The RNA polymerase II core subunit 11 interacts with keratin 19, a component of the intermediate filament proteins

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Abstract We have previously cloned the human RNA polymerase II subunit 11, as a doxorubicin sensitive gene product. We suggested multiple tasks for this subunit, including structural and regulatory roles. With the aim to clarify the human RNA polymerase II subunit 11 function, we have identified its interacting protein partners using the yeast two-hybrid system. Here, we show that human RNA polymerase II subunit 11 specifically binds keratin 19, a component of the intermediate filament protein family, which is expressed in a tissue and differentiation-specific manner. In particular, keratin 19 is a part of the nuclear matrix intermediate filaments. We provide evidence that human RNA polymerase II subunit 11 interacts with keratin 19 via its N-terminal α motif, the same motif necessary for its interaction with the human RNA polymerase II core subunit 3. We found that keratin 19 contains two putative leucine zipper domains sharing peculiar homology with the α motif of human RNA polymerase II subunit 3. Finally, we demonstrate that keratin 19 can compete for binding human RNA polymerase II subunit 11/human RNA polymerase II subunit 3 in vitro, suggesting a possible regulatory role for this molecule in RNA polymerase II assembly/activity.

Key words: RNA polymerase II; Nuclear matrix; Intermediate filament; Leucine zipper; Yeast two-hybrid

1. Introduction

Eukaryotic heteromeric RNA polymerase II (pol II) is a multisubunit enzyme comprised of 10–12 core polypeptides [1]. Despite the fundamental role of pol II in mRNA transcription, little information is available concerning the specific functions of its individual subunits. There is some evidence suggesting that pol II in yeast may modulate transcription by altering its subunit composition in response to nutrient or thermal stress [2–4]. The issue of subunit variation in mammalian pol II has, however, not yet been investigated. We have previously cloned the human RNA polymerase II subunit 11 (hRPB11) and shown the involvement of this protein in dox-mediated cellular toxicity and cellular differentiation [5–7], indicating that hRPB11 may exert a specific role in gene regulation. This subunit contains two amino acid sequences (α motif) with limited homology to the α subunit of *Escherichia coli* RNA polymerase and it interacts with hRPB3, another human RNA pol II α-like subunit [6,8]. The hRPB11 N-terminal α motif is necessary for this interaction and it appears to be the unique contact site of hRPB11 with pol II. The heterodimer hRPB11/3 is considered to be the functional counterpart of the bacterial α subunit homodimer [9,10]. This homodimer plays a role in promoter recognition by interacting directly with sequence-specific upstream DNA elements and in transcriptional regulation by binding to specific sets of transcription activator proteins [8,11,12]. In an effort to study the function of hRPB11, we used this subunit as a bait in the yeast two-hybrid system to identify possible interaction target protein(s). Here, we report the identification of keratin 19 as a novel partner for hRPB11. Keratins are members of the intermediate filament (IF) family of structural proteins [13]. IFs are dynamic structures that are involved in signal transduction from the extracellular matrix to the nucleus [14–18]. Keratins 8, 18 and 19 have been shown to be present in the nuclear matrix-IF (NM-IF) [19,20]. These proteins are bound to nuclear DNA in breast cancer cells supporting the hypothesis that they are involved in the organization of nuclear DNA. In particular, it has been suggested that keratin 19 is involved in the epigenetic activation of new cellular programs, through the rearrangement of the cytoskeleton, which may perturb the NM function [21,22].

Here, we show that keratin 19 shares peculiar homology with the α motif of hRPB3. We provide evidence that hRPB3 and keratin 19 interact with the same region of hRPB11. Moreover, we demonstrate that keratin 19 can compete for binding hRPB11/hRPB3.

2. Materials and methods

2.1. Yeast two-hybrid selection

For two-hybrid screening, the complete open reading frame of human RPB11 was cloned into a *Pst*I restriction site of vector pGBT9 (Clontech, Palo Alto, CA, USA) in frame with the *GAL4* binding domain. Yeast strain HF7c [23], bearing UASg-His3 and UASg-LacZ as reporter genes, was co-transformed with the bait pGBT9-Hrpb11 and with a human skeletal muscle cDNA library (Clontech) fused to the *GAL4* activation domain in the vector pGAD10 (Clontech). Transformation was performed using the lithium acetate method [24]. Cells were plated directly on minimal synthetic defined (SD) medium, 2% glucose, 0.67% Bacto yeast nitrogen base (Difco Laboratories, Detroit, MI, USA), supplemented with the required bases and amino acids, except tryptophan (Trp), leucine (Leu) and histidine (His). Plates were incubated for 7 days at 30°C, then, His^+ transformants were isolated. The His^+ colonies, replica-plated on SD-Leu-Trp-His medium and $LacZ^+$, were identified by a filter-lifting assay for β-galactosidase activity. Plasmid DNA was prepared from candidate clones and electroporated into *E. coli* XLI-blue competent cells (Stratagene, La Jolla, CA, USA). The recovered library-derived plasmids were analyzed as positive candidates. The three constructs

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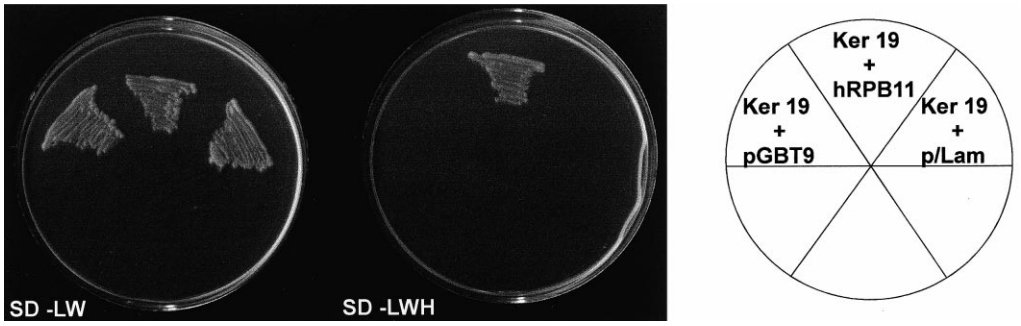

Fig. 1. Human RPB11 binds keratin 19. HF7c yeast cells were co-transformed with the indicated plasmids and plated onto media lacking leucine and tryptophan (−LW) to verify the expression of both bait (Trp^+) and prey (Leu^+) plasmids, onto a −LW plate for assaying β-galactosidase activity (not shown) or onto media lacking leucine, tryptophan and histidine (−LWH) for examining the interaction between bait and prey proteins.

carrying different hRPB11 N-terminal deletions used in a two-hybrid assay have been described elsewhere [6].

2.2. Co-precipitation with GST chimeric proteins, SDS-PAGE and Western blotting analysis

The cDNA of keratin 19 isolated by screening was cloned into the *Eco*RI restriction site of vector pGEX-4T1 (Pharmacia-Biotech) in frame with the GST encoding sequence. LoVo H cells were lysed in 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1% Triton X-100, 1 mM PMSF and 10 μg/ml leupeptin (lysis buffer) for 30 min at 4°C. After centrifugation, supernatants were transferred to a fresh tube and chimeric proteins GST-keratin 19 conjugated to glutathione/Sepharose beads (Pharmacia-Biotech, Milan, Italy) were added and incubated for 90 min at 4°C. Beads were washed three times in lysis buffer, dried and electrophoresis sample buffer was added. The bound proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore) and probed with anti-hRPB11 polyclonal antibody as previously described [5]. Immunoreactivity was detected by chemiluminescence reaction using the ECL kit (Amersham, Milan, Italy).

2.3. In vitro transcription and translation

In vitro transcription and translation were performed using a TNT-coupled reticulocyte lysate system (Promega) and L-[^{35}S]methionine (>1000 Ci/mmol, Amersham) as directed by the manufacturer.

2.4. GST pull-down assays

For protein-protein interaction assays, comparable amounts of resin-bound GST fusion proteins were incubated with in vitro translated proteins in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) for 1 h at 4°C. The resins were then pelleted and extensively washed in the same buffer. The bound proteins were separated by SDS-PAGE and the gel fixed, incubated in Enlighting solution (Du Pont), dried and exposed for fluorography.

3. Results

3.1. Yeast two-hybrid screen

An adult human skeletal muscle cDNA library was screened with hRPB11 as bait by sequential transformation of the appropriate yeast reporter strain HF7c. Of an estimated 1×10^6 transformants screened, 13 clones able to proliferate on media lacking histidine and positive for β-galactosidase staining were obtained. Eight of these positive clones were identified as the pol II subunit hRPB3 [6], one encoded a novel human protein, Che-1 (Fanciulli et al., personal communication), and four were identified as keratin 19. The positives for keratin 19 were encoded by two clones of different length, lacking different keratin 19 N-terminal portions and named ker-cl1 and ker-cl2, respectively. ker-cl1 was represented by three identical clones encoding amino acids 38–400 of keratin 19, while ker-cl2 was represented by a single clone encoding amino acids 140–400 of the same protein. The specificity of hRPB11/keratin 19 interaction was confirmed in a two-hybrid assay performed by co-transforming the ker-cl2 clone with pGBT9/hRPB11 or with pGBT9/lamin and pGBT9 empty vector as a negative control. Fig. 1 shows that, whereas pGBT9/hRPB11 was able to bind keratin19, pGBT9/lamin and pGBT9 empty vector did not show any significant interaction.

3.2. hRPB11 interacts with keratin 19 in vitro

In order to confirm the results obtained with the two-hybrid system, we produced a GST chimeric construct containing ker-cl2, which was used in a co-precipitation assay. The glutathione/Sepharose-GST and GST-ker-cl2 were added to a LoVo H cell lysate that expresses high levels of hRPB11 [5]. Proteins that co-precipitated with the immobilized GST proteins were analyzed by SDS-PAGE and in Western blots for hRPB11. Results showed that, whereas GST alone did not bind hRPB11, GST-ker2 co-precipitates large amounts of hRPB11 (Fig. 2), confirming a specific physical interaction between keratin 19 and hRPB11.

3.3. Keratin 19 interacts with the N-terminal α motif of hRPB11

We have previously demonstrated that hRPB11 interacts with another human pol II subunit, hRPB3, via the N-terminal α motif. To establish the portion of hRPB11 responsible for the interaction with keratin 19, the ability of ker-cl2 to

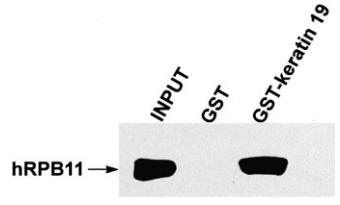

Fig. 2. Interaction of keratin 19 with a GST chimeric construct corresponding to hRPB11. LoVo H cell lysate was incubated with immobilized GST or GST chimeric proteins, as indicated. Interacting proteins recovered were fractionated by 12.5% SDS-PAGE, transferred onto a PVDF membrane and analyzed by Western blotting for hRPB11.

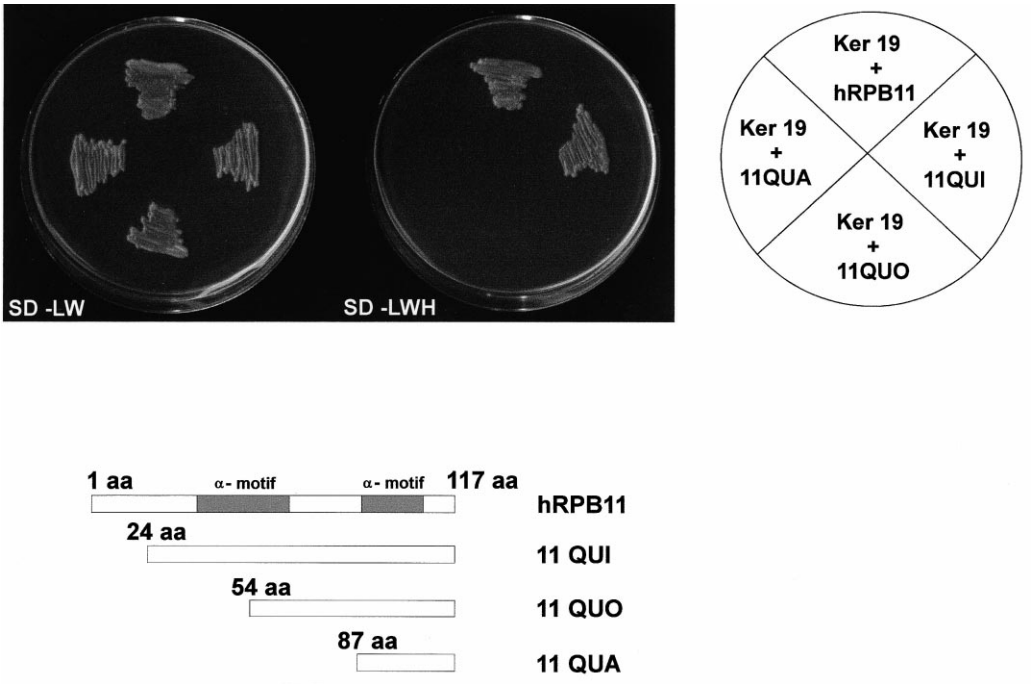

Fig. 3. hRPB11 interacts with keratin 19 by its N-terminal α motif. (A) Interaction analysis between keratin 19 and hRPB11 N-terminal deleted constructs by a two-hybrid assay. (B) Schematic diagram of N-terminal deletions of the hRPB11 RNA polymerase II subunit. The horizontal bars represent the amino acid sequence in each construct.

interact with three progressive amino-terminal truncated hRPB11 derivatives was tested. As shown in Fig. 3, deletion of 24 amino acids at the N-terminus failed to abrogate hRPB11 interaction with pGAD10-ker-cl2, whereas deletion of 54 amino acids containing the N-terminal α motif of hRPB11 completely abrogated the binding capacity. Therefore, hRPB11 requires its N-terminal α motif for interacting with keratin 19.

3.4. Keratin 19 has putative leucine zipper motives sharing significant homology to the α motif of hRPB3

The above results demonstrate that the hRPB11 N-terminal α motif is able to interact with both hRPB3 and keratin 19 proteins. It has been demonstrated that hRPB3 binds hRPB11 essentially via its leucine-rich C-terminal α motif (Fanciulli et al., personal communication). To determine whether a similar domain was also present in keratin 19, the primary structure of keratin 19 was analyzed using ScanProsite software. As shown in Fig. 4A, two putative overlapping leucine zipper motifs were found in keratin 19, with leucine residues involved in periodic repetition at every seventh position. It is noteworthy that amino acid sequence comparison revealed a peculiar homology between the keratin 19 leucine zipper region and the hRPB3 α motif (Fig. 4B).

3.5. Keratin 19 competes with hRPB3 for the same binding site of hRPB11

The indications that keratin 19 and hRPB3 bind the same region of hRPB11 and the homology observed between the keratin 19 leucine zipper region and the hRPB3 α motif led us

A

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          Leucine Zipper I
          ________________
186- LRRVLDELTLARTDLEMQIEGLKEELAYL -214 Keratin 19
                ________________
                Leucine Zipper II
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B

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179- VEADINGLRRVLDELTLARTDLEMQIEGL -207 Keratin 19
     V :.::GL:: L.:L  :: : E:Q : L
245- VLSALSGLKKKLSDLQ-TQLSHEIQSDVL -272 hRPB3
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Fig. 4. Keratin 19 possesses two overlapping leucine zipper domains that share homology with the α motif of hRPB3. (A) The two putative overlapping leucine zipper motifs present in the keratin 19 protein sequence. The leucine residues involved in the periodic repetition at every seventh position are indicated in bold/italic. (B) Amino acid sequence similarity between keratin 19 and hRPB3.

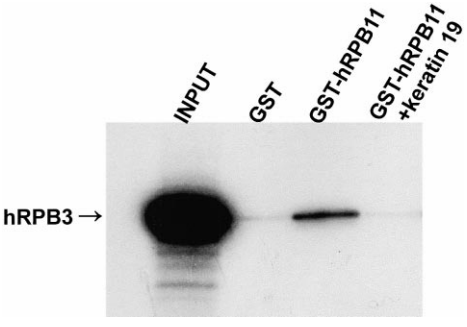

Fig. 5. Keratin 19 competes with hRPB3 for the binding to hRPB11. In vitro translated labelled hRPB3 was subjected to a GST pull-down analysis using beads coated with GST or GST-hRPB11 fusion protein. Prior to GST pull-down, beads were incubated with keratin 19 as indicated.

to hypothesize possible competition between these two proteins for binding to hRPB11. To verify this hypothesis, we tested the ability of GST-hRPB11 to bind ^{35}S-labelled hRPB3 in presence or in absence of keratin 19. Fig. 5 shows that pre-incubation of GST-hRPB11 beads with keratin 19 produced a marked decrease in their ability to interact with hRPB3. These data indicate that hRPB11 interacts with this component of the IF family and that this interaction can dissociate hRPB11 from pol II.

4. Discussion

RNA polymerase is a universal regulator of gene expression. The study of the pol II structure and how structural details encode function is fundamental for an understanding of the molecular mechanisms that regulate RNA polymerase activity [25,26]. hRPB11 is a component of the RNA polymerase multisubunit complex [27,28]. Although RPB11 is required for cell viability in yeast [29], expression of this protein varies in normal human tissues [5]. We have previously shown that doxorubicin affects hRPB11 expression and that this protein is specifically involved in doxorubicin-mediated toxicity. Moreover, we have demonstrated that ectopic expression of hRPB11 affects cell differentiation, changing the cell proliferation rate and transactivating the specific differentiation marker E-cadherin [5]. Taken together all these findings led us to hypothesize a role of hRPB11 in gene regulation. In order to investigate the hRPB11 function, we performed a yeast two-hybrid experiment screening a human muscle cDNA library, using hRPB11 as the bait. The result of this experiment was the isolation of several hRPB11 interacting proteins. In this paper, we report on the interaction of hRPB11 with keratin 19, a component of the IF protein family which forms part of the NM [14–18]. This strong interaction, selected in yeast, was confirmed in a pull-down experiment using human colon carcinoma cell lysate. Furthermore, we demonstrate by a two-hybrid assay that the hRPB11 region involved in contacting keratin 19 contains the N-terminal α motif. This motif appears to be responsible for the interaction between hRPB11 and pol II and is also responsible for the interaction with hRPB3 [6]. Here, we demonstrate that hRPB3 and keratin 19 preferentially compete with the N-terminal α motif on hRPB11 protein for binding. We interpret these data as suggesting that these alternative hRPB11 interactions represent a novel pol II regulatory mechanism. In fact, keratin 19 can bind and dissociates hRPB11 from pol II, thus regulating its function. Keratin 19 possesses several intriguing functional and structural features that further support this hypothesis. Analysis of its primary structure revealed the presence of two canonical overlapping leucine zipper motifs exhibiting significant homology to the basal cell hRPB3 α-like motif. In epithelial cells, keratin 19 is expressed in a small basal cell subpopulation that exhibits stem-cell-like characteristics and appears to have peculiar assembly properties imparting unique characteristics to the basal cells that expresses it [30]. Keratin 19 and keratin 8 and 18 are located to the NM-IF fraction and associate with nuclear DNA in many cell lines [20,21,31,32]. Inappropriate expression of keratin 19 correlates with cellular transformation and with epigenetic activation of new cellular programs via the rearrangement of the cytoskeleton and NM [33,34]. Interestingly, pol II in its hyperphosphorilated form also associates with NM [35] by interacting with several matrix proteins [36,37]. Therefore, the novel interaction between hRPB11 and keratin 19 described here may play a key role in regulating the function of pol II. The details of the mechanism and the potential regulatory role of this interaction await further investigation.

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