The DNA binding activity of Translin is mediated by a basic region in the ring-shaped structure conserved in evolution

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Abstract DNA binding proteins, for the most part, function as dimers or tetramers which recognize their target sequences. Here we show that Translin, a novel single-stranded DNA end binding protein, forms a ring-shaped structure conserved throughout evolution and that this structure is responsible for its DNA binding activity. Point mutations at Leu^{184} and Leu^{191} in the leucine zipper motif of human Translin resulted in loss of the multimeric structure and abrogation of DNA binding. Point mutations at R^{86}, H^{88}, H^{90} to T^{86}, N^{88}, N^{90} in one of the basic regions, however, completely inhibited the DNA binding activity without affecting the multimeric structure. These results support the view that the DNA binding domain of Translin is formed in the ring-shaped structure in combination with its basic region (amino acids 86–97) polypeptides.

Key words: Translin; Leucine zipper; DNA binding domain

1. Introduction

Translin has been identified as a DNA binding protein that specifically recognizes the consensus sequences motifs, ATGCAG and GCCC[A/T][G/C][G/C][A/T], at breakpoint junctions in many chromosomal translocations in human lymphoid neoplasms involving 1p32, 3q27, 5q31, 8q24, 9q34, 9q34.3, 10q24, 11p13, 11q13, 14q11, 14q32, 14q32.1, 17q22, 18q21, 19p13, and 22q11 [1–4]. Like the Ku protein which is essential for DNA double-strand break repair [5], there is some evidence that Translin also binds RNA [6], suggesting a multi-functional aspect. Molecular gene cloning experiments have revealed that the human Translin gene encodes a protein of 228 amino acids with a predicted molecular size of 27 kDa [4]. SDS-PAGE analysis showed that the recombinant Translin migrates as a single band of approximately 27 kDa under reducing conditions and 54 kDa under non-reducing conditions, indicating that Translin polypeptides form dimers. The native molecular weight of Translin, however, was estimated to be approximately 220 kDa by gel filtration chromatography. Therefore, we hypothesized that the native form is a multimeric structure, possibly connected by the heptad repeat of hydrophobic amino acids (leucine zipper motifs) of each dimer. Confirming this hypothesis, our electron microscopic study showed that the native form of Translin is a ring-shaped structure. Crystallographic investigations to determine its three-dimensional character also indicated that the human Translin is a ring-shaped structure with an assembly of eight subunits [7]. It was further determined that this multimeric Translin formed by the subunits is responsible for binding to target sequences situated only at single-strand DNA ends. The Translin genes and proteins are highly conserved among vertebrates and the mouse and chicken homologs are composed of 228 and 229 amino acids respectively, each with an estimated MW of the approximately 27 kDa [8]. The amino acid homologies with human Translin are 99% for mouse and 86% for chicken. In particular, the leucine zipper motifs and their upstream basic regions were found to be highly conserved.

To study whether the ring-shaped structure of Translin has been conserved during evolution, we compared the native forms in man and other vertebrates by electron microscopic studies. Point mutation analyses of both the leucine zipper and upstream basic regions revealed that creation of the DNA binding domain in the ring-shaped structure has essential functional significance.

2. Materials and methods

2.1. Recombinant human, mouse, and chicken Translin

To construct plasmids expressing human, mouse and chicken Translin proteins, cDNAs (EMBL/GenBank accession numbers X78627, X81464 and X95074, respectively) were PCR (polymerase chain reaction) amplified with the following primers: mhTraP (ATGGATCCATGTCTGTGAGCGAGATC) and hTraM (ATAAGCTTCTATTTTTCAACACAAGC), mhTraP and mTraM (ATAAGCTTCTATTTTTCACCACAAGC), chiTraP (ATGGATCCATGTCAGTGAGCGCCATG) and chiTraM (ATAAGCTTCATTTCTCTCCACCTGC) for the human, mouse and chicken forms, respectively. The amplified products were digested with *Bam*HI and *Hin*dIII, and then cloned into *Bam*HI/*Hin*dIII sites of pQE-9 (Qiagen), a bacterial expression vector that contains sequences encoding a six histidine tag. The resulting expression constructs were transformed into the *Escherichia coli* host strain M15[pREP4], and production of recombinant Translin proteins was induced by treatment with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h. The bacteria were then suspended in lysis buffer (50 mM Na phosphate [pH7.0], 300 mM NaCl, 1 mM PMSF(phenylmethylsulfonyl fluoride), 2 mM DTT (dithiothreitol)) and lysed by sonication. The bacterial lysates were centrifuged at $10\,000 \times g$ for 10 min, and the supernatants were dialyzed against the lysis buffer and then applied to a Ni^{2+}-agarose column equilibrated in the same buffer. Proteins were eluted with a linear gradient of 20–200 mM imidazole. Translin proteins were eluted at 100–150 mM imidazole.

2.2. Electron microscopic analysis

To visualize the native forms of human, mouse, and chicken Translin, each of the recombinant Translin samples was prepared on a thin carbon film supported by a mesh copper grid and negatively stained with potassium phosphotungstate adjusted to pH 7.0.

2.3. EMSA (electrophoretic mobility shift assay)

EMSA was performed as described previously [4]. Briefly, recombinant Translin and its mutated proteins (LZ mut, BR mutA, BR mutB) were incubated at room temperature for 20 min with 10 000 cpm of

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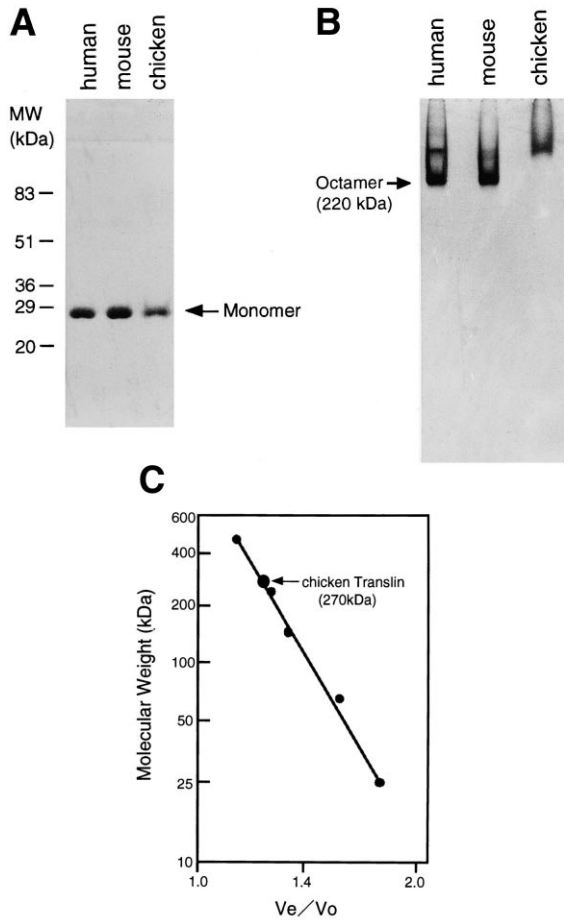

Fig. 1. Evolutionarily conserved structure of native Translin. A: SDS-PAGE analysis of Translin forms in vertebrates. The Translin proteins from man, mouse and chicken were expressed using the pQE-9 plasmid (Qiagen) and purified by nickel chelate chromatography. Each of the purified recombinant Translins (5 μg) was run on a 10% acrylamide SDS-PAGE under reducing conditions followed by staining with Coomassie blue. B: Native PAGE analysis of Translin forms in vertebrates. The Translin proteins (100 μg) from man, mouse and chicken were run on a native 5% acrylamide gel followed by staining with Coomassie blue. The native molecular masses of human and mouse Translin were determined to be 220 kDa by the experimentation as described in Section 2. C: Native molecular weight analysis of chicken Translin protein. The chicken Translin protein was fractionated on a column of Sephadex G-150, equilibrated with 50 mM phosphate buffer (pH 7.6) containing 300 mM NaCl. After gel filtration, portions of each fraction were examined by EMSA using the Bcl-CL1 probe. The standards were ferritin (440 kDa), catalase (230 kDa), aldolase (185 kDa), BSA (68 kDa) and chymotrypsinogen (25 kDa).
←

^{32}P-labeled Bcl-CL1, the target sequence for Translin, in the binding buffer. DNA-protein complexes were then separated on a 5% polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) at 11 V/cm at room temperature for 2 h. The gel was then dried and autoradiographed.

2.4. Native PAGE (polyacrylamide gel electrophoresis) and gel filtration chromatography

To determine whether mutated Translins retain the multimeric structure, native PAGE was performed. Protein samples (100 μg) were run on native 5% acrylamide gels under the same conditions as for EMSA, followed by staining with Coomassie blue. The molecular masses of wild and mutated Translins in the native forms were determined by a combination of Sephadex G-150 gel filtration chromatography and EMSA monitoring of portions of each fraction after the chromatography, as described previously [4].

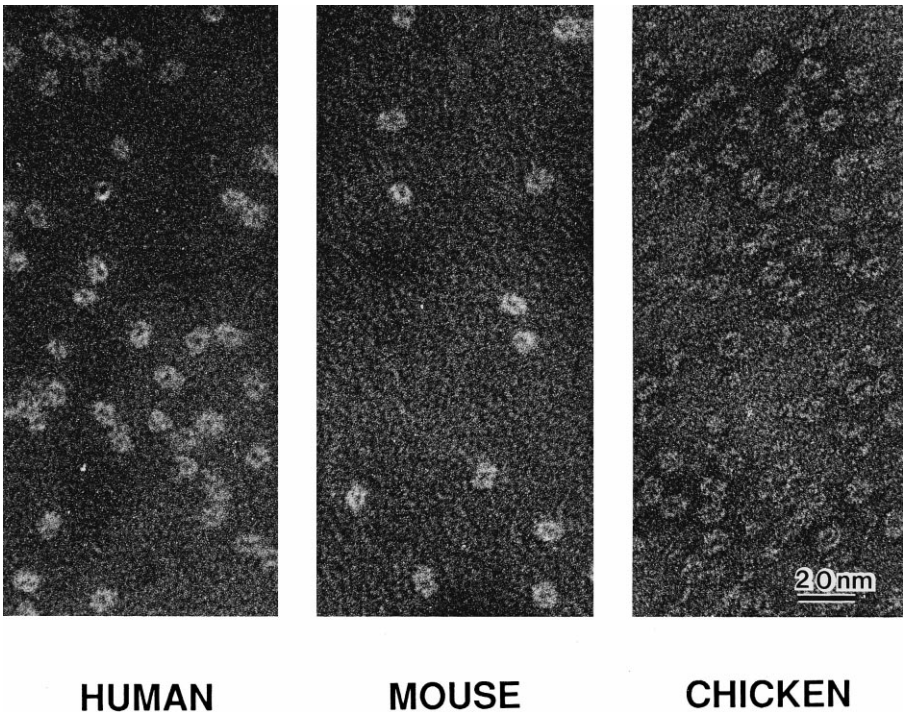

Fig. 2. Electron microscopic visualization of the native forms of human, mouse, and chicken Translin. Each of the recombinant Translin samples was prepared on a thin carbon film supported by a mesh copper grid and negatively stained with potassium phosphotungstate.

3. Results and discussion

3.1. Evolutionarily conserved Translin ring structure

To ascertain whether the ring-shaped structure of Translin is conserved through evolution, the Translin ORFs from human, mouse and chicken were subcloned into a bacterial expression vector pQE-9 and the resulting expression constructs were transformed into the *E. coli* host strain. Translin proteins were produced by induction with IPTG and purified from the bacterial lysates using a Ni^{2+}-agarose column. SDS-PAGE analysis showed all the purified Translins to migrate as single bands of approximately 27 kDa under reducing conditions (Fig. 1A). In our electron microscopic and crystallographic studies [7], the native form of Translin was established to be a ring-shaped structure with an assembly of eight subunits of 27 kDa monomer whose molecular mass is approximately 220 kDa. With native gel electrophoresis, identical mobility corresponding to a molecular weight of 220 kDa was observed for human and mouse Translin, but chicken Translin migrated much more slowly (Fig. 1B). In accordance with this result, the molecular mass of chicken Translin in its native form was estimated to be approximately 270 kDa by Sephadex G-150 gel filtration chromatography (Fig. 1C). Electron microscopic studies clearly revealed that, like the human Translin, the mouse and chicken Translins form ring-shaped structures (Fig. 2). In contrast to human Translin, with its assembly of eight subunits, the large molecular mass of native chicken Translin suggests an assembly of 10 subunits of 27 kDa polypeptides for the chicken protein. In line with this, the sizes of the Translin rings in the human and mouse were found to be almost identical with average diameters of 8.5 and 8.6 nm respectively, while that for the chicken was 9.4 nm.

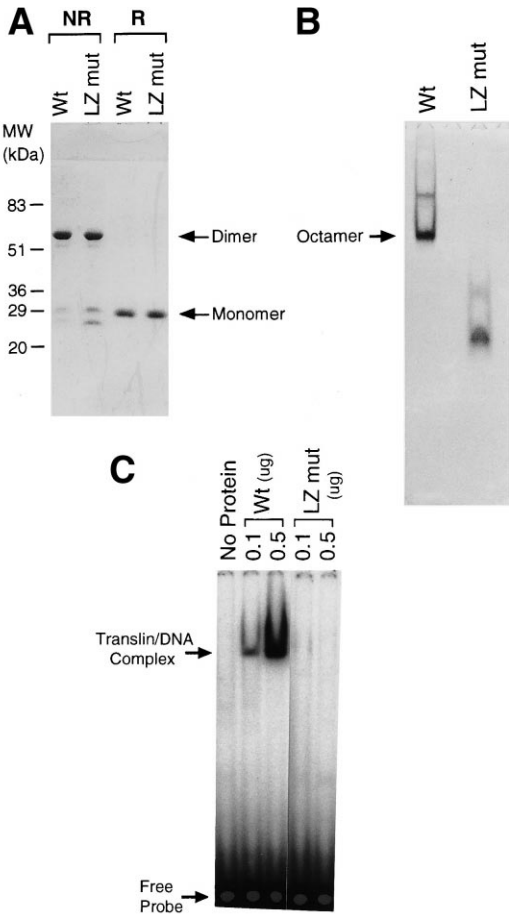

Fig. 3. Role of the leucine zipper motif in the structure and function of Translin. A: SDS-PAGE analysis of the Translin forms mutated in the leucine zipper motif. Aliquots of 5 μg of human Translin (Wt) and its mutated form with leucines at positions 184 and 191 substituted with prolines (LZ mut) were run on a 10% acrylamide SDS-PAGE under non-reducing (NR) and reducing (N) conditions. The gel were then stained with Coomassie blue. B: Disruption of the multimeric Translin structure by mutations in the leucine zipper motif. Aliquots of 100 μg of human Translin (Wt) and its mutated form (LZ mut) were run on a native 5% acrylamide gel followed by staining with Coomassie blue. C: Loss of the DNA binding activity of Translin due to the mutations in the leucine zipper motif. The indicated amounts of Wt and LZ mut Translin were tested for binding to ^{32}P-labeled Bcl-CL1 by EMSA.

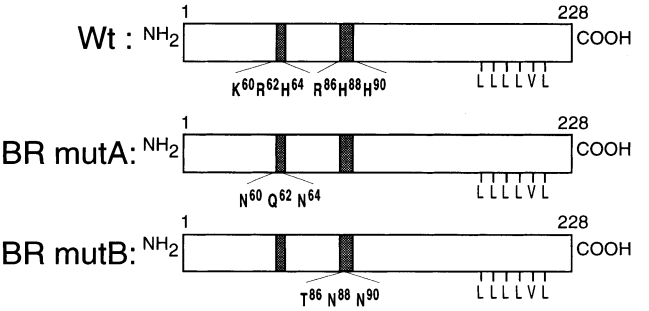

Fig. 4. Schematic representation of human Translin structures mutated in basic regions. The basic regions (gray shades) at amino acids 56–64 and 86–97 of human Translin (Wt) were mutated at positions 60, 62 and 64 (K^{60}, R^{62}, H^{64} to N^{60}, Q^{62}, N^{64}) (BR mutA) or 86, 88 and 90 (R^{86}, H^{88}, H^{90} to T^{86}, N^{88}, N^{90}) (BR mutB). The leucine zipper motif is shown as LLLLVL.

3.2. The multimeric form of Translin is responsible for its DNA binding activity

SDS-PAGE analysis showed that human Translin migrates as a single band of approximately 27 kDa under reducing conditions and 54 kDa under non-reducing conditions (Fig. 3A). To further confirm that the leucine zipper motif at the COOH-terminus (amino acids 177–212) is involved in formation of the native Translin structure, we performed an analysis of point mutations in the leucine zipper motif and their effects were tested. Substitution of the leucines at positions 184 and 191 with prolines (LZ mut) did not affect on the formation of Translin dimers under non-reducing condition (Fig. 3A). However, it disrupted the octameric form detected by native gel electrophoresis (Fig. 3B). In addition, DNA binding activity of the mutated Translin (LZ mut) was completely lost (Fig. 3C), indicating that the leucine zipper facilitates polymerization of the Translin dimer to form the multimeric structure which is responsible for its DNA binding activity.

3.3. Determination of the DNA binding domain of Translin

Although no basic helix-loop-helix (bHLH) region is found upstream of the leucine zipper, the amino acid sequence of Translin contains two relatively short basic regions at amino acids 56–64 and 86–97. To determine which basic regions are required for DNA binding domain, point mutation experiments were carried out. The mutant Translin BR mutA form whose amino acids were mutated at positions 60, 62 and 64 (K^{60}, R^{62}, H^{64} to N^{60}, Q^{62}, N^{64}) (Fig. 4) retained

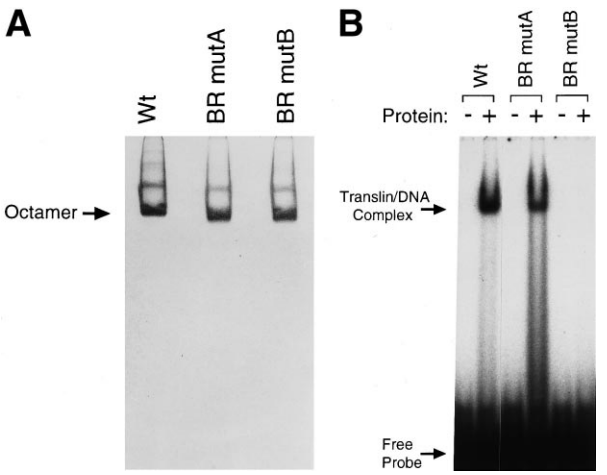

Fig. 5. Loss of the DNA binding activity of Translin by mutations in only one of the basic regions. A: Native PAGE analysis of Translin forms mutated in one of the basic regions. Recombinant human Translin (Wt) and forms mutated in the basic regions (BR mutA, BR mutB) were run on a native 5% acrylamide gel followed by staining with Coomassie blue. B: Role of the basic region of human Translin in its DNA binding activity. Aliquots of 0.5 μg of human Translin (Wt) and its mutated forms (BR mut A and BR mutB) were tested for binding to ^{32}P-labeled Bcl-CL1 by EMSA.

both multimeric structure (Fig. 5A) and DNA binding activity (Fig. 5B). Similarly, the other mutant Translin (BR mutB) whose amino acids were mutated at positions 86, 88 and 90 (R^{86}, H^{88}, H^{90} to T^{86}, N^{88}, N^{90}) (Fig. 4) retained the multimeric structure (Fig. 5A). However, DNA binding activity was completely lost (Fig. 5B). These results clearly indicate that the basic region at amino acids 86–97 is responsible for creating the DNA binding domain of Translin.

In conclusion, the leucine zipper motif at the COOH-terminus is necessary for the function of Translin through formation of the ring-shaped structure. The basic region at amino acids 86–97 of Translin proved to be responsible for creating its DNA binding domain. Recently, Wu et al. also reported that the leucine zipper motif in the C-terminus is essential for the dimerization and the DNA and RNA binding activity of the mouse homologue of Translin [9]. They concluded that the mouse Translin dimer is the minimum structural unit needed for DNA and RNA binding on the basis of results using glycerol gradient centrifugation and EMSA. In our studies with a combination of gel filtration chromatography and EMSA, however, we could detect DNA binding activity of Translin only in fractions corresponding to the molecular mass of the octamer, but not in those for the dimer. The causes of the different outcomes are still obscure, but the discrepancy might be derived from the differences in expression vectors and experimental methods. Further elucidation of Translin actions could be facilitated by perturbation of its DNA binding ability, i.e. overexpression of Translin mutated in its basic regions by transfection or transgenic experiments.

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