Sequence-specific DNA binding activity of rat *cdc37*-related gene product

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Abstract The rat *cdc37*-related gene product (RCdc37), which is possibly involved in the regulation of cell cycle progression, contains a putative basic/leucine zipper (bZIP) domain in its N-terminal portion. In this study, we have identified a rat genomic sequence which can interact with RCdc37 using an in vitro binding assay. The specificity of this interaction was confirmed by gel retardation experiments. These results raise a possibility that RCdc37 might play an important role in the control of cell cycle progression via a sequence-specific DNA binding mechanism.

Key words: Basic/leucine zipper; Cdc37; Cell cycle; 3Y1 cells

1. Introduction

Recently, we have cloned and characterized a novel gene (N17) whose sequence is partially homologous to *Dmcdc37* gene [1]. N17 mRNA level begins to be increased in the late G1 and the same level was retained until just before the G2/M transition, suggesting the cell cycle-regulatory role(s) of N17 gene product in mammalian cell systems [1]. Interestingly, Grammatikakis et al. have cloned a chicken homolog of Cdc37 (CCdc37), as one of the members which can associate with glycosaminoglycan (GAG) [2]. The nucleotide sequence (306–1047) of N17, which corresponds to the coding region of *CCdc37*, is 80% identical to the chicken sequence. The high sequence similarity of N17 not only with chicken *cdc37* but also with *Drosophila cdc37* strongly suggests that N17 might be a mammalian Cdc37-related protein (RCdc37). Although we have previously reported that N17 gene was not able to rescue a yeast *CDC37* mutation, this was not necessarily rule out the possibility of N17 being as one of Cdc37-related proteins [1].

The *CDC37* mutation in *Saccharomyces cereviciae* results in the cell cycle arrest at START, indicating the cell cycle regulatory role(s) of *cdc37* gene product [3,4]. Recent works demonstrate that yeast Cdc37 is required for the association of Cdc28 with G1 cyclins and positively controls the Cdc28 activity [5].

Furthermore, it has been reported that specific GAG fractions are concentrated in the nucleus [6,7] and these nuclear GAGs regulate AP-1-mediated transcription through a direct interaction with AP-1 transcription factor complex [8]. These observations collectively suggest that Cdc37, which can associate with GAG, might be involved in the transcriptional regulation.

In this manuscript, we described that RCdc37 possesses a putative bZIP structure and can interact with a short genomic sequence in a sequence-specific manner.

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2. Materials and methods

2.1. Cell culture

Normal rat fibroblast 3Y1 cells and 3Y1 cells transformed with Rous sarcoma virus (RSV) (SR-3Y1) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were grown in an incubator at 37°C with a 5% CO_2/95% air atmosphere.

2.2. Production of recombinant proteins in Escherichia coli

The full-length *Rcdc37* cDNA was digested with *Eco*RI and *Sph*I or *Eco*RI and *Mva*I and their protruding 5′- or 3′-tails were enzymatically modified. Each restriction fragment was purified from a 5% polyacrylamide gel and subcloned into the *Eco*RI site of pGEX-2T (Pharmacia) whose protruding 5′-termini were enzymatically filled in, to produce pGST-*Rcdc37*(165) and pGST-*Rcdc37*(13) encoding amino acids −20 to 165 and −20 to 13, respectively. Each construct contains the additional 20 amino acids derived from the 5′-untranslated region of *Rcdc37* cDNA. These recombinant plasmids were introduced into *E. coli* JM109. The fusion proteins were induced in the presence of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and purified by glutathione-Sepharose 4B (Pharmacia), under conditions described previously [9].

2.3. Construction of a genomic library and screening for RCdc37 binding sequences

3Y1 genomic DNA was digested completely with *Sau*3AI, ligated into the *Bam*HI site of pTZ18R (Pharmacia) and introduced into *E. coli* JM109 [10]. The resultant bacteria was grown in a mass culture in the presence of ampicillin (50 μg/ml). For screening, 20 μg plasmid DNA was mixed with GST-bound glutathione Sepharose beads in a binding buffer containing 10% glycerol, 5 mM EDTA, 20 mM Tris-Cl pH 7.2, 100 mM NaCl and 0.1% NP40. Following incubation for 1 h on ice, unbound DNA was recovered by centrifugation. Then GST-RCdc37(165)-bound glutathione Sepharose beads were added and maintained for 1 h on ice. The beads were washed five times with washing buffer (identical to the above binding buffer, but containing 2% glycerol). The DNA was released from the beads by incubation for 10 min at 45°C in a solution containing 1% SDS and used to transform JM109 [10]. After the third screening, individual colonies were picked up and plasmid DNA was extracted from each colony. The inserted genomic fragments were purified from the 5% polyacrylamide gels and used for gel retardation assays.

2.4. Gel mobility shift assay

Fusion proteins (0.5 μg) or nuclear extracts (10 μg) derived from 3Y1 or SR-3Y1 cells were mixed with 0.5 μg of poly[d(I-C)] (Boehringer-Mannheim) in 20 μl of a binding buffer containing 110 mM KCl, 4 mM $MgCl_2$, 4 mM Tris-Cl pH 7.6, 4% glycerol, 0.05 mM $ZnCl_2$ and 0.25% bromophenol blue [11]. The reaction mixtures were kept at room temperature for 10 min. The radio-labeled probe was then added and incubation was continued for another 20 min. The complexes were resolved by electrophoresis through neutral 5% polyacrylamide gels.

2.5. Nucleotide sequence determination

The nucleotide sequence of the cloned genomic fragment was determined in both strands by means of dideoxy chain termination method using T7 polymerase provided in the Sequenase version 2.0 kit (United States Biochemicals). The DNA sequence was compared with sequences filed in the EMBL data base.

SSDI 0014-5793(95)01210-9

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RCdc37 (42)  FQKEKEELDRGCRECK-RKVAEFQRKLKELEVAEGGGQVELERLRAEAQQL (92)
yAP1   (69)  RTAQNRAAQRAFRERKERKMKELEKKVQSLESIQQQNEVEATFLRDQLITL (121)
GCN4   (230) KRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER (280)
c-Jun  (266) KRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQL (318)
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Fig. 1. Sequence comparison in the bZIP domain among RCdc37, yAP1, GCN4, and c-Jun. The sequences are aligned to obtain maximum homology. Matched amino acid residues are indicated. Periodic leucine residues are underlined. Numbers in parentheses represent positions relative to the amino-terminal end of each protein.

2.6. Southern blot analysis

High molecular weight genomic DNA was isolated from 3Y1 cells by the standard procedure [10]. Equal amounts of DNA (10 μg) were digested completely with the indicated restriction enzymes, electrophoresed through a 0.8% agarose gel, blotted onto a nylon membrane and cross-linked using UV cross-linker (Stratagene). Hybridization was carried out at 42°C in a solution containing 50% formamide, 6 × standard saline citrate (SSC), 5 × Denhardt's solution, 0.1% SDS and ^{32}P-labeled probe. After hybridization, the filter was washed twice with 2 × SSC/0.1% SDS at room temperature, twice with 0.1 × SSC/0.1% SDS at 50°C and then exposed to autoradiographic film with an intensifying screen at −70°C.

2.7. Nucleotide sequence accession number

The sequence of clone 11 DNA is deposited in DDBJ, EMBL and GenBank nucleotide sequence data bases under accession number D63839.

3. Results and discussion

Genetic study of the phenotypes of the *CDC37* mutation in *S. cereviciae* suggests that Cdc37 plays a key role at or near the G1/S transition [3]. However, the precise mechanism of Cdc37 activity is not fully understood. The predicted amino acid sequence of RCdc37 was compared with those filed in the data base (SWISS-Prot, Release 31.0). Interestingly, we found a short sequence within RCdc37 (residues 42–92) which displays a partial homology with so-called 'basic/leucine zipper (bZIP) domain' of yAP1, GCN4 and c-Jun [12]. The homology in this region of RCdc37 for yAP1, GCN4 and c-Jun was 28.8, 19.2 and 19.2%, respectively (Fig. 1). The arginine residue in the basic region (at position 55 in the case of RCdc37) which has been shown to be essential for DNA binding activity [13] was conserved among these proteins. In addition, RCdc37 contains a highly acidic region near the carboxy terminus. Recent studies have suggested that the acidic residues exist in many transcription factors and play an essential role in the activation of RNA polymerase II transcription initiation [14,15]. These structural features indicate that RCdc37 possibly has a DNA binding ability.

In order to isolate rat genomic DNA fragments which can specifically bind RCdc37, we constructed a 3Y1 genomic library (see section 2). After the three rounds of screening to select for genomic sequences which could interact with GST-RCdc37(165) but not with GST, we have obtained a final enriched library. Genomic DNA inserts were prepared from 24 independent clones, digested with appropriate restriction enzymes and used for gel retardation assays. Among them, the 0.06 kb *Eco*RI fragment (REC11) from clone 11 gave a single retarded band in the presence of GST-RCdc37(165), but not in the presence of GST (Fig. 2A, lane 2 and 4). However, when GST-RCdc37(13) was used for this assay, no shifted band was detected (Fig. 2A, lane 3). Therefore, the additional 20 amino acids derived from the 5′-untranslated region does not participate in this interaction. The specificity of the binding reaction was analyzed by competition with specific and nonspecific DNA. As shown in Fig. 3, the binding reaction was not affected by the unlabeled nonspecific DNA, whereas the intensity of the retarded band was significantly reduced in a dose-dependent

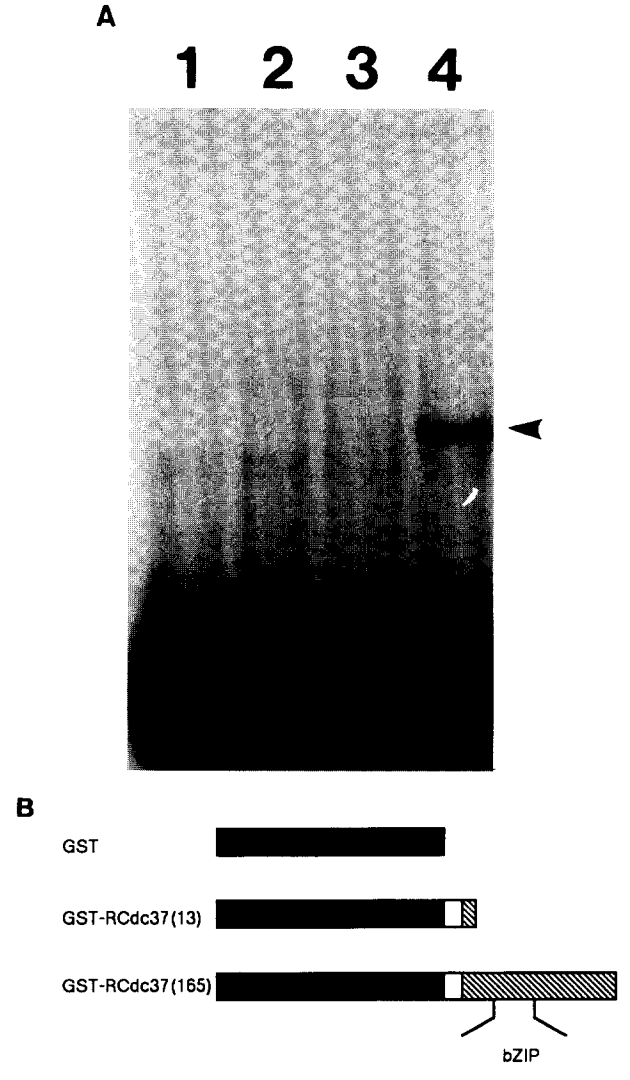

Fig. 2. Gel retardation assay for the DNA binding ability of RCdc37. (A) Identical amounts (0.5 μg) of GST (lane 2), GST-RCdc37(13) (lane 3) or GST-RCdc37(165) (lane 4) were incubated with ^{32}P-labeled REC11 DNA (5,000 cpm). Lane 1 shows the binding reaction without fusion protein. Free and protein-complexed DNA fragments were separated on 5% nondenaturing polyacrylamide gel. Retarded bands were indicated by arrow head. (B) Schematic representation of the fusion proteins used in this assay. GST, 5′-untranslated region of RCdc37 and RCdc37-coding region are shown by filled, open and cross-hatched boxes, respectively. The location of the putative bZIP domain is indicated.

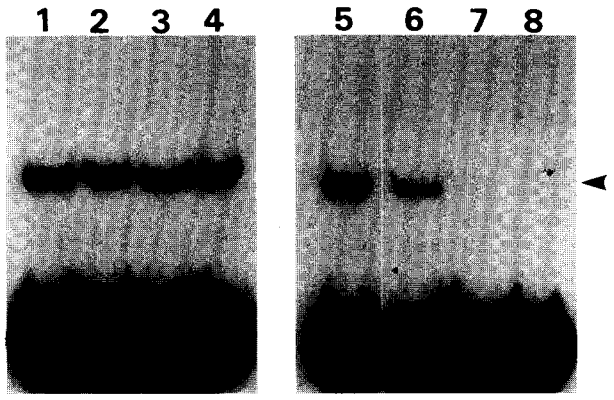

Fig. 3. DNA binding specificity of RCdc37. Identical amounts (0.5 μg) of GST-RCdc37(165) and ^{32}P-labeled REC11 DNA (5,000 cpm) were mixed and incubated with increasing amounts of unlabeled nonspecific (123/124 bp restriction fragments from pBR322) (lane 1–4) or specific (lane 5–8) competitor DNA. The concentration of the competitor DNA was 0- (lane 1 and 5), 1- (lane 2 and 6), 10- (lane 3 and 7) and 100-fold excess over the probe (lane 4 and 8).

manner in the presence of the specific DNA. These results suggest that RCdc37 could bind to the REC11 DNA in a sequence-specific manner.

As described previously [1], the expression of *Rcdc37* gene was significantly increased in SR-3Y1 cells when compared with the parental 3Y1 cells. Therefore, we have performed a gel retardation assay using the crude nuclear extract from 3Y1 or SR-3Y1 cells and compared the REC11 DNA binding activity between the two extracts. As shown in Fig. 4, the retarded band was observed in each extract. The specificity of the binding reaction was confirmed by the competition assay (data not shown). Interestingly, the intensity of the shifted band in SR-3Y1 cells was significantly stronger than that in 3Y1 cells, indicating that there exists a positive relationship between the amount of RCdc37 and the DNA binding activity.

The nucleotide sequence of clone 11 was determined (Fig. 5) and used to screen the database (EMBL, release 42.0). No

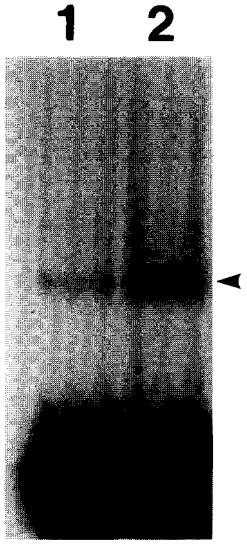

Fig. 4. REC11 DNA-binding activity in the crude nuclear extracts. Equal amount (10 μg) of the crude nuclear extract from 3Y1 (lane 1) or SR-3Y1 cells (lane 2) was incubated in the presence of ^{32}P-labeled REC11 DNA (5,000 cpm) and analyzed by the gel retardation assay.

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  1 GATCATCCGTCTTTTGCTCATCTCTACCCTACACACTTTTCAAAGGCTCT
 51 TAGCAAGGCGGCAACTAACTTTAAGGCTATTTCTTATGAATAATAAAAAA
101 AAAACAGCCTTAAGAGAATTCAATTTTTGCACGGGCCTCTTTGTGGGTGT
151 TGCTATCTTCCATTTAAGCTGTCTGCCATAGAATTCACCTCCCGCTTCCC
201 TGGTAGGAGGTAGAGAAAACAGAGTTTGATTGTCTCTGGTCACAGTCAGC
251 AGGGGGTGCGGTTTGCTGTGGCACAGCAGGCCATAGGCACCCCCACCCCA
301 CCCCCCAATCCCTGGCATCAAGGCGGAATATGGATAGGGTCAGAATCTTT
351 CTGGATC
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Fig. 5. Nucleotide sequence of the clone 11 DNA. The 0.06 kb *Eco*RI fragment (REC11), which can interact specifically with RCdc37, is indicated by underline.

meaningful homology has so far been detected between the insert of clone 11 and any previously described DNA sequences. As Cdc37 is supposed to function as a cell-cycle regulator, it is intriguing to investigate the biological meaning of sequence-specific DNA binding activity of RCdc37 through bZIP structure.

Genomic Southern analysis showed that the radio-labeled clone 11 DNA detects a single band in each digest except for *Eco*RI (Fig. 6). Considering that there are internal *Eco*RI sites in the clone 11 insert, it is possible that the REC11 DNA might exist as a single copy in the rat genome. Northern analysis revealed that the radio-labeled clone 11 DNA could not detect RNA both in 3Y1 and SR-3Y1 cells, indicating that the REC11 DNA does not correspond to the exon sequence.

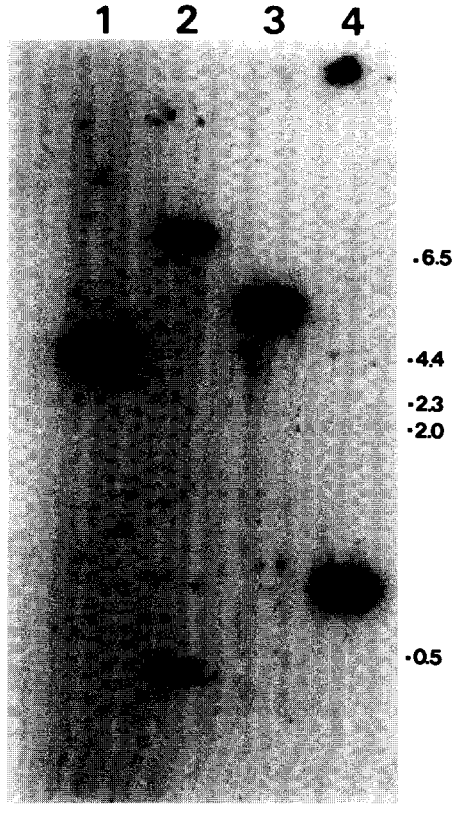

Fig. 6. Genomic Southern analysis. Genomic DNA from 3Y1 cells was completely digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3) or *Bam*HI (lane 4), electrophoresed in a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with radio-labeled clone 11 DNA. Sizes are shown in kilobases.

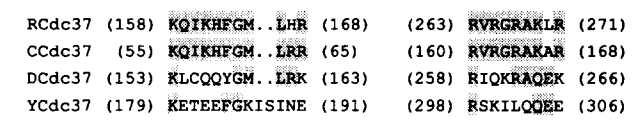

Fig. 7. Sequence comparison of the putative GAG-binding domains of Cdc37. Comparative alignment of the putative GAG-binding domains of RCdc37 with those of chicken (CCdc37), *Drosophila* (DCdc37) and yeast (YCdc37) was shown. Matched amino acid residues are indicated. Numbers in parentheses correspond to the amino acid numbers relative to the amino terminal end of each protein.

Recently, Grammatikakis et al. identified and characterized the chicken homolog of Cdc37 (CCdc37) [2]. Interestingly, CCdc37 could associate with glycosaminoglycan (GAG) and the putative GAG-binding domains were conserved among Cdc37 of different species (chicken, *Drosophila* and yeast). These putative sequences are present also in RCdc37 (Fig. 7), suggesting the GAG-binding ability of RCdc37. It has been suggested that the nuclear GAGs might modulate the AP-1-mediated gene expression through a direct interaction with the AP-1 transcription complex [8]. Thus, it is possible that the nuclear GAGs play an important role to regulate a transcription factor function. The present findings are indicative of a possibility that RCdc37 might be one of the DNA binding proteins whose function is controled by the nuclear GAGs.

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