Cloning and characterization of a novel 90 kDa 'companion' auto-antigen of p62 overexpressed in cancer

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Recently our laboratory identified a cytoplasmic RNA-binding protein p62 which binds to and regulates the expression of IGF II mRNA. p62 was initially shown to be recognized by auto-antibodies in hepatocellular carcinoma (HCC) but now anti-p62 has been described in diverse malignancies. p62 is uniformly expressed in fetal liver and prominently in 33% of HCC nodules, but not detectable in adult liver or normal tissue adjacent to HCC nodules. In this study, a 90 kDa protein (p90), auto-antibodies to which were found associated with anti-p62 responses in the same HCC patient group, was identified by cDNA expression cloning. Indirect immuno-fluorescence showed that, like p62, p90 localized to the cytoplasm in cultured cells and mouse fetal, but not adult liver. Among 11 human gastric cancer tissues examined, p90 was overexpressed in six (55%). Together with other cancer associated auto-antibodies such as anti-p53, anti-p62, anti-Koc, and anti-CENP-F, auto-antibodies to p90 represent a new marker for tumors such as HCC and gastric cancer. Our data support the working hypothesis that auto-antibody production in cancer may be directly linked to aberrant auto-antigen expression.
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Introduction

Primary hepatocellular carcinoma (HCC) frequently occurs in certain regions of Asia and sub-Saharan Africa at a rate of one in 200 new cases a year (Simonetti *et al.*, 1991). Acute and chronic liver diseases such as an unremitting infection with HBV or HCV followed by cirrhosis places patients at increased risk of developing HCC (Kiyosawa *et al.*, 1990). Longitudinal studies in Japanese HCC patients showed that the progression from chronic hepatitis or liver cirrhosis to HCC coincided with the *de novo* appearance of new auto-antibodies with a notable increase in titer of pre-existing antibodies and/or change in antibody specificity (Imai *et al.*, 1993; Zhang *et al.*, 2001b). These observations suggest that the changing auto-immune responses in certain cancer patients could be related to cellular mechanisms associated with malignant transformation. Therefore identifying the auto-antigens in such situations may contribute to elucidating the nature of some of the cellular proteins involved in carcinogenesis.

Using serum antibody from a patient with HCC to screen a cDNA expression library, we identified a 62 kDa auto-antigen (p62) that elicited a humoral immune response in 21% of HCC patients (Zhang *et al.*, 1999). p62 is a mRNA binding protein with two RNA Recognition Motifs (RRM) at the N-terminal domain and four hnRNP K homology (KH) domains extending from mid to C-terminal region. p62 belongs to the family of IGF-II mRNA binding proteins (IMPs) with three distinct gene products IMP-1, IMP-2, and IMP-3 (Nielsen *et al.*, 1999). Nielsen *et al.* (1999) have shown that IMPs bind to the 5′ sequence of leader three IGF-II mRNA and are likely to regulate the expression of IGF-II. The human IMPs have high sequence similarity with other RNA binding proteins such as the *Xenopus* Vg1RBP/Vera protein (Deshler *et al.*, 1998; Havin *et al.*, 1998), the chicken zipcode-binding protein (ZBP-1) (Ross *et al.*, 1997), and the mouse c-*myc* coding region instability determinant binding protein (CRD-BP) (Doyle *et al.*, 1998). These RNA-binding proteins have been implicated in post-transcriptional events such as RNA localization, mRNA stability and translatability. Lastly, the human Koc (hnRNP <u>K</u>omology domain protein <u>o</u>verexpressed in <u>c</u>ancer) protein, identical to IMP-3, was originally isolated by screening for genes differentially expressed in pancreatic cancer (Mueller-Pillasch *et al.*, 1997). Thus, the IMPs are likely to play a role in regulating the expression of many gene products via association with their respective mRNAs and, importantly, their expression may be altered in malignancy (Lu *et al.*, 2001; Mueller-Pillasch *et al.*, 1997).

Our recent immunohistochemical analysis of HCC liver found that 33% (nine out of 27) showed remarkable staining of p62 protein in the cytoplasm of malignant cells in cancer nodules but not in adjacent non-malignant liver cells (Lu *et al.*, 2001). p62

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expression was also detected in scattered cells of cirrhotic nodules in contrast to the uniform expression in all cells of HCC nodules. The observations show that p62 could play a role in abnormal cell proliferation in HCC and cirrhosis by modulating expression of growth factors such as IGF-II (Lu et al., 2001).

Our more recent studies showed that auto-antibodies to p62 and Koc were detected in sera from patients with diverse malignancies including neoplasms of the esophagus, lung, pharynx, uterus, gastrointestinal tract and liver (Zhang et al., 2001a). Importantly, although there were significant sequence similarities between p62 and Koc and the immunodominant epitopes of p62 and Koc were at the amino termini of both antigens, absorption studies showed that the majority of auto-antibodies were not cross-reactive (Zhang et al., 2001a). Auto-antibodies to p62 and Koc were detected in approximately similar frequencies in a variety of malignancies and the immune responses appeared to be independent of each other (Zhang et al., 2001a).

Results

Auto-antibodies to p90 in HCC

In a cohort HCC sera from the Henan Province, China, 21% had auto-antibodies to p62 (Zhang et al., 1999). During this work, it was noted that some patients with antibodies to p62 also had antibody to a 90 kDa protein. In the present study, Western blot analysis was extended by using sera with candidate antibody to p90 and re-examined on side-by-side nitrocellulose strips. Twenty-one of 95 sera (22%) showed reactivity to an apparently common p90 antigen. Figure 1a shows that, in addition to HCC sera, several sera from patients with GC appeared to have antibodies to p90. The p90 protein was detected in various cell lines demonstrating that its expression was not exclusive to MOLT-4 cells (Figure 1b). Expression of endogenous p90 was observed in cancer cell lines such as the commonly used HeLa and normal human keratinocytes. Cell fractionation data showed that p90 was present predominantly in the cytoplasm of MOLT-4 cells (Figure 1b, lane 6).

Isolation and identification of p90 cDNA clones

Since p62 has become a highly interesting cancer auto-antigen comparable to p53 and auto-antigens detected in paraneoplastic syndrome (Tan, 2001), we wanted to identify the molecular nature of the companion-auto-antigen p90 because anti-p90 antibodies have been detected in the same group of patients originally described for anti-p62. Three sera GC15, GC28, and GC29 showed apparently specific reactivity to p90 in Western blot analysis (Figure 1a) were selected to screen a human cDNA expression library constructed from T24 cells. All three sera had cytoplasmic staining on HEp-2 cell substrate but additional reactivities were noted. Figure 1c shows that staining for serum GC28 which gave predominantly diffuse and reticular staining
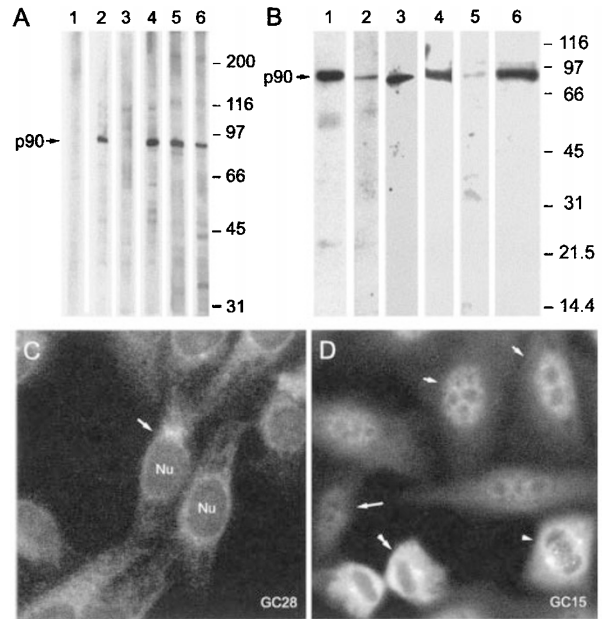

Figure 1 Patients with hepatocellular carcinoma (HCC) and gastric cancer (GC) have auto-antibodies to a 90 kDa cytoplasmic protein (p90) expressed in a variety of human cell lines. (a) Western blot analysis of MOLT-4 whole cell extracts revealed anti-p90 antibodies in sera of representative patients. Lane 1, normal human serum; lane 2, serum from HCC patient S23; lanes 3–6, sera from GC patients GC1, GC15, GC28 and GC29 respectively. (b) Serum S23 recognized endogenous p90 in human cell line T24 (lane 1), HeLa (lane 2), keratinocytes (lane 3), and MOLT-4 (lane 4). Fractionation of MOLT-4 cells into nuclear (lane 5) and cytoplasmic fractions (lane 6) showed that p90 was predominantly in the cytoplasmic fraction. (c and d) show staining of HEp-2 cells by serum GC28 and GC15 both diluted 1:100. GC28 shows predominantly diffuse and reticular staining of the cytoplasm (arrow) and weaker diffuse staining of nuclei (Nu). GC15 shows diffuse cytoplasmic staining in all interphase cells. In addition to anti-p90, GC15 is also known to contain anti-CENP-F antibodies which gave the cell cycle-dependent staining of the nucleus with stronger staining of late S/G2 cells (short arrows), and weaker nuclear staining of G1 cells (long arrow), and bright staining of centromeres in mitotic cells (arrowhead) and newly dividing cells (double arrowheads). Original magnification 400×

of the cytoplasm and very weak diffuse staining of nuclei. In contrast, GC15 shows diffuse cytoplasmic staining in all cells in interphase but, in addition, centromere staining was observed in mitotic cells and newly dividing cells (Figure 1d). The selection of the T24 cell cDNA library was based on availability, our experience with this library, and that p90 is relatively highly expressed in T24 cells (Figure 1b). Initial screening of 800 000 recombinant plaques identified 22 putative p90 clones. After three rounds of purification and screening with the three GC sera, 13 phage clones were isolated and purified to homogeneity. Preliminary sequencing analysis showed that cDNA clones GC281, GC283, and GC291 might be candidate(s) for p90 from probing with sera GC28 or GC29. Two overlapping cDNA clones GC1511 (aa 2188–3210) and GC155 (aa 2394–3210) encoding centromere protein F (CENP-F, GenBank accession

number P49454, total 3210 amino acids) were identified by serum GC15 alone; auto-antibody to CENP-F is known to give cell cycle-dependent nuclear speckled pattern and the staining of centromeres in mitotic cells (Figure 1d). Other cDNA clones did not encode p90 based on criteria described below and will not be discussed further here.

To validate the authenticity of putative p90 cDNA clones, antibody affinity purification and immunoprecipitation of *in vitro* translated products were employed. For affinity-purification of antibodies, 100% pure phages for the candidate clones GC291, GC281 and GC283 were used separately to produce recombinant proteins on plates and transferred to nitrocellulose circles using the same method as library screening. Affinity purified antibodies from serum GC28 to each of the three phage proteins were shown to recognize p90 on Western blots of MOLT-4 cell extracts (Figure 2a). In contrast, control antibodies purified from a CENP-F clone GC1511 did not recognize p90.

The phage clones GC291, GC281, and GC283 were transformed *in vivo* with helper phages to pBK-CMV plasmids which were subsequently used as DNA templates for *in vitro* transcription and translation (TnT). Figure 2b shows the TnT products for clones GC291, GC281, and GC283 as 35/36 kDa doublet, 50 kDa, and 90 kDa, respectively. Sera from all three human anti-p90 sera GC15, GC28, and GC29 showed a clear positive reactivity by precipitating all putative p90 clones but not the unrelated control product

pp75L, whereas a normal human serum showed no reactivity (Figure 2c). The 90 kDa TnT protein product of GC283 comigrated with p90 detected in cell extracts and thus these data suggested clone GC283 might represent a full-length p90 cDNA (Figure 2d).

Sequence analysis of p90 cDNAs

The complete nucleotide sequences of the three putative p90 clones showed that they represented overlapping cDNAs as depicted in Figure 3a. Sequence analysis of GC283 cDNA predicted a single open reading frame with 905 amino acids (Figure 3b) with a predicted molecular mass of 102 201 Da and a pI of 5.85. An in-frame stop codon TAA was identified upstream from the translation start site which is highly consistent with the eukaryotic consensus start sequence (Kozak, 1987); together with the fact that the TnT product comigrated with endogenous p90, the nucleotide sequence analysis confirmed that GC283 encoded the full-length p90 protein. In addition, the 5′-untranslated region is predominately GC-rich. The 3′-noncoding region included two AATAAA polyadenylation signals and six copies of ATTTA sequences, which are frequently found in lymphokine and proto-oncogene mRNAs and are believed to reduce the stability of mRNA (Shaw and Kamen, 1986). Searching GenBank databases using BLAST programs did not generate any significant alignment to established gene products. A human EST KIAA1524 (GenBank Accession number AB040957) reported by the Kazusa
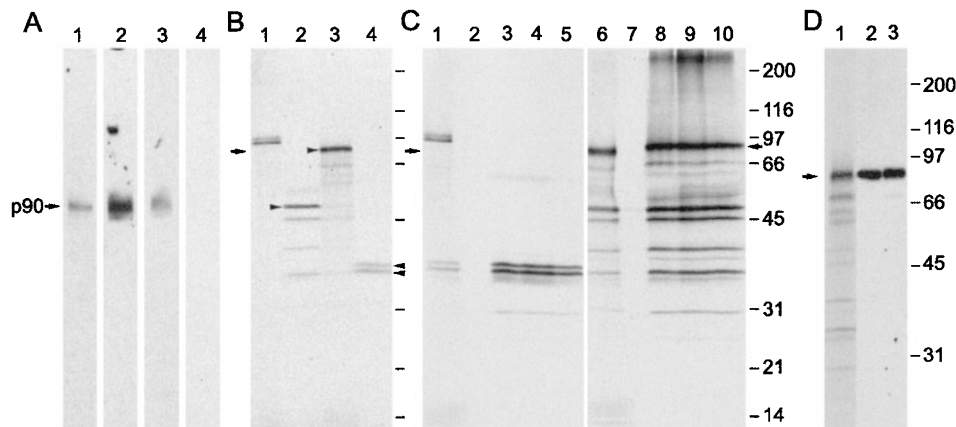

Figure 2 Corroborating evidence that putative p90 cDNA clones isolated from T24 cell cDNA library encode the cellular p90 auto-antigen. (**a**) Western blot analysis shows that antibodies affinity purified from a human anti-p90 serum GC15 using nitrocellulose membranes coated with phage proteins encoded by putative p90 cDNA clones GC291 (lane 1), GC281 (lane 2), and GC283 (lane 3) recognized a 90 kDa protein in MOLT-4 cell extract. Affinity purified antibodies to an unrelated clone GC1511 phage protein show no reactivity (lane 4). (**b**) *In vitro* transcription and translation (TnT) of cDNA clones GC281 (lane 2), GC283 (lane 3), and GC291 (lane 4) incorporating [35S]-methionine into translation products 50, 90 and 35/36 kDa respectively (arrowheads). Lane 1 shows an unrelated translation product protein pp75L as a positive control for the *in vitro* TnT reaction. (**c**) Immunoprecipitation of *in vitro* TnT products by all three human anti-p90 sera GC15, GC28, and GC29. TnT products mixtures of clones GC291 and pp75L (1 : 1 mix, lanes 1 – 5) or clones GC281 and GC283 (1 : 1 mix, lanes 6 – 10) were analysed by immunoprecipitation using a normal human serum (lane 2), GC15 (lane 3), GC28 (lane 4), and GC29 (lane 5). Lanes 1 and 6 represent the respective mix alone without immunoprecipitation for comparison. (**d**) The *in vitro* TnT product of putative full-length p90 clone GC283 (lane 1) co-migrates with endogenous p90 from MOLT-4 (lane 2) and HeLa (lane 3) whole cell extracts recognized by anti-p090 serum GC28. Samples of the 35S-methionine labeled TnT product of GC283 (lane 1) and unlabeled MOLT-4 (lane 2) and HeLa (lane 3) whole cell extracts were separated side-by-side in a 10% separating gel and all three lanes were electrotransferred onto a nitrocellulose membrane. Lane 1 represents the exposure of the 35S-methionine labeled TnT product alone and lanes 2 and 3 show the Western blot results

DNA Research Institute was detected as a partial cDNA (Nagase et al., 2000). Additional human, house, and fish EST clones were detected suggesting that p90 is a conserved protein. A protein producing a weak homology with p90 is an intracellular transport protein USO1 in yeast. No homology was found with the N-terminal two-thirds. The human p90 gene could be localized to human chromosome region 3p13–q13.2 by matching to the human genome draft sequence with the GenBank accession number NT_022434. The complete gene sequence was composed of at least 26 exons spanning 92 kb.

Analysis of p90 protein sequence showed a long coiled-coil region spanning 255 amino acids at the C-terminus (Figure 3c). Since coiled-coil regions are often responsible for protein dimerization, an experiment was designed to determine whether p90 was capable of forming dimer or multimer in vitro. The full-length 90 kDa TnT product which was produced in rabbit reticulocyte lysate was analysed in a Sephacryl S300 gel filtration column and the results showed that p90 migrated as a monomer peak (data not shown). When the full-length amino acid sequence was used to search GenBank protein database, only proteins that are rich in coiled-coils are shown as weak matches. No other known protein motif was identified even when the search was carried out with the N-terminal two-thirds of p90.

Figure 3 Sequence analysis of human p90. (**a**) Sequence analysis of GC283, GC281, and GC291 revealed that they represent overlapping clones as illustrated. Heavy lines represent their open reading frames (ORF). (**b**) Human p90 nucleotide and deduced amino acid sequences. In the 5′-untranslated region, the GC-rich region is boxed and the G and C residues are shown in bold, and the upstream in-frame stop codon TAA is underlined. The methionine start site (Kozak's sequence GGTGCAATGG) and poly(A)+ signals AATAAA in 3′-untranslated region are also underlined. Six copies of ATTTA motif throughout the 3′-untranslated region are double-underlined. A leucine zipper in position 331–352 is shown with leucine residues circled; the proline residue in position 351 is boxed. Two pentapeptides RKELS of unknown significance are detected in the coiled-coil region (double-underlined). (**c**) The Coils and Paircoils programs predicted a probable coiled-coil domain spanning 255 amino acids (boxed region **b**) at the C-terminal one third of p90. These sequence data are available from GenBank under accession number AF334474

Heterologous anti-p90 antibodies gave cytoplasmic staining similar to anti-p62 antibodies

The two NZW rabbits and three BALB/c mice immunized with recombinant p90 fragment aa 618–905 all produced antibodies to p90 as determined by Western blot using MOLT-4 cell extracts (Figure 4a). Double staining experiments using mouse fibroblast cell line 3T3 showed that rabbit anti-p62 (Zhang *et al.*, 1999) gave very similar diffuse cytoplasmic staining as mouse anti-p90 (Figure 4b–e) and human anti-p90 serum GC28 (Figure 4f–h). However, the staining for p90 was slightly more concentrated in the perinuclear region of the cell whereas staining for p62 spread more evenly throughout the cytoplasm and thus appeared stronger in the peripheral region (Figure 4e,h).

Primarily cytoplasmic staining was also observed in mouse embryonic tissues using rabbit anti-p90 antibodies. Figure 5b shows the intense cytoplasmic staining seen in 17.5 and 18.5 day embryonic liver specimen while the control preimmune serum of the same dilution gave barely detectable background staining in comparison (Figure 5a). The expression of p90 in fetal liver was clearly one of the strongest among many organs examined but other tissues including brain, muscle fibers, and epidermal layers also had moderate expres-
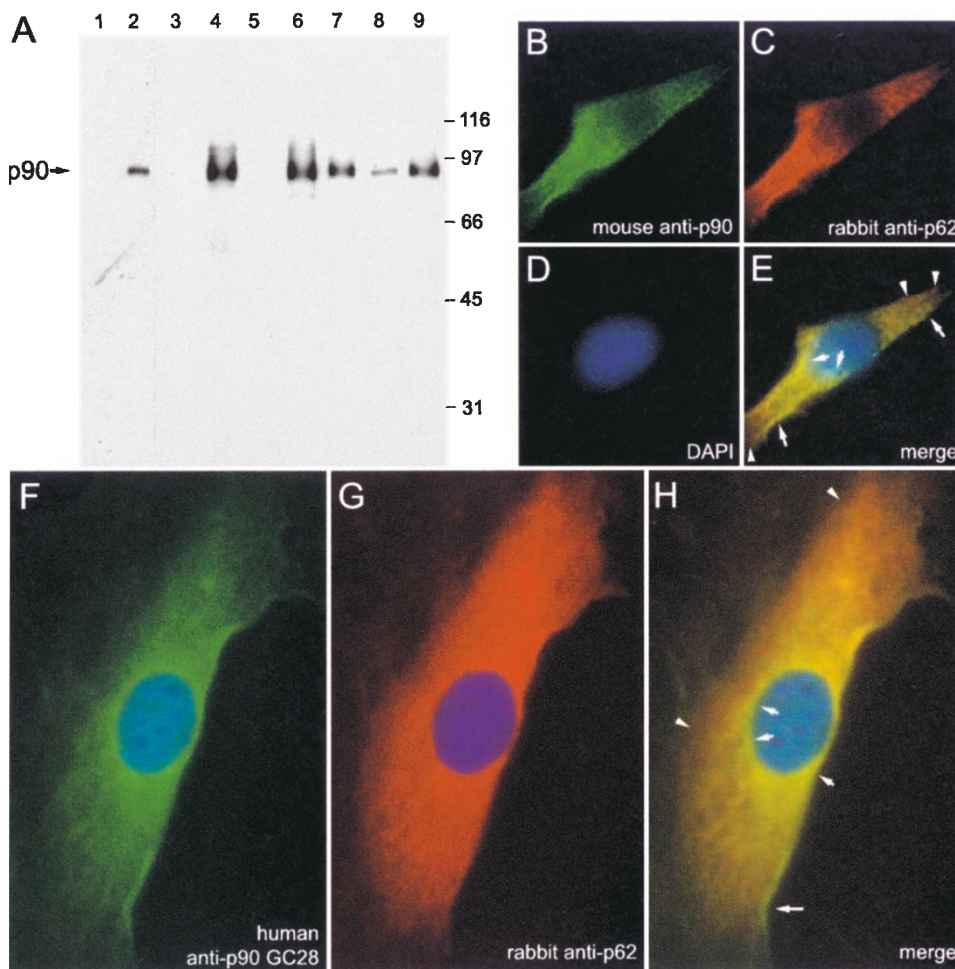
Figure 4 Heterologous antibodies produced to recombinant human p90 show primarily cytoplasmic staining similar to the IGF-II mRNA binding protein auto-antigen p62. BALB/c mice and NZW rabbits were immunized with recombinant p90 C-terminal fragment as described in Materials and methods. (**a**) Western blot analysis shows specific reactivity to p90 in strips of nitrocellulose membrane blotted with MOLT-4 whole cell extract. Lane 1, normal human serum; lane 2, human serum GC28; lanes 3 and 4, pre- and post-immunization sera from rabbit 5180; lanes 5 and 6, pre- and post-immunization sera from rabbit 5181; lanes 7–9, post-immunization sera from mouse #1–3 respectively. All sera were used at 1 : 400 dilution. (**b–e**) Mouse 3T3 cells were double-labeled with anti-p90 from mouse #1 and rabbit anti-p62 antibodies which were detected by FITC-labeled goat anti-mouse Ig (**b**) and Cy3-labeled goat anti-rabbit Ig (**c**) respectively. Cells were counterstained with nuclear dye DAPI (**d**) and a merged image shown in **e**. (**f–h**) 3T3 cells were double-labeled with human anti-p90 serum GC28 and rabbit anti-p62 which were detected by FITC-labeled goat anti-human Ig (**f**) and Cy3-labeled goat anti-rabbit Ig (**g**) respectively. **f–h** show merged images with DAPI. The merged images in **e** and **h** show that, although the staining for anti-p90 and anti-p62 are similar, anti-p90 gave more intense staining in the perinuclear region (short arrows) and peripheral edges of the cell (long arrows) while anti-p62 gave stronger diffuse cytoplasmic staining in primarily peripheral regions (arrowheads). Original magnification 600 ×
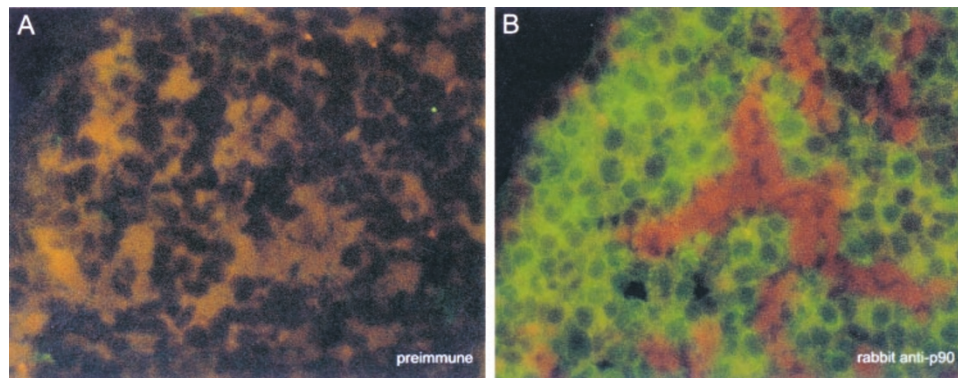
Figure 5 Expression of p90 in fetal mouse liver. Sections of mouse embryo at 17.5 day were stained with diluted rabbit serum at 1 : 400 with preimmune (**a**) and post-immunization sera (**b**) from rabbit 5181. Original magnification 400 ×

Table 1 Autoantibody to p90 and p62 in human cancer and related conditions

	Anti-p90	Anti-p62
Hepatocellular carcinoma (HCC)	21/160 (13.1%)	26/160 (16.3%)
Chronic hepatitis	0/20	0/20
Acute hepatitis	0/31	0/31
HBsAg positive carriers	0/26	0/26
Gastric cancer	3/91 (3.3%)	8/91 (8.8%)
Esophagus cancer	1/20 (5.0%)	4/20 (20.0%)
Colon cancer	0/20	3/20 (15.0%)
Normal controls	0/112	1/112 (0.9%)

HCC sera	Anti-p90 and anti-p62			
	anti-p90+ anti-62+	anti-p90+ anti-p62−	anti-p90− anti-p62+	anti-p90− anti-p62−
95	13 (13.7%)	8 (8.4%)	7 (7.4%)	67 (70.5%)

sion. Like p62, adult mouse liver specimens did not show expression of p90 (data not shown).

Prevalence of anti-p90 auto-antibodies in cancer

Recombinant p90 fragment corresponding to clone GC291 was expressed and used as substrate in ELISA for the detection anti-p90 auto-antibodies in different cancer sera. Table 1 shows the composite data on the prevalence of anti-p90 auto-antibodies based on data from Western blot using MOLT-4 cell extracts and recombinant protein, ELISA using the recombinant p90 fragment, and IP of full-length TnT products. Anti-p90 were detected in 21/160 (13.1%) HCC patients, but not in patients with chronic hepatitis, acute hepatitis, HBsAg carriers, and normal controls (Table 1). Anti-p90 were detected also in 3.3% and 5.0% patients with gastric or esophagus cancer respectively but not in colon cancer. For comparison, the prevalence of anti-p62 antibodies in the same groups of sera is also shown in Table 1. The data show that the prevalence of anti-p62 is somewhat higher than anti-p90 in all patient groups examined. Among 95 HCC sera examined for both anti-p62 and anti-p90, 13 (13.7%) had both antibodies, eight (8.4%) had only anti-p90 and seven (7.4%) had only anti-p62.

Expression of p90 in malignant stomach sections from gastric cancer patient

Among 11 human stomach specimens from gastric cancer patients available for our analysis, six had enhanced expression of p90 (Figure 6). Poorly differentiated and irregularly shaped cells showed uniform strong reactivity by anti-p90 antibody (Figure 6b) but not preimmune serum (data not shown). The squamous epithelial cells adjacent to the cancerous region exhibited no detectable staining. The cytoplasmic localized of p90 protein in malignant cells was consistent with expression observed in mouse 3T3 cells (Figure 4b) and in hepatocytes or mouse embryo (Figure 5b).

Discussion

Soussi (2000) has recently reviewed data complied from 130 publications between 1992–2000 on antibodies to the tumor suppressor protein p53. In brief, p53 antibodies are found predominantly in human cancer patients with a high specificity of 96% but a low sensitivity of only 30%. Antibodies to p53 are predominantly associated with p53 gene missense mutations and p53 accumulation in the tumor. The immune response to p53 is due to a self-immunization process linked to the strong immunogenicity of the p53 protein. Anti-p53 is detected consistently in breast, colon, oral and gastric cancers, in which they have been associated with high-grade tumors and poor survival (Soussi, 2000). Soussi (2000) concluded that the detection of anti-p53 may have promising potential in the early detection of cancer in individuals who are at high risk of cancer, such as exposed workers or heavy smokers.

Here we have identified auto-antibodies to p90 in HCC and GC patients and cloned the full-length cDNA for p90. The important feature is that p90 behaves like p62 as a cytoplasmic auto-antigen expressed in fetal liver and cancer nodules. Although both p62 and p90 have not been shown to be tumor suppressor like p53, anti-p62, and likely anti-p90, are detected in various human cancer (Zhang et al., 2001a).
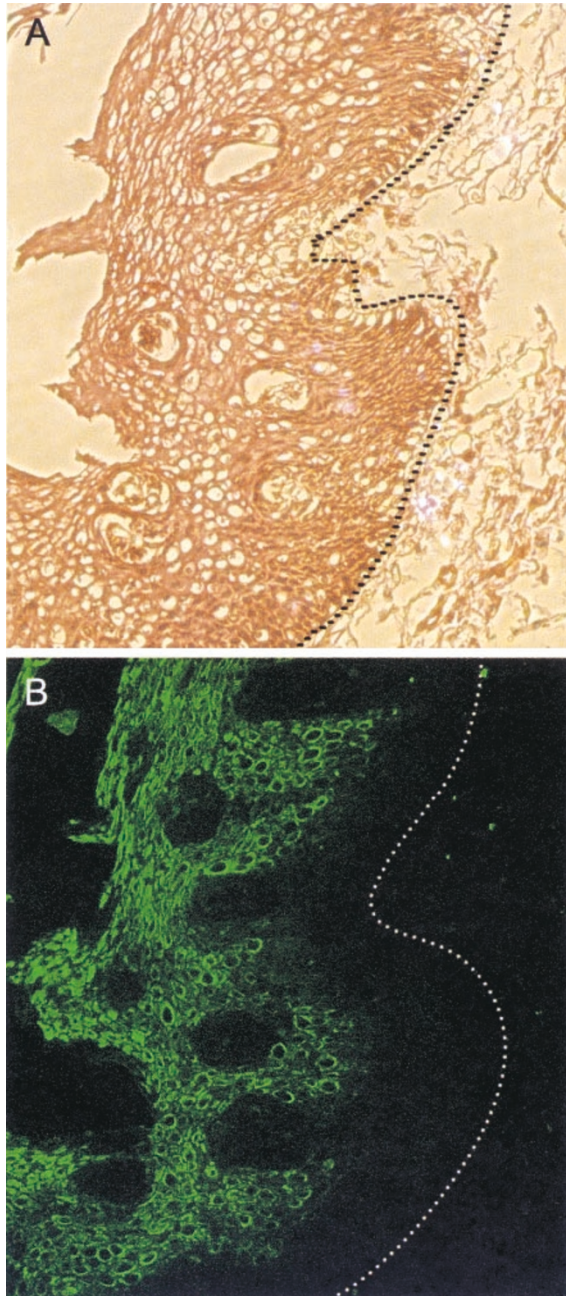
Figure 6 Expression of p90 in gastric cancer. (**a**) H&E staining reveal a section of a poorly differentiated gastric cancer with irregularly shaped tumor cells. (**b**) Immunohistochemical analysis for p90 in an adjacent section as described in Materials and methods. The lining of stomach are delineated by white or black dotted lines for comparison. Original magnification 400 ×

Autoantibodies to p90 and p62 in HCC

The serological profile in HCC patients from China points to a strong association of anti-p90 with anti-p62 antibodies (Table 1). The prevalence of anti-p90 in HCC patients is 21/160 (13.1%) compared to that of anti-p62 (26/160, or 16.3%). As shown in Table 1, 13 patients had both anti-p90 and anti-p62. Thus, among 21 patients with anti-p90 antibodies, 13 (61.9%) also

had antibodies to p62. However, there are also patients with anti-p90 alone or anti-62 alone. Such a 'companion-autoantigen' relationship between two auto-antibodies is reminiscent of well described relationships of anti-DNA and anti-histone antibodies in SLE, anti-Sm and anti-U1-RNP in SLE and mixed connective tissue disease (MCTD) and anti-SS-A/Ro and anti-SS-B/La antibodies in SLE and Sjögren's syndrome (Chan and Buyon, 1994; Hardin, 1986; Tan, 1989). Many lupus auto-antigens such as ribosomal P proteins and Ku p70/p80 identified as components of intracellular complexes are well documented in systemic rheumatic diseases (Tan *et al.*, 1988). The companion–auto-antigen relationship of p62/p90 is novel and not described in our earlier work (Covini *et al.*, 1997; Imai *et al.*, 1993). Also interesting is that auto-antibodies to p90 and p62 were detected in gastric and esophagus cancer but none of the patients had both auto-antibodies. As discussed below, whether p90 and p62 are components of a macromolecular complex or that they are transiently associated in the regulation mRNA expression/transport remains to be determined.

Chronic infection with the hepatitis B virus (HBV) and exposure to aflatoxins (Bressac *et al.*, 1991) in food are the main risk factors for the development of HCC in China. To address whether there was any difference of auto-antibody responses between HCC patients with or without HBV or hepatitis C virus (HCV) infection, the frequency of auto-antibody to p90 was analysed in the 95 HCC sera from patients whose HBV and HCV status were determined previously (Zhang *et al.*, 1998a,b). No correlation was observed between the HBV- or HCV-associated HCC with anti-p90 (data not shown); anti-p62 in HCC were also not linked to the status of HBV and HCV (Zhang *et al.*, 1999). More importantly, *de novo* appearance of antibodies to p62 was demonstrated in three patients during transition to malignancy (Zhang *et al.*, 2001b). Another antibody appearing during the transition period was against CENP-F, a cell cycle-related nuclear protein with maximum expression in the G2 and M phases of the cell cycle and previously shown to have a high association with malignancy (Rattner *et al.*, 1997; Zhang *et al.*, 2001b). As shown in this report, patient GC15 was shown to have anti-CENP-F as well as anti-p90. Although conclusive links between the function of p90 and p62 or CENP-F are not known, our data support that p90 is another member of the growing family of auto-antigens in malignancy.

Feature of p90 and association with p62

A prominent feature of p90 is the 255 amino acid long coiled-coil domain predicted at the carboxyl-terminus. This domain contained at least a major epitope recognized by all human sera containing anti-p90 antibodies. This region of p90 was obviously immunogenic since heterologous antibodies were readily produced in all rabbits and mice used to raise antibodies. Relevant to this observation is that our laboratory has shown that human anti-Golgi auto-

antibodies predominantly recognize a family of peripheral and integral Golgi membrane proteins (golgins) that are all highly enriched in coiled-coil domains suggesting that coiled-coils are potentially important epitopes for selected cytoplasmic auto-antigens (Chan and Fritzler, 1998). Our more recent work identified other auto-antigens with large regions, representing 50% or more of the protein, enriched in coiled-coils including the early endosome antigen EEA1 (Selak et al., 1999) and M phase phosphoprotein-1 MPP1 (Fritzler et al., 2000). A general relationship of auto-antigens with coiled-coil rich proteins remains unclear at this point. However, in order to determine whether coiled-coil regions are specially associated with epitopes recognized by human auto-antibodies, more work is needed to determine whether all epitopes recognized by human auto-antibodies, in this case different human anti-p90 sera, are restricted to the coiled-coil region.

Coiled-coil domains within a protein often suggest their potential for interaction with itself and other proteins forming larger molecular complexes. Our gel filtration analysis of translation product did not demonstrate dimer or multimer formation of p90 in vitro. Furthermore, using extracts from ^{35}S-methionine labeled HeLa cells as substrate for IP, only p90 was immunoprecipitated (data not shown). This suggests that although p90 may be capable of interaction with other macromolecules including p62, the association may not be sufficiently stable under the IP conditions. An alternative interpretation is that since the human anti-p90 antibody and heterologous antibodies raised to the recombinant p90 fragment both recognized the coiled-coil region, any macromolecular complex of p90 involving the coiled-coil domain may not be recognized by these antibodies due to probable steric hindrance. It remains probable that the carboxyl coiled-coil domain is important for protein–protein interaction although we do not have data on its interaction partner(s) at this point. Future work in this direction may involve using p90 as a probe in two-hybrid type experiments to determine its putative interaction partner(s) which may provide more leads to its cellular function (Fields and Song, 1989).

Co-expression of p90 and p62

Currently there is no experimental data to support direct molecular interaction of p90 and p62. In vitro mixing of translation products of full-length p90 and p62 also did not result in the formation of protein complex demonstrable by IP assay (data not shown). Since the conditions for standard IP often employ a mix of three detergents in the incubation and wash buffers to reduce nonspecific binding, it is possible that the failure to detect any protein interaction or associated proteins may be related to the poor stability of the putative complex(es) under these conditions. No interaction was detected even when the incubation and wash buffers were modified to contain only a single detergent – 0.1% NP40 (data not shown). However,

our data do not exclude the probability that p90 and p62 are associated in vivo directly or via intermediate(s) such as protein(s) or specific mRNAs, especially since p62 is a known mRNA binding protein (Nielsen et al., 1999).

Indirect immunofluorescence on cell substrates was used as an alternative method to address the potential association of p90 and p62. Although cancer sera GC15, GC28 and GC29 containing anti-p90 antibodies all gave cytoplasmic staining on HEp-2 cells, weaker nuclear staining was also observed. Using heterologous antibodies raised to recombinant p90, our data showed that the staining for p90 was primarily localized to the cytoplasm. In double staining experiments, using mouse 3T3, human HEp-2, and chicken fibroblasts (data not shown) double stained with different combinations of anti-p62 and anti-p90 antibodies, complete co-localization was never observed but there were clearly large areas of overlap in the cytoplasm. Thus these findings suggest that p62 and p90 may still be associated in vivo.

Relating to HCC, a striking feature on the oncofetal nature of p62 is that p62 is highly expressed in fetal human liver and in nine out of 27 (33%) HCC examined but there was little or no expression in normal adult liver (Lu et al., 2001). From the data published in the HUGE Protein Database (Kikuno et al., 2000) on the EST clone KIAA1524 (Nagase et al., 2000), which represents a partial cDNA of p90 missing the 5′ end, investigators at the Kazusa DNA Research Institute have shown that the expression of KIAA1524 (or p90) mRNA is at about 10-fold higher levels in fetal than adult liver mRNA using a reverse transcriptase–PCR ELISA. In this report, our data showed that the expression of p90 in mouse fetal liver, but not adult liver, was much higher compared to other tissues. Our preliminary analysis of 11 gastric cancer showed that six had enhanced expression of p90. Future work is needed to examine if the expression of p90 is also elevated in other cancer tissues such as HCC and to address whether there is any correlation with the expression of p62 in HCC.

Materials and methods

Sera

A group of 95 sera from patients with HCC from Henan Medical University in Henan Province, People's Republic of China had already been used in a previous study (Zhang et al., 1999). Sera from acute and chronic hepatitis, HBsAg positive carriers, and normals from the same source described (Zhang et al., 1998a,b). Additional sera from patients with HCC, colon, esophageal, or gastric cancer and normal human sera were collected from the serum bank at the Tumor Cell Engineering Laboratory of Xia'men University in the Fujian Province, China. Additional sera from healthy volunteers collected by the General Clinic Research Center at The Scripps Research Institute were also used. The study protocol was approved by the Institutional Review Boards at all institutions.

Western blot analysis of cell lysates

Established cell lines and human keratinocytes were obtained from the American Type Culture Collection and Clonetics (San Diego, CA, USA) respectively and cultured as described (Wang and Chan, 1996). To prepare whole cell extracts, cells grown as a monolayer were harvested and solubilized in Laemmli's sample buffer containing a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN, USA). Cytosolic and nuclear fractions were prepared from MOLT-4 cells using described methods (Braverman *et al.*, 1986).

Cloning of homo sapiens *p90 cDNA*

Patient sera GC15, GC28, and GC29 containing high levels of auto-antibodies to p90 were used separately to screen a lambda ZapExpress® expression cDNA library (Stratagene, La Jolla, CA, USA) constructed from mRNA of T24 cells as previously described (Ochs *et al.*, 1996).

Nucleotide and amino acid sequence analysis

The complete nucleotide sequence was determined using Bigdye terminator sequencing and a semi-automated sequencer model 377 (ABI, Foster City, CA, USA). Both nucleotide and deduced amino acid sequences were analysed for similarity with known sequences using BLAST search (Altschul *et al.*, 1997) and ExPASy Proteomics tools via World Wide Web Internet server (http://www.expasy.ch/www/tools.html). Secondary structure analysis for coiled-coil motifs was conducted with the software program 'COILS' (Lupas, 1997).

Immunoprecipitation (IP) of TnT products

Candidate p90 phagemid clones GC281, GC283, and GC291 were subjected to *in vitro* transcription and translation (TnT) using a rabbit reticulocyte lysate kit (Promega, Madison, WI, USA) following the protocol recommended by the manufacturer. *In vitro* translated proteins were examined for reactivity by sera using IP described (Chan *et al*, 1991; Wang *et al.*, 1999). Controls included an unrelated phosphoprotein pp75 (Wang *et al.*, 1999) produced at TnT products and mixed with a p90 TnT fragment to monitor the specificity of the IP reaction.

Indirect immunofluorescence

Cells were seeded onto eight-chamber well slides at ~30 000 per well and cultured overnight. Cells were fixed at 4°C using a 3:1 mixture of −20°C acetone and methanol for 3–5 min and probed with different antibodies as previously described (Zhang *et al.*, 1999). HEp-2 cell slides from Bion Enterprises, Ltd. (Des Plaines, IL, USA) were also used for the initial staining of human cancer sera.

Immunohistochemistry-ready mouse embryo slides staged at 17.5- and 18.5-day were purchased from Paragon Bioservices, Inc. (Baltimore, MD, USA). Archival paraffin-embedded malignant stomach sections were obtained from gastric cancer patients in Xia'men, China. Antigen retrieval was performed by microwave-heating methods in a citrate-based antigen retrieval solution (BioGenex, San Ramon) according to the manufacturer's recommendation and probed with rabbit antibodies (Lu *et al.*, 2001). Immunofluorescence was observed in a Nikon Eclipse TE300 inverted microscope using 40× or 60× oil immersion objectives.

Affinity purified antibodies from recombinant phage proteins

Candidate phage clones were each plated at the density of 50 000 plaques per plate (diameter 10 cm). An IPTG-coated nitrocellulose membrane was laced on each plate to induce the expression of recombinant proteins. Membranes coated with phage proteins were blocked, washed and then incubated with patient serum at a dilution of 1:50–200. After washing with PBS-Tween, antibodies were eluted in a buffer containing 0.1 M glycine, 150 mM NaCl, 0.1% BSA, pH 2.3, neutralized in 1 M Tris pH 8.7, and concentrated using Amicon microconcentrators (Millipore, Bedford, MA, USA). Affinity purified antibodies were analysed for their specificity using Western blot.

Expression and purification of recombinant p90

Gel purified 1.2 kb *Eco*RI–*Xho*I insert from GC291 was excised from the pBK-CMV plasmid and subcloned into the pET28b expression vector (Novagen, Madison, WI, USA). The pET construct was transformed into bacteria BL21(DE3) (Stratagene) for recombinant protein expression in the presence of 2 mM IPTG. After 4 h incubation, recombinant proteins were extracted and purified using nickel-nitrilotriacetic acid (Ni-NTA) beads affinity column (Qiagen, Valencia, VA, USA).

Production of anti-p90 antibodies

Two NZW rabbits and three BALB/c mice were injected subcutaneously with 1 mg and 50 μg purified recombinant protein derived from clone GC291, respectively, emulsified with an equal volume of CFA (Sigma Chemical Co., St. Louis, MO, USA). A month later, animals were administered a second booster injection of similar dosage with Incomplete Freud's Adjuvant (Sigma). This procedure was repeated for a third and final booster injection. Ten days following the booster injection, blood was collected and analysed by Western blot and indirect immunofluorescence.

Abbreviations

GC, gastric cancer; HCC, hepatocellular carcinoma; IMP, IGF-II mRNA binding protein; IP, immunoprecipitation; TnT, *in vitro* transcription and translation

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