Interaction of human Ku70 with TRF2

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Abstract Ku, a heterodimer of 70- and 80-kDa subunits, plays a general role in the metabolism of DNA ends in eukaryotic cells, including double-strand DNA break repair, V(D)J recombination, and maintenance of telomeres. We have utilized the yeast two-hybrid system to identify Ku70-interacting proteins other than Ku80. Two reactive clones were found to encode the dimerization domain of TRF2, a mammalian telomeric protein that binds to duplex TTAGGG repeats at chromosome ends. This interaction was confirmed using bacterial fusion proteins and co-immunoprecipitations from eukaryotic cells overexpressing TRF2. The transfected TFR2 colocalized with Ku70.

Key words: Yeast two-hybrid; Ku; TRF2; Telomere

1. Introduction

Ku was originally identified as a major autoantigen in patients with autoimmune diseases such as scleroderma-polymyositis [1]. Ku is a heterodimer of 70- and 80-kDa subunits [2,3], and binds to DNA ends, nicks, or single- to double-strand transition [2,3], and serves as a DNA binding component of a DNA-dependent protein kinase (DNA-PK) that phosphorylates certain chromatin-bound proteins in vitro [4,5]. Both Ku and the catalytic subunit of DNA-PK have been shown to be crucial for double-strand-break DNA repair and V(D)J recombination [6–8].

It has been suggested that Ku played an important role in the regulation and maintenance of telomeres as well. In *Saccharomyces cerevisiae*, mutants of either human Ku70 or Ku80 homologues, YKU70/HDF1 and YKU80/HDF2, were shown to have abnormally short telomeres [9,10], and disrupted subnuclear organization of telomeres [11]. Mutations in either Ku subunit led to enhanced instability of telomeres by increasing their sensitivity to either degradation or recombination reactions [12]. Ku has been shown to be an integral component of the telomere in yeast [13] and to bind human telomeric DNA in vitro [14]. Ku was also shown to be associated with the telomere in mammalian cells [15].

Ku is a relatively abundant protein, approximately 5×10^5 molecules per nucleus [16]. Recently, we have shown that Ku was present in the nuclear matrix as well as in the soluble fractions of the human nucleus [17]. In the nuclear matrix, Ku70 appeared to be present in larger amounts than Ku80 [17]. Thus, it appeared that Ku70 might bind other proteins to perform diverse biological functions at least in certain cellular compartments. This possibility prompted us to look for additional interacting proteins of Ku70.

A number of attempts have been made to identify proteins that interact with Ku70 using the yeast two-hybrid system. It was shown that Hdf1p, a yeast homologue of Ku70, interacted with Sir4p, which was required for telomere position effect [18]. In addition, human Ku70 was identified to be an interacting protein of hGCN5 acetyltransferase [19], oncoprotein $p95^{vav}$ [20], and apolipoprotein J [21].

In our yeast two-hybrid system, two reactive clones were found to encode a telomere-specific DNA binding protein, TRF2. TRF2 is one of the telomere-specific DNA binding proteins in mammals [22,23], and is known to protect the telomere from end-to-end fusion [24]. Here we report that the Leu-Ser repeat domain of Ku70 can interact with TRF2. Ku70 may have a role in the regulation and maintenance of telomeres through the interaction with TRF2.

2. Materials and methods

2.1. Yeast two-hybrid screening of human cDNA library

The full-length human Ku70 cDNA was a kind gift from Dr. W.H. Reeves [25]. For the yeast two-hybrid analysis, we employed the system developed by the Brent laboratory [26]. The bait, LexA-Ku70/186, contained partial cDNA corresponding to amino acid residues 200–385 (186 aa) of Ku70. The plasmid was introduced into yeast *S. cerevisiae* strain EGY48 [*MATa, his3, trp1, ura3-52, leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] by a modified lithium acetate method. The yeast transformants carrying the bait were used to screen a HeLa cDNA library as described previously [26]. The bait did not have any intrinsic activity of transcriptional activation for the reporters. The bait–prey interchange experiments were carried out for all positive interactions.

As control baits, LexA-Ku70/139 and LexA-DDX3 were used to determine whether the isolated plasmids showed specificity to Lex-Ku70/186 for interaction with the reporter genes. LexA-Ku70/139 contained amino acid residues 471–609 (139 aa) of Ku70, which encompassed another repeat of leucine at the C-terminus [25]. LexA-DDX3 contained a full-length human DDX3 in which a leucine repeat was also included [27].

To further identify the interaction domains in Ku70, LexA-Ku70/119, which contained a partial cDNA corresponding to amino acid residues 267–385 (119 aa), was tested similarly. To delineate interacting domains of the selected TRF2/B22, four deletion constructs expressing amino acid residues 38–140, 49–197, 120–230, and 180–247 of TRF2 were generated. The full-length TRF2 cDNA clone phTRF216-1 was a kind gift from Dr. T. de Lange [22].

2.2. Expression of recombinant proteins in Escherichia coli

In-frame fusion of the recombinant full-length Ku70 gene was constructed with the pET28 vector (Novagen) for bacterial expression as

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Abbreviations: DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; FITC, fluorescein isothiocyanate

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an amino-terminal $(His)_6$-tagged protein. When the recombinant full-length Ku70 was expressed in bacteria, it was degraded with its N-terminal portion cleaved. To construct a plasmid expressing the N-terminal deletion of the Ku70 gene product, pET28a/Ku70/200-609, a fragment of 1234 bp encompassing amino acids 200–609 was obtained by PCR using a sense primer (5′-CCTTGACTTGATGCACCTGA-3′) and an antisense primer (5′-TCAGTCCTGGAAGTGCTTGG-3′). The PCR product was first cloned into vector pCR2.1 (Invitrogen), then a 1250-bp *Eco*RI fragment from pCR2.1-Ku70/410 was cloned into the *Eco*RI site of pET28a. $Ku70^{\Delta N}$ recombinant protein, which expressed amino acid residues 200–609 and 38 additional amino acids, was partly soluble.

The $(His)_6$-tagged recombinant full-length TRF2 protein was not expressed properly in the bacteria either. To construct a plasmid expressing $TRF2^{\Delta M}$, which was devoid of the Myb-like C-terminus [24], the cDNA encompassing amino acids 1–442 was obtained by PCR with the full-length TRF2 cDNA clone phTRF216-1 using a sense primer (5′-CTGAATTCATGGCGGGAGGAGGC) and an antisense primer (5′-GCAAGCTTCAGGTTGTACTGTCTTC-3′). The PCR products were digested with *Eco*RI/*Hin*dIII and cloned into the *Eco*RI/*Hin*dIII sites of pET28a. $TRF2^{\Delta M}$ fusion protein was also partly soluble.

To construct a plasmid expressing $TRF2^{B22}$, TRF2 insert was generated by liberation from the pJG4-5 prey B22 by *Eco*RI/*Xho*I digestion followed by cloning into the *Eco*RI/*Xho*I sites of pET28a. The product expressed in bacteria was poorly soluble, and was recovered partly by electroelution according to the manufacturer's instruction (Bio-Rad).

2.3. In vitro protein interaction

For in vitro binding assays, 10 μg of purified recombinant $TRF2^{B22}$ was incubated with 20 μg of $Ku70^{\Delta N}$ in the reaction buffer (20 mM HEPES–KOH pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% NP-40, and 0.1% Triton X-100) for 1 h at 4°C. Then, immunoprecipitation was performed with monoclonal anti-Ku70 antibody NMB1 [17]. After application of goat anti-mouse Ig second antibody, immune complexes were precipitated with protein A-Sepharose beads. The precipitates were resolved in 10% SDS–PAGE sample buffer, electrotransferred to nitrocellulose paper, and immunoblotted with anti-T7·Tag antibody. The antigen–antibody complexes were visualized using a NBT/BCIP color reaction (Boehringer-Mannheim) or enhanced chemiluminescence (ECL detection system, Amersham).

To see the effect of TRF2 binding on the association of Ku70 and Ku80, an excessive amount (100 μg) of purified $TRF2^{\Delta M}$ was added to the extracts of 1×10^5 HeLa cells. HeLa cells were extracted in 200 μl extraction buffer (20 mM HEPES–KOH pH 7.9, 420 mM KCl, 25% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, and 0.2% NP-40) for 30 min and centrifuged for 10 min at $14\,000\times g$. The supernatant was dialyzed for 5 h against 100 ml of reaction buffer.

2.4. In vivo interaction in cells with transient transfection of TRF2

COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in 5% CO_2. The $TRF2^{B22}$ cDNA with T7·Tag was inserted into pcDNA3 (Invitrogen). COS cells were transfected with 3 μg of plasmid DNA/60-mm dish using Lipofect-Amine Plus™ reagent (Gibco BRL). At 24 h after transfection, the cell lysates were prepared and analyzed by immunoprecipitation assays.

The whole cell extracts were incubated with monoclonal anti-Ku70, anti-Ku80 [17], and anti-T7·Tag antibodies (Novagen) for 2 h at 4°C, followed by the immunoprecipitation procedure as described above. As a negative control, preimmune mouse serum was incubated with the cell extracts.

2.5. Immunofluorescence microscopy

The $TRF2^{\Delta M}$ cDNA with T7·Tag was inserted into pcDNA3. It was chosen because the prey $TRF2^{B22}$ lacked a nuclear localization signal. COS cells were plated on coverslips and transfected with 1 μg of plasmid DNA/35-mm dish as described above. At 24 h after transfection, cells were fixed in cold methanol at −20°C. As primary antibodies, polyclonal anti-TRF2 antibody and a monoclonal anti-Ku70 antibody, NMB1, were applied. The affinity-purified anti-TRF2 antibody was obtained from antisera of a rabbit, which was immunized using the gel-purified $TRF2^{\Delta M}$ recombinant protein. Alternatively, anti-T7·Tag antibody, and/or polyclonal anti-Ku70 antibodies (Santa Cruz Biotechnology) were applied. After the double immunostaining using rhodamine-conjugated anti-rabbit or anti-goat IgG secondary antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Zymed), cells were viewed using a confocal laser microscope (Zeiss LSM510). As controls, cells were stained with primary or secondary antibody alone. Control slides did not display significant fluorescence in any case.

3. Results and discussion

3.1. Yeast two-hybrid screening

The search for Ku binding proteins using the yeast two-hybrid system has been limited because of the predominant interaction between the two subunits; when full-length cDNA of human Ku70 or Ku80 bait was used, only the expected counterpart, i.e. Ku80 or Ku70, was reported to be detected [28,29]. In an attempt to identify additional Ku70 binding partners, we decided to use the bait of Ku70 partial cDNA in our yeast two-hybrid screening.

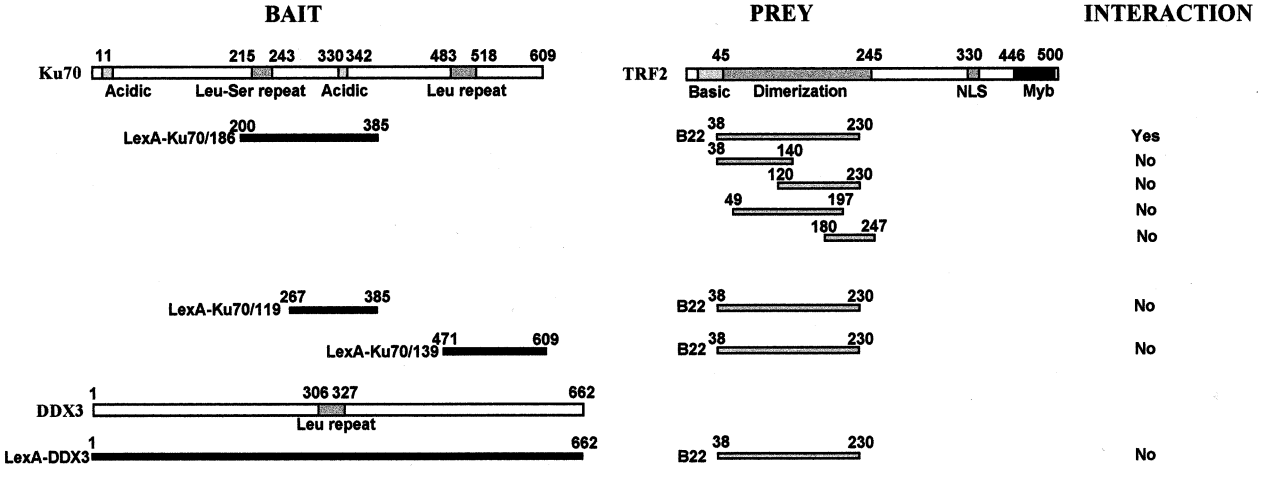

Fig. 1. The yeast two-hybrid system. Domains of Ku70 are shown at the top left. Bars below the lines designate inserts of three baits used for the yeast two-hybrid analysis. Domains of TRF2 are shown in the middle; NLS, nuclear localization signal; Myb, Myb-like DNA binding site. Bars below the lines represent the retrieved prey TRF2/B22 and its truncated forms.

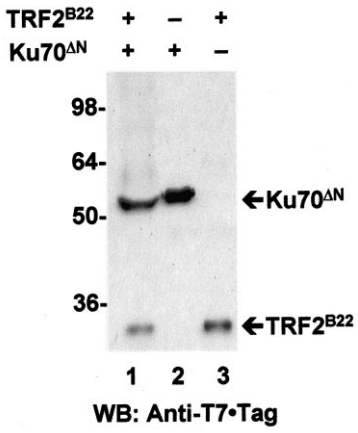

Fig. 2. In vitro interaction of recombinant TRF2 and Ku. Recombinant $Ku70^{\Delta N}$ and $TRF2^{B22}$ proteins were incubated in the reaction buffer at 4°C for 1 h, and immunoprecipitated with anti-Ku70 monoclonal antibody NMB1. As shown in lane 1, the immunoblotting with anti-T7·Tag antibody showed both $Ku70^{\Delta N}$ and $TRF2^{B22}$ indicating co-immunoprecipitation of $TRF2^{B22}$ and $Ku70^{\Delta N}$ in vitro. Lanes 2 and 3 show purified $Ku70^{\Delta N}$ and $TRF2^{B22}$ inputs for reference, respectively.

The bait, LexA-Ku70/186, contained Ku70 partial cDNA corresponding to amino acid residues 200–385, which did not overlap significantly with the published Ku80 binding sites (Fig. 1) [30–32]. In the area, a periodic repeat of leucine and serine (aa 215–243) in every seventh position was included (Fig. 1); thus, it was compatible with the leucine zipper structure [33] except that it was not a repeat of leucine alone. The Leu-Ser repeat abutted a strongly basic region, and displayed significant sequence similarity to a region of v-myc and c-myc proteins [30].

Approximately 3×10^5 independent transformants were pooled and re-spread on the selection medium (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the expression of cDNAs. In the selection medium, a total of 72 colonies showed galactose dependence. The plasmids were extracted by yeast miniprep, the cDNAs were PCR-amplified with primers derived from vector pJG4-5, followed by sequence determination.

DNA sequencing and database searches revealed that the nucleotide sequence of clones B22 and B46 encoded the N-terminal portion of human TRF2 encompassing amino acid residues 38–230 [23,24]. The clones turned out to be identical in DNA sequence as well as in size. This region overlapped with the dimerization site of TRF2 (aa 46–245) [23,24]: the inserts had seven additional amino acid residues at the N-terminus and lacked 15 amino acid residues at the C-terminus of the dimerization site.

It has been proposed that leucine zipper proteins might interact among themselves through the motifs [33]. To further confirm the specificity of the interaction, two additional baits containing similar leucine zipper-like motifs were tested in the two-hybrid system: LexA-Ku70/139 (aa 471–609) and LexA-DDX3. They included the leucine zipper-like domain at the C-terminus of Ku70 [25] and the leucine zipper motif of putative RNA helicase DDX3 [27], respectively. As shown in Fig. 1, TRF2 did not interact with either of them. The results indicated that the association of Ku70/TRF2 was not mediated by non-specific interaction of the Leu-Ser domain of Ku70.

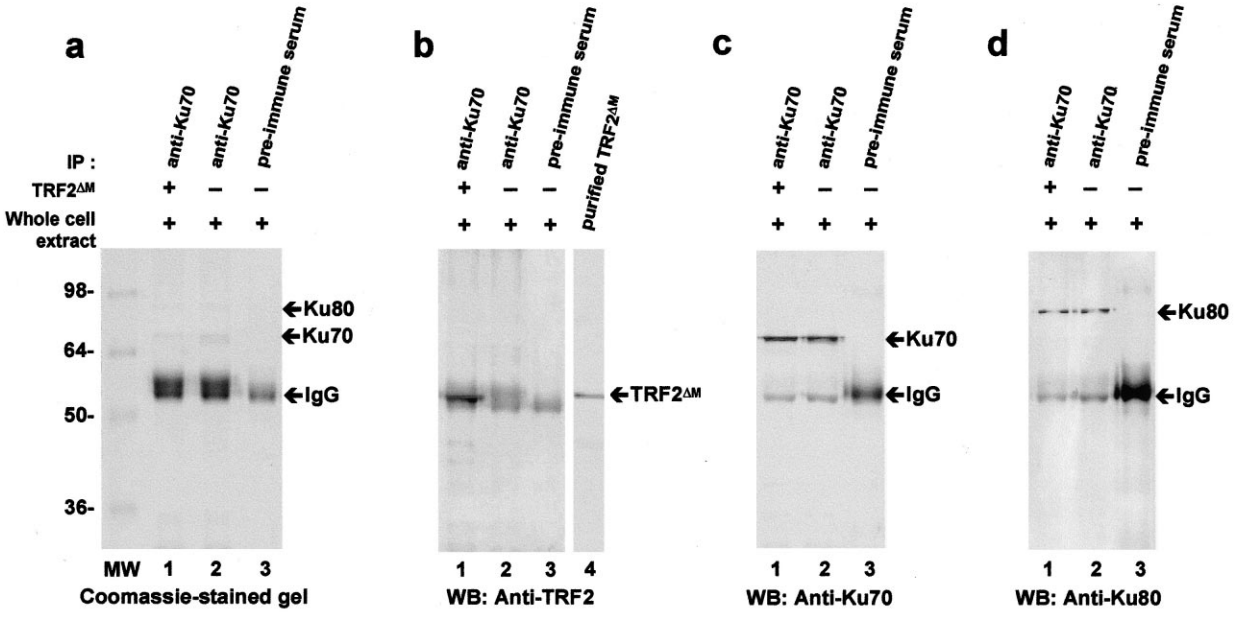

Fig. 3. In vitro interaction of recombinant $TRF2^{\Delta M}$ with native Ku. a: Coomassie-stained gel of immunoprecipitates. An excessive amount of purified recombinant $TRF2^{\Delta M}$ protein was incubated with HeLa cell extracts, and Ku70 was immunoprecipitated with a monoclonal anti-Ku70 antibody and a goat anti-mouse IgG second antibody (lane 1). As a control, the immunoprecipitation was performed under the same condition without $TRF2^{\Delta M}$ (lane 2). In lanes 1 and 2, the IgG heavy chains consisted of both goat (larger) and mouse (smaller) immunoglobulin, while only mouse immunoglobulin heavy chain was present in the control lane 3. In lanes 1 and 2, Ku80 was co-precipitated with Ku70. b: Immunoblotting with anti-TRF2 antibody, the same precipitate as in a. $TRF2^{\Delta M}$, estimated molecular weight 55 kDa, was detected between the goat and mouse heavy chains (lane 1). Lane 4 shows the input $TRF2^{\Delta M}$. c: Immunoblotting with polyclonal anti-Ku70 antisera, the same precipitate. The estimated amount of Ku70 was similar between lanes 1 and 2. d: Immunoblotting with polyclonal anti-Ku80 antisera, the same precipitate. Ku80 was co-immunoprecipitated with Ku70. The estimated amount of Ku80 was similar between lanes 1 and 2.

3.2. Binding domains of Ku70 and TRF2

To narrow down the domain of Ku70 responsible for the interaction with TRF2 further, a truncated bait, LexA-Ku70/119 (aa 267–385) lacking the Leu-Ser repeat was constructed and introduced into the EGY48 strain. As shown in Fig. 1, this deletion completely abolished the interaction with the TRF2. Therefore, the Leu-Ser repeat appeared to be essential for the interaction with TRF2. Our data suggest that the Leu-Ser repeat may be a site through which Ku70 interacts with protein(s) other than Ku80. However, it does not appear to be the only interaction site preserved for the purpose. It has been shown that Ku70 interacts with the oncogene product $p95^{vav}$ through the C-terminus [19].

The preys contained a region which largely overlapped with the dimerization site of TRF2 (aa 46–245). To delineate the domain of TRF2 responsible for the interaction further, we constructed four TRF2 deletion mutants which represented peptides of aa 38–140, 49–197, 120–230, and 180–247 (Fig. 1). None of the truncated preys interacted with Ku70, indicating that the entire region of $TRF2^{B22}$ (aa 38–230) was required for the interaction.

3.3. In vitro interaction between recombinant Ku70 and TRF2

To validate the results obtained with the yeast two-hybrid system, the interaction of recombinant $Ku70^{\Delta N}$ with $TRF2^{B22}$ proteins was examined by immunoprecipitation in vitro. Recombinant $Ku70^{\Delta N}$ and $TRF2^{B22}$ were incubated in the reaction buffer at 4°C for 1 h. Then, Ku70 was immunoprecipitated with monoclonal antibody NMB1 the epitope of which was at the C-terminus of Ku70. As shown in Fig. 2, lane 1, $TRF2^{B22}$ was co-immunoprecipitated with $Ku70^{\Delta N}$ indicating binding in vitro. The purified $Ku70^{\Delta N}$ and $TRF2^{B22}$ are shown in separate lanes (Fig. 2, lanes 2 and 3).

We further examined whether the binding of recombinant

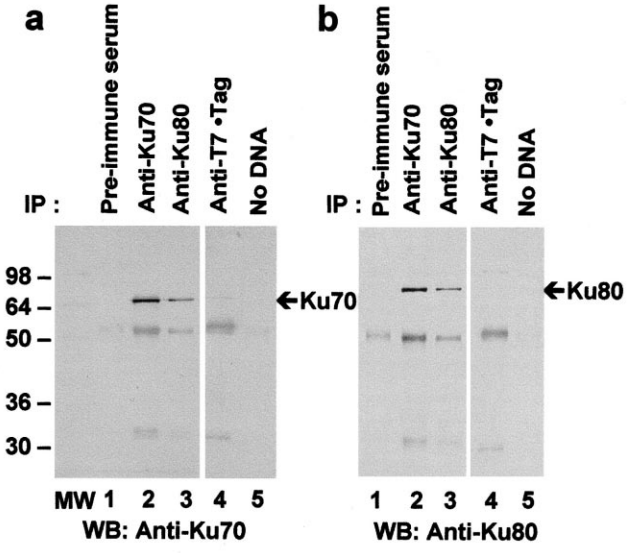

Fig. 4. In vivo interaction of transfected TRF2 and cellular Ku. a: The extracts of COS cells transfected with T7-tagged $TRF2^{B22}$ were immunoprecipitated with anti-Ku70, anti-Ku80, and anti-T7·Tag antibodies, respectively. The immunoblotting was done with a monoclonal anti-Ku70 antibody. Ku70 was co-immunoprecipitated with Ku80 and $TRF2^{B22}$, respectively (lanes 3 and 4). COS cells without transfection were used as negative control (lane 5). b: Immunoblotting with anti-Ku80 antibody, the same precipitates. Ku80 was not co-immunoprecipitated with $TRF2^{B22}$ (lane 4).

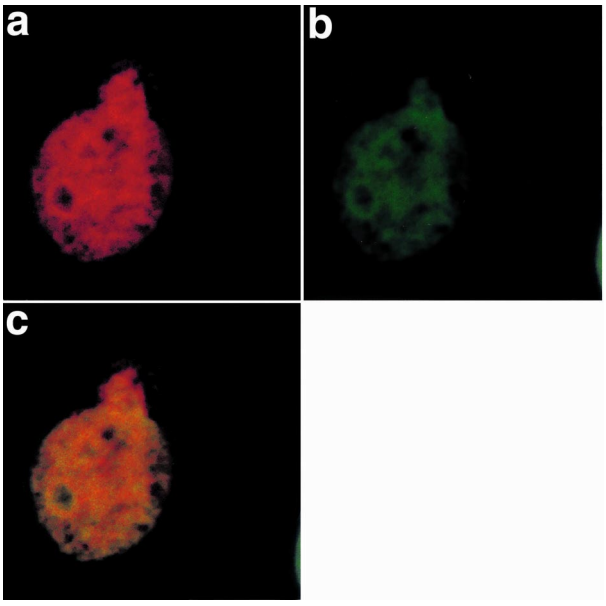

Fig. 5. Double immunofluorescence microscopy of Ku70 and transfected TRF2. COS cells were transfected with pcDNA3/$TRF2^{\Delta M}$. At 24 h after transfection, cells were fixed in cold methanol and then double immunostaining was done using monoclonal anti-T7·Tag antibody and polyclonal anti-Ku70 antibody. Slides were examined using Carl Zeiss confocal microscope LSM510. Both transfected $TRF2^{\Delta M}$ (a, rhodamine-labeled) and native Ku70 (b, FITC-labeled) are distributed in a diffuse reticular pattern with intermixed unstained areas. A merged image (c) shows that they are largely co-localized.

TRF2 would interfere with the association of native Ku70 and Ku80. HeLa cell extracts were incubated with excessive amounts of purified recombinant $TRF2^{\Delta M}$, and Ku70 was immunoprecipitated from the extracts using the monoclonal antibody NMB1 (Fig. 3a, lane 1: Coomassie blue-stained gel). For negative controls, the same immunoprecipitation without additional $TRF2^{\Delta M}$ (lane 2) and immunoprecipitation with normal mouse serum (lane 3) were included. As was shown with $TRF2^{B22}$, $TRF2^{\Delta M}$ also co-immunoprecipitated with Ku70 (Fig. 3b, lane 1). The failure to detect endogenous TRF by immunoblotting could be due to its relatively scant amount in the extract. When the same precipitates were immunoblotted with anti-Ku70 antibody NMB1, the amount of Ku70 was similar to that in the negative control without $TRF2^{\Delta M}$ (Fig. 3c, lanes 1 and 2). Ku80 was also detected similarly regardless of the addition of excessive $TRF2^{\Delta M}$ (Fig. 3d, lanes 1 and 2). Thus, it appeared that the presence of excessive TRF2 did not interfere with the association of Ku70 and Ku80 in vitro.

3.4. In vivo interaction and co-localization of Ku70 and transfected TRF2

To verify the interaction in vivo, we performed a series of co-immunoprecipitation experiments using COS cells which overexpressed $TRF2^{B22}$ fused to a T7·Tag. As was expected, Ku70 and Ku80 co-precipitated by either anti-Ku70 or anti-Ku80 antibodies (Fig. 4a,b, lanes 2 and 3). When $TRF2^{B22}$ was immunoprecipitated using anti-T7·Tag antibody, Ku70 was co-precipitated (Fig. 4a, lane 4) whereas Ku80 was not (Fig. 4b, lane 4). The results suggested that $TRF2^{B22}$ interacted with Ku70 but not with Ku80 directly in vivo.

To determine whether Ku70 and TRF2 co-localized in the nucleus, COS cells were transiently transfected with a mammalian expression plasmid encoding T7-tagged TRF2$^{\Delta M}$. Upon immunostaining with either polyclonal anti-TRF2 antibody or monoclonal anti-T7·Tag antibody, about 5% of cells displayed expression of transfected TRF2$^{\Delta M}$ in a diffuse, reticular pattern (Fig. 5a). Ku70 also displayed a similar immunofluorescence pattern (Fig. 5b) as was described previously [17]. The Ku70 staining pattern was similar between the cells with or without TRF2$^{\Delta M}$ expression. With double immunofluorescence staining, it was shown that the transfected TRF2$^{\Delta M}$ mostly co-localized with native Ku70 (Fig. 5c).

Our results suggest that Ku and TRF2, which are both essential for the maintenance of telomeres [9–12,24], form a functional complex in vivo. Ku has been shown to play a crucial role in DNA double-strand-break repair for any particular nucleotide sequence or end configuration [6–8]. Since telomeres contain DNA double-strand breaks essentially, the 'non-specific' repair function of Ku would have to be controlled in a way that end-to-end fusion of chromosomes is prevented. Considering that Ku interacts with other proteins such as Sir at the telomere [18], the Ku function at the telomere appears to be regulated in a complicated network of protein-to-protein interactions in which TRF2 would certainly be a key factor.

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