Protein 4.1R binding to eIF3-p44 suggests an interaction between the cytoskeletal network and the translation apparatus


Erythroid protein 4.1 (4.1R) is an 80-kd cytoskeletal protein that stabilizes the membrane-skeletal network structure underlying the lipid bilayer. Using the carboxyl terminal domain (22/24 kd) of 4.1R as bait in a yeast 2-hybrid screen, we isolated cDNA clones encoding a polypeptide of eIF3-p44, which represents a subunit of a eukaryotic translation initiation factor 3 (eIF3) complex. The eIF3 complex consists of at least 10 subunits that play an essential role in the pathway of protein translation initiation. Northern blot analysis revealed that eIF3-p44 (approximately 1.35 kb) is constitutively expressed in many tissues. The essential sequence for this interaction was mapped to the carboxyl-terminus of 4.1R (residues 525-622) and a region (residues 54-321) of eIF3-p44. The direct association between 4.1R and eIF3-p44 was further confirmed by in vitro binding assays and coimmunoprecipitation studies. To characterize the functions of eIF3-p44, we depleted eIF3-p44 from rabbit reticulocyte lysates either by anti-eIF3-p44 antibody or by GST/4.1R-80 fusion protein. Our results show that the eIF3-p44 depleted cell-free translation system was unable to synthesize proteins efficiently. The direct association between 4.1R and eIF3-p44 suggests that 4.1R may act as an anchor protein that links the cytoskeleton network to the translation apparatus. (Blood. 2000;96:747-753) © 2000 by The American Society of Hematology
translation initiation factor 3 (eIF3), was further analyzed. eIF3 is a large translation initiation complex that contains at least 10 subunits. It plays a central role in the binding of the initiator methionyl-tRNA and mRNA to the 40S ribosomal subunit to form the 40S initiation complex. In this study, we show that 4.1R associates with eIF3-p44 both in vitro and in vivo, which suggests possible interactions between the cytoskeleton network and the translation apparatus.

Materials and methods

Yeast 2-hybrid screen

Yeast 2-hybrid screening was conducted using the yeast strain Y190, which contains the HIS3 and lacZ reporter genes required for GAL4-dependent transcriptional activation. The carboxyl 22/24-kd domain (residues 462-641) of 4.1R (4.1R-22) was subcloned in frame to the GAL4 DNA binding domain (GAL4-BD) in the pAS2-1 vector (Clontech, Palo Alto, CA). This construct was used as bait to screen human lymphocyte and testis cDNA libraries (Clontech) fused to a GAL4 activation domain (GAL4-AD) in the pACT2 vector. The 2 plasmids were then cotransformed into Y190 yeast. The transformants were selected on SD minimal medium containing 25 mmol/L 3- amino-1,2,4-triazole, but without the addition of leucine (−Leu), tryptophan (−Trp), and histidine (−His), as described by the manufacturer (Clontech). Positive colonies (ie, Leu +, Trp +, His +) were further tested for β-galactosidase activity using colony-lift and liquid assays, as described by the manufacturer (Clontech).

To identify the minimum regions of 4.1R and eIF3-p44 that bind to each other, constructs containing various portions of 4.1R were subcloned into the pAS2-1 vector (Figure 1A), and different eIF3-p44 constructs (Figure 1B) were inserted into the pACT2 vector. Yeast cells (Y187) were simultaneously transformed with the 2 constructs and assayed for β-galactosidase activity.

Northern blot analysis and cDNA library screening

A blot filter containing 2 µg poly (A)+ RNA derived from multiple human tissues was purchased from Clontech. The blot was hybridized to a cDNA probe derived from the 0.9-kb fragment of eIF3-p44 corresponding to amino acid residues 54-321 (eIF3-p44-1) under previously described conditions. The same blot was stripped and reprobed with human β-actin cDNA to quantify RNA loading.

To obtain the full-length eIF3-p44 cDNA, the same probe was used to screen a human testis cDNA library (Clontech). The conditions for screening and isolation were previously described.

Plasmids and antibodies

eIF3-p44-1 (residues 54-321), originally identified from a yeast 2-hybrid screen (Figure 1A), and eIF3-p44 (residues 1-321), a full-length eIF3-p44 cDNA isolated from a cDNA library, were used as templates to generate GST/eIF3-p44-1 and GST/eIF3-p44 constructs, respectively. The cDNA fragments spanning the coding regions of eIF3-p44-1 and eIF3-p44 were polymerase chain reaction-amplified and fused in frame to GST in the pGEX-2T vector. Sense mRNA was generated from the T7 promoter within the vector. Sense mRNA was translated in a coupled in vitro transcription/translation system (TNT rabbit reticulocyte lysate; Promega, Madison, WI) in the presence of 35 S-labeled methionyl–tRNA and mRNA to the 40S ribosomal subunit to form the 40S initiation complex. In this study, we show that 4.1R associates with eIF3-p44 both in vitro and in vivo, which suggests possible interactions between the cytoskeleton network and the translation apparatus.

In vitro binding assay

The cDNA encoding an 80-kd 4.1R isoform was constructed in the pSG5 vector (Strategene, La Jolla, CA). Synthetic sense-capped mRNA was generated from the T7 promoter within the vector. Sense mRNA was translated in a coupled in vitro transcription/translation system (TNT rabbit reticulocyte lysate; Promega, Madison, WI) in the presence of 35S-methionine to radiolabel newly synthesized proteins.

Equal amounts of the labeled 80-kd 4.1R protein were incubated with affinity-purified GST or GST/eIF3-p44 fusion proteins previously immobilized on glutathione–agarose beads as described. After incubation, the immobilized complexes were washed 3 times with bead binding buffer (50 mmol/L potassium phosphate, pH 7.5, 150 mmol/L KCl, 1 mmol/L MgCl2, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride). Bound protein complexes were analyzed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.
Cell culture, transfection, and Western blot analysis

Molt-4 cells (a human lymphoid cell line) were maintained in RPMI-1640 medium, as previously described.34 SiHa cells (a human cervical carcinoma cell line) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The cDNA encoding human eIF3-p44-1 (residues 54-321) was inserted in frame into a cytomegalovirus promoter-driven FLAG epitope-tagged expression vector (Kodak, New Haven, CT), designated FLAG-eIF3-p44-1. SiHa cells (1 x 10⁶) were transiently transfected with or without 10 µg FLAG-eIF3-p44-1 cDNA, as previously described.23

Twenty-four hours after transfection, cells were washed in PBS and lysed in 1 mL ice-cold EBC buffer (50 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl, 0.5% NP-40, 1 µg aprotinin, 1 µg leupeptin, and 2 mmol/L phenylmethlysulfonyl fluoride) for 30 minutes on ice, and the lysate was cleared by centrifugation. Solubilized proteins (10 µg) were separated on a 10% SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and detected by anti-FLAG mAb, or anti-β-actin mAb. Secondary antibodies were goat antirabbit or goat antimouse IgG conjugated with horseradish peroxidase (Promega). Immunoreactive polypeptides were then detected by the Western Exposure Chemiluminescent Detection System (Pierce, Rockford, IL) as previously described.23

Coimmunoprecipitation assay

Coimmunoprecipitation of 4.1R and eIF3-p44 was performed using Molt-4 cell extracts. Cells were lysed in EBC buffer, and the lysate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant (approximately 5 mg) was precleared with protein A–Sepharose beads (Sigma). Immunoprecipitation was carried out with preimmune serum (2 µg), anti-eIF3-p44 antibodies, or anti-FLAG mAb overnight at 4°C, and incubated with protein A–Sepharose beads for another 2 hours. Immunoprecipitates were washed 3 times with EBC buffer. Samples were resuspended in 20 µL SDS sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10 µg/mL bromophenol blue) and were boiled at 95°C for 5 minutes. The samples were then centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatants were separated on 7.5% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, the immunoreactive proteins were detected by anti-N-4.1R or anti-C-4.1R antibodies, as described above. For reverse immunoprecipitation, the cell lysates were immunoprecipitated with anti-C-4.1R antibodies and analyzed by immunoblotting with anti-FLAG-p44 antibodies.

In vitro depletion assay

To deplete eIF3-p44, rabbit reticulocyte lysates (12.5 µL) were preincubated for 2 hours at 4°C with preimmune serum (2 µg), anti-eIF3-p44 antibody (2 µg) previously coupled to protein A–Sepharose beads, immobilized GST (10 µg), or GST/4.1R-80 fusion proteins (10 µg) previously conjugated to glutathione–agarose beads. Samples were centrifuged at 5000 g for 5 minutes at 4°C and washed 3 times with EBC buffer. Samples were then centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatants were separated on 7.5% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, the immunoreactive proteins were detected by anti-N-4.1R or anti-C-4.1R antibodies, as described above. For reverse immunoprecipitation, the cell lysates were immunoprecipitated with anti-C-4.1R antibodies and analyzed by immunoblotting with anti-FLAG-p44 antibodies.

Results

4.1R and eIF3-p44 interact in a yeast 2-hybrid assay

We previously showed that pICln (a protein involved in cellular volume regulation) binds to the 30-kd domain of 4.1R in a yeast 2-hybrid system.23 Using a similar approach, we set out to identify proteins that interact with the 22/24-kd domain of 4.1R (4.1R-22). The cDNA encoding the 22/24-kd domain, fused in frame to the Gal4 DNA-binding domain (Gal4-BD), was used as bait to screen human lymphocyte and testis cDNA libraries fused to the Gal4 activation domain (Gal4-AD). We screened approximately 3 x 10⁶ transformants, and 63 positive clones were obtained.

A search of the GenBank database revealed that at least 4 known genes (NuMA, eIF3-p44, Sec 14–like protein, and 26S proteasome subunit p55) were found from the screen. Among these known proteins, we further examined NuMA and eIF3-p44. NuMA is a nuclear mitotic apparatus protein that had previously been isolated in a yeast 2-hybrid assay using the full-length 135-kd 4.1R as bait.23 This clone was also independently isolated 17 times in our screen. The other known gene that interacts with the 22/24-kd domain of 4.1R (residues 462-641; Figure 1A) is eIF3-p44. The interaction of 4.1R and eIF3-p44 in the yeast 2-hybrid assay appears to be specific: eIF3-p44-1/pACT2 did not bind to the empty vector pAS2-1 containing only unused Gal4-BD (Figure 1A). The 4.1R-22/pAS2-1 also did not interact with the unused Gal4-AD vector, pACT2 (Figure 1B).

Cloning and Northern blot analysis of eIF3-p44

Using yeast 2-hybrid screening, we isolated a cDNA clone (p44-1; Figure 1B) encoding a eukaryotic translation initiation factor 3 subunit (eIF3-p44).30,31 Clone p44-1 represents part of the coding sequence and the 3' untranslated region (UTR) of eIF3-p44, including the stop codon TAA followed by the polyadenylation signal AATAAA. Northern blot analysis revealed that the eIF3-p44 transcript (1.35 kb) was detectable in all tissues examined and was predominantly expressed in testis, heart, pancreas, and skeletal muscle (Figure 2). To obtain the cDNA that covers the entire coding region of eIF3-p44, we screened a human testis cDNA library using eIF3-p44-1 cDNA as a probe. Several full-length clones were isolated, and their sequences were confirmed on both strands.

Interaction between 4.1R and eIF3-p44 occurs through the 22/24-kd domain of 4.1R and amino acids 54-321 of eIF3-p44

To determine the region of 4.1R that interacts with eIF3-p44, constructs containing different regions of 4.1R in the pAS2-1 vector and eIF3-p44-1 in the pACT2 vector were cotransformed...
Figure 3. In vitro binding assay indicates that elf3-p44 binds to 4.1R. The elf3-p44 cDNA was subcloned into the pGEX2T vector and expressed as a GST/elf3-p44 fusion protein in Escherichia coli. (A) Purified GST (lane 2) and GST/elf3-p44 fusion protein (lane 3) were visualized by Coomassie blue staining. (B) Binding of GST/elf3-p44 to 4.1R-80 (80-kd isoform) in vitro. 35S-methionine-labeled 4.1R-80 (lane 3) was incubated with affinity-purified GST (lane 2) or GST/elf3-p44 fusion protein (lane 3) previously coupled to glutathione–agarose beads. After incubation, the colony-lift assay (data not shown) to examine for positive interactions. Thus, the 98 amino acids of 4.1R derived from the 22/24-kd domain (4.1R-2) are required and sufficient for interaction with elf3-p44.

To map the region of elf3-p44 that binds to 4.1R, we expressed different domains of elf3-p44/pACT2 clones and cotransformed them into Y187 with 4.1R-22/pAS2-1. As shown in Figure 1B, only p44 (encoding the entire coding region of elf3-p44) and p44-1 (residues 54-321) showed substantial binding to 4.1R-22, whereas p44-2 (residues 54-185) revealed a weak interaction. In contrast, peptide segments of elf3-p44 encompassing amino acids 185-321 (p44-3) or 231-321 (p44-4), which contain the RNA recognition motif, did not interact with 4.1R-22. Therefore, we believe that the entire structure of elf3-p44, particularly the region spanning amino acids 54-321, are necessary and important for the maintenance of the correct conformation for elf3-p44 binding to 4.1R. Taken together, these results indicate that the interacting regions of these 2 proteins are restricted to the amino acids residues 525-622 of 4.1R and 54-321 of elf3-p44.

4.1R interacts with elf3-p44 in vitro

The direct interaction between 4.1R and elf3-p44 was further analyzed using an in vitro binding assay. The full-length elf3-p44 cDNA was constructed in frame into the pGEX2T vector. The GST/elf3-p44 fusion protein was expressed and purified with glutathione–agarose beads (Figure 3A, lane 3). Immobilized GST/elf3-p44 protein was incubated with 35S-methionine-labeled 4.1R (80-kd isoform), and retention on the beads was analyzed by SDS-PAGE. As shown in Figure 3B, GST/elf3-p44 fusion protein interacted with the labeled 80-kd 4.1R (Figure 3B, lane 3). In contrast, the control GST fusion protein failed to bind to labeled 4.1R (Figure 3B, lane 2). Consistent with our results from the yeast 2-hybrid assays, these results further confirm that elf3-p44 specifically interacts with 4.1R in vitro.

4.1R interacts with elf3-p44 in vivo

Polyclonal antibodies against elf3-p44 were raised to further characterize the interaction between 4.1R and elf3-p44. To test the specificity of this antibody, we transiently transfected FLAG-elf3-p44-1 into SiHa cells. The cell extract was prepared 24 hours after transfection and analyzed by anti-FLAG mAb or anti-elf3-p44 polyclonal antibodies. Figure 4 shows that FLAG-elf3-p44-1 (residues 54-321) was recognized by both anti-FLAG (Figure 4A, lane 1) and anti-elf3-p44 (Figure 4B, lane 1) antibodies, whereas, the endogenous elf3-p44 in both transfected (Figure 4B, lane 1) and untransfected (Figure 4B, lane 2) SiHa and Molt-4 (Figure 4B, lane 3) cells was only detected by anti-elf3-p44 antibody. Because the SiHa cells were transfected with a N-terminal-truncated clone, FLAG-elf3-p44-1 (residues 54 to 321), a low molecular weight band was detected in transfected cells (Figure 4B, lane 1).

To examine whether endogenous 4.1R is associated with elf3-p44 in vivo, we performed a co-immunoprecipitation assay. Cell lysates prepared from Molt-4 cells were first immunoprecipitated with either preimmune serum or anti-elf3-p44 antibodies. Coprecipitated proteins were then detected by anti-N-4.1R (against the headpiece 209 amino acids of the 135-kd isoform) (Figure 5A) or anti-C-4.1R (against the C-terminal 22/24-kd domain) (Figure 5B) antibodies. As shown in Figure 5, panels A and B (lane 2), no endogenous 135-kd or 80-kd 4.1R was detected when preimmune serum was used for the immunoprecipitation experiment. In contrast, the 135-kd isoform of 4.1R was coprecipitated with anti-elf3-p44 antibodies and was detected by anti-N-4.1R antibody (Figure 5A, lane 3). Similarly, both 135-kd and 80-kd 4.1R isoforms were coprecipitated with anti-elf3-p44 and immunoreacted with anti-C-4.1R antibody (Figure 5B, lane 3). The in vivo association of 4.1R and elf3-p44 was further confirmed by reverse immunoprecipitation (Figure 5C). Cell lysates were first immunoprecipitated with either preimmune serum (Figure 5C, lane 2) or anti-C-4.1R antibody (Figure 5C, lane 3), and this was followed by immunoblotting of co-precipitated proteins with anti-elf3-p44 antibody. Because the heavy chains of the immunoglobulins comigrate with 4.1R under reducing conditions on SDS-PAGE, immunoprecipitates were dissolved in sample buffer lacking β-mercaptoethanol to maintain the disulfide bonds between the light and heavy chains of the immunoglobulins. As shown in Figure 5, panel C, elf3-p44 was pulled down by anti-C-4.1R antibody (lane 3) but not by pre-immune serum (lane 2). Taken together, these results indicate that elf3-p44 interacts with 4.1R in vivo.

Cell-free translation system deficient in elf3-p44 was unable to synthesize proteins efficiently

To verify that elf3-p44 plays a role in protein biosynthesis, we attempted to deplete elf3-p44 activity in reticulocyte lysates using anti-elf3-p44 antibodies (Figure 6) or GST/4.1R-80 fusion protein.
Figure 5. 4.1R interacts with eIF3-p44 in vivo. Cell lysates prepared from Molt-4 cells were immunoprecipitated with preimmune serum (lane 2) or anti-eIF3-p44 antibody (lane 3). Bound protein complexes were analyzed by immunoblotting with antibodies against the N-terminal portion of 135-kd 4.1R (A, anti-N-4.1R) or against the C-terminal 22/24-kd domain of 4.1R (B, anti-C-4.1R antibody). Lane 1 was loaded with Molt-4 cell lysates (10 µg) and immunoblotted with anti-N-4.1R (A), anti-C-4.1R (B), or anti-eIF3-p44 antibody (C). The 135-kd 4.1R isoform was specifically detected by anti-N-4.1R antibody (lane 3), whereas 2 alternative splicing isoforms of 4.1R (135-kd and 80-kd) were recognized by anti-C-4.1R antibody (B, lane 3). (C) Reverse immunoprecipitation. Cell lysates prepared from Molt-4 cells were immunoprecipitated with preimmune serum (lane 2) or anti-C-4.1R antibody (lane 3) and analyzed by immunoblotting with anti-eIF3-p44 antibody. Lane 1 represents a positive control in which Molt-4 cell extracts were immunoblotted with anti-eIF3-p44 antibody. Samples were dissolved in SDS sample buffer containing β-mercaptoethanol (A, B) or lacking β-mercaptoethanol (C, lanes 2 and 3).

Similar effects were also observed when immobilized GST/4.1R-80 fusion protein was used to deplete eIF3-p44. As seen in Figure 7, the eIF3-p44 in reticulocyte lysate was significantly removed by the addition of 10 µg GST/4.1R-80 fusion protein (Figure 7B, lane 3) immobilized on glutathione–agarose beads, and the eIF3-p44-depleted lysate produced substantially decreased luciferase mRNA translation (Figure 7C, lane 3). In contrast, immobilized GST alone did not significantly remove eIF3-p44 (Figure 7B, lane 2), and the GST-treated lysate was not defective in mRNA translation (Figure 7C, lane 2). Furthermore, the addition of increasing amounts (0.25 to approximately 2 µg) of soluble GST/4.1R-80 fusion protein (Figure 8A), but not GST protein (2 µg; Figure 8C, lane 2), resulted in increasing loss of the protein translation efficiency of luciferase mRNA (Figure 8B). Taken together, these results suggest that eIF3-p44 can be depleted by either anti-eIF3-p44 antibodies or its interaction protein (4.1R) and that eIF3-p44-depleted or GST/4.1R-80 treated lysates do not efficiently synthesize proteins.

Discussion

We and others have shown that 4.1R is composed of multiple isoforms that are heterogeneous in size and subcellular localization. Many of these isoforms are present in nonerythroid cells or in erythroid cells at different developmental stages. Their biologic significance, however, has not yet been well characterized. In the current study we identified eIF3-p44, a subunit of the eukaryotic initiation factor 3 (eIF3) complex, as a binding target of 4.1R. The interaction between the carboxyl terminal domain (22/24 kd) of 4.1R and eIF3-p44 was first discovered using the yeast 2-hybrid system and then confirmed by in vitro binding, in vitro depletion, and coimmunoprecipitation studies.

The finding that eIF3-p44 directly interacts with a cytoskeletal protein (4.1R) was informative. The interaction between eukaryotic translation components during the early phase of protein synthesis...
has been well characterized.37 Eukaryotic translation initiation begins with the assembly of a preinitiation complex, consisting of a small ribosome subunit (40S), eIF1A, eIF3, and eIF2-GTP-tRNA. Next this complex binds to mRNA in a reaction requiring adenosine triphosphate and the mRNA mG-cap binding protein complex (eIF4A, eIF4B, eIF4E, and eIF4G) and then scans toward the 3′ end until it forms a stable complex at the first AUG initiation codon. eIF4G serves as a scaffold protein for the assembly of eIF4E and eIF4A and recruits Mnk1 to phosphorylate eIF4E.38

eIF3 is a large, multi-subunit complex (approximately 600 kd) that plays a central role in the initiation of translation. It was originally isolated from rabbit reticulocyte lysates, and it contains at least 10 different protein subunits ranging from 35 to 170 kd.32 Among these protein subunits, eIF3-p44 binds specifically to eIF3-p170 and contains a consensus RNA recognition motif near its carboxyl terminus.30 eIF3 binds to 40S ribosomal subunits, resulting in the dissociation of 80S ribosomes. eIF3 also stabilizes initiator methionyl-tRNA binding to 40S subunits and participates in mRNA binding through its interaction with eIF4G.32 Interestingly, 4.1R was reported to be an actin-associated protein that binds to the spectrin–actin complex through its 10-kd domain.26–28 Purified 4.1R also interacts with tubulin39 and myosin.40 In the current study, we show that eIF3-p44 binds to both 80- and 135-kd 4.1R isoforms (Figure 5) and that this interaction is through the carboxyl terminal 22/24-kd (residues 525-622) domain of 4.1R.32,38 The involvement of 4.1R in the cytoskeleton and translational apparatus protein (NuMA)29 and a centrosome protein (CPAP),46 among other proteins will eventually provide a more complete understanding of the molecular mechanism of how phosphorylation regulates the 4.1R-linked cytoskeleton to the translational apparatus.

Recent studies have shown that mRNA and polysomes are associated with the cytoskeleton and that this association may influence the transport, anchoring, and translation of mRNA.35,36 For example, the elongation factor 1 alpha (EF-1α) is abundantly expressed and constitutes 1% to 2% of the total proteins in normal growing cells. It catalyzes the GTP-dependent binding of aminoacyl-tRNA to ribosomes, and it regulates the faithfulness and rate of growing cells. It catalyzes the GTP-dependent binding of aminoacyl-tRNA, and it regulates the faithfulness and rate of growing cells.

References

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