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The LIM domains of *hic-5* protein recognize specific DNA fragments in a zinc-dependent manner *in vitro*

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ABSTRACT

***hic-5* protein is a member of the LIM protein family, containing four LIM domains in its C-terminal region. It is mainly localized in focal adhesions and shows striking similarity to paxillin in its LIM domains, although the function of these LIM domains has remained elusive. In the present study, we found that full-length and the C-terminal half of *hic-5* protein, including four LIM domains, bound to DNA in a zinc-dependent manner *in vitro*. Mouse genomic fragments that specifically bound to the *hic-5* protein were isolated by successive rounds of *hic-5* protein–DNA complex immunoprecipitation and PCR amplification. Seven independent clones were isolated, which contained high amounts of G+A and/or a long A/T tract. A DNA binding protein blot assay revealed the specificity of the interaction between *hic-5* protein and the DNA fragment. Using a series of truncated forms of the *hic-5* LIM domains, each of the four LIM domains was found to contribute to DNA binding in a distinctive manner.**

INTRODUCTION

The *hic-5* gene was originally isolated from mouse osteoblastic cells as one of the TGFβ1-inducible genes, encoding a polypeptide with a molecular weight of ~55 kDa (1) whose prominent feature is the presence of four LIM domains in its C-terminal half. The LIM domain is a unique cysteine-rich motif that defines a double zinc finger structure with a consensus sequence CXXCX16–23HXXCXXCXXCX16–21CXX(D/H/C) and which is found in a variety of proteins with diverse functions and subcellular distributions, including transcription factors, components of adhesion plaques and actin-based cytoskeletal components (2). The members of the LIM proteins can be classified into several groups; a LIM homeodomain family, LIM only protein, LIM kinase, a GTPase activating protein (GAP) family and the zyxin family, which includes enigma and paxillin. Spectroscopic analysis demonstrated that the LIM domain defines a specific zinc binding structure and that zinc coordination is required for proper folding of the LIM domain (3). In spite of this structural information, it is controversial as to whether the LIM domain is involved in protein–protein or protein–nucleic acid interactions. Accumulating evidence has demonstrated that the LIM domains serve as an interface for protein–protein interactions, although the interacting molecules identified so far are so diverse that it is impossible to deduce the determinant for specificity or selectivity of the interactions. For example, the LIM domain of cysteine-rich protein (CRP) interacts with that of zyxin (4), but the LIM domain of LIM homeodomain protein (Lhx/Xlim-1) interacts with a LIM domain binding factor (Ldb1) that contains no LIM motif (5). The protein enigma interacts with the insulin receptor and Ret/ptc2 (6) and its homolog, named ENH, binds to certain members of the protein kinase C family (7).

On the other hand, a certain similarity in structure has been pointed out between the LIM consensus and DNA binding-type zinc fingers, such as the GATA transcription factor family and steroid hormone receptor superfamily (8). From this similarity, together with the above mentioned diversity of protein recognition by the LIM domain, it is likely that the LIM domain also functions as a protein–nucleic acid interface.

hic-5 protein belongs to the zyxin family and has striking similarity with paxillin in its LIM domains (9). Paxillin is a phosphoprotein which interacts with tyrosine kinases of the src family as well as with focal adhesion kinase and vinculin at focal adhesions (10). Brown *et al*. showed that LIM 3 of paxillin is essential for localization in focal adhesions (11), but the function of the LIM domains in *hic-5* protein has not yet been determined. Interestingly, zyxin, which is another member of the family and is a low abundance phosphoprotein that accumulates with integrins at focal adhesions, has recently been shown to have a functional nuclear export signal (NES) and shuttles between the nucleus and cytoplasmic focal adhesions (12). The existence of almost the same NES amino acid sequence as zyxin in *hic-5* protein and the observation that treatment of cells with leptomycin B, an inhibitor of nuclear export, induced nuclear accumulation of *hic-5* protein (in preparation) tempted us to examine the DNA binding ability of the LIM domains in *hic-5* protein. In the present communication, we also attempted to isolate DNA sequences that specifically bound to *hic-5* protein. Our results suggest that the LIM domains of this protein bind to DNA in a zinc- and sequence-dependent manner.

MATERIALS AND METHODS

The nucleotide sequences reported in this paper have been submitted to the GenBank with accession nos: AF056072 for clone 98; AF056073, clone 10; AF056074, clone 101;

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AF056075, clone 19; AF056076, clone 29; AF056077, clone 78; AF056078, clone 97.

Construction of recombinant proteins

Three types of prokaryotic expression vectors for *hic-5* were constructed using the pET-16b vector (Novagen, Madison, WI). As a nearly full-length *hic-5* expression vector containing nt 288–1596 of the *hic-5* cDNA, the previously described pET-L5 plasmid was used (1). For construction of expression vectors of C-terminal truncated (pET-N) or N-terminal truncated (pET-C) *hic-5* protein, a *Nsp*I–*Hin*fI fragment (nt 190–779) of *hic-5* cDNA or a fragment of nt 756–1553 flanked by an *Eco*RI adaptor were obtained. The fragments were blunted and ligated with *Bam*HI linker for in-frame insertion into the expression vector. After *Bam*HI digestion, *Bam*HI linker linked cDNA fragments were subcloned into the *Bam*HI site of the pET-16b vector. For construction of deletion mutants, desired *hic-5* fragments were prepared using restriction enzymes or PCR. These fragments were cloned into the *Bam*HI site of the pGEX-5X-1 vector. These mutant fragments contain the following nucleotide sequences of *hic-5* cDNA; LIM 1–4 (nt 827–1598), LIM 1–3 (nt 827–1333), LIM 1–2 (nt 827–1160), LIM 1–2 X [nt 827–1164 followed by 124 bp, which were derived from the cloning vector, pVZ-1 (13), and translated into 42 amino acids, X, unrelated to *hic-5* protein], LIM 1 (nt 822–992), LIM 2–4 (nt 971–1725), LIM 3–4 (nt 1160–1725), LIM 4 (nt 1335–1523).

To engineer the expression vector of the LIM region of human paxillin, a cDNA fragment encoding the LIM domains (nt 1042–1748) was obtained by PCR and subcloned into the *Eco*RI site of the pGEX-5X-1 vector.

The BL21 strain of *Escherichia coli* (14) was transformed with expression vectors for the respective proteins. Logarithmically growing cultures were induced to produce the protein by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h.

DNA cellulose binding of the *hic-5* protein

Escherichia coli cells harboring the prokaryotic expression vector of *hic-5* and that had been induced to produce *hic-5* protein in the presence of IPTG were suspended in lysis buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM DTT) containing 0.1 mg/ml lysozyme and 0.1% Triton X-100 and then lysed with three bursts of sonication (140 W, 10 s). The insoluble fraction was collected by centrifugation and washed with lysis buffer containing 2 M urea to partially purify *hic-5* protein. This fraction contained most of the *hic-5* protein expressed with a small amount of contamination (confirmed by silver staining after SDS–PAGE). After solubilizing in lysis buffer containing 4 M urea, the proteins were renatured by successive dialysis against lysis buffer containing 2, 1 and 0 M urea with 1 mM $ZnCl_2$ or 1 mM EDTA for several hours at 4°C and then used in the binding assay as described (15). In brief, 0.4 mg protein were incubated in binding buffer (50 mM Tris–HCl, pH 7.5, 12 mM α-thioglycerol, 10% glycerol, 0.1 M NaCl) with 0.1 g double-stranded (native) or single-stranded (denatured) DNA–cellulose (Pharmacia LKB Biotechnology) for 24 h at 4°C. The DNA–cellulose had been preincubated with 3% BSA in binding buffer. After washing with binding buffer containing 0.003% NP-40 and 2% Triton X-100, the material bound to DNA–cellulose was eluted with 1.25% SDS and resolved by SDS–PAGE. The *hic-5* protein was then detected by western blotting using an antibody (αC86) as described previously (1).

Isolation of DNA sequences bound to *hic-5* protein

Genomic DNA from mouse osteoblastic cells (MC3T3) was digested with *Mbo*I or *Hae*III. The digested DNA fragments (average size 200–300 bp) were mixed and ligated to the UNI-Amp Adapter (Clontech, Palo Alto, CA). In 125 µl binding buffer (50 mM Tris–HCl, pH 7.5, 12 mM α-thioglycerol, 10% glycerol, 0.1 M NaCl, 0.1 mM $ZnCl_2$) containing 5 µg poly(dI-dC), 1 µg adapter-linked DNA fragments were incubated with 6 µg bacterially produced and partially purified *hic-5* protein at room temperature for 1 h. DNA fragments bound to the *hic-5* protein were mixed with 1.25 ml immunoprecipitation buffer (0.1 M HEPES, pH 7.5, 0.3 M KCl, 10 mM $MgCl_2$, 20 mM $ZnCl_2$, 2% Triton X-100, 0.1% SDS) containing anti *hic-5* antibody (1011) and then incubated on ice for 1 h. The DNA–*hic-5*–antibody complex was precipitated by adding 25 µg protein A–Sepharose in binding buffer and incubating for 1 h at 4°C. Bound DNA fragments were separated from free DNA by centrifugation. After removal of the supernatant, the immunoprecipitate was washed four times with immunoprecipitation buffer. The DNA fragments bound to *hic-5* protein were incubated in dissociation buffer (0.5 M Tris–HCl, pH 9.0, 20 mM EDTA, 10 mM NaCl, 0.2% SDS) at 50°C for 1 h, extracted with phenol–chloroform and precipitated with ethanol. Recovered DNA fragments were then amplified by PCR using UNI-Amp primers (Clontech). The bound and amplified DNA fragments were used as the substrate for additional rounds of binding to *hic-5* protein. After four rounds of binding/elution, selected DNA fragments were cloned into the pCR II vector (Invitrogen, San Diego, CA). Individual clones were screened by immunoprecipitation–PCR (IP–PCR) as described above, except that the cloned plasmids were used for substrate DNA. Amplified fragments were analyzed by electrophoresis on 1% agarose gels.

Protein blot assay to detect DNA binding

This assay was performed essentially as described elsewhere (16). In brief, pellets of *E.coli* BL21 harboring recombinant *hic-5* or paxillin cDNA as described above were lysed in lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM DTT) containing 0.1% Triton X-100 and 0.1 mg/ml lysozyme. Proteins were separated by SDS–PAGE and blotted to nitrocellulose filters. Radiolabeled DNA probe was added to the preincubated filter (10^5 c.p.m./filter) and the filters were incubated for 5 h at 4°C. After the binding reaction, the filters were washed with reaction buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.1 mM $ZnCl_2$) containing sonicated *E.coli* genomic DNA or yeast tRNA for 3 h five times, each for 15 min, air dried and autoradiographed.

For DNA probes, the *hic-5* binding fragments were cloned into pCR II vector as described above. Cloned fragments were digested with *Hin*dIII and *Xho*I, resolved electrophoretically and purified from a 1% agarose gel. These fragments were labeled with Klenow fragment and [α-^{32}P]dCTP. The labeled probe was purified by phenol–chloroform extraction and ethanol precipitation in the presence of ammonium acetate.

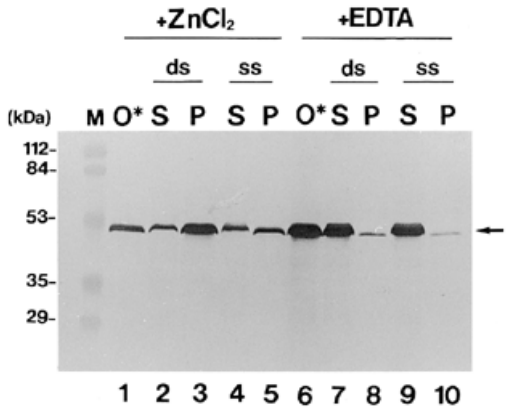

Figure 1. DNA binding activity of *hic-5* protein. The bacterially produced and partially purified *hic-5* protein was incubated with double-stranded (ds, lanes 2, 3, 7 and 8) or single-stranded (ss, lanes 4, 5, 9 and 10) DNA–cellulose in the presence of $ZnCl_2$ (lanes 1–5) or EDTA (lanes 6–10). The bound (lanes 3, 5, 8 and 10) and unbound fractions (lanes 2, 4, 7 and 9) were collected by centrifugation and, following SDS–PAGE electrophoresis, they were western blotted using an antibody against *hic-5* protein. Lanes 1 and 6 were input proteins prepared in the presence of $ZnCl_2$ or EDTA respectively. M, O*, S and P indicate molecular weight markers, original input, supernatant and pellet fraction, respectively. The arrow indicates the position of *hic-5* protein.

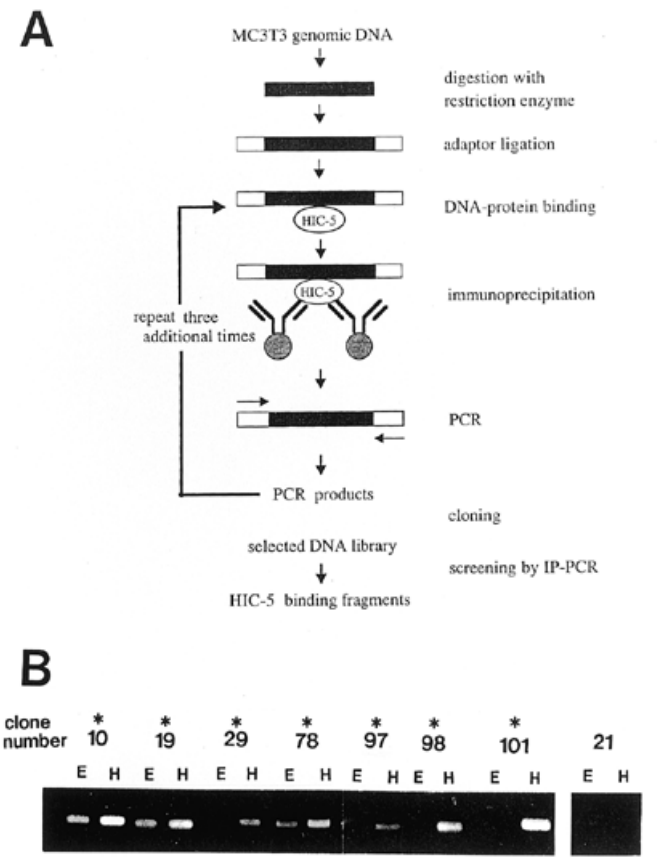

Figure 2. Screening procedure for *hic-5* protein binding fragments by immunoprecipitation and PCR. (**A**) A schematic presentation of the immunoprecipitation–PCR (IP–PCR) method used for isolating mouse genomic DNA fragments that bind to *hic-5* protein. (**B**) Isolated clones were tested for their binding activity with *hic-5* protein using the IP–PCR method. For each clone, H indicates the result of the binding reaction with *hic-5* protein and E indicates the control experiment using an *E.coli* protein extract that contain no *hic-5* protein. In this experiment, seven binding clones were identified. Asterisks show the *hic-5* binding clones. Some portions of clones 10, 19 and 78 were precipitated by *E.coli* proteins, but this may be caused by their property to bind to some proteins in a non-specific manner.

RESULTS

Affinity of *hic-5* protein for DNA

We first investigated whether *hic-5* protein has the ability to bind nucleic acids *in vitro* using bacterially produced and partially purified protein. *hic-5* protein was incubated with double-stranded or single-stranded DNA–cellulose and the fraction of *hic-5* protein bound or unbound to the DNA–cellulose was analyzed by western blotting using an anti-*hic-5* antibody. Figure 1 shows that a significant amount (~80% of imput) of the *hic-5* protein was recovered in the bound fraction when it was incubated with double-stranded DNA. In addition, this binding was dependent on the presence of zinc ions. These results suggest that the Zn fingers composing the LIM domains of *hic-5* protein were capable of binding to double-stranded DNA.

Isolation of *hic-5* protein binding sequences from mouse genomic DNA

The DNA binding ability of *hic-5* protein tempted us to isolate the potential *hic-5* protein binding sequences from mouse genomic DNA. The strategy we used was previously described and has already been used successfully for a similar purpose in several studies (17–20). First, as outlined in Figure 2A, total mouse genomic DNA was fragmented into average sizes of 200–300 bp and inserted into the cloning vector. The cloned DNA fragments were mixed with recombinant *hic-5* protein, immunoprecipitated with an antibody (1011) and amplified by PCR. This process was repeated, and after four rounds, a progressive enrichment of *hic-5* protein binding fragments was accomplished, as displayed by Southern blotting analysis of a series of PCR products (data not shown).

To identify the enriched sequences, the DNA fragments were cloned into a plasmid vector, randomly selected and hybridized to the PCR products obtained by four round selection. We selected the clones which gave the strongest signals and tested them further for *hic-5* protein binding in a similar manner to the enrichment procedure described above. In this process, we found that seven plasmids containing cloned fragments were selectively immunoprecipitated in the presence of *hic-5* protein (Fig. 2B).

We sequenced the fragments of these seven clones. The most outstanding feature of the sequences was the presence of an extraordinarily long poly(A)-like tract in the middle of five out of the seven fragments (Fig. 3, underlined). Another feature seen in all of the fragments was a high G+A content, ranging from 60 to 70%, composed of many reiterations of GA dinucleotides. Homology search analysis showed that the sequences preceding the long A tract in clones 19, 29, 97 and 98 were the 3′-end half of mouse B1 sequences, which correspond to human Alu sequences. Additionally, in three clones (19, 29 and 98), this 3′-end of the Alu sequence was followed by the 5′-end of another Alu sequence, being separated by the long A tracts underlined in Figure 3. Among these clones, the fragment named 101 was used for further analysis.

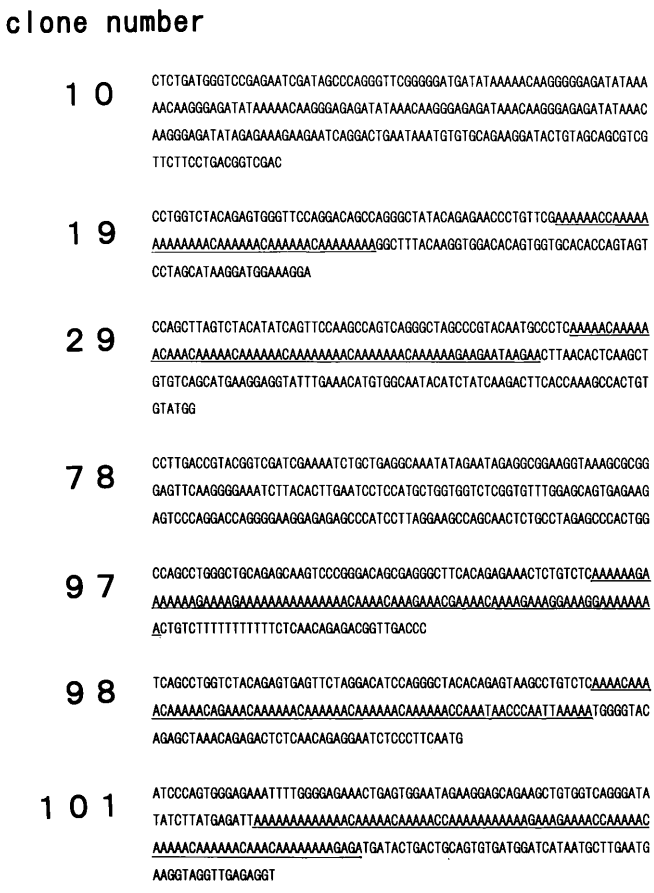

Figure 3. Nucleotide sequences of the *hic-5* binding clones. Underlined parts indicate A-rich tracts.

Specificity of the interaction between *hic-5* protein and the binding fragments

We next examined the specificity of the interacton between *hic-5* protein and the binding fragments using the DNA binding protein blot assay as previously described. In this assay, the protein extract from *E.coli* expressing *hic-5* protein was electrophoretically separated by SDS–PAGE, transferred to a membrane and incubated with end-labeled 101 fragment in the presence or absence of competitor *E.coli* DNA. Figure 4A and B shows that binding of fragment 101 to *hic-5* protein was remarkably resistant to an increasing presence of non-specific *E.coli* DNA competitor when compared with its binding to randomly selected proteins, designated a–c, which were endogenous *E.coli* proteins that non-specifically bind to DNA. In Figure 4C, sequence-specific competitors were added simultaneously with non-specific *E.coli* DNA competitor. In this experiment, the sequence specificity between *hic-5* protein and the 101 fragment was further confirmed, since binding was competed out by unlabeled 101 fragment in a dose-dependent manner but not by fragment 5-3, which was a randomly cloned fragment showing no selective binding to *hic-5* protein. These results suggest that *hic-5* protein and the 101 fragment interact with each other in a sequence-specific manner *in vitro*. Additionally, the DNA binding ability of *hic-5* protein was compared with that of paxillin, which has LIM domains highly homologous (62% identity at the amino acid sequence level) to those of *hic-5* protein. The 101 fragment also bound to paxillin in this assay in the presence of a low concentration of non-specific *E.coli* DNA competitor (5 μg/ml). However, binding was competed out at an *E.coli* DNA concentration as low as 10 μg/ml, whereas binding to *hic-5* protein persisted at >20 μg/ml (Fig. 4D). This result suggests that the LIM domains in *hic-5* protein have a unique DNA binding property. However, the possibility cannot be totally excluded that paxillin also binds specifically to certain DNA fragments other than 101.

The presence of a long A/T tract in five out of the seven *hic-5* protein binding sequences implies that *hic-5* protein bound to a poly(A) tract such as those present in the 3′-end of mRNAs. This possibility was examined by protein blot assay using riboprobes transcribed from the fragments, but *hic-5* protein did not show any specific binding to riboprobes containing poly(A) tracts (data not shown).

Determination of the DNA binding domain in *hic-5* protein

To determine the domains responsible for the DNA binding activity of *hic-5* protein, we constructed prokaryotic expression vectors of N- and C terminal-truncated forms of the protein and performed a DNA binding protein blot assay together with a nearly full-length (dF) *hic-5* protein as described above (Fig. 5A). The dF and C forms of the protein bound to the 101 fragment and, interestingly, the N-terminal truncated form (C form) showed remarkably higher affinity for the fragment than the dF form. Furthermore, this binding of dF and C forms to the fragment was zinc ion-dependent, which is consistent with the results in Figure 1. On the other hand, the C-terminal truncated form (N form) hardly bound to the fragment. These results suggest that the LIM domains of *hic-5* protein mainly contribute to DNA binding ability and that the N-terminal portion of the protein negatively affects DNA binding.

To define the DNA binding domains in the C-terminal half of *hic-5* protein in further detail, we constructed a series of deleted forms of the *hic-5* LIM domains as illustrated in Figure 6A. The DNA binding ability of these deleted forms was examined by DNA binding protein blot assay. As summarized in Figure 6A, two of these constructs, LIM 1–3, in which the LIM domain at the very C-terminal end of the protein was deleted, and LIM 1, containing only the LIM domain at the N-terminus, completely lost DNA binding, although almost equal levels of the proteins were expressed in *E.coli* and were blotted on the membrane, as shown in Figure 6B and C. Besides the main products from the expression vectors, proteins of lower molecular weight that bound the DNA probe were found in several bands, as shown in Figure 6C, and these are likely to be degraded products of *hic-5* protein. In conclusion, these results suggest that the DNA binding ability of *hic-5* protein resides in LIM 2, LIM 4 or the pair LIM 1 and LIM 2. The function of LIM 3 in DNA binding is unclear at this stage, because it seemed to have a negative effect on DNA binding in the construct LIM 1–3 but not in the other constructs.

DISCUSSION

Although the function of the LIM domains is still obscure, several lines of evidence have emerged showing that it functions as a

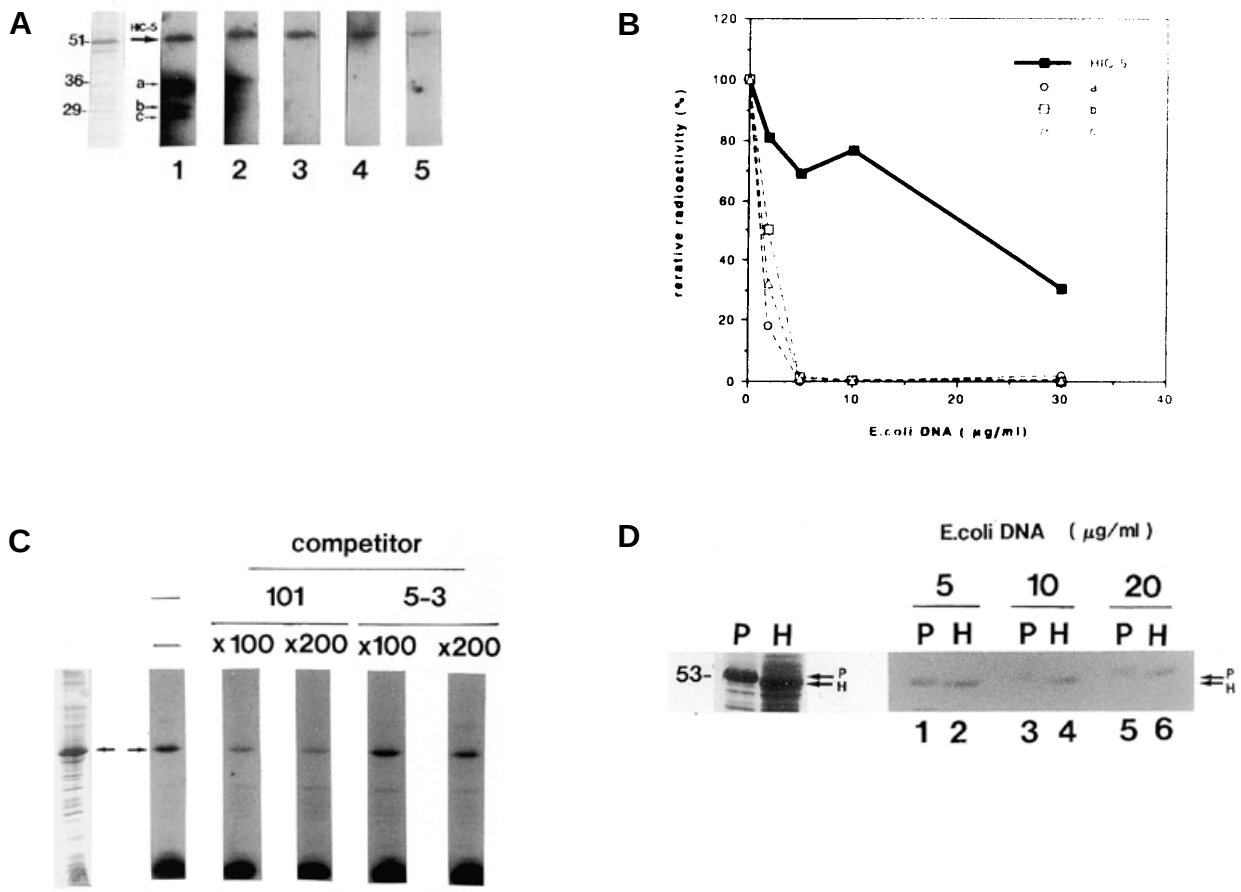

Figure 4. Binding specificity between *hic-5* protein and an isolated clone. (**A**) The protein extract from *E.coli* harboring the *hic-5* protein was resolved by 12% SDS–PAGE and blotted onto nitrocellulose filters. The DNA binding protein blot assay were performed with the end-labeled clone 101 fragment in the absence or presence of unlabeled *E.coli* DNA as a non-specific competitor (lanes 1–5, 0, 2, 5, 10 and 30 μg/ml, respectively). In the left end panel, the protein extract was separated by SDS–PAGE and stained with Coomassie brilliant blue. The arrows a–c indicate the position of randomly selected proteins a–c on 12% SDS–PAGE. (**B**) Competition for binding by *E.coli* DNA. The radioactivity of labeled 101 DNA fragments bound to each protein was determined with a BAS2000 image analyzer and the radioactivity relative to that in the absence of the *E.coli* DNA was determined. Closed squares, percentage of radioactivity bound to *hic-5* protein; open circles, to protein a; open squares, to protein b; open triangles, to protein c. (**C**) Binding of *hic-5* protein to a specific sequence. A DNA-binding protein blotting assay was performed with the end-labeled 101 fragment probe in the presence of 10 μg/ml unlabeled *E.coli* genomic DNA without further competitor DNA (lane –) or with a 100-fold (lanes ×100) or 200-fold (lanes ×200) molar excess each of the 101 and 5-3 fragments. In the left end panel, the protein extract was separated by SDS–PAGE and stained with Coomassie brilliant blue. The arrows indicate the band of *hic-5* protein. (**D**) Comparison of DNA binding activity between *hic-5* protein and paxillin. The protein extract from *E.coli* harboring the LIM domain of *hic-5* protein (GST–LIM 1–4) (lanes 2, 4 and 6) or paxillin (GST–paxillin LIM) (lanes 1, 3 and 5) was subjected to the DNA binding protein blot assay using the end-labeled 101 fragment as probe in the presence of 5–20 μg/ml *E.coli* genomic DNA as indicated. In the left end panel, the protein extract containg GST–paxillin LIM (lane P) or that of GST–LIM 1–4 of *hic-5* (lane H) was separated by SDS–PAGE and stained with Coomassie brilliant blue. The arrows labeled P indicate the position of GST–paxillin LIM and those labeled H indicate GST–LIM 1–4 of *hic-5*. Strong signals in the fast migrating fractions came from sequence-non-specific binding of the probe to small molecular weight proteins in *E.coli*.

protein–protein interface. In this study, we found that *hic-5* protein could bind to DNA *in vitro* in a zinc-dependent manner and that the LIM domains were responsible for the activity. The requirement for zinc ions for binding, which suggests a strict dependency of binding on the secondary structure of the LIM domains retained by zinc ions, implies that the DNA binding ability was not artificial but inherent in the LIM domains of *hic-5* protein. A comparison of DNA binding of *hic-5* protein with that of paxillin, whose LIM domains are highly homologous to those of *hic-5* protein, further supports the assumption that the LIM domains of *hic-5* protein have a unique DNA binding property (Fig. 4D). Deletion analysis of the four LIM domains suggested that either LIM 1–2 or LIM 4 were necessary for binding, while LIM 3 seemed to have negative effects on binding. These results, although observed in an *in vitro* DNA binding protein blot assay, are the first demonstration that a certain LIM domain can bind to DNA.

With regard to the binding sequence of *hic-5* protein, we could enrich several DNA fragments from the mouse genome as putative *hic-5* protein binding sequences. The sequence specificity of *hic-5* protein binding to these fragments is demonstrated in Figure 4. Thus, *hic-5* protein seemed to recognize the DNA fragments in a sequence-specific manner. Although we roughly delineated the *hic-5* protein binding region within the fragments using the DNA binding protein blot assay, we could not determine the consensus sequences in more detail because the interaction between *hic-5* protein and the DNA fragments was not detectable in a conventional gel shift assay. It is known that a certain type of interaction between DNA and protein is observable in the DNA

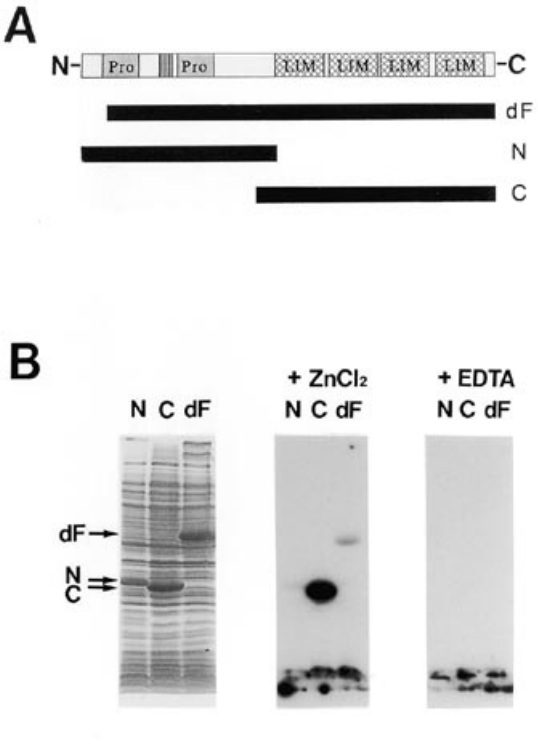

Figure 5. Zinc-dependent DNA binding activity of full-length and C-terminal LIM domains. (**A**) A schematic representation of the truncated forms of *hic-5* protein. Dotted and striped areas show proline-rich regions and an acidic region, respectively. Checked boxes show the four LIM domains. Thick lines indicate the region expressed from each construct: dF, a nearly full-length *hic-5* protein; N, a C-terminal truncated protein; C, an N-terminal truncated protein. (**B**) Protein extracts from *E.coli* harboring the dF, N and C constructs were electrophoretically separated and blotted onto a nitrocellulose filter. The filter was probed with the end-labeled 101 fragment in the presence of 10 μg/ml unlabeled *E.coli* genomic DNA and either 1 mM $ZnCl_2$ (middle panel) or 50 mM EDTA, 10 mM DTT (right panel). The left panel shows the Coomassie blue staining pattern after SDS–PAGE. The arrows indicate the positions of each protein.

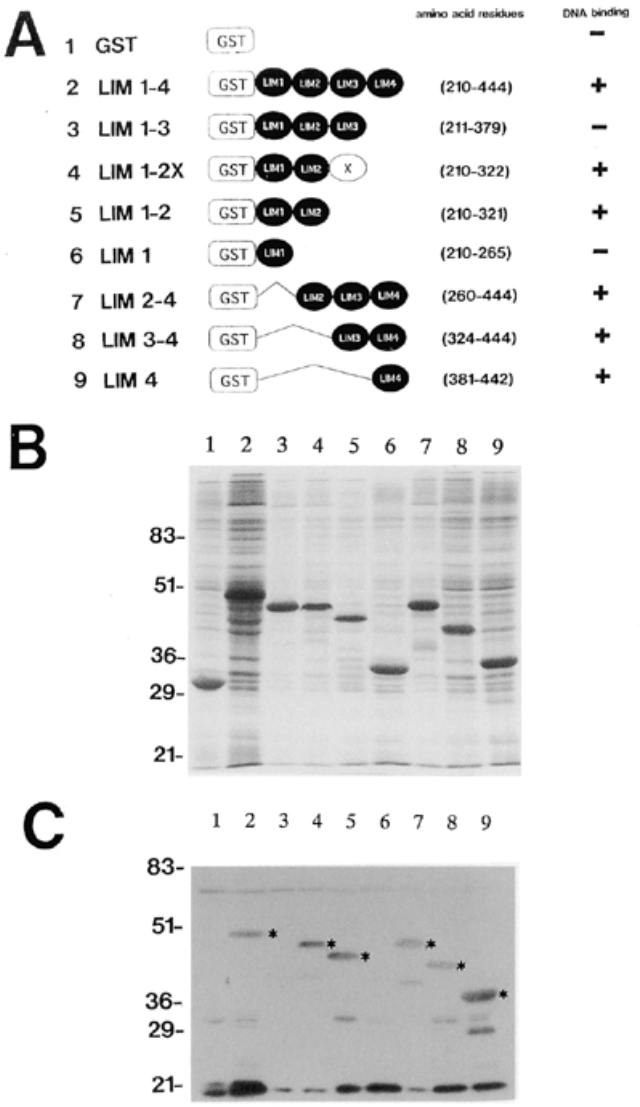

Figure 6. Deletion analysis of DNA binding by the LIM domains of *hic-5* protein. (**A**) A schematic representation of the GST–*hic-5* fusion protein derived from the LIM domains of *hic-5* protein. X in lane 4 indicates 42 amino acids derived from the cloning vector. (**B**) Coomassie brilliant staining of protein extracts from *E.coli* harboring each of the deleted forms, electrophoretically separated (lane 1, GST; lane 2, GST–LIM 1–4; lane 3, GST–LIM 1–3; lane 4, GST–LIM 1–2 X; lane 5,s GST–LIM 1–2; lane 6, GST–LIM 1; lane 7; GST–LIM 2–4; lane 8, GST–LIM 3–4; lane 9, GST–LIM 4) and blotted onto a nitrocellulose filter. (**C**) The filter was probed with end-labeled 101 fragment in the presence of 5 μg/ml unlabeled *E.coli* DNA.

binding protein blot assay as used in the present experiments but not in a system in which the DNA–protein complex is subjected to electrophoresis. The DNA binding proteins showing this property are exemplified by ARBP (21) and SAF-A (22). In these cases, the proteins are not thought to recognize the sequence itself but the secondary structures intrinsic in the sequences. The interaction of DNA and *hic-5* protein is likely to be one such case.

Sequencing the *hic-5* protein binding fragments isolated thus far revealed the unique properties of a high G+A content and the presence of a long A/T tract, supporting the above-mentioned idea that *hic-5* protein recognizes a unique secondary DNA structure. It is well known that the Alu sequence is accompanied by a poly(A)-like tract at the 3′-end, but it is usually <20 bp long (23). In this respect, the sequences of clones 19, 29, 97 and 98 are thought to be part of a unique B1 family with a long poly(A)-like tract, while the other clones, 10, 78 and 101, may be relatives. Although the significance of the B1-like sequences in *hic-5* protein binding remain to be resolved, the DNA of a certain member of the mouse B1 family is reported to adopt a unique Z form secondary structure (24). One of these aspects or their combination in the sequences may contribute to recognition of the fragments by *hic-5* protein.

The significance of the DNA binding ability of *hic-5* protein is unclear at this stage. Since forced expression of *hic-5* increases expression of several genes, as reported previously (2), *hic-5* might affect some nuclear function, including transcriptional regulation, through its DNA binding. Alternatively, the DNA binding ability of *hic-5* protein may somehow be deleterious to cells and thus *hic-5* protein has to be dispersed in the cytoplasm by the potential NES. In any case, further analysis of *hic-5* protein binding to DNA and its effect on cellular functions at the molecular level might shed some light on the mechanism of cellular senescence and immortalization.

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