

## TRiC-P5, a novel TCP1-related protein, is localized in the cytoplasm and in the nuclear matrix

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### SUMMARY

We have recently reported the cloning of a novel protein, TRiC-P5, with significant homology with protein 1 of the t-complex (TCP1). In the present study, the cellular localization of TRiC-P5 in Raji cells has been determined using an antiserum raised against a 18.5 kDa fusion protein. Results from cell fractionation and immunoblot studies indicate that TRiC-P5 is mainly localized in the cytoplasm. In addition, a significant part of TRiC-P5 is also found in the nucleus where it is attached to the nuclear matrix, a complex filament network involved in essential cellular

functions such as DNA replication, and RNA transcription and maturation. Immunofluorescence experiments using the anti-TRiC-P5 antibodies confirm these results. We also provide evidence that, in the cytoplasm, TRiC-P5 is part of a large protein complex, most probably the TCP1-ring complex (TRiC), a hetero-oligomeric ring complex that plays a role of molecular chaperone in the folding of actin and tubulin.

Key words: nuclear matrix, nucleus, chaperone, TCP1, TRiC-P5

### INTRODUCTION

Although many essential roles have been postulated for the nuclear matrix, few proteins involved in these processes have been characterized so far. To identify such proteins, we isolated messenger RNAs coding for nuclear matrix proteins, by immunoprecipitation of Raji cell polysomes with a serum raised against nuclear matrix proteins (Laliberté et al., 1984). A nuclear matrix cDNA library was constructed with these immunoselected mRNAs (Shapiro and Young, 1981; Kraus and Rosenberg, 1982). Using this library, we reported the isolation of the cDNA for the nuclear matrix protein mitotin (Todorov et al., 1991). Recently, we investigated another cDNA clone, called 5C8, from this human library. The full-length cDNA clone was isolated from a lambda zap II mouse MEL cDNA library (Joly et al., 1994). To our surprise, no significant homology with known nuclear matrix proteins like lamins or matrisins was found in the Genbank or EMBL databases. However, there was a significant nucleotide homology (48%) with mouse t-complex protein 1b (TCP1b; Willison et al., 1986). Many reports have identified TCP1 as a subunit of a hetero-oligomeric ring complex that plays the role of molecular chaperone in the folding of tubulin and actin (Lewis et al., 1992; Yaffe et al., 1992; Horwich and Willison, 1993). Frydman et al. (1992) and Rommelaere et al. (1993) also found that the other members of this ring complex are structurally related to TCP1. The deduced amino acid sequence of our cDNA presented a nearly perfect match with tryptic

peptides from the P5 subunit of bovine TCP1-ring complex described by Frydman et al. (1992). For this reason we called this novel protein mTRiC-P5, for mouse TCP1-ring complex protein no. 5 (Joly et al., 1994).

Molecular chaperones are proteins that assist folding and oligomeric assembly of newly synthesized proteins. They recognize and bind unfolded polypeptides to prevent premature folding and aggregation (Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Horwich and Willison, 1993). Electron microscopy has shown that molecular chaperones of many species ranging from bacteria to man are organized in a large oligomeric ring complex (Trent et al., 1991; Lewis et al., 1992; Frydman et al., 1992; Phipps et al., 1993; Mummert et al., 1993). In *Escherichia coli* and in the thermophilic archaeobacterium *Sulfolobus shibatae*, this ring complex is composed of GroEL and the thermophilic factor 55, respectively (Trent et al., 1991; Langer et al., 1992). Recently, many laboratories have characterized eukaryotic cytosolic molecular chaperones (Lewis et al., 1992; Yaffe et al., 1992; Gao et al., 1992; Frydman et al., 1992). These cytosolic chaperones are also organized in a double-ring complex but seem to have a hetero-oligomeric structure composed of proteins of molecular mass ranging from 52 to 65 kDa.

Nuclear molecular chaperones are not well characterized, except for nucleoplasmin, a protein that binds histones and transfers them to DNA. Since the cDNA clone of TRiC-P5 was originally isolated from a nuclear matrix protein-enriched cDNA library, we decided to investigate more closely the

cellular localization of TRiC-P5 in the Raji cell line, from which the cDNA library was derived. Our results indicate that in this cell line the protein is found in both the cytoplasm and the nuclear matrix.

## MATERIALS AND METHODS

### Cell culture

Burkitt lymphoma Raji cells were propagated in suspension and cultured in RPMI-1640 (Bethesda Research Laboratories, Bethesda, MD) supplemented with 10% fetal calf serum (Flow Laboratories Inc., McLean, Virginia) and 0.1% gentamycin (Bethesda Research Laboratories, Bethesda, MD). For labeling, the cells were either incubated overnight with 10  $\mu$ Ci/ml of [<sup>35</sup>S]methionine (sp. act. 1,000 Ci/mmol; Amersham Canada Ltd, Oakville, Ontario, Canada), or labeled for short periods with 2 mCi/ml of [<sup>35</sup>S]methionine in methionine minus RPMI buffer, followed by chase in normal medium containing 0.3 mM methionine with or without 50  $\mu$ g/ml cycloheximide.

### Sucrose-gradient centrifugation

Raji cells ( $1 \times 10^8$ ) in log phase were washed 2 times with phosphate buffered saline (PBS), and incubated at 0°C for 5 minutes in Harm's buffer (Lewis et al., 1992) with 0.5% NP-40 and protease inhibitors (1 mM PMSF, 1 mM benzamidine, 25  $\mu$ g/ml aprotinin, 25  $\mu$ g/ml leupeptin). Cells were homogenized by vortexing for 1 minute and then centrifuged at 3,000 *g* for 10 minutes at 4°C. The supernatant was layered onto a prechilled sucrose gradient (10% to 40%, w/w) and centrifuged as described by Lewis et al. (1992). The gradient was fractionated into 16 fractions (of 1 ml) from the bottom. The protein concentration of each fraction was determined by the Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada). Western blot analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) of each fraction were done as described below.

### RNA preparation

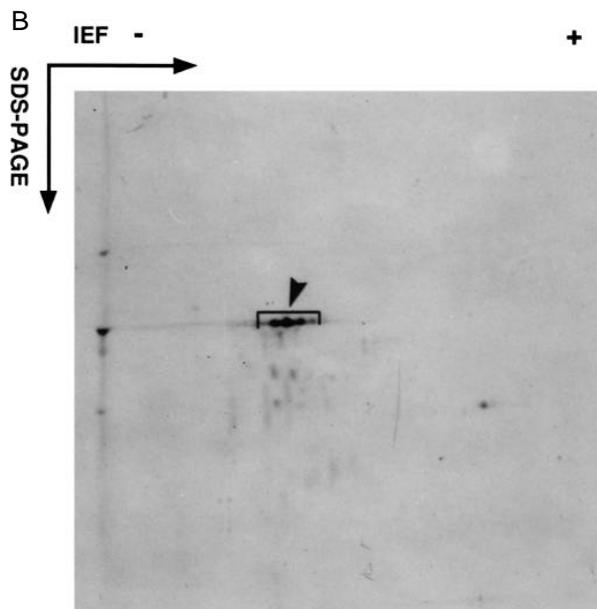
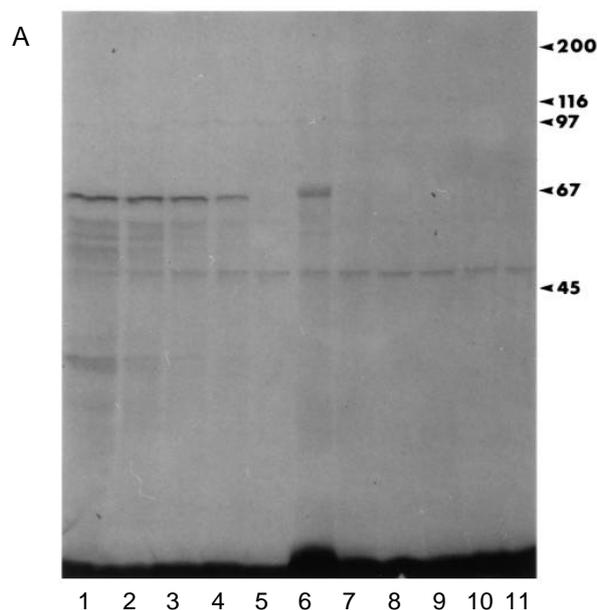
Total RNA was isolated using the guanidium-isothiocyanate-cesium chloride centrifugation procedure (Chirgwin et al., 1979). About  $1 \times 10^8$  cells were lysed in 10 ml of 4 M guanidine-isothiocyanate, 25 mM sodium citrate, pH 7.0, 100 mM  $\beta$ -mercaptoethanol buffer by vortexing for 2 minutes and by forcing the cells 5 times through a 20

gauge needle. RNA was pelleted by centrifugation through a 5.7 M CsCl, 100 mM EDTA (pH 7.0) cushion using a SW41 rotor (Beckman Instruments, Fullerton, CA) at 125,000 *g* for 18 hours at 20°C. Poly(A)<sup>+</sup> RNAs were purified with the poly(A-T)-tract system (Promega corporation, Madison, WI).

For synthesis of in vitro transcribed RNA, complete mouse cDNA (Joly et al., 1994) was inserted in the pBluescript II SK<sup>-</sup> vector (Stratagene, CA). Vector was linearized with the appropriate restriction enzymes. Sense or anti-sense RNAs were transcribed with T3 or T7 RNA polymerase according to the manufacturer's directions.

### Hybrid selection

Hybrid-selection experiments were done as previously described (Parnes et al., 1981; Studier et al., 1990; Todorov et al., 1991). Plasmid DNA was isolated from clone 5C8 and fixed on nitrocellulose filters. The filters were hybridized with 200  $\mu$ g/ml of Raji cell poly(A)<sup>+</sup> RNA isolated in a solution containing 65% formamide, 20



**Fig. 1.** (A) In vitro transcription and in vitro translation of the mouse TRiC-P5. Sense and anti-sense RNAs were generated from the pBluescript II sk<sup>-</sup> plasmid containing the complete mouse cDNA clone (Joly et al., 1994) by in vitro transcription with the T7 RNA polymerase, or with the T3 RNA polymerase. The RNAs generated were used in in vitro translation experiments. The in vitro translated products were analyzed by SDS-PAGE and fluorography. Molecular mass markers are indicated (in kDa). Lanes 1-4, sense RNA: 5  $\mu$ g, 2.5  $\mu$ g, 1  $\mu$ g, 0.5  $\mu$ g. Lane 5, control without RNA. Lane 6, control with globin. Lanes 7-10, anti-sense RNA: 5  $\mu$ g, 2.5  $\mu$ g, 1  $\mu$ g, 0.5  $\mu$ g. Lane 11, control without RNA. We can see in lanes 1 to 4 that a band migrating at a relative molecular mass of 63 kDa is generated by the sense RNA. (B) Hybrid selection and in vitro translation of the human TRiC-P5. Two-dimensional gel migration of the in vitro translated product of mRNA hybrid-selected by clone 5C8. Nitrocellulose filters containing clone 5C8 were hybridized with poly(A)<sup>+</sup> RNA isolated from Raji cells. The RNAs that hybridized with the insert of clone 5C8 were eluted from the filters and used for in vitro translation experiments. The arrowhead shows the in vitro translated product analyzed by two-dimensional gel electrophoresis and fluorography. Molecular mass and pI for the in vitro translated products were determined by comigration with known proteins. The protein migrates as isovariants with isoelectric points between 6.6 and 7.0 and a relative molecular mass of 63 kDa.

mM PIPES, pH 6.5, 0.4 M NaCl, 0.2% SDS and 100 µg/ml yeast tRNA. Hybridization was performed at 50°C for 5 hours. Filters were washed at 65°C, first with 10 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 1 mM EDTA, 0.5% SDS, followed by 0.2× SSC, 0.2% SDS and finally 0.2× SSC. The hybridized RNAs were eluted by boiling the filters in water containing 100 µg/ml tRNA and were precipitated with ethanol.

### In vitro translation

Protein synthesis was carried out in the presence of 1 mCi/ml of [<sup>35</sup>S]methionine (Amersham Canada Ltd, Oakville, Ontario, Canada), using the rabbit reticulocyte lysate kit (Bethesda Research Laboratories, Bethesda, MD) and hybrid selected or in vitro transcribed RNAs. In vitro translation products were analyzed by migration in two-dimensional gel electrophoresis (see below) and fluorography. Molecular mass and pI for the in vitro translated products were determined by comigration with known proteins.

### Serum preparation

The 801 bp insert of the human 5C8 cDNA (see Fig. 1) was cloned into Studier's vector (Studier et al., 1990), resulting in a fusion of 5C8 coding sequences in appropriate frame and orientation. Transformed bacteria were induced with IPTG, to overexpress the fusion protein corresponding to the C terminus of the human TRiC-P5. Bacterial proteins were extracted. Total proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. A polypeptide of the expected molecular mass (18.5 kDa) was observed. This 18.5 kDa peptide was cut, electro-eluted from the gel and injected into rabbits to produce antibodies. The serum was collected after the second boost. Antibodies were immunoaffinity purified using the 18.5 kDa peptide fixed on nitrocellulose. The antibodies were eluted with 0.1 M glycine-HCl, pH 2.6, quickly neutralized with 1 M K<sub>2</sub>HPO<sub>4</sub>, and stored frozen at -80°C.

### Cellular fractionation

Unless described, all procedures were performed on ice. Cells were harvested by centrifugation and cell pellets were washed twice with PBS. Raji cells were incubated for 10 minutes on ice in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 3 mM CaCl<sub>2</sub>, 10 mM KCl, 1 mM PMSF (Mellon and Bhorjee, 1982). Cytoplasmic and nuclear fractions were separated by mechanical homogenization with a Dounce homogenizer and centrifugation at 400 g for 10 minutes. The supernatant was centrifuged at 15,000 g for 30 minutes to remove insoluble proteins from the cytoplasmic fraction, and soluble proteins were precipitated by addition of three volumes of ethanol. To prepare nuclear matrices, the isolated nuclei were washed twice with the isolation buffer and digested for 60 minutes with 250 µg/ml of DNase I (Boehringer Mannheim Canada, Laval, PQ, Canada). After a 20 minute centrifugation at 400 g, the nuclei were resuspended in 10 mM Tris-HCl, pH 7.4, 0.2 mM MgCl<sub>2</sub>, 1 mM PMSF buffer, and 4 M NaCl, in the same buffer, was added drop by drop to a final NaCl concentration of 1.8 M. Finally, 1% Triton X-100 was added to the suspension to remove the nuclear membranes. The resulting nuclear matrix fraction was collected by a 10,000 g centrifugation and washed twice in PBS. After SDS-PAGE, proteins were either detected by staining with Coomassie Blue or transferred onto nitrocellulose membranes (0.45 µm, Schleicher and Schuell) at 40 V during 16 hours at 4°C.

### Protein electrophoresis and transfer

Proteins were separated on 8% SDS-PAGE (Laemmli, 1970) or by two-dimensional gel electrophoresis (O'Farrell, 1975; Dagenais et al., 1984). After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue R-250 or transferred onto nitrocellulose membranes (Schleicher and Schuell) with the transblot apparatus (Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada) for immunodetection as described by the manufacturer.

### Immunodetection

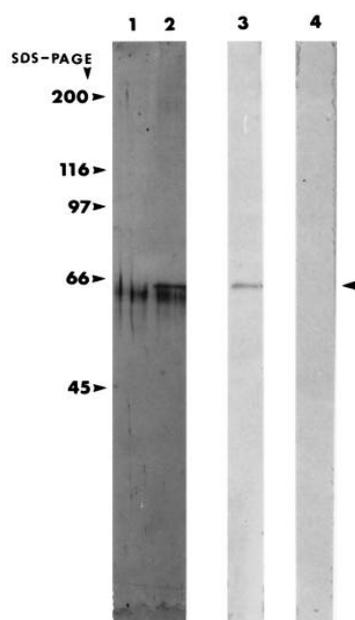
After transfer, the membranes were first stained with Ponceau Red (Carbajal et al., 1986) for protein or molecular mass identification. For immunodetection, membranes were incubated for 1 hour at room temperature in PBS containing 0.5% casein (BDH Inc., St-Laurent, Québec, Canada) to block non-specific sites, and washed 6 times with PBS alone. The membranes were successively incubated and washed as before for 2 hours with anti-TRiC-P5 antiserum (diluted 1:1000 in PBS, casein buffer) or immunoaffinity-purified antiserum (1:100), and for 2 hours with anti-rabbit IgG (diluted 1:2000) linked to peroxidase (Bethesda Research Laboratories, Bethesda, MD).

To detect intermediate filament proteins, a mouse monoclonal antibody was used at 1:250 (Pruss et al., 1981) and was detected by an anti-mouse IgG/IgM (diluted 1:500) linked to peroxidase (Jackson Laboratories). The complexes were revealed with chloronaphthol 0.03% (ICN Biomedicals Ltd, St-Laurent, Québec, Canada) in PBS containing 0.15% peroxide (BDH Inc., St-Laurent, Québec, Canada).

### Immunoprecipitation

Translation products were incubated overnight at 4°C with the appropriate antiserum, followed by addition of a suspension of IgGSorb (The Enzyme Center Inc.), and further incubations for 1 hour at room temperature, and 1 hour at 4°C. The IgGSorb was then washed several times with lysis buffer (20 mM KPO<sub>4</sub>, pH 7.5, 0.1 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 20 mM methionine, 1 mM PMSF, 1 mM benzamidine, 25 µg/ml leupeptin, 25 µg/ml aprotinin), containing 0.5 M NaCl, followed by lysis buffer alone and, finally, by TBS buffer containing 1 mM PMSF. The bound proteins were eluted by boiling the IgGSorb in SDS-PAGE sample buffer.

For Raji cells, 5.3×10<sup>6</sup> cells were washed in PBS and lysed in lysis



**Fig. 2.** Specificity of the anti-TRiC-P5 serum generated using a recombinant protein. Raji cell proteins (50 µg) were separated by SDS-PAGE (lanes 2,3,4) and submitted to western blot analysis. Lane 1 contains no protein. Separated tracks were incubated with either the total serum (lanes 1 and 2), the antibodies immunopurified on the 18.5 kDa fusion peptide (lane 3), or the antibodies immunopurified on albumin (lane 4), as primary antibodies. The serum detects a single band at 63 kDa (lane 2 and 3), although there is some non-specific labeling at 60 kDa with the total serum (lane 1). The position of TRiC-P5 is indicated by an arrowhead. Molecular mass standard positions are indicated (in kDa).

buffer. Samples were sonicated 3 times for 30 seconds and kept under agitation for 1 hour at 4°C. After ultracentrifugation for 1 hour at 40,000 *g*, 700 µl of supernatant was incubated with 10 µl of preimmune serum during 16 hours at 4°C. After addition of 150 µl of 20% IgGSorb, the incubation was continued for 1 hour at room temperature. IgGSorb was removed by a 20 minute centrifugation at 16,000 *g*. Each supernatant was incubated overnight at 4°C with the specific anti-TRiC-P5 antiserum (1:100), followed by addition of 50 µl of the IgGSorb suspension, and further incubation for 1 hour at room temperature. The IgGSorb was then washed 3 times with lysis buffer, and 3 times with PBS containing 1 mM PMSF. The bound proteins were eluted by boiling the IgGSorb in SDS-PAGE sample buffer.

### Immunofluorescence microscopy

Coverslips were coated with 0.01% poly-L-lysine (Sigma Chem. Co.). Whole cells or subcellular fractions were deposited on the coverslips and fixed twice in -20°C methanol for 5 minutes. The coverslips were washed 2 times with PBS. To permeabilize the cells, the specimens were incubated for 4 minutes with 0.4% Triton X-100 in PBS. They

were pretreated for 30 minutes in PBS containing 0.1% casein and 0.1% Tween-20. The specimens were reacted for 1 hour at 37°C with the appropriate antiserum diluted in PBS/casein/Tween-20 and extensively washed. Subsequently, the cells were incubated with an anti-rabbit IgG linked to FITC (Boehringer Mannheim Canada, Laval, PQ, Canada), or to anti-mouse IgG linked to Texas Red in PBS. The coverslips were extensively washed with PBS and mounted in a solution of 90% glycerol, 10 mM Tris-HCl, pH 8.6. Preparations were examined with a fluorescence microscope.

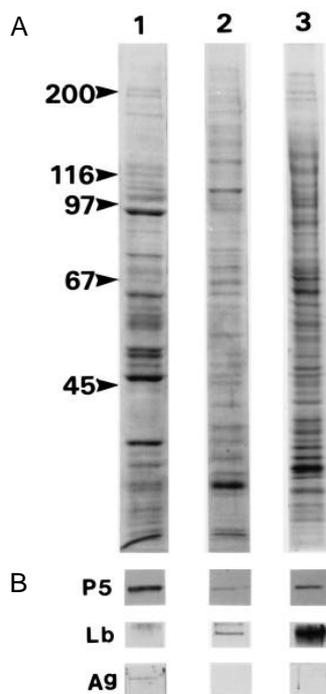
The following antibodies were used in our experiments: the anti-TRiC-P5 antiserum at 1:500 dilution, an antiserum against BHK-21 nuclear matrix proteins at 1:500 dilution (Laliberté et al., 1984), and an anti-lamin B antibody at 1:100 dilution (from Dr Yves Raymond).

## RESULTS

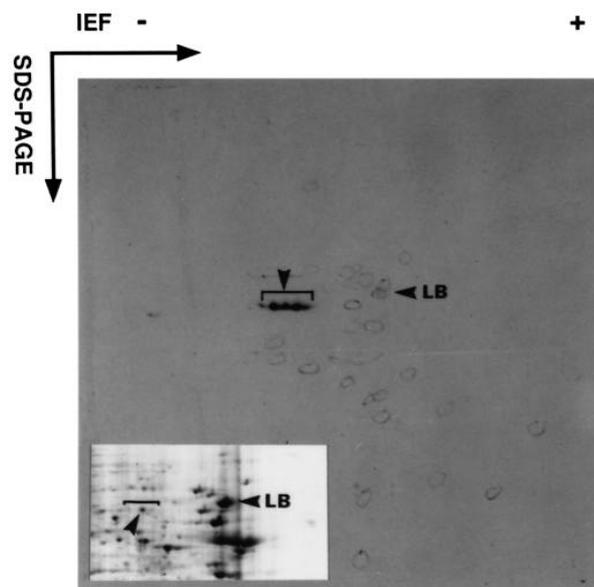
### Protein characterization

To identify novel nuclear matrix proteins, we isolated messenger RNAs coding for nuclear matrix proteins by immunoprecipitation of polysomes with a serum against nuclear matrix proteins (Laliberté et al., 1984). Clone 5C8 from this library has a insert of 801 bp. Mouse cDNA for mTRiC-P5 was isolated using this 5C8 insert (Joly et al., 1994). The 801 bp 5C8 fragment was sequenced, and the deduced amino acid sequence was compared with that of mTRiC-P5. An overall 97% amino acid identity was found between the partial human clone (from Raji cells) and the mouse clone (from MEL cells).

A single protein migrating at 63 kDa on SDS-PAGE is generated by *in vitro* transcription/translation experiments using murine TRiC-P5 2-kb cDNA (Fig. 1A, lanes 1-4). An *in vitro* translated product of the same molecular mass is obtained



**Fig. 3.** Subcellular localization of the TRiC-P5 protein by western blot analysis. Exponentially growing Raji cells were fractionated into cytoplasmic (lane 1) and nuclear (lane 2) fractions. The nuclear fraction was further subfractionated to separate a nuclear matrix fraction (lane 3); 70% of the total proteins were found in the cytoplasm and 30% in the nucleus. Nuclear matrix proteins represent 2.5% of the nuclear proteins. Samples containing 50 µg of proteins from each fraction were separated by SDS-PAGE. (A) Proteins were stained by Coomassie Blue. Molecular mass standard positions are indicated (in kDa). (B) TRiC-P5 at 63 kDa was detected by immunoblotting with the immunoaffinity purified antiserum (P5). TRiC-P5 can be detected in all three fractions. Lamin B was detected at 68 kDa with the intermediate filament protein monoclonal antibody (Lb). Lamin B was detected only in the nucleus and the nuclear matrix. Neurofilament protein was detected at 200 kDa with the intermediate filament protein monoclonal antibody (Ag). The neurofilament protein was detected only in the cytoplasm.



**Fig. 4.** Two-dimensional gel analysis of TRiC-P5 in nuclear matrix. Raji cell nuclear matrix proteins (60 µg) were separated on two-dimensional gels and analyzed by western blot with the immunoaffinity purified anti-TRiC-P5 antibodies as primary antibodies. TRiC-P5 and lamin B (LB) positions are indicated by arrowheads. Proteins detected by Ponceau Red staining are lightly circled. Inset: Coomassie Blue staining.

with Raji cell mRNAs hybrid-selected by the 5C8 insert (Fig. 1B). The comigration of murine and human *in vitro* translated products indicates that the protein TRiC-P5 is conserved between both species. The *in vitro* translated product migrates as isoforms with pI values in the range 6.6-7.0 on two-dimensional gels (Fig. 1B).

A polyclonal antiserum was generated in a rabbit injected with a 18.5 kDa fusion protein produced by inserting the 801 bp 5C8 fragment in Studier's vector (Studier et al., 1990). In immunoblotting, the antiserum recognized a polypeptide migrating at 63 kDa that was not recognized by the preimmune serum, although there was some labeling at 60 kDa (Fig. 2, lanes 1, 2). To enhance the specificity, the antiserum was immunoaffinity-purified by binding to the fusion protein fixed onto nitrocellulose followed by elution from the membrane. The immunoaffinity-purified antiserum recognized a single 63 kDa protein in immunoblotting analysis (Fig. 2, lane 3).

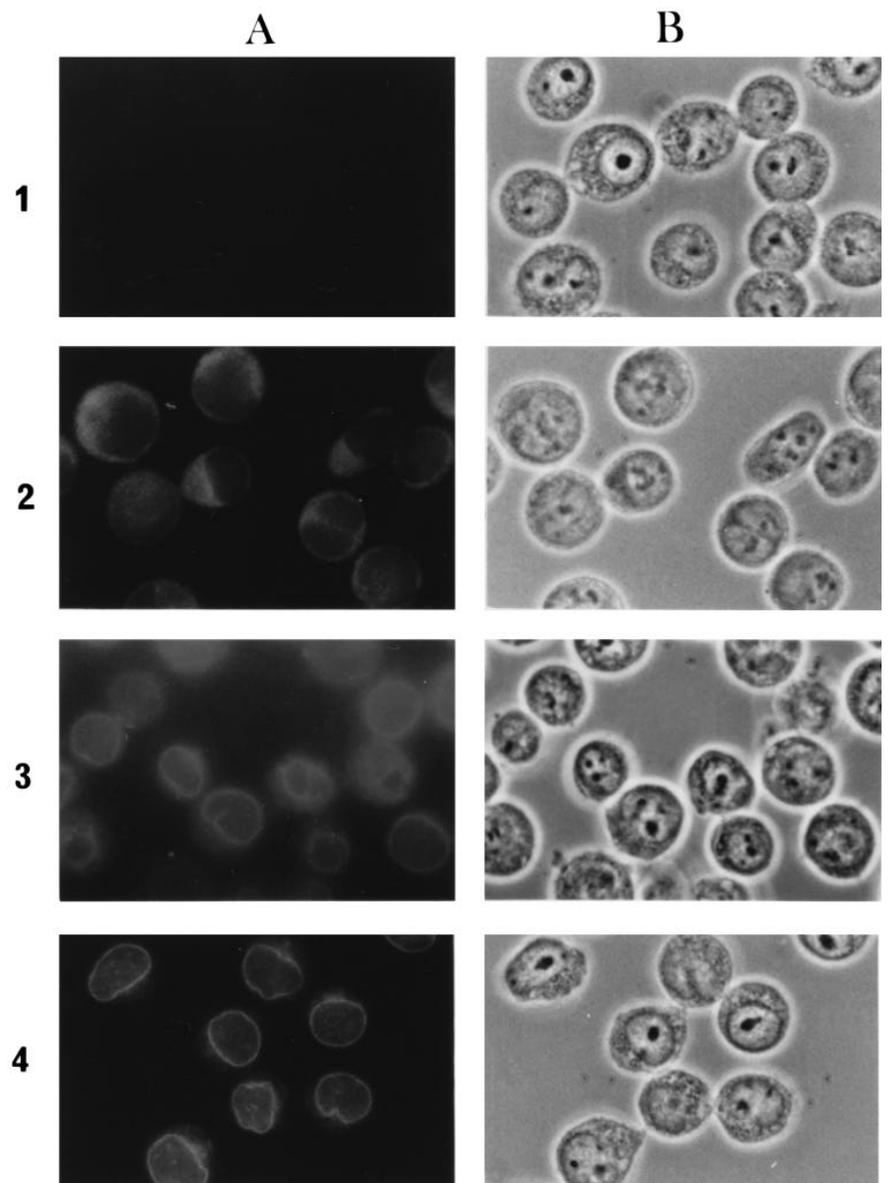
Both, the *in vivo* synthesized protein and the *in vitro* translated product migrate at an apparent molecular mass of 63 kDa

(Figs 1, 2). This indicates that TRiC-P5 *in vivo* does not undergo post-translational processing resulting in a change in mobility detectable by SDS-PAGE. This conclusion was further supported by results from pulse-chase experiments. The protein TRiC-P5 was pulse-labeled with [<sup>35</sup>S]methionine in exponentially growing Raji cells, and after various chase times, the protein was immunoprecipitated with total antiserum. The mobility of the 63 kDa labeled protein stays the same on SDS-PAGE from 0 to 24 hours post-synthesis (results not shown). No difference was observed in the relative abundance of TRiC-P5 during the different chase times.

The half-life of TRiC-P5 is greater than 24 hours, as determined by similar pulse-chase experiments in the presence of protein synthesis inhibitors (data not shown).

### Subcellular localization of the TRiC-P5 protein

To determine the subcellular localization of the TRiC-P5 protein, exponentially growing Raji cells were fractionated into cytoplasmic and nuclear fractions. Isolated nuclei were further

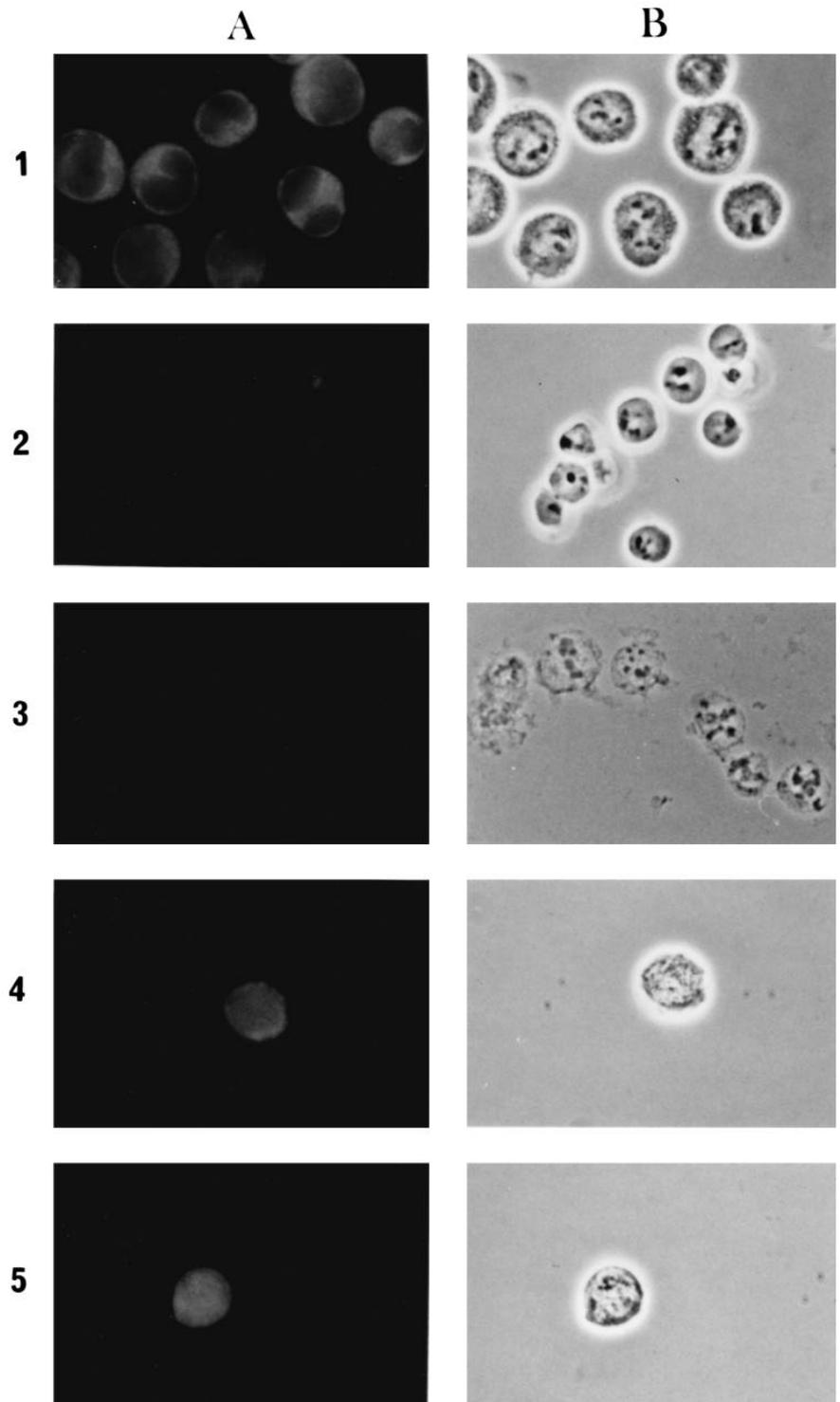


**Fig. 5.** Cellular localization by immunofluorescence on Raji cells. Raji cells were deposited on coverslips and analyzed by various serums. In (A) we can see the immunofluorescence result; in (B) the phase-contrast. (1) Preimmune serum; (2) anti-TRiC-P5 antiserum; (3) anti-lamin B antiserum; (4) anti-BHK nuclear matrix antiserum.  $\times 1,000$ .

extracted with non-ionic detergent, low-salt buffer, DNase I and high-salt buffer to generate a nuclear matrix fraction. The fractions obtained were assayed for the presence of TRiC-P5 protein by SDS-PAGE and immunoblotting. The bulk of TRiC-P5 protein (>90%) was found in the cytoplasm (Fig. 3B, lane 1). A significant amount of protein (<10%) was also found in the nucleus (Fig. 3B, lane 2). Inside the nucleus, TRiC-P5 is a component of the nuclear matrix, since it was not extracted

by sequential treatments of the nuclei with detergent, nucleases or high-salt buffers (Fig. 3B, lane 3).

To verify the possibility of cross-contamination of the sub-cellular fractions, intermediate filament proteins were detected by a monoclonal antibody that recognized an epitope shared by all intermediate filament-type proteins (Pruss et al., 1981). As expected, lamin B was detected only in the nucleus and in the nuclear matrix (Fig. 3B, lanes 2, 3). A 200 kDa neurofila-



**Fig. 6.** Immunofluorescence of Raji cell fractions with the anti-TRiC-P5 antiserum. Raji cells were fractionated and each fraction was deposited on coverslips and analyzed with the anti-TRiC-P5 antiserum. In (A) we can see the immunofluorescence result; in (B) the phase-contrast. (1) Raji cells; (2) isolated nuclei; (3) nuclei after DNase I digestion; (4) nuclei after DNase digestion and high-salt treatment; (5) Nuclear matrices obtained after extraction with the non-ionic detergent Tween-80.  $\times 1,000$ .

ment protein described by Murphy et al. (1993) in human lymphocytes was found only in the cytoplasmic fraction (Fig. 3B, lane 1).

To confirm the association of TRiC-P5 with the Raji cell nuclear matrix, nuclear matrices were extracted and proteins were separated by two-dimensional gel electrophoresis. Nuclear matrix proteins were detected by Coomassie Blue staining. A single protein was recognized by immunoblotting with the immunoaffinity-purified antiserum (Fig. 4). This protein migrated as isovariants with pI values in the range 6.6-7.0, exactly like the *in vitro* translated product obtained after hybrid selection (compare Figs 1B, 4), indicating that it is the same protein.

### Indirect immunofluorescence microscopy in interphase cells

For immunofluorescence experiments, Raji cells were sedimented on poly-L-lysine-coated coverslips, fixed, permeabilized, stained with preimmune or immune sera, and visualized with FITC- or Texas Red-labeled secondary antibodies. With the anti-TRiC-P5 antiserum, fluorescence was localized exclusively in the cytoplasm, no staining was observed into the

nucleus (Figs 5.2, 6.1). The immunofluorescence pattern in the cytoplasm was not uniform, and cap-like structures were observed in many cells. No detectable staining was seen with the preimmune serum (Fig. 5.1).

The periphery of the nucleus was labeled by a serum against lamin B. The staining revealed that Raji cell nuclei often have a kidney-like shape and are generally placed in an acentric position within the cell (Fig. 5.3). The anti-nuclear matrix antiserum, used to prepare the cDNA library from which the 5C8 cDNA clone was isolated, gave rise to a bright staining of the nuclear periphery and a punctate staining of intra-nuclear components (Fig. 5.4).

Immunofluorescence with the anti-TRiC-P5 antiserum was performed on isolated nuclei or isolated nuclei extracted with non-ionic detergent, low-salt buffer, DNase I and high-salt buffer to generate nuclear matrices. The antiserum did not stain the nuclei of unextracted cells (Fig. 6.1). No fluorescence staining was observed in isolated nuclei (Fig. 6.2) or in isolated nuclei extracted with DNase I (Fig. 6.3). These nuclei were uniformly stained after further extraction with high-salt buffer (Fig. 6.4). This procedure removed essentially all the chromatin, leaving the nuclear matrix and the nuclear envelope. The pattern and intensity of this fluorescence staining was not altered after removal of the nuclear membranes with non-ionic detergent, and staining was clearly visible in the isolated nuclear matrices (Fig. 6.5).

### Association of TRiC-P5 with a large protein complex

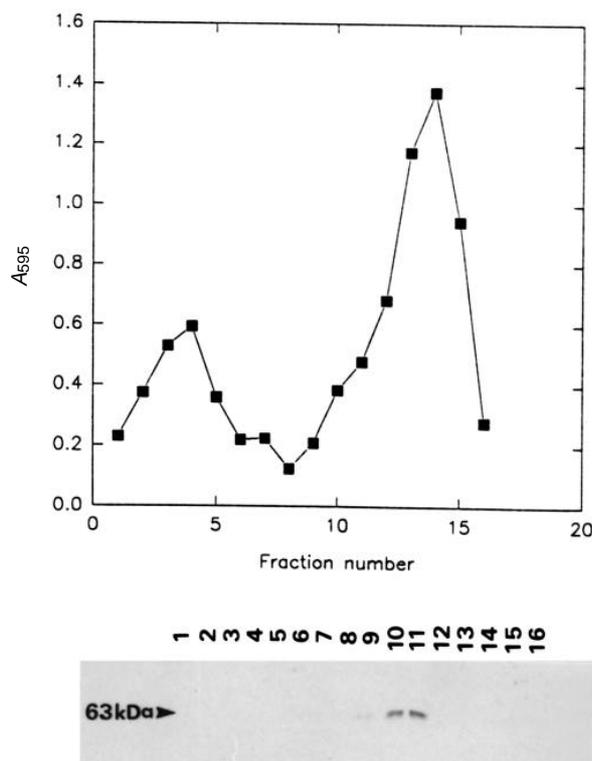
Raji cell homogenates were centrifuged on sucrose gradients. Gradient fractions were analyzed for their protein content and protein samples taken from the fractions were separated by SDS-PAGE. The presence of TRiC-P5 protein in the gradient fractions was revealed by immunoblotting. The sedimentation value of TRiC-P5 was evaluated at 18.1-19.5% sucrose, which corresponds to a molecular mass of approximately 800 kDa (Fig. 7).

### DISCUSSION

We have recently cloned a mouse cDNA encoding a novel protein with homology to TCP1, a cytoplasmic protein expressed mainly in the testis. This novel protein is conserved in mouse and human, since the human protein synthesized by *in vitro* translation of hybrid-selected Raji cell mRNAs has the same molecular mass as the mouse protein synthesized by *in vitro* translation and *in vitro* transcription. The molecular mass can be estimated at 63 kDa, which is similar to the theoretical value of 60.5 kDa deduced from the amino acid sequence of the mouse cDNA (Joly et al., 1994).

To collect more information on the protein, we have generated a polyclonal rabbit antiserum against the carboxy-terminal segment of the human TRiC-P5 protein. In western blotting and immunoprecipitation assays, the antiserum specifically recognized a 63 kDa protein in Raji cells, which is stable and does not seem to be modified by any apparent post-translational modification.

On two-dimensional gels, the human TRiC-P5 migrates as isovariants between pI 6.6 and 7.0, whether the protein was synthesized *in vitro* or *in vivo*. The presence of these isovari-



**Fig. 7.** Sucrose density centrifugation analysis of the TRiC-P5 complex. A homogenate of Raji cells was layered onto a sucrose gradient and centrifuged. Sixteen fractions of 1 ml were recovered from the bottom and the proteins were analyzed by SDS-PAGE. The graph represents protein content of each fraction. Analysis of TRiC-P5 in each fraction was done by western blot using the immunoaffinity-purified anti-TRiC-P5 antibodies. A 63 kDa protein is detected mainly in fractions 10 and 11 corresponding to 18.1 and 19.5% sucrose, respectively. This position corresponds to a relative molecular mass of 800 kDa when compared with known proteins run in parallel gradients.

ants could be related to phosphorylation, since the amino acid sequence contains potential sites for phosphorylation; however, preliminary results indicate that the protein is not phosphorylated.

Two approaches: cellular fractionation followed by immunoblotting, and indirect immunofluorescence microscopy, were used for determining the subcellular localization of TRiC-P5. Both approaches clearly indicate that the large majority of the TRiC-P5 protein is localized in the cytoplasm. In this subcellular compartment, the TRiC-P5 protein is part of a large protein complex. This result is in accordance with the cellular localization reported for TCP1. A disparity between the estimated molecular mass of the observed complex for TRiC-P5 (800 kDa) and that reported for the TCP1-ring complex (970 kDa) is probably simply due to the fact that the molecular mass has been estimated by two different techniques.

Not all of the TRiC-P5 is present in the cytoplasm. Immunoblotting indicates that TRiC-P5 is also found in the nucleus, where it is attached to the nuclear matrix. Immunofluorescence on the other hand fails to detect any nuclear staining but the epitopes recognized by the antiserum are revealed after removal of chromatin. These results and the fact that we used a nuclear matrix antiserum to construct the human library confirm that the TRiC-P5 protein is present in the nuclear matrix. TRiC-P5 was found also in the nuclear matrix of other cell types (data not shown), indicating that the situation described above is not a characteristic unique to Raji cells. We do not know for the present how TRiC-P5 is organized in the nuclear matrix. More experimental data will be needed to determine whether, in the nuclear matrix, TRiC-P5 is also part of a complex or is in a monomeric form. It is interesting to note that a potential nuclear localization signal, RRVRK, similar to the one described for the nuclear protein NuMa (Yang et al., 1992), and like the nuclear localization signal consensus K-R/K-X-R/K (X is any amino acid; Chelsky et al., 1990), is present in the mouse amino acid sequence (position 312-316; Joly et al., 1994). This sequence is also found in the TCP1 protein, which may be found in the nucleus (Horwich and Willison, 1993).

In the cytoplasm, TCP1 and probably TRiC-P5 are part of a hetero-oligomeric ring complex that plays the role of a molecular chaperone in the folding of tubulin and actin (Lewis et al., 1992; Yaffe et al., 1992; Horwich and Willison, 1993). Actin is distributed not only in the cytoplasm but also in the nucleus where filamentous actin (F-actin) has been shown to be part of the nuclear matrix. The function of nuclear actin is still controversial, it may play a role in transcription and/or in processing of nascent RNPs. By analogy with its potential role in the cytoplasm, nuclear TRiC-P5 may act as a molecular chaperone in the proper folding of nuclear actin and/or other nuclear matrix proteins. We are currently investigating the possibility of interaction between TRiC-P5 and nuclear matrix proteins.

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