Molecular Cloning of a Novel Member of the Eukaryotic Polypeptide Chain-Releasing Factors (eRF)

ITS IDENTIFICATION AS eRF3 INTERACTING WITH eRF1*

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Yeast GST1 gene, whose product is a GTP-binding protein structurally related to polypeptide chain elongation factor-1a (EF1a), was first described to be essential for the G1 to S phase transition (GSPT) of the cell cycle, and the product was recently reported to function as a polypeptide chain release factor 3 (eRF3) in yeast. Although we previously cloned a human homologue (re-named as GSPT1) of the yeast gene, it has remained to be determined whether GSPT1 also functions as eRF3 or if another GSPT may have such a function in mammalian cells. In the present study, we isolated two mouse GSPT genes, the counterpart of human GSPT1 and a novel member of the GSPT gene family, GSPT2. Both the mouse GSPTs had a two-domain structure characterized as an amino-terminal no-homologous region (approximately 200 amino acids) and a carboxyl-terminal conserved eukaryotic elongation factor-1a-like domain (428 amino acids). Messenger RNAs of the two GSPTs could be detected in all mouse tissues surveyed, although the level of GSPT2 message appeared to be relatively abundant in the brain. The mouse GSPT1 was expressed in a proliferation-dependent manner in Swiss 3T3 cells, whereas the expression of GSPT2 was constant during the cell-cycle progression. Immunoprecipitation assays in COS-7 cells expressing flag epitope-tagged proteins demonstrated that not only GSPT1 but also GSPT2 were capable of interacting with eRF1. Such interaction between GSPT2 and eRF1 was also confirmed by yeast two-hybrid analysis. Taken together, these data indicated that the novel GSPT2 may interact with eRF1 to function as eRF3 in mammalian cells.

Cell-cycle progression is regulated by a variety of genes at the G1 to S phase transition (1, 2). Among them, the yeast GST1 gene, whose product is a GTP-binding protein structurally related to EF1a, was first isolated based on the ability to complement a temperature-sensitive gst1 mutation of Saccharomyces cerevisiae (3). GST1 is a cdc-like mutant whose execution point is distal to the mating factor-sensitive step. In this mutant, DNA synthesis was substantially arrested at the non-permissive temperature. It thus appeared that the GST1 gene product play an essential role at the G1 to S phase transition in the yeast cell cycle. On the other hand, SUP35 (SUP2) was cloned by another group investigating omnipotent suppressor mutant of S. cerevisiae, and the gene turned out to be identical to GST1 (4). Omnipotent suppressor is a class of nonsense suppressors that is recessive and effective toward all three types of nonsense codons (5). Mutations in the GST1/SUP35 gene were shown to increase the level of translational ambiguity (6–8), suggesting that this gene may also function as a positive regulator of translational accuracy in yeast. We previously cloned a human homologue (renamed as GSPT1) of the yeast gene and reported that there is a functional conservation of the gene from yeast to human; GSPT1 could complement the gst1 mutation of S. cerevisiae (9). However, the relationship between the yeast cell-cycle control and translational regulation by the GST1/SUP35 gene product has remained to be determined.

In eukaryotic protein synthesis, termination codons are directly recognized by a polypeptide chain releasing factor, eRF1, to release synthesized polypeptide chain from ribosome, and another releasing factor, eRF3, is essentially required for the ribosomal binding of eRF1. Although the molecular entity of eRF3 has remained unknown, the termination factor appears to act as a GTP-binding protein based on the analogy of prokaryotic RF3 (10, 11). Recently, it was reported in S. cerevisiae (12) and Xenopus laevis (13) that the product of the GST1/SUP35 gene forms a ternary complex with SUP45, which is another member of omnipotent suppressors to function as eRF3. These findings allowed us to postulate that the previously identified human GSPT1 may also function as eRF3. However, our further studies revealed the existence of another GSPT gene in mammalian cells (14). In the present paper, we have isolated two mouse GSPT genes, the counterpart of human GSPT1 and a novel member of the GSPT1 gene family, GSPT2. The two GSPTs were clearly distinct from each other in terms of the amino acid sequences, the expression during cell-cycle progression, and

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tissue distribution. Analyses using immunoprecipitation technique and yeast two-hybrid system indicated that not only GSPT1 but also the novel GSPT2 may form a binary complex with eRF1 to function as eRF3.

**EXPERIMENTAL PROCEDURES**

**RNA Blot Hybridization Analysis**—Total cellular RNA was prepared from cells or tissues using the acid guanidinium thiocyanate-phenol-chloroform method (15). For Northern blotting, 20 μg of RNA was denatured by heating at 65 °C for 5 min in 2.2 mM formaldehyde, 50% (v/v) formamide, and size-fractionated by 1.2% agarose gel containing 2.2% formaldehyde. The RNA was then transferred directly to nylon membranes. Hybridization was carried out overnight at 42 °C in a solution containing 40% formamide, 5× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate), 3× Denhardt's solution (1× Denhardt's solution = 0.002% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone), 50 mM sodium phosphate (pH 6.8), and 100 μg/ml of heat-denatured salmon sperm DNA. Filters were washed twice at room temperature in 2× SSC buffer, twice at 68 °C in 1× SSC buffer for 10 min per wash, and subjected to autoradiography. The plasmid carrying the 3° Cf or 3rh nasolution containing 0.5 M ammonium acetate, 0.2% SDS, and 1 mM EDTA. The RNA was recovered by ethanol precipitation.

**Ribonuclease Protection Assay.**—Randomly primed cDNA prepared from total RNA of various tissues was amplified by PCR using the sequence-specific primer, G13 (GSPT1) or G23 (GSPT2), and the commercial mixture for the PCR. Conditions of the PCR were held within the range of quantitative amplification to provide a relative measure of RNA content in the various samples. The synthetic oligonucleotide used were: G5/A-TTGAAAGGAGGAGATTCCCTC; G13/AAAGTAAAGAGGGCGGTGTGAAATGCGTTTGCA; and G23/TGGGCAATGTTGCGTGTTCA.

**DNA Transfection into COS-7 Cells and Immunoprecipitation.**—Although mouse eRF1 (Sf46 homologue) has not been cloned yet, eRF1 is highly conserved from Xenopus to human. Thus, human eRF1 (TB3–1), of which cDNA had been cloned from total RNA of HL60 cells by means of reverse transcription-PCR based on the sequence described previously (19) with recent correction (20), was used as a counterpart in the present assay. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies, Inc.) containing 5% horse serum and maintained at 37 °C in 95% air and 5% CO₂, 10 μg each of various cDNAs, in which GSPT1, GSPT2, or TB3–1 had been fused in-frame with the flag epitope sequence of pFlag-CMV-2 expression vector (Eastman Kodak Co.), was added to the cells grown at a density of approximately 80% confluence per 90-mm dish with LipofectAMINE-2× (in Opti-MEM I (Life Technologies, Inc.) according to the standard procedure. After a 76-h culture, the transfected cells were harvested and then lysed by shaking for 30 min in 0.5 ml of an extraction buffer consisting of 20 mM Na-Hepes (pH 7.5), 75 mM NaCl, 1% Nonidet P-40, 15 mM sodium fluoride, 1 mM NaVO₃, 10 mM NaPO₄, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml of aprotinin. The lysate was centrifuged at 15,000 × g for 40 min, and the supernatant was precleared by incubating at 4 °C for 40 min with 10 μl of anti-mouse IgG agarose beads (50% slurry, American Qualex Inc.). After removal of the agarose beads, the supernatant was further incubated at 4 °C for 90 min with 20 μl of the agarose beads (50% slurry) conjugated with anti-flag tag monoclonal antibody M2 (Kodak Scientific Imaging System). The agarose beads were washed three times with 1 ml of the extraction buffer, and proteins were eluted from the beads by adding 40 μl of SDS sample buffer (heating at 95 °C) of the samples was separated by SDS-polyacrylamide gel electrophoresis (8% polyacrylamide), and the proteins were transferred to a polyvinylidene difluoride membrane (Fluorotrans, Pall BioSupport Division). The membrane, being shaken after being incubated in TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 5% (w/v) bovine serum albumin, was incubated with rabbit antisera raised against recombinant human GSPT1 or GSPT2 (1:5,000 each), and then further incubated with 125I-labeled protein A in TBS containing 5% bovine serum albumin. The radioactivity retained on the membrane was visualized with the Fuji BAS 2000 bioimaging analyzer.

**Yeast Two-hybrid Assay System.**—The cDNAs encoding TB3–1, GSPT1, and GSPT2 were fused in-frame with the GAL4 DNA-binding domain and/or transactivation domain in pGBT9 and pGAD24 expression vectors. A series of deletion and chimeric mutants of GSPTs were constructed using a PCR-based strategy. PCR products encoding amino-terminal and carboxyl-terminal truncations of GSPT2 were subcloned into pGBT9 in-frame with the GAL4 DNA-binding domain. For chimeric constructs, PCR products encoding amino-terminal regions of GSPT2 and the carboxyl-terminal region of GSPT1 were fused and subcloned into pGADT7 in-frame with the GAL4 DNA-binding domain. The two-hybrid vectors thus obtained were cotransformed into the SFY526 yeast host strain. Transformed colonies were replated on tryptophan- and leucine-deficient medium. Quantitative β-galactosidase assays were carried out as described previously (21). In brief, densities of cell culture grown at 30 °C in SD medium lacking leucine and tryptophan were measured by optical density at 600 nm, and then the cells were harvested by centrifugation at 9000 × g for 30 min in 0.1 ml of Z buffer (82 mM sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM MgSO₄), and permeabilized by freezing in liquid nitrogen and thawing at 37 °C. After cells were diluted with 0.7 ml of Z buffer containing 40 μM β-mercaptoethanol, reactions were started by adding 0.16 ml of 0.5 mM nitrophenyl-β-D-galactopyranoside (4 mg/ml in Z buffer), incubated at 30 °C for 10 min to 10 h, stopped by adding 0.4 ml of 1 M Na₂CO₃, then assayed by

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measurement of $A_{420}$, $b$-galactosidase activity was calculated in Miller units (22) as $1,000 \times (A_{420}/A_{600})^3$ culture volume (milliliter)$^3$ reaction time (min).

RESULTS

Molecular Cloning of Two Closely Related Mouse cDNAs That Are Homologous to Human GSPT1 Gene—In the course of investigating the expression of GSPT1 gene, we noticed there were at least two transcripts in mouse Swiss 3T3 cells, which were cross-hybridized with human GSPT1 cDNA probe; one was the same length (2.7 kb) as human GSPT1 transcript, and the other was approximately 2.6 kb (data not shown). Because the two transcripts were also found in mouse FM3A cells, we screened the FM3A cDNA library (approximately $6 \times 10^5$ primary recombinants) at reduced stringency with the human cDNA as a probe. Twelve positive clones were detected and isolated, and the DNA prepared was subcloned into pUC119 and analyzed by restriction endonuclease mapping, resulting in two types of the restriction maps.

Two independent clones, pGM19 and pGM1, which contain inserts of approximately 2.1 and 2.3 kb, respectively, were thus chosen and subjected to DNA sequencing analyses. The insert of pGM19 was composed of 2097 base pairs with an open reading frame of 1650 nucleotides (550 amino acids) starting from the 5'-end of the sequence followed by a stop codon at position 1653. The insert of pGM1 had 2311 nucleotides, which encodes an open reading frame of 612 amino acids with the putative translation termination codon at position 1839. The nucleotide sequences inserted in pGM19 and pGM1 were similar to each other and also to that of human GSPT1 (9). To isolate the full-length cDNAs, 5'-RACE PCR was performed on a mouse brain cDNA library. Fragments amplified by RACE PCR extended 255 and 235 nucleotides further upstream of the cloned pGM19 and pGM1, respectively. These nucleotides encode other methionines corresponding to the initiation methionine identified in human GSPT1 (23).

Differential Expression of GSPT1 and GSPT2 in Mouse Tissues and Cell Lines—To investigate the relationship between the present two mouse GSPT genes, their expressions were analyzed by an RNase protection assay with specific riboprobes (cRNA) constructed to the coding regions of the carboxyl-terminal GSPT1 and amino-terminal GSPT2. As shown in Fig. 2A, there was a marked increase in GSPT1 transcript at 5 h upon stimulation of Swiss 3T3 cells with 5% fetal bovine serum, which was followed by a gradual decrease. In contrast, GSPT2 transcript did not fluctuate over the 25-h period after the serum stimulation. Similar results were obtained in the cells stimulated with phorbol ester (Fig. 2B). Thus, GSPT1 gene appeared to be inducible in response to growth stimulation, whereas GSPT2 is constantly expressed not dependent on the stimulation in the fibroblastic cells. We also examined the tissue distribution of GSPT1 and GSPT2 mRNAs in mouse. Both GSPT1 and GSPT2 were expressed in all tissues tested.
though GSPT2 transcript was relatively enriched in the brain (Fig. 3). The same results were obtained upon analysis by the RNase protection assay (data not shown).

**Interaction of GSPT Gene Products with eRF1**—Recent studies in yeast (12) and *X. laevis* (13) have shown that Sup35p can interact with Sup45p (eRF1) to function as a polypeptide chain releasing factor, eRF3, in protein synthesis. To extend this notion to mammalian cells and assess the implication of the two mouse GSPT gene products in the termination reaction of protein synthesis, the association of GSPT1 and GSPT2 with eRF1 was investigated by immunoprecipitation assays in COS-7 cells expressing flag epitope-tagged proteins. The sequence of a flag monoclonal-antibody epitope was attached to the open reading frames of GSPT1, GSPT2, and eRF1 cDNAs to produce proteins that were composed of the flag epitope fused to the amino termini of the gene products. The expression vectors composed of the flag-tagged cDNAs under the control of CMV promoter were transiently transfected into COS-7 cells, and the cell extracts were immunoprecipitated with anti-flag monoclonal antibody. As shown in Fig. 4, an endogenous 54-kDa peptide, which was recognized by anti-TB3–1 (eRF1) antiserum, could be co-immunoprecipitated with the flag-tagged GSPT1 or GSPT2 (Panel B, lanes 1 and 2). On the other hand, several anti-GSPT antiserum-reactive peptides of approximately 90 kDa were observed upon immunoprecipitation of the flag-tagged TB3–1 (Panel A, lane 3).

Such direct interaction between eRF1 and GSPT was further confirmed by the yeast two-hybrid assay system (24, 25). The varying length of GSPT2 and the full-length TB3–1 gene were cloned downstream of the GAL4-binding (pGBT9 vector) and activation (pGAD424 vector) domains, respectively, and the resulting plasmids were transformed into the *S. cerevisiae* SFY526 cells. The activity of the β-galactosidase reporter was assayed quantitatively by the standard procedure (21). As shown in Fig. 5A, there was strong interaction between the full-length mouse GSPT2 (1–632) and the human eRF1. Significant interaction between GSPT1 and eRF1 was also detectable if the more sensitive yeast strain Y190 was used instead of SFY526 (data not shown).

Because the mouse GSPT2 gene product significantly interacted with eRF1, we further analyzed the functional domains with the varying length of GSPT2 in the yeast two-hybrid assay system. When 35, 109, and 135 amino acids were deleted from the amino terminus of GSPT2 (136–632), (136–632), and (136–632), respectively), their interactions with eRF1 were progressively reduced. Moreover, GSPT2 gene products (36–427) deleted with carboxyl-terminal 205 amino acid residues also failed to interact with eRF1. These results suggested that not only the amino-terminal region of GSPT2 but also its carboxyl-terminal region is involved in its association with eRF1.
In the present study, we have identified a novel member of the GSPT gene family, GSPT2, which may function as the polypeptide chain release factor, eRF3, in the termination of mammalian protein synthesis. This is the first demonstration showing the heterogeneity of GSPT- (or eRF3-) related genes in eukaryotic cells. Mouse GSPT1 and GSPT2 genes were clearly distinct from each other in terms of their amino acid sequences (especially in their amino termini) expression during cell-cycle progression, and tissue distribution. In contrast to GSPT1, the expression of the novel GSPT2 gene was constant during the cell-cycle progression of 3T3 cells and was relatively abundant in mouse brain. Our previous study on the chromosome mapping of the GSPT1 gene suggested the existence of a homologous gene on the X chromosome (14), which may represent the locus of human GSPT2 gene.

As has been reported previously, the yeast GST1/SUP35 gene product consists of two domains, the amino- and carboxy-terminal ones (Fig. 5). The amino-terminal domain consisting of 257 amino acid residues is unique for the yeast GST1/SUP35 and is also reported to be essential for the inheritance of the [psi+] phenotype (26, 27). On the other hand, the carboxy-terminal domain (428 amino acids) is similar to EF1-a and essential for the translation termination (28). Recently, Jean-Jean et al. (23) reported that the 5’-end of the open reading frame of human GSPT1 gene is longer than previously reported by 138 codons, indicating that the length of the amino-terminal domain (209 amino acids) of human GSPT1 is now close to that of the yeast GST1/SUP35 gene product. The two mouse GSPTs also had the amino-terminal domain consisting of approximately 200 amino acids. Thus, eukaryotic GSPT gene products appeared to be characterized as at least the two-domain structure, a unique amino-terminal region and the conserved EF1-a-like carboxy-terminal domain.

A striking difference between mouse GSPT1 and GSPT2 is observed in the amino-terminal region, though their carboxy-terminal domains are highly homologous in the primary structure. In the present study, interaction of eRF1 with the GSPT gene products was assessed using co-immunoprecipitation and yeast two-hybrid assays. The results indicated that both GSPT1 and GSPT2 were able to bind eRF1. When the amino-terminal region of GSPT2 was deleted, the interaction was impaired. In addition, the carboxy-terminal region of GSPT2 was also required for the interaction; GSPT2 deleted with the carboxy-terminal 205 amino acids failed to bind eRF1. Thus, the data presented here clearly demonstrated that the GSPT2 gene product interacts with eRF1 directly and that at least two distinct regions of GSPT2 are required for its binding to eRF1.

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In the present study, we have demonstrated significant interaction between GSPT gene products and eRF1. However, there was no obvious difference in the eRF1 binding properties between the two GSPTs. In prokaryotic cells, two release factors, RF1 and RF2, which correspond to eRF1, catalyze the termination reaction at UAA and UAG codons and UAA and UGA codons, respectively (29). In addition, a nonessential factor, RF3, appears primarily to function as an enhancer predominantly for the RF2-mediated termination at UGA codon (30, 31). In eukaryotic cells, however, only eRF1 has been identified as the peptidyl release factor for the three translation codons, and both eRF1 and eRF3 appear to be essential and required to form a functional release factor complex (32-33). However, we cannot totally rule out the possibility that a RF2-like termination factor(s) other than eRF1 may exist and interact efficiently with either of the GSPT gene products in eukaryotic cells. This notion might be supported by the findings that several genes related to eRF1/SUP45 were present in human genome (34).

In a previous study (9), we reported that human GSPT1 is capable of complementing the temperature-sensitive growth of the yeast GST1 mutant, though the exact role of the yeast GST1/SUP35 gene product in the regulation of cell-cycle progression has remained unknown. This suggests that in mammalian cells either of the GSPT genes may participate in the regulation of cell cycle. Given that mouse GSPT1 gene expression is up-regulated at the G1 to S phase transition, in which GSPT2 gene expression is constant, GSPT1 might interact with a protein(s) other than eRF1-like factor, which regulates cell-
cycle progression. The GST1 gene, which exists in a single copy in yeast, might duplicate in evolution to generate functionally specialized GSPT genes (GSPT1 and GSPT2) in mammals. These possibilities concerning the presence of a novel interaction molecule(s) to GSPT gene products and the relationship between translation termination and cell-cycle regulation are currently under investigation in our laboratory.

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