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Monoclonal antibodies against human translation termination factor eRF3 and their utilization for sub-cellular localization of eRF3

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Eukaryotic translation termination is triggered by peptide release factors eRF1 and eRF3. eRF1 recognizes the stop codon and promotes nascent peptide chain release, while eRF3 facilitates this peptide release in a GTP-dependent manner. In addition to its role in termination, eRF3 is involved in normal and nonsense-mediated mRNA decay. Despite extensive investigation, the complete understanding of eRF3 function have been hampered by the lack of specific anti-eRF3 monoclonal antibodies (Mabs). The purpose of the study was production of recombinant eRF3a/GSPT1, development of anti-eRF3a/GSPT1 Mabs and their utilization for eRF3a/GSPT1 sub-cellular localization. Plasmid encoding C-terminal part of human GSPT1/eRF3a was constructed. Purified protein, which was predominantly present in the inclusion bodies, was used for the development of Mabs. Characterization of the regions recognized by Mabs using GSPT1/eRF3a mutants and its visualization in the 3D space suggested that Mabs recognize different epitopes. Consistent with its function in translational termination, immunostaining of the cells with developed Mabs revealed that the endogenous GSPT1/eRF3a localized in endoplasmic reticulum. Taking into account the important role of eRF3 for the fundamental research one can suggests that developed Mabs have great prospective to be used as a research reagent in a wide range of applications.

Keywords: eRF3/endoplasmic reticulum localization/monoclonal antibody/plasmid construction.

Abbreviations: ATCC, American Type Culture Collection; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; BCA, bicinchoninic acid protein assays; BSA, Bovine serum albumin; ECL, Enhanced Chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; eRF, eukaryotic Release Factor; FCS, fetal calf serum; GST, glutathione-S-transferase; HAM's medium, a growth medium for mammalian cells; IPTG, (isopropyl-beta-D-thiogalactopyranoside; kDa, kilodalton; Mab, monoclonal antibody; NMD, nonsense-mediated mRNA decay; PABP, poly(A) binding protein; PBS, phosphate buffered saline; PBST, PBS Tween-20 solution; PEG, Polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene Fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Translation termination, the last step of protein synthesis, usually requires ribosome, a stop codon in the messenger RNA and polypeptide release factors. In higher eukaryotes, translation termination is governed by eRF1 and eRF3 factors (*1*). eRF1, the Class I release factor that recognizes the stop codon in the A site of ribosome, triggers the release of the nascent peptide chain (*2*). eRF1 is omnipotent for the three stop codons: UAA, UAG or UGA. The Class II release factor eRF3 belongs to GTPase superfamily, and it enhances the termination efficiency by stimulating the eRF1 activity in a GTP-dependent manner (*3*, *4*). Complex formation between eRF3 and eRF1 promotes GTP binding to eRF3 (*5–7*), with eRF1 functioning as cofactors that stabilize the GTP conformation (*8*). Release factors also play important roles in biological processes other than the translation termination. eRF3 is involved in normal and Nonsense-mediated mRNA decay (NMD) via two different pathways: through its association with cytoplasmic poly(A) binding protein (PABP) and Upf1 (*9–13*). It was shown that *in vitro* eRF3 inhibits multimeric PABP–PABP interactions in the poly(A) tail of mRNAs. These may destabilize the association of PABP with 3′-poly(A) tail, suggesting that eRF3 may also play an important role in the degradation of mRNAs and/or the regulation of translation efficiency mediated through initiation factors (*10*, *14*).

On the basis of sequence analysis and functional properties mammalian eRF3 is divided into the N- and C-terminal domains (*1*). The conserved C-terminal region is homologous to those of elongation factor eEF1A (*15*). It is responsible for translation termination activity and is essential for viability (*4*, *16*). In contrast, the N-terminal non-homologous domain is not essential for translation termination, but it is

important for binding to PABP (*4*, *15*, *17*, *18*). The interaction of the N-terminus of eRF3 with the C-terminal domain of PABP, which is evolutionarily conserved, seems to link the termination event with the initiation process in protein biosynthesis (*4*, *17*). The crystal structure of the C-terminal part of eRF3 of the yeast *Schizosaccharomyces pombe* has been described recently (*19*).

Two distinct genes encoding eRF3 called GSPT1/eRF3a and GSPT2/eRF3b were identified in the human, mouse and rat genomes (*20*, *21*). The eRF3a gene contains several intronic sequences, whereas the eRF3b gene has none. At the mRNA level, eRF3a and eRF3b differ in tissue distribution and in expression during cell cycle progression (*22*, *23*). The encoded proteins, eRF3a and eRF3b, share 87% identity with a striking difference in the amino-terminal region and highly homology in their carboxy-terminal domains. All those proteins exhibit GTP binding motifs (G1–G4) similarly to GTP binding motifs of other G proteins. GSPT1/eRF3a is the major factor acting in translation termination in mammals and clearly demonstrates that eRF3b can substitute for eRF3a in this function (*21*).

Despite extensive investigation the complete understanding of eRF3 function been hampered by the lack of monoclonal antibodies (Mabs). The availability of the Mab, which recognizes specific epitopes allow studying the protein functions. One of the approaches of the investigation of the protein functions is based on its cellular localization. The goal of the present study is production of recombinant human translation termination factor RF3a/GSPT1, development of the highly specific Mab against RF3a/GSPT1, characterization of its binding site and cellular localization of RF3a/GSPT1 by immunofluorescence using newly developed antibody.

Materials and Methods

Plasmid constructs coding for the eRF3a mutants were prepared in Engelhardt Institute of Molecular Biology, Moscow, RF as described (*24*). Plasmid constructs coding for human eRF3a, human eRF3b and their expression in *Escherichia coli* were performed in Engelhardt Institute of Molecular Biology, Moscow, RF as described (*25*).

Anti-eRF3 serum (directed against *Xenopus laevis* eRF3 missing the first 74 amino acid residues) was kindly provided by Dr G. Zhouravleva, St Petersburg State University, RF (*26*).

Rabbit polyclonal anti-S3A ribosomal protein antibody was obtained from Abnova, USA.

Monoclonal anti-β-tubulin antibody and isotype-specific kit for antibody characterization were purchased from Sigma Aldrich. Fluorescence labelled secondary antibodies were purchased from Jackson Immunoresearch Laboratories (PA, USA). RPMI, HAM's F12 media, Trypsin/EDTA and fetal calf serum (FCS) were obtained from Invitrogen.

Vectashield mounting medium containing DAPI was obtained from Vector Laboratories, Inc., CA, USA. Kit for bicinchoninic acid-based protein (BCA) assay was purchased from Interchim, France. Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Little Chalfont, UK. Unless otherwise specified all other chemical reagents were purchased from Sigma Aldrich.

Cell culture

The cell lines were purchased from American Type Culture Collection (ATCC). Human HeLa cells (ATCC CCL-2^{TM} cervical adenocarcinoma cell line) were grown at 37°C in $RPMI_{1640}$ medium supplemented with 10% FCS, streptomycin (100 mg/l) and penicillin (16 mg/l), 2 mM L-glutamine. A549 cell line (ATCC CCL 185, human type II pneumocytes) (*27*) was maintained in Kaighn's modified HAM's F12 medium supplemented with 10% FCS, streptomycin (100 mg/l) and penicillin (16 mg/l), 2 mM L-glutamine and 1.5 g/l sodium bicarbonate. Trypsin/EDTA was used for sub-culturing of the cells.

Cell extract preparation

Harvested cells were suspended in buffer containing 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 1 mM PMSF supplemented with 50 M leupeptine, 50 M pepstatin, 50 M chymostatin and 50 mM NaF. The cell suspension was disrupted by sonication at 4°C and centrifuged at 15,000 g (Beckman) for 15 min. The protein concentration was measured by BCA protein assay. The supernatant was used for immunoprecipitation and western blot analysis.

Expression and production of the C-terminal part of human eRF3a

Plasmid construction. Plasmid pGEX2T/heRF3C encoding C-terminal part of human GSPT1/eRF3a (from 367 to 637 amino acid) was constructed by sub-cloning of a BamH1–EcoR1 fragment of eRF3a cDNA (kindly provided by Dr Jean-Jean O, Curie University, Paris, France) containing 887 bp from nucleotide 1332 to 2169 (including non-translating region). Human eRF3aC cDNA was amplified by PCR with Tag polymerase (Promega) using as a forward primer, 5′-ccggatcctggagcaatgagag-3′, (BamH1 underlined) and as a reverse primer, 5′-ccgaattctgcagggtcatca-3′ (EcoR1 underlined). The amplified PCR product was purified by QIAquick PCR purification kit (Qiagen) and inserted in frame into BamH1–EcoR1 restriction sites of the pGEX2T vector, designed for the expression of glutathione-*S*-transferase (GST) fusion. The sequence of the sub-cloned insert was verified by DNA sequencing (Genset SA).

Expression and purification of the GST-heRF3C protein

BL21(DE3) strain of *E. coli* was transformed with pGEX2T/heRF3aC plasmid and grown in 100 ml of LB medium in the presence of 50 g/ml of ampicillin at 37°C until OD_{600} reached 0.6. Overexpression of the recombinant GST-heRF3aC protein was induced using 1 mM IPTG at 25°C for 6 h. Purification of GST-heRF3aC, which was predominantly present in the inclusion bodies, was performed as described (*28*). Briefly, cells were harvested by centrifugation and resuspended in 5 ml of a solution containing 50 mM Tris 7.5, 1 mM EDTA, 100 mM NaCl and 2 mg of lysozyme for 20 min at 4°C. Afterwards, 5 ml of Triton X-100 was added and the mixture was incubated for 10 min, following the addition of 1 mg of DNAse and incubation for 20 min at room temperature. After centrifugation at 12,000 *g* for 10 min at 4°C (Beckman) the pellet was resuspended in 1.25 ml of 0.1 M Tris–HCl, pH 8.5, containing 6 M urea and the sample was centrifuged for 15 min at 10,000 *g*. The supernatant was diluted 1 : 10 with 50 mM KH_2PO_4 (pH 10.7), 1 mM EDTA (pH 8.0), 50 mM NaCl and incubated at the room temperature for 30 min. During the incubation, the pH of the solution was maintained at 10.7. For protein renaturation the pH was adjusted to 8.0 and the incubation was continued for 30 min. After centrifugation at 10,000*g* for 15 min GST-heRF3C protein was purified by affinity chromatography using Glutathione-Sepharose 4B column. For the expression of GST protein, BL 21 (DE3) strain of *E. coli* was transformed with pGEX2T and grown in 100 ml of LB medium in the presence of 50 mg/ml of ampicillin at 37°C for 3 h. The protein was purified using a Glutathione-Sepharose 4B column.

Expression of GSPT1/eRF3a mutants

The preparation of plasmid constructs and the expression of deletion GSPT1/eRF3a mutants ($eRF3a^{2–478}$, $eRF3a^{531–637}$, $eRF3a^{471–617}$, $eRF3a^{478–637}$ and $eRF3a^{139–606}$) was described in detail in the previous publication (*24*). The numbers indicate the remaining amino acids of full-size GSPT1/eRF3a that were included in the deletion mutant. The pGADGH/eRF3a plasmid constructs expressing corresponding GSPT/eRF3a as a C-terminal part of the fusion protein with the yeast GAL4 transactivation domain (GAL4AD) have been described beforehand (*24*). For the expression of GSPT1/eRF3a mutants the yeast cells were transformed with a construct expressing GAL4AD fused to one of the deletion mutants of GSPT1/eRF3a.

Transformants were selected on leucine-deficient medium and grown for 36 h at 30°C until OD reach 0.6. After centrifugation at 4000 g (Beckman) for 5 min, the pellet was washed twice with 1 ml of H_2O, then 50 µl of the buffer containing Tris 20 mM pH 7.5, 50 mM Nacl, 10 mM MgCl, 2 mM DTT, 1 mM PMSF supplemented with 50 M leupeptine, 50 M pepstatin, 50 M chymostatin and 50 mM NaF was added and vortexed with washed beads three times for 30 s. After centrifugation at 4000 g (Beckman) for 10 min at 4°C the volume 50 µl of supernatants containing 2 mg/ml of proteins were mixed with 12.5 µl of 3× of Laemmli solution and used for western blot analysis. The deletion GSPT1/eRF3a mutant proteins consist of the part of GSPT1/eRF3a fused to Gal4AD.

Development of monoclonal anti-human GSPT1/eRF3a antibody

Immunization, fusion and selection of Mabs. Animal experiments were carried according to the ethical standards formulated in the Declaration of Helsinki. An emulsion of equal volumes of GST-heRF3aC and Freund's complete adjuvant was injected twice at weekly intervals intraperitoneally into Balb/c mice (2 mg/mice). At 10, 11 and 12 days mice were boosted with 4 mg of GST-heRF3aC in PBS. At Day 14, the splenocytes were fused with mouse myeloma X63 Ag8 653 cells as described. The hybridoma clones secreting Mab were identified by indirect Enzyme Linked Immunosorbent Assay (ELISA). To exclude the clones producing anti-GST antibody the hybridoma supernatants were tested as follows: 50 µl of either GST or GST-heRF3aC solution at 200 ng/ml was incubated in the wells of a 96-well microtitre plates for 12 h at 4°C. After washing with PBS—0.05% Tween-20 (PBST) solution, followed by the incubation with 200 µl PBS–1% BSA for 1 h at 37°C, 50 µl of supernatant was added and incubated for 1 h at 37°C. After washing, 50 µl of rabbit peroxidase labelled anti-mouse immunoglobulin were added to the wells for 1 h at 37°C. The reaction was developed by *o*-phenylendiamine (0.5 mg/ml) in citric acid buffer, pH 4.9 in the presence of 0.05% H_2O_2. Absorbance was recorded at 492 nm using a Titertek Multiscan. Hybridoma producing supernatants that interact with GST-heRF3aC protein in the absence of interaction with GST were cloned twice by the method of serial dilution and screened by ELISA.

Purification of Mabs. Mabs were purified from culture supernatant or ascitic fluid (Balb/c mice) by affinity chromatography using Protein G-Sepharose beads. Briefly, the pH of the culture supernatant or ascites was adjusted to 8.0 by using 1 M Tris–HCl (pH 8.0) and passed through the column with beads. The beads were washed with 100 mM Tris–HCl (pH 8.0) and then with 10 mM Tris–HCl (pH 8.0). Mabs were eluted with 100 mM glycine (pH 3.0) and the pH was neutralized using 1 M Tris–HCl, pH 8.0.

Isotype determination. The Mab isotypes were determined by the radial immunodiffusion method using isotype-specific kit (*29*). Briefly, a hot solution of 2% agarose in Tris barbital buffer was mixed with 6% solution of PEG and poured on a glass plate. Holes were punched in a circular pattern around a central hole. The amount of 10 µl of purified Mabs (50 µg/ml) was placed in a round centre well and 5 µl isotype-specific reagents were placed in surrounding wells. The antigen–antibody reaction was then noted by the visible immunoprecipitin bands at the point of reactivity. The isotype specificity was verified by indirect ELISA using isotype-specific kit (Sigma). Briefly, 100 µl of purified Mabs (50 µg/ml) was adsorbed in the well of micro plate. The immobilized antibody was then probed with unlabeled isotype-specific antisera followed by enzyme-tagged secondary antibody.

Western blot analysis

Western blot analysis was performed as previously described (*30*). The cell extracts were prepared as mentioned above. Equal amounts of protein were mixed with two times of Laemmli sample buffer (62.5 mM Tris–HCl pH 6.8; 8.4% SDS, 5% mercaptoethanol, 8.5% glycerol, 0.25% bromophenol blue) and maintained at 100°C for 7 min before loading 20 µg of cell extract per lane onto 15% SDS–polyacrylamide gels. The proteins were separated by electrophoresis according to Laemmli (*31*) and then electrophoretically transferred onto a 0.2 µm Immuno-Blot PVDF membrane. The rabbit serum or the newly developed anti-eRF3 Mabs were applied for 4 h followed by incubation for 1 h either with goat anti-rabbit or rabbit anti-mouse polyclonal antibody (1 : 1000) coupled with peroxidase. ECL reagents were used for visualization of the immunoreactive protein bands. To verify the quantity of loaded protein, each membrane was re-blotted with a mouse monoclonal anti-β-tubulin antibody. Western blot analysis was performed three times using samples from three different and independent experiments. Only films representing equal amount of the proteins, as shown by the anti-β-tubulin antibody, were selected for further analysis.

Immunoprecipitation

One of the developed Mab or normal mouse immunoglobulins (50 µg/10 µl) were pre-incubated with 20 µl of Protein A-Sepharose (Invitrogen, CA, USA) for 1 h at 4°C. Following washing with PBS, the beads were added to 500 µl of the cell extract and incubated overnight at 4°C. Then the beads were washed three times with the buffer containing 50 mM of Tris–HCl, pH 7.4, 100 mM NaCl, 0.1% NP40, 1 mM DTT, supplemented with 1 mM PMSF, 50 M leupeptine, 50 M pepstatin, 50 M chymostatin and 50 mM of NaF, centrifuged for 5 min at 12,000 *g* and analysed by western blotting. The other one of developed Mab was used as the primary antibody.

Immunofluorescence and co-localization

Immunofluorescence was performed as previously described (*32*). Briefly, either A549 or HeLa cells were placed on glass slides and were grown overnight. The cells were fixed in a freshly made solution containing two volumes of formaldehyde, 19 volumes of acetone and 19 volume of MeOH for 20 min at −20°C. The slides were then incubated in 3% BSA, followed by a solution of 10% normal goat serum. After washing, primary monoclonal anti-GSPT1/eRF3a (1 : 300, 10 µg/ml) were applied for 4 h at room temperature, followed by incubation with fluorescein isothhiocyanate conjugated rabbit anti-mouse (Jackson Immunoresearch Laboratories, PA, USA) for 2 h. In some experiments, only the secondary antibody was used as a negative control. The slides were viewed with a fluorescence microscope using standard fluorescein isothiocyanate and rhodamine filter sets after staining of the nuclei with 25 µl of Vectashield mounting medium containing DAPI (Vector Laboratories, Inc, CA, USA). For co-localization experiments with anti-GSPT1/eRF3a Mab, rabbit anti-S3A ribosomal protein polyclonal antibody (1 : 300, Abnova) was used to stain for endoplasmic reticulum. Afterwards, Texas Red-conjugated goat anti-rabbit antibodies (1 : 400, Jackson Immunoresearch Laboratories, PA, USA) were applied for 2 h and then the slides were stained and observed using a fluorescence microscope as described above.

Molecular modelling

The modelling and display of amino acids of interest were realized using molecular modelling tools. The representations were deduced from crystallographic data for C-terminal part of *S. pombe* eRF3 (215–662 amino acid residues), accession code 1R5B in Protein Data Bank (*19*) and from crystallographic data for complex of human eRF1 and C-terminal part of human GSPT1/eRF3a (440–637 amino acid residues), accession code 3E1Y in Protein Data Bank, using the life science modelling and simulation suite of applications Discovery Studio 2.1 (Accelrys Inc., San Diego, USA, 2008).

Results

Purification of recombinant GSPT1/eRF3a

A plasmid encoding C-terminal part of human GSPT1/eRF3a (from 367 to 637 amino acids) (Fig. 1) was constructed and used for the expression and purification. The sequence of the human GSPT1/eRF3a and the region corresponding to the C-terminal domain from 367 to 637 amino acids (highlighted in grey colour) are presented on Fig. 1. Electrophoresis of *E. coli* lysates with plasmid pGEX2T/heRF3aC, carrying C-terminal part of the protein revealed a 54 kDa protein band in the lysates of transfected cells treated with IPTG. The GST moiety adds ~26 kDa to the molecular mass. The molecular weight of the expressed protein

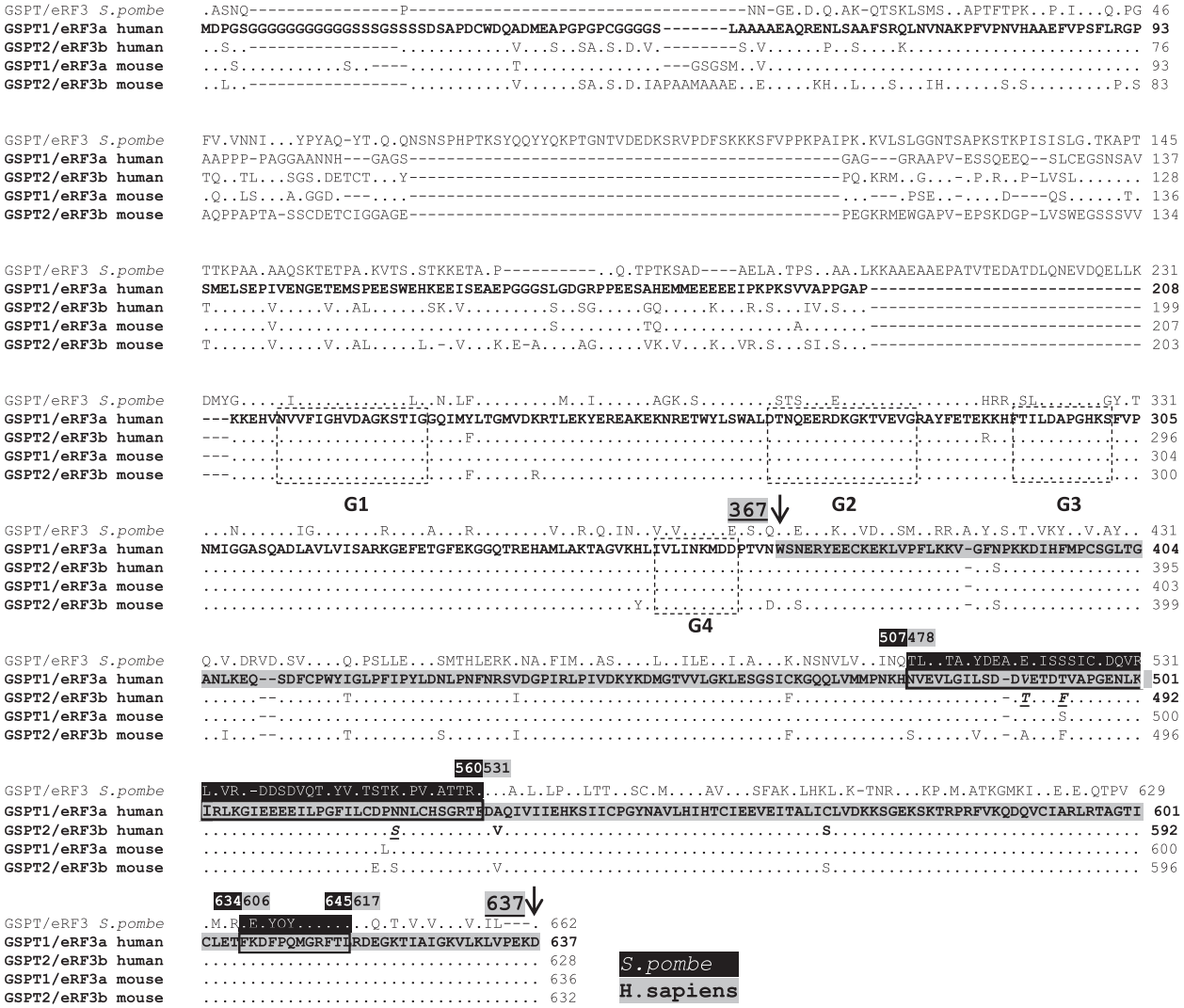

Fig. 1 Alignment of the amino acid sequences of *S. pombe*, human and mouse GSPT/eRF3 proteins. The alignment of the amino acid sequences of *S. pombe* eRF3 (NP_588225.1), human GSPT1 (NP_002085.2), human GSPT2 (NP_060564.2), mouse GSPT1 (NP_666178.2) and mouse GSPT2 [NP_ 032205.2 proteins (*35*)] was optimized by computer analysis (T-coffee Multiple Sequence Alignment Program). Identical amino acids are shown by dots. The conservative domains of human and mouse G1–G4 involved in GTP binding are indicated by dashed boxes. The C-terminal part of GSPT1/eRF3a protein (from 367 to 637 amino acid residues) used for the immunization is highlighted in grey colour. The regions of human GSPT1/eRF3a (from 478 to 531 and 606 to 617 amino acid residues) containing the epitopes recognized by Mabs 14 and 16 are indicated by the black frame. The regions of *S. pombe* eRF3 from 507 to 560 and from 634 to 645 amino acid residues corresponding to the regions from 478 to 531 and from 606 to 617 amino acid residues for human GSPT1/eRF3a are highlighted in black colour. Amino acid residues of GSPT2/eRF3b, which are different from those of GSPT1/eRF3a in the region from 478 to 531, are underlined.

corresponded to molecular weight deduced from amino acid sequence of fusion eRF3aC-GST protein. The protein band with the same molecular weight was absent in cell extract from the non-induced or non-transformed cells (data not shown). Purification of eRF3aC-GST (shown as eRF3a-GST), which was predominantly present in the inclusion bodies, was performed as described in 'Material and Methods' section. After purification the 54 kDa eRF3a-GST protein was more than 95% pure as assessed by SDS–PAGE and Coomassie blue staining (Fig. 2A).

Hybridoma characterization

The sequences of human and mouse GSPT/eRF3 are aligned in Fig. 1. Since there is a high homology between human and mouse C-terminal GSPT1/eRF3a protein, which consequently renders human eRF3aC a weak immunogen, the immunogenicity of human eRF3aC was increased by utilizing fusion GST-eRF3a protein. From an initial screening of 1000 hybridoma cultures, 5 clones showed immunoreactivity with GSPT1/eRF3a and absence of the reaction with GST by ELISA and western blot analysis. The isotypes of the 4 Mabs (Mabs 14, 16, 81 and 116) corresponded to IgG and the fifth Mab (Mab 21) corresponded to IgM as determined by the radial immunodiffusion method and indirect ELISA. Mabs 81, 116 and 21 with low efficiency in ELISA and western blot analysis have not been considered for the further analysis. Mab 14 with IgG_{2b} isotype and Mab 16 with IgG_1 isotype were selected for the present work.

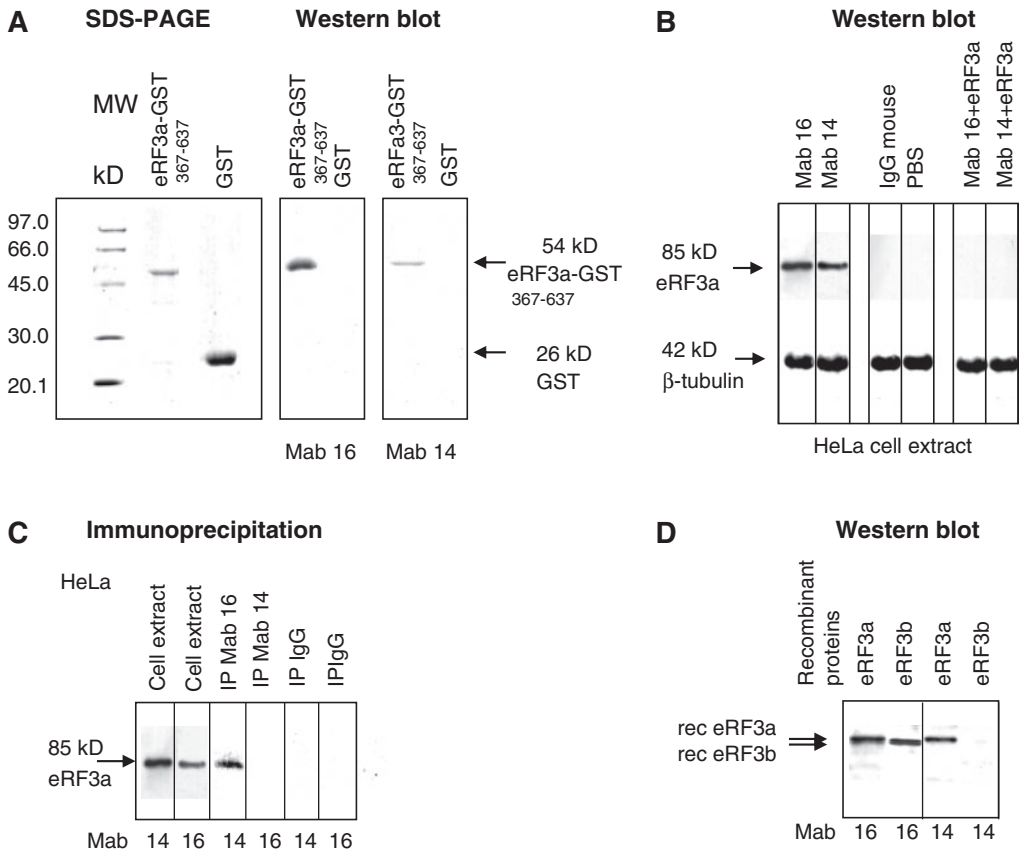

Fig. 2 Preparation of recombinant eRF3a-GST protein and characterization of Mabs 14 and 16. (A) Preparation of recombinant eRF3-GST protein. Construction of plasmid pGEX2T/heRF3aC, encoding C-terminal part of human GSPT1/eRF3a from 367 to 637 amino acids (shown in the figure as eRF3a-GST), protein expression and purification from the inclusion bodies was achieved as described in 'Materials and Methods' section. Twelve per cent SDS–PAGE of purified eRF3a-GST and GST proteins followed by Coomassie blue staining (A) revealed 54 and 26 kDa bands, respectively (arrows at the right side). At the left side molecular weight standards are shown. Western blot analysis of purified eRF3a-GST protein either Mab 16 or Mab 14 identified single 54 kDa band corresponding to eRF3a-GST protein. The arrows at the right side indicate 54 kDa eRF3a-GST and 26 kDa GST. A representative result from four experiments is shown. (B) Western blot analysis of cell extract with Mabs. The cell extract was prepared as described in 'Materials and Methods' section. Western blot analysis of HeLa cell extract with Mabs 16 and 14 revealed the single 85 kDa band (arrow at the left side). No protein bands were detected when the normal immunoglobulin or PBS were used instead of Mab. Pre-incubation of the purified Mab with 10-fold excess of recombinant eRF3a resulted in the abolition of 85 kDa band. The membrane was re-blotted with anti-β-tubulin antibody; the arrow at the left indicates β-tubulin. One representative result from four experiments is shown. (C) Immunoprecipitation of cellular GSPT1/eRF3a by Mabs. The extract of HeLa cells was pre-incubated either with Mab 16 or Mab 14, or normal mouse immunoglobulin bound to Protein A Sepharose. Resulting immunoprecipitates were examined by western blot analysis with the help of another anti-eRF3 Mab as described in 'Materials and Methods' section. In the control row, the whole-cell extract was loaded. The arrow at the left side indicated cellular 85 kDa GSPT1/eRF3a. (D) Western blot analysis of recombinant GSPT1/eRF3a and GSPT2/eRF3b with Mabs. Plasmid constructs coding for human GSPT1/eRF3a, human eGSPT1/RF3b and their expression in *E. coli* were performed in Engelhardt Institute of Molecular Biology, Moscow, RF as described (*25*).Western blot analysis of recombinant proteins GSPT1/eRF3a and GSPT2/eRF3b with Mabs 14 and 16 was performed as described in 'Material and Methods' section. The arrows at the left side indicated GSPT1/eRF3a and GSPT2/eRF3b.

Western blot analysis of GST-eRF3a protein using either Mab 16 or Mab 14 revealed a band with apparent MW of 54 kDa corresponding to the GST-eRF3a fusion protein. None of the antibodies recognize GST (Fig. 2A).

Mabs 14 and 16 recognize GSPT1/eRF3a from the cells extracts

Western blot analysis of extracts prepared from HeLa cells using Mabs 14 and 16 showed that both Mabs recognize a single band with an apparent molecular weight of 85 kDa corresponding to the human GSPT1/eRF3a. Molecular weight of detected GSPT1/eRF3a in western blot analysis is higher than theoretically calculated one and corresponds to the observation of others (*33*). No band was observed when normal IgG was used as the primary antibody. Replacement of the antibody by PBS resulted in the disappearance of the specific band. Pre-incubation of the Mabs with 10-fold excess of recombinant GST-eRF3a abolished the specific signal (Fig. 2B). Similar results were obtained with A549 cells (data not shown).

To further characterize the Mabs, we tested if Mabs 14 and 16 could immunoprecipitate GSPT1/eRF3a. As shown on Fig. 2C, Mab 14 detected the single band with the apparent molecular weight of 85 kDa band corresponding to GSPT1/eRF3a in the immunoprecipitate from HeLa cells extract incubated with Mab 16. Mab 16 does not detect any band in the immunoprecipitate of the cell extract incubated with Mab 14. No band was observed in the immunoprecipitate from HeLa cells extract incubated with normal IgG (Fig. 2C).

Similar results were obtained with A 549 cells (data not shown).

Analysis of the regions recognized by Mabs 14 and 16

Western blot analysis of full-length recombinant proteins GSPT1/eRF3a and GSPT2/eRF3b using Mabs show that Mab 16 recognize both GSPT1/eRF3a and GSPT2/eRF3b, while Mab 14 recognize only GSPT1/eRF3a but not GSPT2/eRF3b (Fig. 2D).

In order to characterize the regions recognized by Mabs, a western blot analysis of mutant GSPT1/eRF3a proteins fused to Gal4AD expressed in yeast was performed. The construct containing the full-size GSPT1/eRF3a protein served as the positive control. As shown in Fig. 3, both Mabs recognize full-size eRF3a, eRF3a$^{478–637}$ and eRF3a$^{471–617}$mutants. These results clearly show the region recognized by both Mabs is localized in C-terminal part of GSPT1/eRF3a between 478 and 617 amino acid residues. None of the Mabs interact with eRF3a$^{2–478}$ and eRF3a$^{531–637}$mutants. The absence of the interaction cannot be due to the low expression of the protein, since rabbit anti-eRF3 serum recognizes eRF3a$^{2–478}$ and eRF3a$^{531–637}$mutant in western blot analysis (Fig. 3). These results suggest that Mabs 14 and 16 recognize the C-terminal part of GSPT1/eRF3a up to 531 amino acid residues. Taking collectively the results of western blot analysis with the deletion mutants show that the regions recognized by both Mabs 16 and 14 are localized between 478 and 531 amino acid residues. The difference in the interaction of two Mabs was observed in the eRF3a$^{139–606}$ mutant. In contrast to Mab 16 recognizing eRF3a$^{139–606}$ mutant, the interaction between this eRF3a$^{139–606}$ mutant and Mab 14 was much weaker (Fig. 3). The difference in the recognition of eRF3a$^{139–606}$ mutant by both Mabs may be explained by the existence of the functional epitope recognized by Mab 14, which includes two distant parts localized within 478–531 and 606–617 amino acid sequences.

Visualization of the epitopes using crystal structure of human and S. pombe eRF3 C-terminal parts

To verify whether epitope recognized by Mab 14 consists of two distant amino acid sequences, regions from 478 to 531 and from 606 to 617 amino acid residues were visualized in 3D space. The crystal structure of human eRF1 in complex with the C-terminal part of human GSPT1/eRF3a (residues 440–637) has been reported recently, accession code 3E1Y (*34*). Using crystallographic data the molecular modelling allows visualization of the regions corresponding to human eRF3a$^{478–637}$ and eRF3a$^{471–617}$ deletion

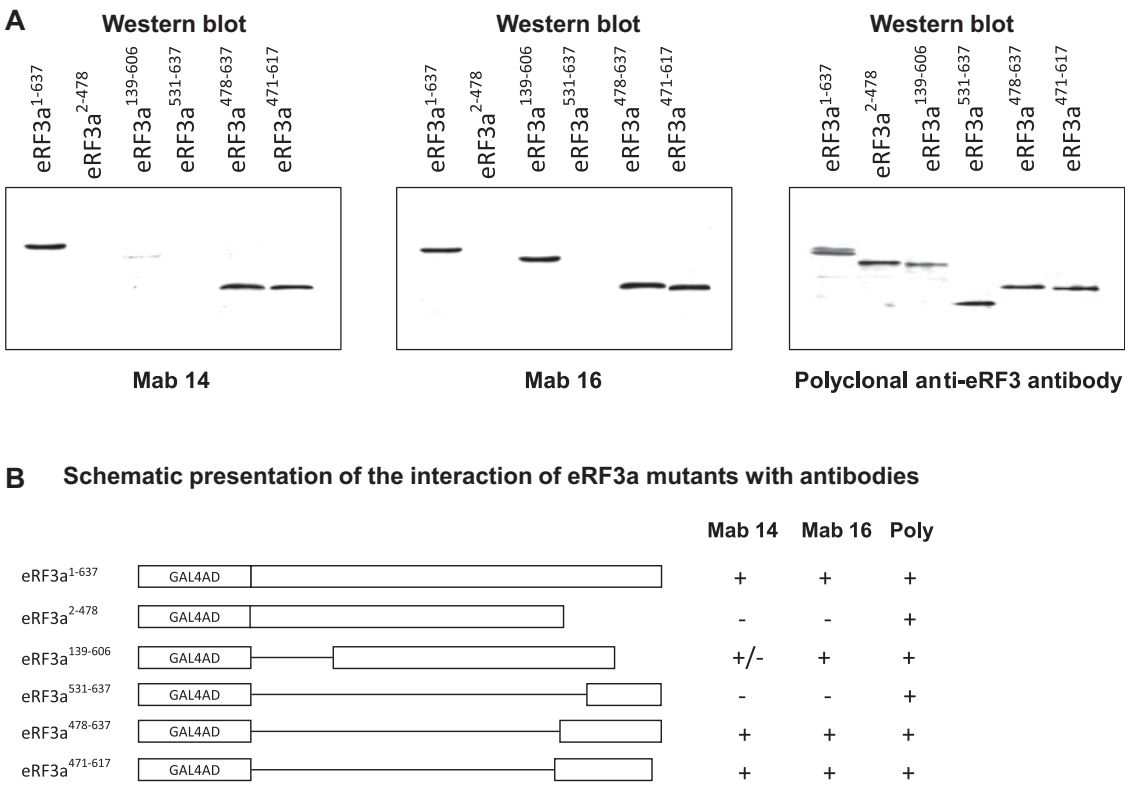

Fig. 3 Interaction of GSPT1/eRF3a deletion mutants with Mabs. (A) Western blot analysis of GSPT1/eRF3a deletion mutants with Mabs 16, 14 or anti-eRF3 serum. Expression of GSPT1/eRF3a mutants and western blot analysis with Mabs 16 and 14 were performed as described in 'Materials and Methods' section. At the left side molecular weight standards are shown. The names of GSPT1/eRF3a mutants are presented on the top. The numbers of mutants indicate the remaining amino acids of full-size GSPT1/eRF3a, which were included in the presented mutant. The arrows indicate immunoreactive bands. (B) Schematic presentation of the interaction of GSPT1/eRF3 mutants with Mabs or anti-eRF3 serum. Graphical images of GSPT1/eRF3a mutants, which were used for the analysis, are shown. Boxes indicate the remaining amino acid sequences. The ability of the various mutants to interact with Mabs 16, 14 or anti-eRF3 serum, as follows from the results of western blot analysis, is indicated on the right.

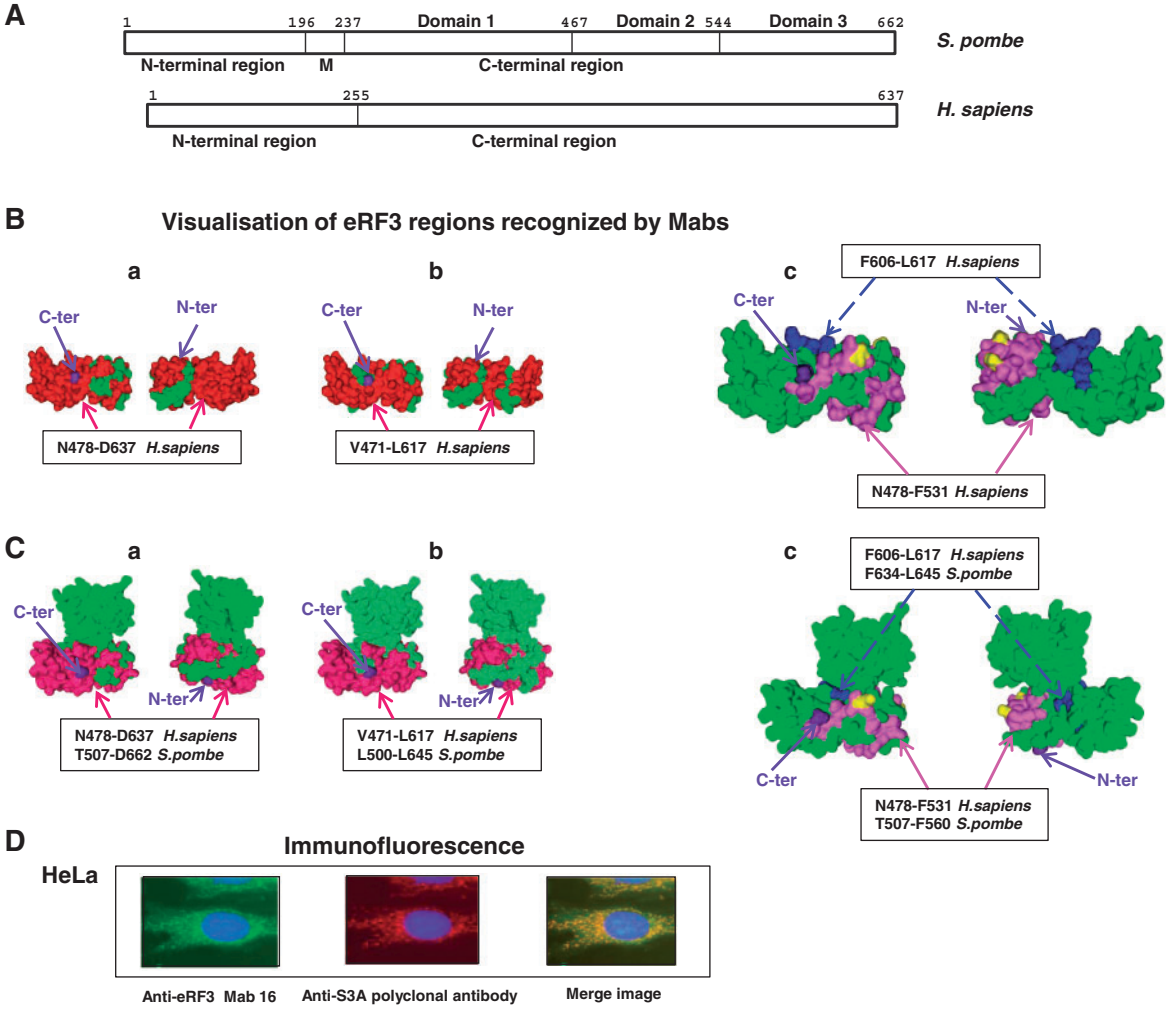

Fig. 4 Visualization of the regions recognized by Mabs in the 3D space. (A) Domain organization of human and *S. pombe* eRF3 proteins. Schematic representation of the domain organization of *S. pombe* and *H. sapiens* GSPT/eRF3 proteins. *S. pombe* eRF3 consists of N, M and C parts and contains 662 amino acid residues. Human GSPT/eRF3 consists of N and C parts and contains 637 amino acid residues. (B) Visualization of the regions of human GSPT1/eRF3a recognized by Mabs. The representations were deduced from crystallographic data for complex of human eRF1 and C-terminal part of human GSPT1/eRF3a (440–637 amino acid residues), accession code 3E1Y in Protein Data Bank. (a, b) Molecular surface of human GSPT1/eRF3a 3D structure show the regions corresponding to human eRF3a$^{478–637}$ and eRF3a$^{471–617}$ deletion mutants recognized by both Mabs (red colour). (c) The regions containing the epitopes recognized by Mabs are presented in magenta for the region from 478 to 531 and in dark blue for the region from 606 to 617 amino acids residues. The panel displays ventral and dorsal views of surface structure. Amino acid residues of GSPT1/eRF3a, which are different from those of GSPT2/eRF3b in the region from 478 to 531 amino acid residues, are shown in yellow colour. The positions of N-terminal (440) and C-terminal (637) amino acid residues are indicated by the arrow. The C-terminal amino acid residue is shown in violet colour; the N-terminal amino acid residue is not visible in proposed orientation of the molecule. (C) Visualization of *S. pombe* eRF3 regions, which correspond to human GSPT1/eRF3a regions recognized by Mabs. The representations were deduced from crystallographic data for C-terminal part of free protein *S. pombe* eRF3 (215–662 amino acid residues), accession code 1R5B in Protein Data Bank. (a, b) Molecular surface of eRF3 3D structure show the regions, which correspond to the regions of human eRF3a$^{478–637}$ and eRF3a$^{471–617}$ deletion mutants recognized by both Mabs (red colour). (c) The regions of *S. pombe* eRF3 corresponding to the regions of human GSPT1/eRF3a, which contain the epitopes recognized by Mabs, are presented in magenta for the region from 478 to 531 amino acids and in dark blue for the region from 606 to 617 amino acids. The panel displays ventral and dorsal views of surface structure. The amino acid numbers of *S. pombe* eRF3, which corresponds to the amino acid numbers of human GSPT1/eRF3a are indicated. Amino acid residues of GSPT1/eRF3a, which are different from those of GSPT2/eRF3b in the region from 478 to 531 amino acids, are shown in yellow colour. The positions of N-terminal (215) and C-terminal (662) amino acid residues (shown in violet colour) are indicated by the arrow. (D) Intracellular localization of GSPT1/eRF3a using Mabs. HeLa cells were grown to confluence on the glass slides. After fixation in formaldehyde/acetone/MeOH solution at –20°C, the cells were treated with Mab 16, then polyclonal anti-S3A ribosomal protein antibody, followed by the incubation with FITC-conjugated rabbit anti-mouse afterwards with Texas Red-conjugated goat anti-rabbit antibodies. The slides were viewed with a fluorescence microscope with standard fluorescein isothhiocyanate and rhodamine filter sets using 400× magnifications. Merge image indicated endoplasmic reticulum localization of GSPT1/eRF3a. One representative experiment from four triplicate experiments is shown.

mutants recognized by both Mabs (red colour) [Fig. 4B(a, b)]. The region 139–606 amino acids, which corresponds to eRF3a$^{139–606}$ deletion mutant cannot be visualized since the crystal structure of the full size eRF3 is not yet solved. The structure of the C-terminal part of human GSPT1/eRF3a [Fig. 4B(c)] shows two regions recognized by Mabs (deduced from the results of immunoblotting with deletion

mutants): from 478 to 531 (magenta colour) and from 606 to 617 amino acid residues (blue colour). It reveals their adjacent position in 3D space.

Taking into account the interaction of Mab 16 with recombinant GSPT1/eRF3a and GSPT2/eRF3b, while the interaction of Mab 14 only with GSPT1/eRF3a, we have identified three amino acids, which are different for both proteins sequences in the region between 478 and 531 amino acid residues. V489 of eRF3a is replaced by T480 of eRF3b; T493 of eRF3a is replaced by F484 of eRF3b and N522 of eRF3a is replaced by S513 of eRF3b. These three amino acids are indicated by underlined letters in italic on Fig. 1 and marked in yellow colour on Fig. 4B(c). Nevertheless, it was suggested that GSPT1/eRF3a undergoes conformational changes upon binding to eRF1, thus we use the crystal structure of free protein *S. pombe* C-terminal part of eRF3, which was solved recently, accession code 1R5B (*19*) to confirm our observations of adjacent position of 478–531 and 606–617 amino acid residues regions in 3D space. Taking into account the high similarity between the C-terminal parts of eRF3 from *S. pombe* and human, we made a hypothesis that the 3D structures of these two proteins should be similar. The knowledge of crystallographic structure of the C-terminal part of *S. pombe* GSPT/eRF3 (residues 215–662) and multiple sequence alignment allows to visualize the regions, which correspond to the regions of the human GSPT1/eRF3a (478–637, 471–617, 478–531 and 606–617 amino acid residues) recognized by both Mabs. Taking into account the difference between the lengths of *S. pombe* eRF3 (662 amino acids residues) and the human GSPT1/eRF3a (637 amino acids residues) in addition to the high homology in the C-terminal part of the molecules (Fig. 1), multiple sequence alignment was used to determine the positions of the amino acids of regions recognized by newly developed Mabs. Consequently, there is a shift in the number of *S. pombe* and corresponding human amino acids residues (Fig. 1) (*19*). 3D structure of the C-terminal part of *S. pombe* eRF3 [Fig. 4B(a, b)] show the regions, which correspond to the regions of human eRF3a$^{478–637}$ and eRF3a$^{471–617}$ deletion mutants recognized by both Mabs (red colour). The region, which corresponds to eRF3a$^{139–606}$ deletion mutant cannot be visualized since the crystal structure of the full size eRF3 is not yet solved. Visual representation of the regions corresponding to the regions of human GSPT1/eRF3a recognized by Mabs 14 and 16: from 478 to 531 (in blue colour) and from 606 to 617 amino acids residues (in magenta colour) is shown in Fig. 4C(c). It confirms their adjacent position in 3D space. Amino acid residues of GSPT1/eRF3a, which are different from those of GSPT2/eRF3b in the region corresponding to the region of human GSPT1/eRF3 from 478 to 531 amino acid residues, are indicated by the yellow colour [Fig. 4B(c)].

Sub-cellular immunofluorescence localization of GSPT1/eRF3a using our newly developed Mabs

To determine the sub-cellular localization of GSPT1/eRF3a, we stained the cells with the newly developed Mabs. Immunofluorescence with either Mab 16 (Fig. 4D) or Mab 14 (data not shown) revealed punctuated perinuclear staining of GSPT1/eRF3a in HeLa cells, characteristic of endoplasmic reticulum localization (Fig. 4D). Pre-treatment of the Mab with 10-fold excess of recombinant eRF3a resulted in abolishing of the staining; immunofluorescence only with second antibody did not show any staining (data not shown). To confirm ER localization of GSPT1/eRF3a with our developed Mabs, double immunostaining with Mabs and ER marker anti-S3A ribosomal protein polyclonal antibody was performed. As shown in Fig. 4D, complete overlap between staining of GSPT1/eRF3a with Mabs and anti-S3A antibody staining was observed. Similar results were obtained with A549 cells.

Discussion

The functional C-terminal region of GSPT/eRF3 comprises GTP-binding domain (Fig. 1, G1–G4 domains) (*35*). The crystal structure of human eRF1 in complex with the C-terminal part of human GSPT1/eRF3a (440–637 amino acid residues) and the crystal structure of the C-terminal part (215–662 amino acid residues) of free protein eRF3 of the yeast *S. pombe* has been described recently (*19*, *34*). The availability of the crystal structure of the C-terminal part of GSPT1/eRF3 allowed to obtain the tools for the study of the molecular mechanisms of this release factor in translation termination.

The C-terminal part of eRF3 of yeast *S. pombe* can be further divided into three domains. Domain 1 (residues 237–467) represents the GTPase domain that binds the guanine nucleotide, and in common with other GTPases. Domain 1 is connected to domain 2 (residues 468–554) by a long stretch of peptide with a single helical turns in the middle. Domain 2 is connected to domain 3 (residues 555–662) by a short extended stretch of peptide. We utilized the 3D structure of human (from the complex with eRF1) and *S. pombe* free protein eRF3 for the visualization of the regions recognized by the newly developed Mabs against human GSPT1/eRF3a.

One of the aims of this work was to develop Mabs, which specifically recognize human GSPT1/eRF3a. To reach this goal we constructed plasmid pGEX2T/heRF3ac coding for C-terminal part of a human GSPT1/eRF3a. Expressed in *E. coli* fusion protein contains GST part linked to C-terminal part of GSPT1/eRF3a (eRF3a-GST). Since there is a high homology between human and mouse C terminal GSPT1 (eRF3), which consequently determined human C-terminal part of eRF3 as a weak immunogen, its immunogenity was increased by using purified fusion eRF3a-GST protein for immunization. Additionally, we used the method for hyper immunization of mice, which also augmented the immunogenity of the protein (*36*). It was shown that Mab 16 recognized both recombinant proteins GSPT1/eRF3a and GSPT2/eRF3b, while Mab 14 recognized only GSPT1/eRF3a, additionally both Mabs recognized GSPT1/eRF3a in extracts from human cells. However, we detected the band only in the cell extracts precipitated by Mab 16. This result is consistent with the observation of Chauvin *et al.* (*20*).

They reported that endogenous eRF3b could not be detected by anti-eRF3b antibody in any human cell lines, because eRF3b protein may be expressed at a low level in all cell types. The analysis of interaction of eRF3a mutants with Mabs 14 and 16 allows to characterize the binding sites recognized by these antibody. It was observed that N-terminal part of GSPT1/eRF3a, containing up to 478 amino acid residues (eRF3a$^{4–478}$ mutant) was not included in the epitope recognized by both Mabs in contrast to the anti-eRF3 serum, which recognizes eRF3a$^{4–478}$mutant. This is in an agreement with the experimental procedure, since C-terminal part of eRF3a including 360–637 amino acids residues was used for the development of Mabs. The interaction of Mabs 14 and 16 with eRF3a$^{478–637}$mutant and eRF3a$^{471–617}$mutant indicates that epitopes, recognized by both Mabs, are localized in C-terminal part of GSPT1/eRF3a between 478 and 617 amino acid residues. Further from N side eRF3a$^{531–637}$mutant abrogated the binding of both Mabs, while interacts with anti-eRF3 serum. The results suggested that Mabs 14 and 16 recognize the part of eRF3a localized between 478 and 531 amino acid residues. According to the alignment of human GSPT1/eRF3a and GSPT2/eRF3b sequences, there are three different amino acids in the region between 478 and 531 amino acids (Fig. 1). Taking into account that Mab 16 recognizes both GSPT1/eRF3a and GSPT2/eRF3b, while Mab 14 recognizes only GSPT1/eRF3a, we could speculate that these amino acids or some of them may play the important role in the abolishing of the interaction between eRF3b and Mab 14.

Since Mab 16 reacted strongly with eRF3a$^{139–606}$ mutant, while the interaction of Mab 14 was very weak, it suggested that of C-terminal part of eRF3a up to 606 amino acid residues negatively influenced the recognition of this mutant by Mab 14. Since Mab 14 recognizes eRF3a$^{478–617}$ mutant, it appears that the region including the sequence from 606 to 617 amino acid residues is essential for the formation of the epitope recognized by Mab 14. The region which is localized between 478 and 530 amino acid residues may be situated close in space to the region including amino acid sequence from 606 to 617, forming one continuous epitope for Mab 14 (*19*). To verify this hypothesis both regions were localized in 3D space using the crystallographic data of the complex of human eRF1 with the C-terminal part (440–637 amino acid residues) of human GSPT1/eRF3a. Visualization of the two regions (from 478 to 531 and from 606 to 617 amino acid residues) reveals their adjacent position in 3D space (Fig. 4B and C). Taking into account the possibility of the conformational alteration of human GSPT1/eRF3a in the complex with eRF1, the adjacent position of these two sequences was confirmed using the crystal structure of free protein *S. pombe* eRF3. Thus, the data and visualization appears to support the possibility that these sequential regions come together on the surface of the molecule to form one continuous region, which includes the epitope recognized by Mab 14. The structural epitope of a protein usually covers a 5–10 nm^2 (500–1000 Å^2) surface area involving 14–20 amino acid side chains. Only few of these side chains are responsible for the majority of the binding affinity, and the functional epitope may only involve 4–14 antigen side chains. Difference in the capacity Mabs 16 and the 14 to precipitate GSPT1/eRF3a from the cell extract, divergence in the interaction with GSPT1/eRF3a and GSPT2/eRF3b, additionally to the discrepancy in the recognition of GSPT1/eRF3a deletion mutants clearly show that Mabs 16 and 14 recognize the two different functional epitopes. Analysis of the region recognized by Mabs 16 and 14 revealed that Mabs recognize the C-terminal part of eRF3 outside of GTP-binding domains that in turn excludes the recognition of the other G proteins.

Knowledge of the sub-cellular location of a protein reveals the potential role it plays in a variety of cellular processes. Co-localization of studied protein (detected by antibody specifically recognized this protein) and cellular organelle (identified by organelle-specific antibody) using immunofluorescence is powerful tool for protein sub-cellular localization. Consistent with its function in translational termination and its association with ribosomes, immunostaining of the cells with newly developed Mabs and co-immunostaining with ER marker anti-S3A ribosomal protein polyclonal antibody revealed that the endogenous eRF3/GSPT1 localized in endoplasmic reticulum. This result is in agreement with the observation of others, which have demonstrated ER localization of the endogenous GSPT1/eRF3a as well as ER localization of a construct encoding C-terminal FLAG-tagged full-length GSPT1/eRF3a protein (*37*).

Additionally to its role in translation termination, normal and NMD, eRF3 is likely involved in cancer development: overexpression of GSPT1/eRF3a in intestinal type gastric tumours was reported (*22*). Involvement of GSPT1/eRF3a in tumourigenesis may be resulted from an increase in the translation efficiency of specific oncogenic transcripts, cell cycle deregulation or apoptosis alteration (*22*, *37*, *38*). Taking into account the important role of eRF3 for the fundamental research, one can suggests that newly developed Mabs have great prospective to be used as a research reagent in a wide range of applications. These antibodies are also the strong candidates to be investigated for further as potential diagnostic tool in clinic.

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Conflict of interest

None declared.

References

1. Kisselev, L.L. and Buckingham, R.H. (2000) Translational termination comes of age. *Trends Biochem. Sci.* **25**, 561–566
2. Frolova, L., Legoff, X., Rasmussen, H.H., Cheperegin, S., Drugeon, G., Kress, M., Arman, I., Haenni, A.L., Celis, J.E., Philippe, M., Justesen, J., and Kisselev, L. (1994) A highly conserved eukaryotic protein family possessing properties of polypeptide-chain release factor. *Nature* **372**, 701–703
3. Stansfield, I., Jones, K.M., Kushnirov, V.V., Dagkesamanskaya, A.R., Poznyakovski, A.I., Paushkin, S.V., Nierras, C.R., Cox, B.S., Teravanesyan, M.D., and Tuite, M.F. (1995) The products of the *Sup45* (Erf1) and *Sup35* genes interact to mediate translation termination in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 4365–4373
4. Zhouravleva, G., Frolova, L., Legoff, X., Leguellec, R., Ingevechtomov, S., Kisselev, L., and Philippe, M. (1995) Termination of translation in eukaryotes is governed by 2 interacting polypeptide-chain release factors, Erf1 and Erf3. *EMBO J.* **14**, 4065–4072
5. Pisareva, V.P., Pisarev, A.V., Hellen, C.U.T., Rodnina, M.V., and Pestova, T.V. (2006) Kinetic analysis of interaction of eukaryotic release factor 3 with guanine nucleotides. *J. Biol. Chem.* **281**, 40224–40235
6. Kononenko, A.V., Mitkevich, V.A., Dubovaya, V.I., Kolosov, P.M., Makarov, A.A., and Kisselev, L.L. (2008) Role of the individual domains of translation termination factor eRF1 in GTP binding to eRF3. *Proteins:Struct. Funct. Bioinf.* **70**, 388–393
7. Hauryliuk, V., Zavialov, A., Kisselev, L., and Ehrenberg, M. (2006) Class-1 release factor eRF1 promotes GTP binding by class-2 release factor eRF3. *Biochimie* **88**, 747–757
8. Hauryliuk, V., Hansson, S., and Ehrenberg, M. (2008) Cofactor dependent conformational switching of GTPases. *Biophys. J.* **95**, 1704–1715
9. Cosson, B., Berkova, N., Couturier, A., Chabelskaya, S., Philippe, M., and Zhouravleva, G. (2002) Poly(A)-binding protein and eRF3 are associated in vivo in human and *Xenopus* cells. *Biol. Cell* **94**, 205–216
10. Hoshino, S., Imai, M., Kobayashi, T., Uchida, N., and Katada, T. (1999) The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3′-poly(A) tail of mRNA - Direct association of eRF3/GSPT with polyadenylate-binding protein. *J. Biol. Chem.* **274**, 16677–16680
11. Kozlov, G. and Gehring, K. (2010) Molecular basis of eRF3 recognition by the MLLE domain of poly(A)-binding protein. *PloS One* **5**, e10169
12. Ivanov, P.V., Gehring, N.H., Kunz, J.B., Hentze, M.W., and Kulozik, A.E. (2008) Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. *EMBO J.* **27**, 736–747
13. Kobayashi, T., Funakoshi, Y., Hoshino, S., and Katada, T. (2004) The GTP-binding release factor eRF3 as a key mediator coupling translation termination to mRNA decay. *J. Biol. Chem.* **279**, 45693–45700
14. Amrani, N., Ghosh, S., Mangus, D.A., and Jacobson, A. (2008) Translation factors promote the formation of two states of the closed-loop mRNP. *Nature* **453**, 1276–1U85
15. Atkinson, G.C., Baldauf, S.L., and Hauryliuk, V. (2008) Evolution of nonstop, no-go and nonsense-mediated mRNA decay and their termination factor-derived components. *BMC Evol. Biol.* **8**, 290
16. Teravanesyan, M.D., Kushnirov, V.V., Dagkesamanskaya, A.R., Didichenko, S.A., Chernoff, Y.O., Ingevechtomov, S.G., and Smirnov, V.N. (1993) Deletion analysis of the *Sup35* gene of the yeast *Saccharomyces cerevisiae* reveals 2 nonoverlapping functional regions in the encoded protein. *Mol. Microbiol.* **7**, 683–692
17. Inge-Vechtomov, S., Zhouravleva, G., and Philippe, M. (2003) Eukaryotic release factors (eRFs) history. *Biol. Cell* **95**, 195–209
18. Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N., and Katada, T. (2002) A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in cap/poly(A)-dependent translation. *J. Biol. Chem.* **277**, 50286–50292
19. Kong, C.G., Ito, K., Walsh, M.A., Wada, M., Liu, Y.Y., Kumar, S., Barford, D., Nakamura, Y., and Song, H.W. (2004) Crystal structure and functional analysis of the eukaryotic class II release factor eRF3 from S. pombe. *Mol. Cell* **14**, 233–245
20. Chauvin, C., Salhi, S., Le Goff, C., Viranaicken, W., Diop, D., and Jean-Jean, O. (2005) Involvement of human release factors eRF3a and eRF3b in translation termination and regulation of the termination complex formation. *Mol. Cell. Biol.* **25**, 5801–5811
21. Jakobsen, C.G., Segaard, T.M., Jean-Jean, O., Frolova, L., and Justesen, J. (2001) [Identification of a novel termination release factor eRF3b expressing the eRF3 activity in vitro and in vivo]. *Mol. Biol.* **35**, 672–681
22. Malta-Vacas, J., Aires, C., Costa, P., Conde, A.R., Ramos, S., Martins, A.P., Monteiro, C., and Brito, M. (2005) Differential expression of the eukaryotic release factor 3 (eRF3/GSPT1) according to gastric cancer histological types. *J. Clin. Pathol.* **58**, 621–625
23. Chauvin, C., Salhi, S., and Jean–Jean, O. (2007) Human eukaryotic release factor 3a depletion causes cell cycle arrest at G(1) phase through inhibition of the mTOR pathway. *Mol. Cell. Biol.* **27**, 5619–5629
24. Merkulova, T.I., Frolova, L.Y., Lazar, M., Camonis, J., and Kisselev, L.L. (1999) C-terminal domains of human translation termination factors eRF1 and eRF3 mediate their in vivo interaction. *FEBS Lett.* **443**, 41–47
25. Jakobsen, C.G., Segaard, T.M., Jean-Jean, O., Frolova, L., and Justesen, J. (2001) [Identification of a novel termination release factor eRF3b expressing the eRF3 activity in vitro and in vivo]. *Mol. Biol.* **35**, 672–681
26. Chabelskaya, S., Gryzina, V., Moskalenko, S., Le Goff, C., and Zhouravleva, G. (2007) Inactivation of NMD increases viability of sup45 nonsense mutants in Saccharomyces cerevisiae. *BMC Mol. Biol.* **8**, 71
27. Lieber, M., Smith, B., Szakal, A., Nelsonrees, W., and Todaro, G. (1976) Continuous tumor-cell line from a human lung carcinoma with properties of type-li alveolar epithelial cells. *Int. J. Cancer* **17**, 62–70
28. Tassan, J.P., Jaquenoud, M., Fry, A.M., Frutiger, S., Hughes, G.J., and Nigg, E.A. (1995) *In vitro* assembly of a functional human Cdk7-cyclin-H complex requires Mat1, a novel 36 Kda ring finger protein. *EMBO J.* **14**, 5608–5617
29. Ouchterlony, O. and Nilsson, L.A. (1986) *Handbook of Experimental Immunology, Immunochemistry*. pp. 32.1–32.50, Blackwell Scientific Publications, Oxford Ed
30. Berkova, N., Lair-Fulleringer, S., Femenia, F., Huet, D., Wagner, M.C., Gorna, K., Tournier, F., Ibrahim-Granet, O., Guillot, J., Chermette, R., Boireau, P., and Latge, J.P. (2006) *Aspergillus fumigatus* conidia inhibit tumour necrosis factor- or staurosporine-induced apoptosis in epithelial cells. *Int. Immunol.* **18**, 139–150
31. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* **227**, 680–685

32. Alekseeva, L., Huet, D., Femenia, F., Mouyna, I., Abdelouahab, M., Cagna, A., Guerrier, D., Tichanne-Seltzer, V., Baeza-Squiban, A., Chermette, R., Latge, J.P., and Berkova, N. (2009) Inducible expression of beta defensins by human respiratory epithelial cells exposed to *Aspergillus fumigatus* organisms. *BMC Microbiol.* **9**, 33
33. Frolova, L.Y., Simonsen, J.L., Merkulova, T.I., Litvinov, D.Y., Martensen, P.M., Rechinsky, V.O., Camonis, J.H., Kisselev, L.L., and Justesen, J. (1998) Functional expression of eukaryotic polypeptide chain release factors 1 and 3 by means of baculovirus insect cells and complex formation between the factors. *Eur. J. Biochem.* **256**, 36–44
34. Cheng, Z., Saito, K., Pisarev, A.V., Wada, M., Pisareva, V.P., Pestova, T.V., Gajda, M., Round, A., Kong, C.G., Lim, M., Nakamura, Y., Svergun, D.I., Ito, K., and Song, H.W. (2009) Structural insights into eRF3 and stop codon recognition by eRF1. *Genes Dev.* **23**, 1106–1118
35. Hoshino, S., Imai, M., Mizutani, M., Kikuchi, Y., Hanaoka, F., Ui, M., and Katada, T. (1998) Molecular cloning of a novel member of the eukaryotic polypeptide chain-releasing factors (eRF) - Its identification as eRF3 interacting with eRF1. *J. Biol. Chem.* **273**, 22254–22259
36. Kusov, I.I., Nastashenko, T.A., Kazachkov, I.A., Donets, M.A., Grishina, G.K., Berkova, N.P., and Balaian, M.S. (1991) [The antigenic properties of hepatitis A virus particles possessing different physicochemical characteristics]. *Vopr Virusol* **36**, 203–206
37. Hegde, R., Srinivasula, S.M., Datta, P., Madesh, M., Wassell, R., Zhang, Z.J., Cheong, N.E., Nejmeh, J., Fernandes-Alnemri, T., Hoshino, S., and Alnemri, E.S. (2003) The polypeptide chain-releasing factor GSPT1/eRF3 is proteolytically processed into an IAP-binding protein. *J. Biol. Chem.* **278**, 38699–38706
38. Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43–53