RFP is a DNA binding protein associated with the nuclear matrix

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ABSTRACT

We reported that the *RFP* gene encodes a protein with putative zinc finger domains and was involved in the activation of the *ret* proto-oncogene. To further characterize the RFP protein, we developed a polyclonal antibody against the product synthesized from a fragment of the *RFP* cDNA expressed in *Escherichia coli*. Western blot analysis showed that RFP was identified as a 58 kDa protein in cell lysates from four human and rodent cell lines and from mouse testis. In addition, a unique 68 kDa protein was detected in the testis. Using AH7974 (rat ascites hepatoma) and Raji (human Burkitt lymphoma) cells, we demonstrated strong association of RFP with the nuclear matrix. Furthermore, RFP solubilized from the nuclear matrix had DNA-binding activity although it appears to bind more preferentially to double-stranded DNA than to single-stranded DNA. These results thus suggest that RFP may play a role in molecular processes which occur in the nuclear matrix.

INTRODUCTION

RFP was identified as a gene involved in the generation of the *ret* transforming gene activated by DNA rearrangement (1, 2, 3). The amino acid sequence of RFP protein indicated that it contains a new cysteine-rich motif (4) similar to the well known zinc finger motif identified in a variety of eukaryotic regulatory proteins such as *Xenopus* 5s gene transcription factor (TFIIIA) (5) and human SP1 (6). This new motif was represented as C-X₂-C-X₁₁₋₂₇-C-X-H-X₂-C-X₂-C-X₇₋₄₉-C-P-X-C (designated C₃HC₄ motif) and found in at least 17 other proteins. They include the human V(D)J recombination-activating gene, *RAG-1* (7), the human interleukin 2 receptor regulatory gene, *RPT-1* (8), the human autoantigen, SS-A/Ro (9) and the *PML/Myl* gene found fused to the retinoic acid receptor gene in acute promyelocytic leukemia (10, 11). In addition, a second cysteine-histidine rich motif (C-X₂-H-X₇-C-X₇-C-X₂-C-X₅-H-X₂-H) was present in

RFP (3). Similar sequence was also found in RPT-1, SS-A/Ro, *PML/Myl* and two other C₃HC₄motif-containing genes, *Xenopus* nuclear factor 7 (XNF7) (12, 13) and T18 oncogene (14). The conservation of these motifs suggests that many of these proteins could bind zinc/divalent metal ions and nucleic acids. Alternatively, they may be involved in specific protein-protein interaction (4, 15).

We reported that the *RFP* gene was expressed at high levels in a variety of human and rodent tumor cell lines and in germ cells of mouse testis. In addition, a unique transcript was observed in testis RNA. This unique transcript was shown to be expressed maximally in postmeiotic round spermatids, indicating the regulation of its expression during spermatogenesis (3). These results thus suggested that the RFP protein is present at high levels in mitotically and meiotically dividing cells and might play a role in cell growth and male germ cell development.

In the present study we characterized the RFP protein. The protein was identified as a 58 kDa nuclear protein in several human and rodent cell lines and in mouse testis. We also show that RFP is tightly associated with the nuclear matrix and has DNA-binding activity.

MATERIALS AND METHODS

Cell lines

The human tumor cell lines, Raji (Burkitt lymphoma) and SK-N-MC (primitive neuroectodermal tumor) were grown in RPMI 1640 and Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum, respectively. AH7974 (rat ascites hepatoma) cells were described by T.Koizumi et al (16). NIH3T3 (mouse fibroblast) cells were maintained in DMEM with 7% calf serum.

Construction of plasmid

RFP cDNA was digested with *Sau*3AI restriction endonuclease to obtain a cDNA fragment comprising amino acids 330 to 488 of the RFP protein. The fragment was inserted into the *Bam*HI site of pET-3c expression vector as described previously (17,

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18). The coding sequence of the cDNA fragment was fused to the first 11 amino acids of the gene 10 protein (the major capsid protein of T7) plus an arginine derived from the *Bam*HI linker. The recombinant plasmid was sequenced to confirm that the cDNA fragment was inserted in the proper orientation.

Antibodies

The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) strain carrying a single copy of the gene for T7 RNA polymerase under the control of the *lacUV5* promoter. The induction of the RFP protein was performed with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) as described previously (18).

After accumulation of the fusion protein, the cells were lysed by freezing and thawing, and the viscosity was rendered by sonication. The lysate was centrifuged at $15,000\times g$ for 30 min and the pellet containing the insoluble fusion protein was applied to SDS-polyacrylamide gels. The proteins were recovered by electroelution. Rabbits were immunized five times subcutaneously with 500 μ g of the purified protein in Freund's adjuvants.

Cell lysate and fractionation

Total cell extracts were prepared from cell lines and mouse tissues. 2×10^7 cells were suspended in 1 ml of SDS-sample buffer (20 mM Tris-HCl (pH 6.8), 2 mM EDTA, 2% SDS, 10% sucrose, 20 μ g/ml bromophenol blue (BPB), 80 mM dithiothreitol (DTT)) and sheared through a 26 gauge needle until they were no longer viscous.

AH7974 cells were used for cell fractionation. They were incubated in hypotonic buffer (25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM phenylmethyl-sulfonyl fluoride (PMSF)) on ice for 5 min and homogenized in a tight-fitting Dounce homogenizer with 5 strokes. The concentration of sucrose in the homogenate was immediately adjusted to 0.25 M. The homogenate was centrifuged at low speed ($800\times g$) for 5 min, and the pellet was suspended in isotonic buffer (25 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM PMSF). After centrifugation at the low speed for 5 min, the pellet was referred to as the nuclear fraction. After the first centrifugation, the supernatant was centrifuged at $100,000\times g$ for 30 min to obtain the membrane pellet. The resulting supernatant was referred to as the cytosolic fraction.

Preparation of nuclei and nuclear matrix

Nuclei were prepared from AH7974 and Raji cells according to the method of T.Koizumi et al. (16). AH7974 cells (10–20 g wet weight) or Raji cells (1–2 g wet weight) were swollen in 2 volumes of a hypotonic solution containing 5 mM $MgCl_2$, 1 mM $NaHCO_3$, and 1 mM PMSF and disrupted in a Dounce homogenizer with 5–10 strokes. The concentration of homogenate was immediately adjusted to 0.25 M of sucrose. The crude nuclei were collected by centrifugation of the isotonic homogenate at $800\times g$ for 10 min. Following one wash with isotonic buffer, the nuclear pellet was resuspended in 50 volumes of 2.1 M (in case of AH7974) or 2.3 M (in case of Raji) sucrose containing 5 mM $MgCl_2$ and centrifuged at $52,000\times g$ for 60 min (Beckman SW27 rotor). Purified nuclei pelleted at the bottom of the tubes were washed twice with 0.25 M sucrose containing 5 mM $MgCl_2$. The purity of the isolated nuclei was confirmed by electron microscopic examination and by determination of marker enzyme activities (16).

From the nuclei thus obtained the nuclear matrix was prepared according to the method of Smith et al. (19) with slight modification. Nuclei were resuspended in isotonic buffer (25 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM PMSF) at a concentration of 2.5 mg DNA/ml and digested with 35 unit/ml of bovine pancreatic DNaseI (Takara-Shuzo Co., Kyoto, Japan) at 4°C for 16 hrs. The digested nuclei were extracted four times with LS buffer (10 mM Tris-HCl (pH 7.4), 0.2 mM $MgCl_2$, 1 mM PMSF) to yield the soluble low-salt bulk chromatin and the insoluble low-salt matrix (LS matrix). LS matrix was then extracted three times with HS buffer (10 mM Tris-HCl (pH 7.4), 2 M NaCl, 0.2 mM $MgCl_2$, 1 mM PMSF) to yield the high-salt matrix (HS matrix). Finally to obtain the nuclear matrix, HS matrix was extracted once with 1% TritonX-100 in LS buffer, and washed twice with LS buffer. Centrifugations were performed at $1,000\times g$ for 15 min during the initial LS buffer extractions and at $6,000\times g$ for 15 min for all subsequent extractions.

Western blotting

Proteins separated on SDS-polyacrylamide gels were transferred to nitrocellulose membranes. Filters were blocked in PBS containing 5% calf serum for 3 hrs at room temperature and incubated with antibodies at 4°C overnight. After washing three times with PBS containing 0.05% Tween20, the filters were reacted with biotin-labeled rabbit IgG(H+L)-specific goat antibodies (Vectastain, Burlingame, CA). Then avidin-biotin peroxidase complex (ABC) (Vectastain) was incubated for 45 min at room temperature. Color development was performed using POD immunostain set (Wako Pure Chemical Ind., Osaka, Japan).

DNA-cellulose column chromatography

Nuclear matrix described above was suspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 0.1% Nonidet-P40 (NP40) and 0.5 M NaCl, sonicated for 30 sec and centrifuged at $40,000\times g$ for 5 min. The pellet was resuspended in the same buffer, sonicated and centrifuged in the same way twice. The supernatants of these three centrifugations were combined and diluted in 4 volumes of loading buffer (20 mM Tris-HCl (pH 7.4), 0.01% NP40, 5 mM EDTA, 10% glycerol, 1 mM DTT). The final NaCl concentration was adjusted to 100 mM. Before applying on the column, the diluted supernatant was sonicated and centrifuged again. The resulting supernatant was then applied to a double-stranded DNA-cellulose, single-stranded DNA cellulose or control cellulose column (1 $\times$ 5 cm) equilibrated with the loading buffer (20). The column was then eluted stepwise with the loading buffer containing different concentrations of NaCl (0.2–2.0 M). Fractions were then dialyzed against the loading buffer and analyzed for RFP by Western blotting as described above.

Centrifugal elutriation

Centrifugal elutriation was performed using a standard chamber of Beckman J-6M/E centrifuge (Beckman, Palo Alto, CA). All operations were performed at 0 to 4°C in 0.2% bovine serum albumin (BSA) in PBS. Cells (2×10^8) in 5 ml of RPMI 1640 containing 10% fetal calf serum were loaded at a rotor speed of 1500 rpm and 100 ml fractions were collected. The flow rates for each fraction were: fraction 1, 10 ml/min; fraction 2, 12 ml/min; fraction 3, 14 ml/min; fraction 4, 16 ml/min; fraction 5, 18 ml/min; fraction 6, 20 ml/min; fraction 7, 22 ml/min;

fraction 8, 24 ml/min. An aliquot from each fraction was stained with propidium iodide (50 μ g/ml) and analyzed for DNA content using Epics profile cell sorter (Coulter Corporation, Hialeah, FL). The main bulk of each cell fraction was collected and used for Western blotting.

RESULTS

Identification of RFP protein

RFP cDNA fragment comprising amino acids 330 to 488 (Figure 1a) was inserted into the *Bam*HI site of pET-3c vector and expressed as described previously (18). As shown in Figure 1b (lane 2), an approximately 20 kDa fusion protein was produced in *E. coli* in the presence of induction with isopropyl- β -D-thiogalactopyranoside (IPTG). The fusion protein was purified by eluting from SDS-polyacrylamide gels (Figure 1b, lane 3) and used for immunization. The antibody obtained was shown to react with the immunized protein by Western blotting (Figure 1b, lane 5).

We have previously reported that the *RFP* gene is transcribed as 2.4 and 2.8 kb mRNAs in human tumor cell lines and as 2.4 kb mRNA in rodent tumor cell lines (2, 3). Western blot analysis showed that the antibody specifically reacted with a 58 kDa band in all cell lines tested, including Raji (human Burkitt lymphoma), SK-N-MC (human primitive neuroectodermal tumor), NIH3T3 (mouse fibroblast) and AH7974 (rat ascites hepatoma) (Figure 2). The apparent molecular weight was consistent with that predicted

from the amino acid sequence of *RFP* cDNA, suggesting that the antibody generated here recognizes the RFP protein.

When we examined the expression of the RFP protein in normal mouse tissues, the same 58 kDa band was detected in the testis but not in the liver, spleen and kidney (Figure 3). This result also confirmed our previous data in which the *RFP* mRNAs were shown to be expressed at high levels in germ cells of mouse testis. In addition, a 68 kDa band was observed in the lysate from the testis. This band may represent an RFP product translated from the 2.8 kb transcript found specifically in the testis (3). Other bands seen in the blots are probably non-specific because they are present in the lanes stained with control serum.

Localization of RFP protein in the nuclear matrix

We next examined the subcellular localization of the RFP protein. AH7974 cells were fractionated into nuclear, membrane and soluble cytosolic fractions. As shown in Figure 4a, RFP was predominantly detected in the nuclear fraction although it was present at a low level in the cytosolic fraction. To investigate its precise location in the nucleus, the RFP protein was analyzed in various fractions generated during the preparation of nuclear matrix (Figure 4b). The nuclei was isolated from AH7974 cells through sucrose gradient and the immunoreactive RFP protein was readily detectable in them (Figure 4b, lane a). The RFP protein was hardly solubilized from the nuclei by exhaustive digestion with DNaseI (Figure 4b, lane b) and low salt (Figure 4b, lane c) treatment, and was retained in the low salt matrix (Figure 4b, lane d). In the process of the high-salt treatment, RFP was also recovered in the fraction of high salt matrix (Figure 4b, lane f) although approximately 20~30% of the protein was eluted in the supernatant (Figure 4b, lane e). Finally to obtain the nuclear matrix, the sample was treated with 1% TritonX-100. RFP was undetectable in the soluble fraction (Figure 4b, lane g), indicating that most of the protein is tightly associated with the nuclear matrix (Figure 4b, lane h). Similar experiment using Raji cells also showed the localization of RFP protein in the nuclear matrix (data not shown).

Figure 1. RFP protein produced in *E. coli*. (a) Schematic illustration of *RFP* cDNA. A 0.47 kb *Sau*3AI cDNA fragment (a solid bar) comprising amino acids 330 to 488 of the *RFP* protein was inserted into the *Bam*HI site of pET-3c expression vector. A putative C₃HC₄ motif, cysteine-histidine rich motif and coiled coil structure are indicated. (b) Expression of the RFP protein. Cultures of BL21(DE3) carrying the plasmids were grown and induced with IPTG as described in materials and methods. Three hours later, 30 μ l samples were electrophoresed in a 13% SDS-polyacrylamide gel. Lanes represent cultures of BL21(DE3) with the plasmid containing the *RFP* cDNA fragment in the opposite (lane 1) or proper (lane 2) orientation. The purified protein recovered by electroelution was shown in lane 3. Aliquots (2 μ l/lane) of induced cultures (lane 2) were analyzed by Western blotting. The antibodies used were normal rabbit serum (lane 4) and anti-RFP polyclonal antibody (lane 5). kDa, kilodalton.

Figure 2. Detection of RFP protein in different cell lines by Western blotting. Lysates containing 20 μ g of proteins from Raji human Burkitt lymphoma (lanes 1 and 2), SK-N-MC human primitive neuroectodermal tumor (lanes 3 and 4), NIH3T3 mouse fibroblast (lanes 5 and 6) and AH7974 rat ascites hepatoma (lanes 7 and 8) cells were separated on 8% SDS-polyacrylamide gels and analyzed by Western blotting. The antibodies used were normal rabbit serum (lanes 1, 3, 5 and 7) and anti-RFP polyclonal antibody (lanes 2, 4, 6 and 8). kDa; kilodalton.

Figure 3. Detection of RFP protein in various mouse tissues by Western blotting. Lysates containing 20 μ g of proteins from testis (lanes 1 and 2), liver (lanes 3 and 4), spleen (lanes 5 and 6), and kidney (lanes 7 and 8) were reacted with normal rabbit serum (lanes 1, 3, 5 and 7) and anti-RFP polyclonal antibody (lanes 2, 4, 6 and 8). kDa; kilodalton.

Figure 4. Localization of the RFP protein in the nuclear matrix (a) Subcellular localization of the RFP protein. AH7974 cells were fractionated into nuclear (N), membrane (M), and cytosolic (C) fractions. The lysate from each fraction was reacted with anti-RFP polyclonal antibody as described in Figure 2. (b) Western blot analysis of fractions generated during preparation of the nuclear matrix. (Upper panel) Schematic illustration of preparation of the nuclear matrix. (Lower panel) Western blotting with anti-RFP antibody. (lane a), isolated AH7974 nuclei; (lane b), supernatant after DNase I treatment; (lane c), supernatant after low salt treatment; (lane d), low salt matrix; (lane e), supernatant after high salt treatment; (lane f), high salt matrix; (lane g), supernatant after TritonX-100 treatment; (lane h), nuclear matrix. Equivalent aliquot of each fraction was reacted with anti-RFP antibody.

Figure 5. DNA-cellulose column chromatography of RFP protein eluted from the nuclear matrix. (a) Extraction of RFP protein from the nuclear matrix. The nuclear matrix (lane 1) was sonicated in the presence of 1 mM EDTA and then centrifuged. The RFP protein was detected predominantly in the supernatant (lane 3) but not in the pellet (lane 2). (b, c, d) DNA-cellulose column chromatography. The RFP protein solubilized from the nuclear matrix (derived from 180 mg protein of AH7974 nuclei) was applied onto double-stranded (b) or single-stranded (c) DNA-cellulose column or control cellulose column (d). Lanes 1 indicate flow-through fractions. Lanes 2 to 5 indicate fractions eluted with the loading buffer containing 0.2, 0.5, 1.0, and 2.0 M NaCl, respectively. The eluates were then analyzed by Western blotting with anti-RFP antibody.

Figure 6. Cell cycle analysis of RFP protein. (a) Flowcytometric analysis of DNA ploidy. Raji cells were separated by centrifugal elutriation and stained with propidium iodide. The G1 phase (Fr. 2), S phase (Fr. 4) and G2/M phase (Fr. 7)-rich fractions are shown. Control represents Raji cells before separation. 2N and 4N DNAs are indicated on the abscissa. (b) Equivalent aliquots of each fraction and control cells were reacted with anti-RFP polyclonal antibody.

DNA binding activity of RFP protein eluted from the nuclear matrix

Since the RFP protein contains cysteine-rich sequences similar to the zinc finger motif, it is expected to bind to nucleic acids. To investigate this possibility, we extracted the RFP protein from the nuclear matrix of AH7974 by EDTA treatment and sonication. As shown in Figure 5a, approximately 90% of RFP was solubilized by this method. The RFP protein extracted from the nuclear matrix (derived from 180 mg protein of AH7974 nuclei) was applied to three kinds of columns; double-stranded DNA cellulose column, single-stranded DNA cellulose column and control cellulose column. Potential DNA-binding proteins

were eluted stepwise with the loading buffer containing 0.2 M, 0.5 M, 1.0 M, or 2.0 M NaCl. Then 2% volume of each fraction was analyzed by Western blotting with the anti-RFP antibody. RFP was detectable in all fractions of the eluate from double-stranded DNA-cellulose column although it appeared to be eluted maximally between concentrations of 0.5 M to 1.0 M NaCl (Figure 5b). When we used a single-stranded DNA-cellulose column, RFP was eluted mainly at a concentration of 0.5 M NaCl (Figure 5c, lane 3). Although a 85 kDa protein was also observed in the same lane, this may represent an RFP-related protein which can interact with single-stranded DNA. In contrast, RFP was undetectable in each fraction from control cellulose column (Figure 5d). These results indicated that RFP has DNA binding activity and that its association with double-stranded DNA was stronger than that with single-stranded DNA.

Cell cycle analysis of RFP protein

It is interesting to investigate whether the content of the RFP protein varies at different stages of the cell cycle. Thus Raji cells were separated into fractions of progressively increasing cell size by centrifugal elutriation. The DNA ploidy of each fraction was analyzed by a fluorescence-activated cell sorter after staining DNA with propidium iodide (Figure 6a). The G1 phase (fraction 2), S phase (fraction 4) and G2/M phase (fraction 7)-rich fractions were chosen and analyzed for the RFP protein. As shown in Figure 6b, no significant difference in the expression level was observed among these three fractions.

DISCUSSION

In this study, we have characterized the RFP protein by Western blotting with a polyclonal antibody against its polypeptide expressed in *E. coli*. RFP was identified as a protein with an apparent molecular weight of 58 kDa in cell lysates from mouse, rat and human cell lines. The same protein was also detected in mouse testis but not in the liver, spleen and kidney. These results were in agreement with our previous study (3) in which RFP mRNAs were shown to be highly expressed in a variety of tumor cell lines and mouse testis. In addition, a 68 kDa protein was specifically observed in the testis. We reported that a 2.8 kb unique transcript is present in germ cells of mouse testis and is highly expressed particularly in round spermatids (3). Thus it is possible that the 68 kDa protein is translated from this 2.8 kb message although analysis of its amino acid sequence is necessary. Alternatively, it may represent another protein which has sequence homology to the RFP protein.

As predicted from the amino acid sequence of RFP containing the cysteine-rich motifs, cell fractionation experiment showed that the RFP protein is present predominantly in the nucleus. We also demonstrated the strong association of RFP with the nuclear matrix and its DNA-binding activity, although a population (20–30%) of RFP was solubilized by high salt treatment during the process of nuclear matrix preparation. It is well known that many molecular events such as attachment of supercoiled DNA loops (21), DNA replication (22, 23), transcription (24) and RNA processing (25) occur in the nuclear matrix. In particular, the sites called matrix attachment regions (MARs) (26), at which the nuclear matrix interacts with DNA, appear to be important for these events. To date, a number of proteins with DNA-binding activity were reported to be associated with the nuclear matrix, including DNA polymerase α (27), topoisomerase II (28), c-myc (29), and corticosteroid receptors (30). In addition, a nuclear matrix protein named matrin protein F/G was shown to contain

putative zinc finger motifs (31). Although the RFP protein did not show any homology to these proteins, the fact that RFP localizes in the nuclear matrix and can bind to DNA suggests its potential role in the nuclear processes mentioned above.

The amino acid sequence of RFP contains a new cysteine-rich motif (C_3HC_4 motif) which has recently been identified in a number of proteins (4). In addition, a second Cys/His rich motif was present in RFP. The similar motif was found in some of the proteins with the C_3HC_4 motif, including RPT-1 (8), SS-A/Ro (9), PML/Myl (10, 11), T18 (14) and XNF7 (12). These proteins also share a predicted coiled coil structure immediately C-terminal to the Cys/His rich motif (Figure 1a) (32). Thus they appear to constitute a subfamily of the proteins with the C_3HC_4 motif. Furthermore, it is of considerable interest that three of the six proteins with the Cys/His-rich motif and coiled coil structure (RFP, PML/Myl and T18) were involved in the activation of the proto-oncogenes; *ret*, retinoic acid receptor and *B-ras*, respectively (2, 3, 14, 32). All of the three domains (C_3HC_4 , Cys/His-rich and coiled coil) in RFP, PML/Myl and T18 were retained in the sequence of the oncogenic fusion proteins, suggesting their functional importance for the transforming activity (32). In this regard, it is notable that the RFP protein is expressed at high levels in a variety of tumor cell lines but not in normal tissues except the testis (3). In addition, we showed that RFP is consistently expressed during the cell cycle. Thus it seems likely that RFP plays a role in cell growth. Determination of binding sequence of RFP to DNA and of its precise localization in the nuclear matrix could provide a new insight into the physiological function(s) of RFP.

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