

Results

Detection of Autoantibody against Cytoplasmic Antigen p62. Initial studies focused on the analysis of serum specimens from 95 HCC patients from Henan Province. Many of these sera contained antibodies reactive with a 62-kD cellular antigen that was present in low concentration in MOLT-4 cell extracts but in higher concentration in extracts from other cell lines. Fig. 1 shows Western blotting of three such sera against whole cell extracts from MOLT-4, T24, and HepG2. Lanes 1, 5, and 9 are normal human serum showing negative reactions. Lanes 2, 6, and 10 show the reactivities of HCC serum YZ. This serum showed

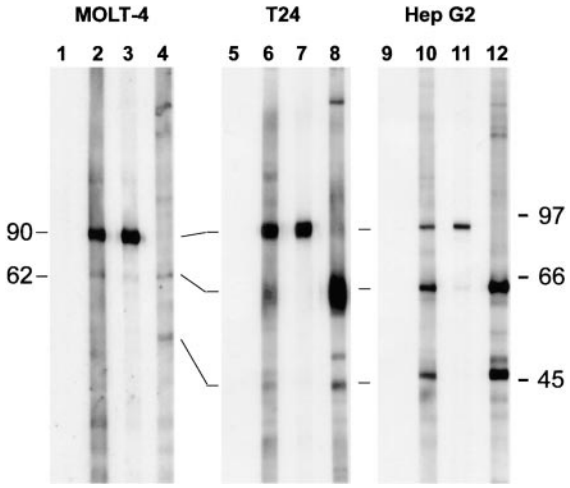
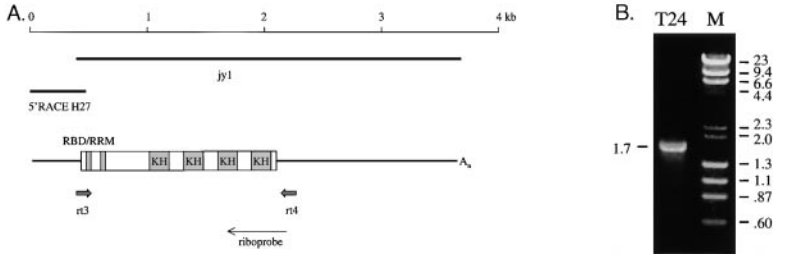


Figure 1. Reactivity of three HCC sera in Western blotting against whole cell extracts from MOLT-4, T24, and HepG2 cell lines. Lanes 1, 5, and 9 were normal human sera. Serum YZ (lanes 2, 6, and 10) showed strong reactivity with a 90-kD protein in MOLT-4 cells and reacted weakly with a 62-kD protein in MOLT-4 cells and T24 cell extracts. A strong reaction with the 62-kD protein was detected with HepG2 cell extracts, together with a strong reaction with a 50-kD protein. Sera YL (lanes 3, 7, and 11) and CH (lanes 4, 8, and 12) demonstrate other types of reactions (see text). These representative data demonstrate that HCC sera are heterogeneous in their antibody repertoires and that different cell lines apparently have different expressions of 90-, 62-, and 50-kD proteins.

strong reactivity with a 90-kD band in MOLT-4 cell extracts and weak reaction with a 62-kD band. With T24 cell extract, the 62-kD band was slightly stronger, but in HepG2 cell extract the 62-kD reactivity was as strong or stronger than the 90-kD reactivity. In addition, a good signal was observed at 50 kD. Serum YL from a different patient contained antibodies primarily against the 90-kD antigen and displayed very weak reactivity with the 62-kD antigen (lanes 3, 7, and 11). A third serum, CH, appeared to have antibodies primarily against the 62- and 50-kD antigens (lanes 4, 8, and 12). In addition, serum CH, as well as YZ, demonstrated weak reactivity with other antigens of higher and lower molecular sizes, but the predominant reactivities detected were against the 90-, 62-, and 50-kD proteins. These data demonstrated that different cell lines showed different levels of expression of these intracellular proteins, with T24 expressing higher levels of the 62-kD and HepG2 expressing higher levels of both 62- and 50-kD antigens than MOLT-4.

cDNA Cloning of the 62-kD Autoantigen. The above data suggested that in order to isolate cDNA clones encoding the 62-kD antigen, T24 or HepG2 cDNA expression libraries would be the preferred cell lines. HCC serum YZ was used to immunoscreen a T24 λzap cDNA expression library. One positive clone was isolated from 3.0×10^5 recombinant plaques, purified to homogeneity, and subcloned in vivo into pBK-CMV. This clone, designated JY1, was a cDNA of 3.3 kb and was shown to have an apparent coding sequence with a termination codon and a long 3′ UTR with a poly A tail. 5′-RACE methodology was used to obtain overlapping 5′ clones using the Marathon-Ready cDNA repertoire derived from human colorectal adenocarcinoma SW480 cell line. Two overlapping independent clones were obtained and analyzed, with the longest clone, H27, containing a cDNA insert of 374 bp. An in-frame TGA stop codon at position 320 bp upstream of a methionine start codon was found. The presumptive full length cDNA is shown in Fig. 2 C. The cDNA has a 5′-UTR of 435 bp, an ORF of 1,668 bp, and a 3′-UTR of 1,564 bp. RT-PCR was performed using T24 cell line



C.

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1    TATATCATGTAAGCTAGTCACACCTATTTGAATTCACTTTTCTTTGTTTCTTGATTTCTTATGGCTATTATTGCAACAATCAAACAAATTTTGAATACCTTTTGTGAGCTAGCACTGTGG
121  AAATATGAGGGAAGTTTATTGTGTTCTTACTTTAAATAGTTTGCAGTTTTATTGTGTATACAAATGTATGCATGTGAAATATACAGATTTTAAAAAAGCAGATCATTAAAGATTAGGTCA
241  TAAGGTGTAGTCTGTACATGGAGGAAGAGCTAAGTTGATTCAGAATAACATGGTTAGATTTCGAAGCTAGCCTGGATTTGAATCTTGGATCCACCACTGACTAGCTATGTAAGCTTAGTC
361  AAGCAACTCAACGGAGGAGGCGAGGAGCGCCGGGTACCGGGCCGGGGGAGCCGCGGGCTCTCGGGGAAGAGACGGATGATGAACAAGCTTTACATCGGGAACCTGAGCCCCGCCGTCACC
1                                                                                                    M  M  N  K  L  Y  I  G  N  L  S  P  A  V  T
481  GCCGACGACCTCCGGCAGCTCTTTGGGGACAGGAAGCTGCCCCTGGCGGGACAGGTCCTGCTGAAGTCCGGCTACGCCTTCGTGGACTACCCCGACCAGAACTGGGCCATCCGCCGCCATC
16   A  D  D  L  R  Q  L  F  G  D  R  K  L  P  L  A  G  Q  V  L  L  K  S  G  Y  A  F  V  D  Y  P  D  Q  N  W  A  I  R  A  I
601  GAGACCCTCTCGGGTAAAGTGGAATTGCATGGGAAAATCATGGAAGTTGATTACTCAGTCTCTAAAAAGCTAAGGAGCAGGAAAATTCAGATTCGAAACATCCCTCCTCACCTGCAGTGG
56   E  T  L  S  G  K  V  E  L  H  G  K  I  M  E  V  D  Y  S  V  S  K  K  L  R  S  R  K  I  Q  I  R  N  I  P  P  H  L  Q  W
721  GAGGTGTTGGATGGACTTTTGGCTCAATATGGGACAGTGGAGAATGTGGAACAAGTCAACACAGACACAGAAACCGCCGTTGTCAACGTCACATATGCAACAAGAGAAGAAGCAAAAATA
96   E  V  L  D  G  L  L  A  Q  Y  G  T  V  E  N  V  E  Q  V  N  T  D  T  E  T  A  V  V  N  V  T  Y  A  T  R  E  E  A  K  I
841  GCCATGGAGAAGCTAAGCGGGCATCAGTTTGAGAACTACTCCTTCAAGATTTCCTACATCCCGGATGAAGAGGTGAGCTCCCCTTCGCCCCCTCAGCGAGCCCAGCGTGGGGACCACTCT
136  A  M  E  K  L  S  G  H  Q  F  E  N  Y  S  F  K  I  S  Y  I  P  D  E  E  V  S  S  P  S  P  P  Q  R  A  Q  R  G  D  H  S
961  TCCCGGGAGCAAGGCCACGCCCCTGGGGGCACTTCTCAGGCCAGACAGATTGATTTCCCGCTGCGGATCCTGGTCCCCACCCAGTTTGTTGGTGCCATCATCGGAAAGGAGGGCTTGACC
176  S  R  E  Q  G  H  A  P  G  G  T  S  Q  A  R  Q  I  D  F  P  L  R  I  L  V  P  T  Q  F  V  G  A  I  I  G  K  E  G  L  T
1081 ATAAAGAACATCACTAAGCAGACCCAGTCCCGGGTAGATATCCATAGAAAAGAGAACTCTGGAGCTGCAGAGAAGCCTGTCACCATCCATGCCACCCCAGAGGGGACTTCTGAAGCATGC
216  I  K  N  I  T  K  Q  T  Q  S  R  V  D  I  H  R  K  E  N  S  G  A  A  E  K  P  V  T  I  H  A  T  P  E  G  T  S  E  A  C
1201 CGCATGATTCTTGAAATCATGCAGAAAGAGGCAGATGAGACCAAACTAGCCGAAGAGATTCCTCTGAAAATCTTGGCACACAATGGCTTGGTTGGAAGACTGATTGGAAAAGAAGGCAGA
256  R  M  I  L  E  I  M  Q  K  E  A  D  E  T  K  L  A  E  E  I  P  L  K  I  L  A  H  N  G  L  V  G  R  L  I  G  K  E  G  R
1321 AATTTGAAGAAAATTGAACATGAAACAGGGACCAAGATAACAATCTCATCTTTGCAGGATTTGAGCATATACAACCCGGAAAGAACCATCACTGTGAAGGGCACAGTTGAGGCCTGTGCC
296  N  L  K  K  I  E  H  E  T  G  T  K  I  T  I  S  S  L  Q  D  L  S  I  Y  N  P  E  R  T  I  T  V  K  G  T  V  E  A  C  A
1441 AGTGCTGAGATAGAGATTATGAAGAAGCTGCGTGAGGCCTTTGAAAATGATATGCTGGCTGTTAACACCCACTCCGGATACTTCTCCAGCCTGTACCCCCATCACCAGTTTGGCCCGTTC
336  S  A  E  I  E  I  M  K  K  L  R  E  A  F  E  N  D  M  L  A  V  N  T  H  S  G  Y  F  S  S  L  Y  P  H  H  Q  F  G  P  F
1561 CCGCATCATCACTCTTATCCAGAGCAGGAGATTGTGAATCTCTTCATCCCAACCCAGGCTGTGGGCGCCATCATCGGGAAGAAGGGGGCACACATCAAACAGCTGGCGAGATTCGCCGGA
376  P  H  H  H  S  Y  P  E  Q  E  I  V  N  L  F  I  P  T  Q  A  V  G  A  I  I  G  K  K  G  A  H  I  K  Q  L  A  R  F  A  G
1681 GCCTCTATCAAGATTGCCCCTGCGGAAGGCCCAGACGTCAGCGAAAGGATGGTCATCATCACCGGGCCACCGGAAGCCCAGTTCAAGGCCCAGGGACGGATCTTTGGGAAACTGAAAGAG
416  A  S  I  K  I  A  P  A  E  G  P  D  V  S  E  R  M  V  I  I  T  G  P  P  E  A  Q  F  K  A  Q  G  R  I  F  G  K  L  K  E
1801 GAAAACTTCTTTAACCCCAAAGAAGAAGTGAAGCTGGAAGCGCATATCAGAGTGCCCTCTTCCACAGCTGGCCGGGTGATTGGCAAAGGTGGCAAGACCGTGAACGAACTGCAGAACTTA
456  E  N  F  F  N  P  K  E  E  V  K  L  E  A  H  I  R  V  P  S  S  T  A  G  R  V  I  G  K  G  G  K  T  V  N  E  L  Q  N  L
1921 ACCAGTGCAGAAGTCATCGTGCCTCGTGACCAAACGCCAGATGAAAATGAGGAAGTGATCGTCAGAATTATCGGGCACTTCTTTGCTAGCCAGACTGCACAGCGCAAGATCAGGGAAATT
496  T  S  A  E  V  I  V  P  R  D  Q  T  P  D  E  N  E  E  V  I  V  R  I  I  G  H  F  F  A  S  Q  T  A  Q  R  K  I  R  E  I
2041 GTACAACAGGTGAAGCAGCAGGAGCAGAAATACCCTCAGGGAGTCGCCTCACAGCGCAGCAAGTGAGGCTCCCACAGGCACCAGCAAAACAACGGATGAATGTAGCCCTTCCAACACCTG
536  V  Q  Q  V  K  Q  Q  E  Q  K  Y  P  Q  G  V  A  S  Q  R  S  K  *  556
2161 ACAGAATGAGACCAAACGCAGCCAGCCAGATCGGGAGCAAACCAAAGACCATCTGAGGAATGAGAAGTCTGCGGAGGCGGCCAGGGACTCTGCCGAGGCCCTGAGAACCCCAGGGGCCGA
2281 GGAGGGGCGGGGAAGGTCAGCCAGGTTTGCCAGAACCACCGAGCCCCGCCTCCCGCCCCCCAGGGCTTCTGCAGGCTTCAGCCATCCACTTCACCATCCACTCGGATCTCTCCTGAACTC
2401 CCACGACGCTATCCCTTTTAGTTGAACTAACATAGGTGAACGTGTTCAAAGCCAAGCAAAATGCACACACCTTTTTCTGTGGCAAATCGTCTCTGTACATGTGTGTACATATTAGAAGGG
2521 AAGATGTTAAGATATGTGGCCTGTGGGTTACACAGGGTGCCTGCAGCGGTAATATATTTAGAAATAATATATCAAATAACTCAACTAACTCCAATTTTTAATCAATTATTAATTTTTTT
2641 TTCCTTTTAAAGAGAAAGCAGGCTTTTCTAGACTTTAAAGAATAAAGTCTTTGGGAGGTCTCACGGTGTAGAGAGGAGCTTTGAGGCCACCCGCACAAAATTCACCCAGAGGGAAATCTC
2761 GTCGGAAGGACACTCACGGCAGTTCTGGATCACCTGTGTATGTCAACAGAAGGGATACCGTCTCCTTGAAGAGGAAACTCTGTCACTCCTCATGCCTGTCTAGCTCATACACCCATTTCT
2881 CTTTGCTTCACAGGTTTTAAACTGGTTTTTTGCATACTGCTATATAATTCTCTGTCTCTCTCTGTTTATCTCTCCCCTCCCTCCCCTTCTTCTCCATCTCCATTCCTTTTGAATTT
3001 CCTCATCCCTCCATCTCAATCCNGTATNTACGCACCCCCCCCCCCAGGCAAAGCAGTGCTCTGAGTATCACATCACACAAAAGGAACAAAAGCGAAACACACAAACCAGCCTCAACTTA
3121 CACTTGGTTACTCAAAAGAACAAGAGTCAATGGTACTTGTCCTAGCGTTTTGGAAGAGGAAAACAGGAACCCACCAAACCAACCAATCAACCAAACAAAGAAAAATTCCACAATGAAAG
3241 AATGTATTTTGTCTTTTTGCATTTTGGTGTATAAGCCATCAATATTCAGCAAAATGATTCCTTTCTTTAAAAAAAAAAAATGTGGAGGAAAGTAGAAATTTACCAAGGTTGTTGGCCCAG
3361 GGCGTTAAATTCACAGATTTTTTTAACGAGAAAAACACACAGAAGAAGCTACCTCAGGTGTTTTTACCTCAGCACCTTGATCTTGTGTTTCCCTTAGAGATTTTGTAAAGCTGATAGTTG
3481 GAGCATTTTTTTATTTTTTTAATAAAAATGAGTTGGAAAAAAAATAAGATATCAACTGCCAGCCTGGAGAAGGTGACAGTCCAAGTGTGCAACAGCTGTTCTGAATTGTCTTCCGCTAGC
3601 CAAGAACCTATATGGCCTTCTTTTGGACAAACCTTGAAAATGTTTATTTAAAAAAAAAAAAAAAAA  3667
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Figure 2. Full length cDNA and corresponding protein sequence of p62. (A) Serum YZ was used to immunoscreen a T24 λzap cDNA library, and a partial cDNA clone designated JY1 with an insert of 3.3 kb was isolated. Using 5′-RACE methodology, a further cDNA clone 5′ to JY1, named H27, was isolated. The domain structure of p62 with the NH_2-terminal consensus RNA–binding domain and four KH domains is shown. (B) RT-PCR was performed using T24 cell line RNA. A 1.7-kb isolate was obtained consistent with the size of the putative ORF of p62. (C) The nucleotide and deduced amino acid sequence of p62 is shown. The CS-RBD located in the NH_2-terminal region and four KH domains extending from the middle region to the COOH-terminal region are underlined. p62 cDNA contains 3,667 bp, and the ORF has 556 amino acids. Two polyadenylation sequences in the 3′-untranslated region are underlined. The cDNA sequence has been submitted to GenBank under accession number AF057352.

Table I. *Similarity and Identity of p62 and Related Proteins and Other KH Domain–containing Proteins*

	Similarity (Identity)					
	Koc	ZBP1	B3	FMR1	hnRNP K	hnRNP X
			%			
p62	80.3 (66.5)	83.9 (70.5)	82.7 (69.7)	39.8 (16.2)	48.0 (25.7)	41.5 (17.4)
Koc		83.1 (75.6)	90.3 (84.1)			
ZBP1			89.0 (78.7)			

Percent identity and similarity of homologous protein sequences were calculated using GAP program (Genetics Computer Group). Koc, KH domain–containing protein overexpressed in cancer; ZBP1, β-actin mRNA zipcode–binding protein; B3, *Xenopus laevis* KH domain–containing transcription factor B3; FMR1, the fragile X mental retardation gene; hnRNP K, heterogeneous nuclear ribonucleoprotein K protein; hnRNP X, heterogeneous nuclear ribonucleoprotein X protein.

RNA as template with one pair of designed primers (rt3 and rt4, shown in Fig. 2 A). The RT-PCR products were of ~1.7 kb, compatible with the size of the ORF in the isolated cDNA clone (Fig. 2 B). The ORF codes for 556 amino acids with a predicted molecular mass of 62 kD and a calculated pI of 8.59.

p62 Has RNA-binding Motifs and Is Highly Homologous to Three Other RNA-binding Proteins. The 62-kD protein contained two types of RNA binding motifs, the consensus sequence RNA–binding domain (CS-RBD) and four hnRNP K homology (KH) domains (38). The CS-RBD domain was located in the NH_2-terminal region, and the four KH domains extended from the middle region to the COOH-terminal region (Fig. 2). Three proteins were found to have high degrees of homology to p62 at both nucleotide and protein levels. Table I compares percent similarity and identity of p62 with the other three proteins. KH domain–containing protein overexpressed in cancer (Koc), a putative oncogene (39), had 66.5% identity and 80.3% similarity to p62. Zipcode binding protein (ZBP1), a β-actin mRNA-binding protein in chicken (40), had a 70.5% identity and 83.9% similarity to p62, and B3 (*X. laevis* TFIIIA–binding protein), an oocyte factor that binds to a developmentally regulated cis element in the TFIIIA gene (41), showed 69.7% identity and 82.7% similarity to p62. Table I also shows that other KH domain–containing proteins such as FMR1 (42), hnRNP K (43), and hnRNP X (44) showed much lower levels of homology. The sequences of p62, Koc, ZBP1, and B3 are shown in Fig. 3 A, demonstrating the CS-RBD and four hnRNP K homology domains.

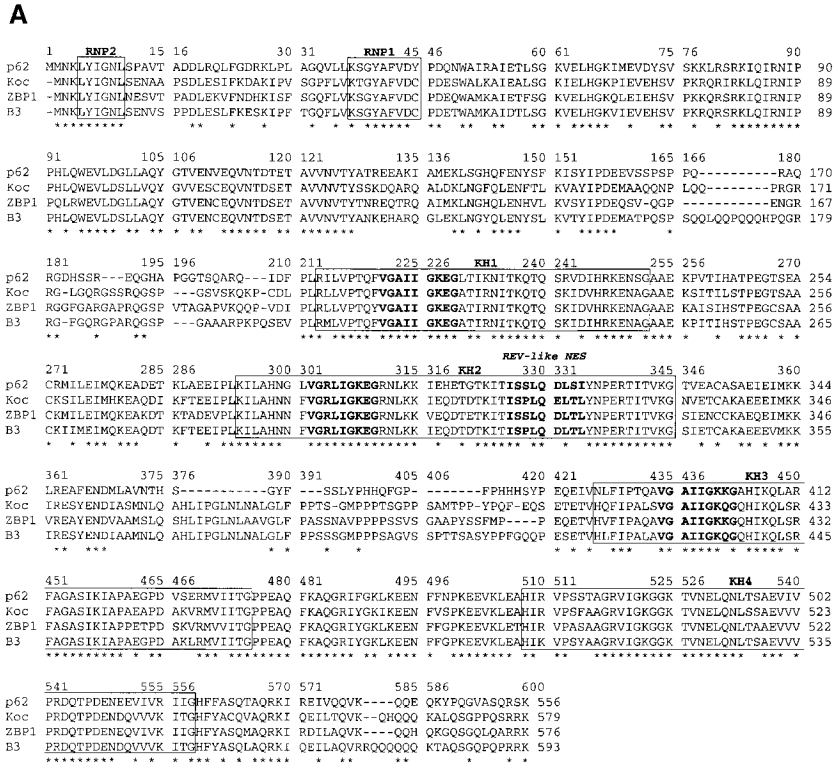

Figure 3. Similarity between p62 and three RNA-binding motif proteins Koc, ZBP1, and B3. (A) The identity between p62 and the other three proteins are shown (*). (B) The great similarity of the domain structures is shown. The RNA-binding domains with two conserved RNP regions, RNP1 and RNP2, and four KH domains are boxed. A nine–amino acid sequence (VGAIIGKE/KG) of unknown function in the first three KH domains is shown in bold as is the REV-like nuclear export signal in the second KH domain.

In addition, a nine–amino acid sequence (VGAIIGKE/KG) of unknown function previously reported in ZBP1 (40) was also found in the first three KH domains of the other three proteins. A potential REV-like nuclear export signal also found in ZBP1 protein (40) was present in position 308–319 of p62. The sequence alignments of the four proteins are depicted in Fig. 3 A, and their domain structures are shown in Fig. 3 B.

Recombinant p62 Protein Was Recognized by Human Autoantibodies and Comigrated with Cellular 62-kD Protein. The p62 recombinant protein was expressed using the pET28a vector in *E. coli* and purified using nickel affinity column chromatography. A 62-kD polypeptide was detected by Coomassie blue staining together with one prominent lower molecular mass product (Fig. 4 A, lane 1). HCC sera containing antibodies to the 62-kD cellular antigen reacted strongly with the 62-kD recombinant protein and also with the lower molecular weight product (Fig. 4 A, lane 3). In contrast, HCC sera containing only 90-kD autoantibodies such as serum YL and normal human serum (Fig. 4, lane 2) were nonreactive. In vitro–translated products of p62 cDNA showed that the polypeptide migrated at 62-kD (Fig. 4 B, lane 6) and was immunoprecipitated by human anti-p62 prototype serum (Fig. 4 B, lane 4) but not normal human serum (Fig. 4 B, lane 5). The in vitro–translated products (Fig. 4 C, lane 8) comigrated with the 62-kD antigen detected by Western blotting of HepG2 cell extract (Fig. 4 C, lane 8a).

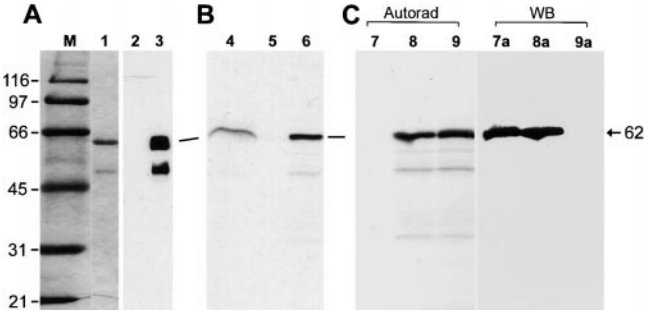

Figure 4. p62 recombinant protein expressed as a 6× histidine tag protein in *E. coli* was purified using nickel column chromatography. (A) Molecular mass markers (lane M). A 62-kD peptide that corresponded to the size of the ORF of p62 was detected in SDS-PAGE with Coomassie blue staining (lane 1). HCC serum YZ, which was used for cDNA cloning, was reactive with p62 recombinant protein in Western blot analysis (lane 3), but the recombinant protein was not reactive with normal human serum (lane 2) or HCC sera that did not contain detectable antibodies to the 62-kD protein (not shown). A lower molecular size product, presumably a degradation product, detectable by Coomassie blue staining was also reactive with HCC serum (lanes 1 and 3). (B) Immunoprecipitation of in vitro–translated p62 cDNA. Anti-p62 prototype serum (lane 4), normal human serum (lane 5), and in vitro–translated products alone (lane 6). (C) Comigration of the in vitro–translated products of p62 with the 62-kD cellular protein in HepG2 cells. The panel (lanes 7–9) was processed by autoradiography and the same panel (7a–9a) used subsequently for Western blot analysis. Lanes 7/7a contained HepG2 cell extracts alone, lanes 8/8a contained a mixture of in vitro–translated radiolabeled products and HepG2 cell extracts, and lanes 9/9a contained in vitro–translated radiolabeled products alone. The in vitro–translated radiolabeled product of p62 cDNA comigrated exactly with cellular p62.

Immunization with p62 Recombinant Protein. To further confirm that the recombinant protein was identical to the 62-kD cellular protein, four female New Zealand White rabbits were immunized with p62 recombinant protein. Rabbit immune sera immunoprecipitated in vitro–translated products (Fig. 5 A, lane 3). In addition, Fig. 5 B shows that the cellular 62-kD protein was immunoprecipitated by immune rabbit serum (lane 10) in a fashion similar to human anti-p62 prototype sera (lanes 7 and 8), using extracts from [^{35}S]methionine–labeled HeLa cells as the antigen preparation. Immune rabbit sera were also reactive in Western blotting with 62-kD proteins from T24, HepG2, and A549 cells (data not shown). Fig. 5 B shows that other proteins were also immunoprecipitated, but many of these were also immunoprecipitated by preimmune rabbit serum and normal human sera and are presumed to be nonspecific precipitates.

Subcellular Localization of p62 Protein. It had been previously observed by immunofluorescent histochemistry that HCC sera with antibodies to the 62-kD antigen were negative for staining of the nucleus but positive for cytoplasmic staining. Immune rabbit antiserum affinity-purified from recombinant protein showed cytoplasmic staining, as depicted in Fig. 6 B. The pattern of human HCC prototype serum YZ is shown in Fig. 6 A. The fine details of cytoplasmic staining of human serum YZ and rabbit immune sera are somewhat different, with rabbit serum showing a coarser pattern of cytoplasmic staining than human serum. This could be related to the fact that human sera are polyclonal and might contain other autoantibodies. The relevant finding was that both human HCC sera with anti–p62-kD antigen reactivity and rabbit immune sera were reactive with antigens that were cytoplasmic in location.

p62 mRNA Expression in Tissues and Cell Lines. Using a 480-bp fragment corresponding to the COOH-terminal

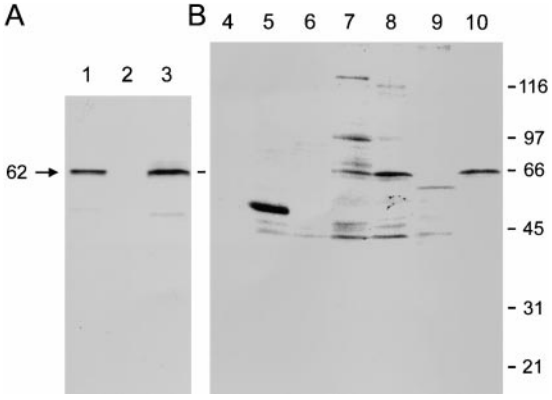

Figure 5. Human anti–62-kD antibody and rabbit anti–recombinant p62 antibodies recognize recombinant antigen. (A) Immunoprecipitation using in vitro–translated products of p62. Lane 1, in vitro–translated products; lane 2, preimmune rabbit serum (No. 3366); lane 3, immune rabbit serum (No. 3366). (B) Immunoprecipitation using [^{35}S]methionine–labeled HeLa cell extracts. Lane 4, normal human serum; lane 5, anti-SSB/La prototype serum, which recognizes an irrelevant 48-kD autoantigen used as a positive control for the assay; lane 6, HCC serum without p62 autoantibodies; lanes 7 and 8, p62 prototype sera; lane 9, preimmune rabbit serum (No. 3366); lane 10, immune rabbit serum (No. 3366).

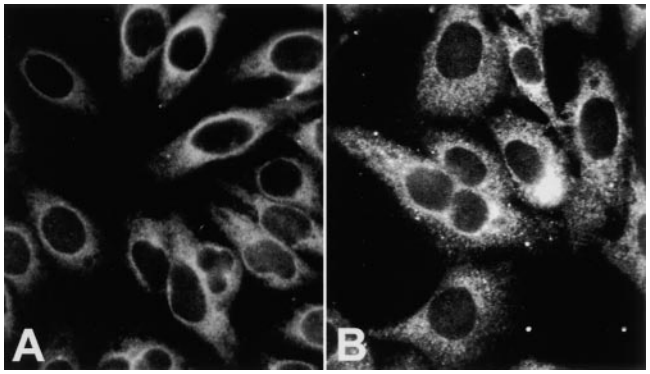

Figure 6. p62 is a cytoplasmic protein, as revealed by immunofluorescence analysis of HEp-2 cell substrate using human p62 prototype serum YZ (A) and affinity-purified rabbit anti–p62 serum No. 3366 (B).

domain of p62 as probe (Fig. 2 A), two major forms of p62 transcripts were detected in Northern blotting using commercially available poly A^+ RNA from a number of human tissues and cell lines. Fig. 7 demonstrates that there was a 3.7-kb transcript (lower arrow) that was reactive with the probe, as well as a 5.2-kb transcript (upper arrow). The 3.7-kb transcript of p62 was found in heart and placenta, whereas brain, lung, liver, kidney and pancreas gave much

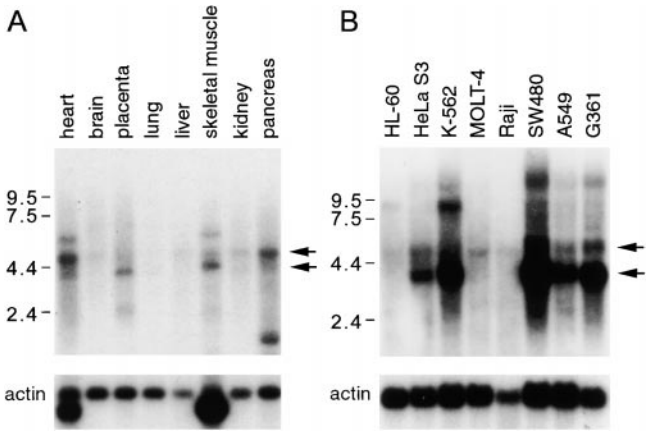

Figure 7. Northern blot analysis of p62 mRNA. The nylon membrane blotted with poly A^+ RNA isolated from multiple human tissues (A) and human cancer cell lines (B) was purchased from Clontech. Each lane contained 2 μg of purified poly A^+ RNA. An antisense riboprobe was generated from the 480-bp fragments corresponding to the COOH-terminal domain of p62 (see Fig. 2 A) and hybridized to the membrane at a concentration of 1.0×10^6 cpm of the probe per milliliter of hybridization solution. Two transcripts of 3.7 and 5.2 kb were detected in human tissues (A, arrows) and cancer cell lines (B, arrows). The 3.7-kb transcript of p62 was found in heart and placenta, but its occurrence was lower or negative in brain, lung, liver, kidney, and pancreas. A signal for skeletal muscle migrated somewhat more slowly than the 3.7-kb transcript (see text). High levels of the 3.7-kb transcript were detected in HeLa, K-562, SW480, A549, and G361 cell lines, but low expression was observed in HL-60, MOLT-4, and Raji cell lines. The 3.7-kb transcript expression corresponded to detectable cellular p62 protein (see text), but the significance of the 5.2-kb RNA is unknown. A 2.0-kb human β-actin cDNA probe was used as a control for monitoring RNA levels. As described in the manufacturer's (Clontech) instructions, there are two forms of β-actin mRNA, a 2- and 1.6–1.8-kb form, in heart and skeletal muscle.

lower signals or were negative for p62 expression. A signal for skeletal muscle migrated somewhat more slowly than the 3.7-kb transcript (see data showing the probe corresponding to the NH_2-terminal domain below). High levels of the 3.7-kb transcript were also detected in HeLa, K-562 (chronic myelogenous leukemia), SW480 (colorectal adenocarcinoma), A549 (lung carcinoma), and G361 (melanoma) cell lines, whereas low expression was observed in HL60 (promyelocytic leukemia), MOLT-4 (lymphoblastic leukemia), and Raji (Burkitt's lymphoma) cell lines. The presence of the 3.7-kb transcript in these cell lines showed good correlation with the presence of a 62-kD polypeptide antigen in extracts of these cell lines, whereas cell lines negative for the 3.7-kb transcript contained negligible or barely detectable amounts of 62-kD polypeptide. The data are not shown for all cell lines, but as can be seen in Fig. 1, MOLT-4 cell extracts contained low levels of p62 antigen and low levels of 3.7-kb transcript (Fig. 7 B). The Northern blot data just described were confirmed with an antisense riboprobe generated from a 687-bp fragment corresponding to the NH_2-terminal domain of p62, and the same membrane shown in Fig. 7 A was stripped and rehybridized with this probe. The 3.7- and 5.2-kb transcripts were specifically detected by this probe, a result similar to the observations shown in Fig. 7 A with the exception that the "slower" 3.7-kb band in skeletal muscle was not detected. From these observations, we propose that both the 3.7- and 5.2-kb transcripts represent cellular mRNAs encoding the 62-kD protein. The identity of the 5.2-kb transcript has not been determined, but it could represent an alternative mRNA for the 62-kD protein with extra 5′ upstream or 3′ untranslated sequences.

Analysis of Sera from Humans with Different Conditions. An enzyme-linked immunosorbent assay system was developed using p62 recombinant protein as antigen and a total of 242 sera from humans with different conditions were examined for reactivity. Table II shows that detectable antibodies to p62 antigen were present in 21.1% of patients in a group of patients with HCC from Henan Province. However, in sera from patients with conditions that are known to be precursor diseases to HCC, including asymptomatic HBsAg, acute

Table II. *Prevalence of p62 Autoantibody in Human Sera from Henan Province by ELISA Screening*

Categories	Prevalence patients with p62 antibody/ total patients with disease
Hepatocellular carcinoma	20/95 (21.1%)
Asymptomatic HBsAg carrier	0/26
Acute hepatitis	0/31
Chronic hepatitis	0/20
Normal human sera	0/30
Normal human sera*	0/40

*This group of sera was derived from the San Diego area.

hepatitis, and chronic hepatitis, no antibodies to p62 were detected. Normal human sera from patients who came from Henan Province or the San Diego area were also negative for p62 antibodies.

Discussion

Study of human tumor antigens recognized by the autologous host has had a long history, and T cell–recognized epitopes on human tumor cells have been extensively characterized (30). However, antibody-defined tumor antigens have been receiving greater attention recently, and several centers have used a method called SEREX (serological analysis of recombinant cDNA expression libraries), libraries of cDNA constructs made from mRNA extracted from tumor tissue (29) and screening of such libraries with autologous serum. From these studies, several novel as well as previously defined tumor antigens have been identified (30). Our approach was not restricted to use of cDNA libraries from autologous tumors because our previous studies had shown that antibodies in HCC sera were reactive with antigens expressed by a variety of tissue culture cell lines (23–25). The autoantigens identified included some previously recognized in autoimmune diseases such as lupus and scleroderma (22, 23). However, an unusual feature in the HCC model was that novel antigen-antibody reactions were detected in some patients during transformation from chronic liver disease to malignancy. Therefore, use of such sera to isolate and identify antigens might reveal special signatures of cancer cells. A novel protein with alternative splicing factor motifs (25) and a cell cycle–related nuclear protein with WD-40 motifs (26) have been isolated. These novel autoantigens, including p62 reported here, are not restricted to cancer cells but are also expressed in normal cells, an observation which has also been reported for cancer antigens in melanoma. These antigens have been called differentiation antigens (28). At this point, it is unclear why some differentiation antigens become capable of provoking autoimmune responses, but possibilities which have been raised in the case of p53 include gene mutations, gene product overexpression, and unusual complexes with other cellular proteins such as heat shock proteins (27, 45).

In addition to the remarkably high percentage of similarity and identity between p62 on the one hand and ZBP1 and Koc on the other, there are several features of these proteins that are of interest. ZBP1 is a chicken protein of 68 kD which was identified through its property of binding to a conserved element in the 3′ untranslated region of β-actin mRNA (40). Rabbit antibodies raised against ZBP1 polypeptides were shown to bind to β-actin mRNA in the leading edge of the lamella in cells such as chicken embryo fibroblasts and 3T3 fibroblasts (40). Studies are in progress to determine whether recombinant p62 is capable of binding in vitro to the "zipcode" element of β-actin mRNA as is the case with ZBP1.

The Koc protein was isolated following analysis of differential gene expression in pancreatic cancer tissue (39). High transcript levels of Koc were found not only in pancreatic cancer tissue but also in soft tissue sarcoma, gastric cancer, and colon cancer. Differences in mRNA expression of Koc in different tissues has been reported (39). We have also observed different transcript levels of p62 in different tissues and, in addition, high levels of expression of p62 transcript in some cell lines (HeLa, K-562, SW480, A549, and G361) but low expression in others including the promyelocytic leukemia HL-60, lymphoblastic leukemia MOLT-4, and Burkitt's lymphoma Raji. It is perhaps of some interest that the two human members (p62 and Koc) of this putative family of RNA-binding proteins appear to be associated in some way with cancer. It has recently been reported that two different Koc-homologous genes were also identified by SEREX methodology (46), but sequence information concerning these genes and their relationship to cancer are not yet available.

Previously, we reported that several patients with HCC mount de novo immune responses to nuclear antigens at the time of conversion from chronic hepatitis or liver cirrhosis to HCC (24) and that the autoantibodies that were produced against intracellular antigens might be regarded as immune system reporters of abnormal intracellular molecular events. Novel proteins that have been detected include HCC1 (25), a putative member of the SR family of alternative splicing factors, and SG2NA, a protein highly expressed in the S and G2 phases of the cell cycle (26). p62 appears to be another such molecule, but unlike the antibody responses to HCC1 and SG2NA, which were observed in individual patients, the autoimmune response to p62 was detected in >20% of one group of HCC patients, suggesting that a shared stimulus might be inciting the immune responses. This shared stimulus could be environmental in nature, but this is as yet only conjecture. An important study that could not be performed at this time was the analysis of HCC tissues to determine whether there were abnormalities in the p62 gene or in its expression, as tissue specimens were not available in this retrospective study. This will be the focus of a prospective study in newly identified HCC patients with autoantibodies to p62.

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Note added in proof. While this paper was in press, an article by J. Nielsen et al. was published (*Mol. Cell. Biol.* 19: 1262–1270) describing a cDNA called IMP-2 (insulin-like growth factor II mRNA binding protein) that was completely identical to p62 except for an insertion of 43 amino acids between the KH2 and KH3 domains.

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