Molecular cloning of a novel GTP-binding protein induced in fish cells by rhabdovirus infection

Eun-Hee Lee[a], Hyun-Ju Kim[b], Jeong-Jae Park[b], Jeong-Yun Choi[b], Wha-Ja Cho[b], Seung-Ju Cha[b], Chang-Hoon Moon[b], Jeong-Min Park[b], Won-Joon Yoon[b], Byung-Ju Lee[b], Dong-Hun Lee[c], Ho-Sung Kang[a], Mi-Ae Yoo[a], Han-Do Kim[a], Jeong-Woo Park[b,*]

[a]*Department of Molecular Biology, Pusan National University, Pusan 609-735, South Korea*
[b]*Department of Biological Science, University of Ulsan, Ulsan 680-749, South Korea*
[c]*Division of Life Sciences, Chungbuk National University, Cheongju, Chungbuk 361-763, South Korea*

Received 11 May 1998

Abstract We have cloned and sequenced a cDNA encoding GTP-binding protein from a fish cell, CHSE-214. The clone was 1493 bp long and contained an open reading frame encoding 364 amino acids. It has the five sequence motifs G1–G5 that are conserved in all GTP-binding proteins. Its amino acid sequences are strikingly different from those of the well-characterized G-proteins. However, sequences closely related to this protein are found in various kinds of species including human, *Arabidopsis*, *Drosophila* and archaebacteria, suggesting a novel subfamily within the superfamily of the GTP-binding proteins. Northern analysis indicates that this gene is constitutively expressed at a low level in normal cells but is induced by fish rhabdovirus infection at about 24 h post infection and disappears thereafter. Based on these observations, we propose that this protein represents an evolutionarily conserved novel subfamily of GTP-binding proteins which may play an important role in fish rhabdovirus infection.

Key words: Novel GTP-binding protein; Fish; Rhabdovirus

1. Introduction

GTP-binding proteins constitute a large superfamily of regulatory molecules. This superfamily comprises at least three subfamilies [1] including the large heterotrimeric G-protein (e.g. transducin), the small monomeric GTP-binding proteins (e.g. Ras), and the GTPase involved in protein synthesis (e.g. elongation factor Tu). The large heterotrimeric G-protein is a membrane-bound signal-transducing G-protein and is composed of α subunits (39–52 kDa), β subunits (35–36 kDa), and γ subunits (7–10 kDa) [2].

GTP-binding proteins have been found to play important roles in virus infection. The heterotrimeric GTP-binding protein is coupled to a coreceptor of human immunodeficiency virus type 1 (HIV-1) and can function in HIV-1 entry [3,4]. Infection with hypovirus reduces the accumulation of heterotrimeric GTP-binding protein in the chestnut blight fungus and reduces levels of fungal virulence [5,6]. Ras cooperates with simian virus 40 large T antigen [7], and hepatitis C virus core protein [8] to transform rat embryo fibroblasts. The dominant inhibitory Ras can delay Sindbis virus-induced apoptosis in neuronal cells [9]. The Ras-Raf pathway is activated in HIV-infected monocytic cells and participates in HIV-induced activation of NF-κB [10]. The Arf-like GTP-binding protein takes part in the generation of post-Golgi vesicles carrying envelope glycoproteins of vesicular stomatitis virus [11].

Recently a novel group of GTP-binding proteins has been identified. Most of them have been identified fortuitously by genomic or cDNA cloning in *Halobacterium cutirubrum* [12], *Thermoplasma acidophilum* [13], *Methanococcus jannaschii* [14], *Caenorhabditis elegans* [15], the fission yeast *Schizosaccharomyces pombe* [16], *Drosophila melanogaster* [17], *Xenopus laevis* [18], *Arabidopsis thaliana* (unpublished), and *Saccharomyces cerevisiae* (unpublished). However, two related genes were cloned in an attempt to isolate cDNA for developmentally regulated proteins in the central nervous system of the mouse [19,20] and for selectively inhibited proteins in human fibroblasts transformed by SV40 [21]. These proteins harbor the five characteristic regions G1–G5 that are believed to play a role in the interaction with GTP. Based on their sizes, this group resembles the α subunits of the heterotrimeric G-proteins. However, apart from their size, they do not display significant similarity with the well-characterized G-proteins. Their function is still unknown but their high degree of conservation suggests that they play an essential role in the control of cell growth and differentiation.

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus that causes an acute disease in wild and hatchery-reared salmonid fish. In this study we report the cDNA cloning of a novel GTP-binding protein that showed increased expression in fish cells by IHNV infection.

2. Materials and methods

2.1. Cell and virus

A fish cell line, CHSE-214 (Chinook salmon embryo), was grown in Eagle's minimum essential medium (EMEM) at 18°C, supplemented with 10% fetal bovine serum (Gibco-BRL, USA) and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively, Gibco-BRL). A Korean strain of IHNV, IHNV-PRT [22], was grown in CHSE-214 cells. Cells were infected by IHNV-PRT at a multiplicity of infection of 10 at 4°C for 1 h, washed twice with serum-free media and grown in EMEM supplemented with 10% fetal bovine serum and antibiotics at 18°C.

2.2. Subtracted probe

Subtracted mRNA was prepared with the slight modification of the method of Hara et al. [23]. Briefly, at 24 h post infection (p.i.), total RNA was extracted from IHNV-infected CHSE-214 cells using the guanidinium thiocyanate-acid phenol-chloroform method [24]. Total RNA (500 μg) from mock-infected CHSE-214 cells was mixed with 500 μl of DEPC-water, 500 μl of 2×binding buffer (20 mM Tris-HCl

*Corresponding author. Fax: (82) (52) 259-1694.
E-mail: jwpark@uou.ulsan.ac.kr

The nucleotide sequences reported in this paper have been deposited in GenBank with accession number AF035177.

PII S0014-5793(98)00641-3

pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS) and 30 μl of 10% (w/v) oligo(dT)$_{30}$-Latex suspension (Qiagen, USA). The mixture was heated at 65°C for 3 min and then incubated at room temperature for 30 min. After centrifugation at 12 000×*g* for 2 min, the precipitate was washed twice with washing buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) by centrifugation. The precipitate was resuspended with 400 μl of RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 2 mM each of dNTPs, 10 mM DTT) containing 1000 U of M-MLV reverse transcriptase (Gibco-BRL) and incubated at 42°C for 50 min. The mixture was then heated at 90°C for 3 min and rapidly cooled. The free RNA dissociated from cDNA-oligo(dT)$_{30}$-Latex was removed by centrifugation. The precipitate was washed twice with 200 μl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) by centrifugation. After blocking the free oligo(dT) residues on oligo(dT)$_{30}$-Latex with (dA)$_{30}$ oligonucleotide, the cDNA-oligo(dT)$_{30}$-Latex was hybridized with 1 μg of mRNA prepared from IHNV-infected CHSE-214 cells by incubation at 55°C for 20 min in hybridization buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% SDS) containing 2 μg of oligo(dT)$_{12-18}$. The reaction mixture was centrifuged at room temperature for 10 min and the supernatant was collected. This subtractive hybridization was repeated four times in total.

The subtracted mRNA was transcribed into cDNA using M-MLV reverse transcriptase (Gibco-BRL). A poly(C) tail was added to the 3′ end of the cDNA using terminal transferase (Gibco-BRL). The dC-tailed cDNA was amplified by PCR with (dG)$_{20}$ primer and (dT)$_{30}$ primer in the presence of [α-^{32}P]dCTP and ^{32}P-labeled PCR product was used as probe to screen a cDNA library.

2.3. Construction of cDNA library and screening

The CHSE-214 cells were harvested 24 h after IHNV infection. Total nucleic acids were extracted using the guanidinium thiocyanate-acid phenol-chloroform method and mRNA was purified using oligo(dT)$_{30}$-Latex suspension (Qiagen). The cDNAs were synthesized using a cDNA cloning kit (Gibco-BRL; Superscript plasmid system). The cDNA was ligated into the *Sal*I and *Not*I sites of the pSPORT1 vector and transfected into *Escherichia coli* strain DH5α according to the manufacturer's instruction, and screened by hybridization with ^{32}P-labeled PCR product.

2.4. 5′ RACE (rapid amplification of cDNA ends) cloning of a full-length novel GTP-binding protein

5′ RACE amplification was carried out by using 5′ RACE kit (Gibco-BRL) according to the manufacturer's instruction. CHSE-214 cells were harvested 24 h after IHNV infection, and total RNA was extracted. 1 μg of the total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco-BRL) and the first gene-specific primer, 5′-CACGTCTGCTGTCCTGGCAACAGCA-3′. A poly(C) tail was added to the 3′ end of the cDNA using terminal transferase (Gibco-BRL). The dC-tailed cDNA was amplified by PCR with 5′ RACE-abridged anchor primer and the second gene-specific primer, 5′-CAGCAATGACCTGCCGACCTCGACC-3′. The PCR was allowed to proceed for 35 cycles of 94°C (1 min), 55°C (1 min) and 72°C (1 min). The PCR product was re-amplified by nested PCR with universal amplification primer and a nested gene-specific primer, 5′-CGACCCTTACCTTGGGAAGCTCCTT-3′. The PCR reaction was performed by 35 cycles of 94°C (1 min), 50°C (1 min) and 72°C (1 min). The 5′ RACE amplification product was cloned into pGEM-T vector (Promega, USA).

2.5. DNA sequencing and sequence analysis

DNA sequencing was performed at the Basic Science Research Center, Korea on an automatic DNA sequencer (Applied Biosystems, USA) according to the dye terminator procedure. The DNA sequences and the deduced amino acid sequences were compared with sequences of all the species retrieved from the EMBL/GenBank databank using Gα of heterotrimeric GTP-binding protein as the outgroup. Sequences were aligned using CLUSTAL W [25] and then the phylogenetic tree was constructed with the TreeView [26].

2.6. Northern blot analysis

Total RNA was extracted from mock- and IHNV-infected CHSE-214 cells and rainbow trout tissues and RNA samples (20 μg) were transferred to nylon membranes (Amersham; Hybond-N). Hybridization was done with [α-^{32}P]CTP-labeled probes prepared by random oligonucleotide priming method [27]. The probe used was the 5′ RACE amplification product of 413 bp.

3. Results and discussion

To investigate the cellular genes induced in IHNV-infected CHSE-214 cells, a subtractive probe was constructed with the mRNA present in IHNV-infected cells but not in the mock-infected cells using oligo(dT)$_{30}$-Latex. The probe was used for screening of cDNA library constructed from IHNV-infected cells. In order to discriminate the clone containing virus-specific cDNA from cell-specific cDNA, these clones were re-screened using cDNA probes prepared from genomic RNA of IHNV. We could isolate several clones that contained cell-specific cDNA and were induced by IHNV infection. One was a novel GTP-binding protein. To isolate a full-length cDNA clone for the putative novel GTP-binding protein, 5′ RACE was conducted. A full-length gene was amplified at the 5′ end by RACE-PCR and sequenced on both strands by utilizing restriction fragments to make a series of subclones. The full-length cDNA was 1493 bp in length with an open reading frame (ORF) of 1095 bp encoding a 364-aa protein (GenBank accession number AF035177).

A database search was performed to determine the degree of homology of this protein with other G-proteins. The most closely related protein was a human novel GTP-binding protein (89% sequence identity, 96% similarity) whose expression was down-regulated in SV40 transformation in the fibroblasts [21]. In addition, this protein is closely related to a novel group of GTP-binding proteins that have been identified in mouse [19,20], *H. cutirubrum* [12], *T. acidophilum* [13], *M. jannaschii* [14], *C. elegans* [15], *S. pombe* [16], *D. melanogaster* [17], *X. laevis* [18], *A. thaliana* (unpublished), *S. cerevisiae* (unpublished), and *P. sativum* (unpublished).

To figure out the structural features of the fish novel GTP-binding protein, its deduced amino acid sequence was compared with those of other novel GTP-binding proteins using the CLUSTAL W program (Fig. 1). In addition, a phylogenetic tree was constructed with the amino acid sequences of the GTP-binding proteins (Fig. 2). All the novel GTP-binding proteins mentioned above share 30–89% sequence identity at the amino acid level, possess a similar size (364–399 amino acids except *C. elegans*, 573 amino acids) and have five conserved regions G1–G5 that are critical in the interaction with GTP. All the proteins contain the same G2 motif (YEFTTL) which is usually conserved within a particular subfamily of GTP-binding proteins, but not between different subfamilies [1]. However, the novel group of GTP-binding proteins shows very low structural similarity with the human α subunit of heterotrimeric GTP-binding protein (3–11% sequence identity) illustrated by the phylogenetic tree. Based on these characteristics, it is evident that the novel GTP-binding proteins cannot be assigned to known trimeric GTP-binding protein and thus Schenker et al. [21] suggested a new subfamily within the superfamily of GTP-binding proteins. The novel GTP-binding proteins can be classified into at least four phylogenetically distinct subgroups: subgroup 1 from *Drosophila*, mouse and *Xenopus*; subgroup 2 from plant such as *Arabidopsis* and pea (*P. sativum*); subgroup 3 from fish, human, *C. elegans* and *S. pombe*; subgroup 4 from archaea such as *Methanococcus*, *Halobacterium* and *Thermoplasma*. It is interesting that fish

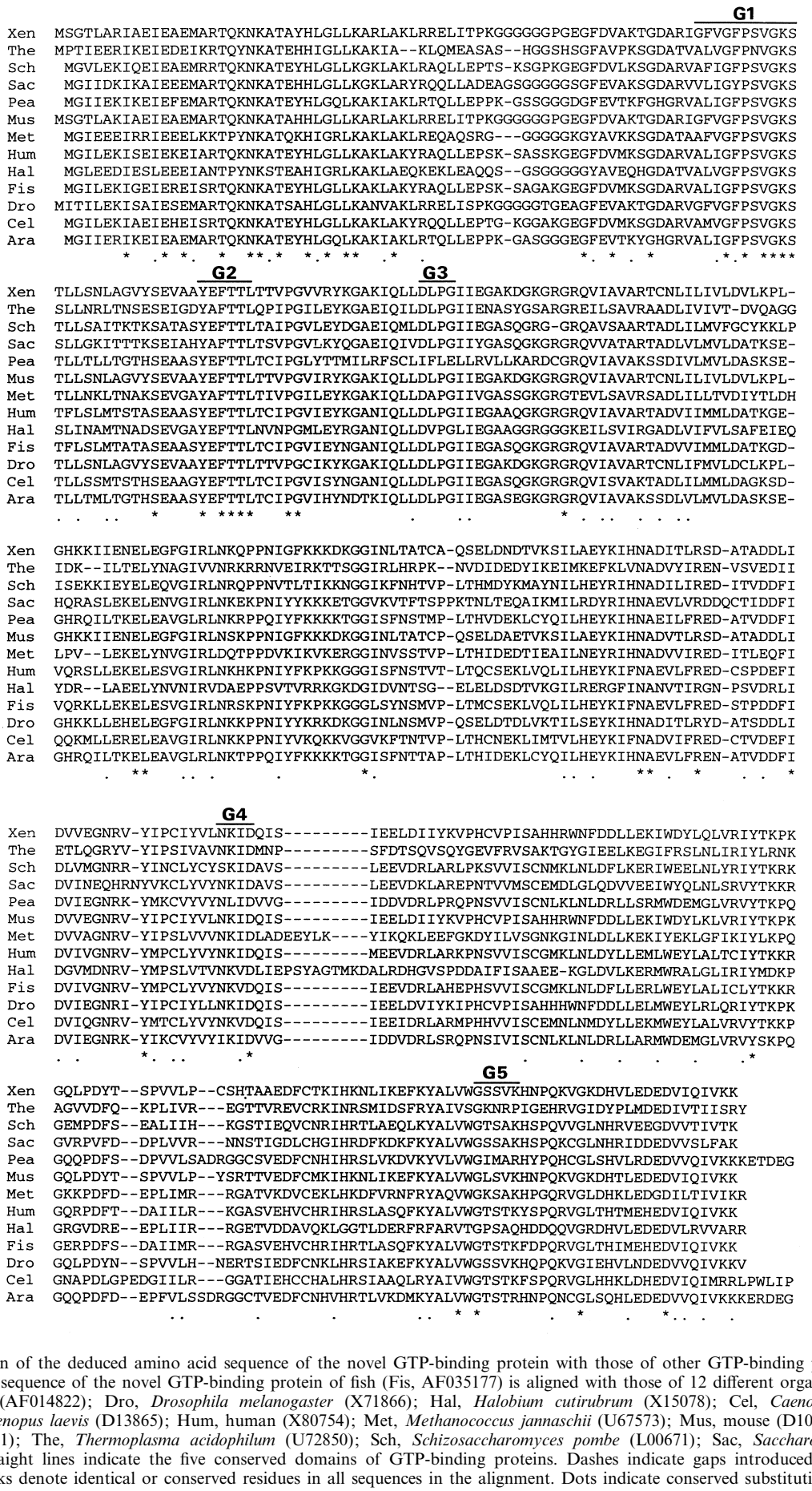

Fig. 1. Comparison of the deduced amino acid sequence of the novel GTP-binding protein with those of other GTP-binding proteins. The deduced amino acid sequence of the novel GTP-binding protein of fish (Fis, AF035177) is aligned with those of 12 different organisms: Ara, *Arabidopsis thaliana* (AF014822); Dro, *Drosophila melanogaster* (X71866); Hal, *Halobium cutirubrum* (X15078); Cel, *Caenorhabditis elegans* (L14745); Xen, *Xenopus laevis* (D13865); Hum, human (X80754); Met, *Methanococcus jannaschii* (U67573); Mus, mouse (D10715); Pea, *Pisum sativum* (AF014821); The, *Thermoplasma acidophilum* (U72850); Sch, *Schizosaccharomyces pombe* (L00671); Sac, *Saccharomyces cerevisiae* (Z72958). The straight lines indicate the five conserved domains of GTP-binding proteins. Dashes indicate gaps introduced to optimize the alignment. Asterisks denote identical or conserved residues in all sequences in the alignment. Dots indicate conserved substitutions.

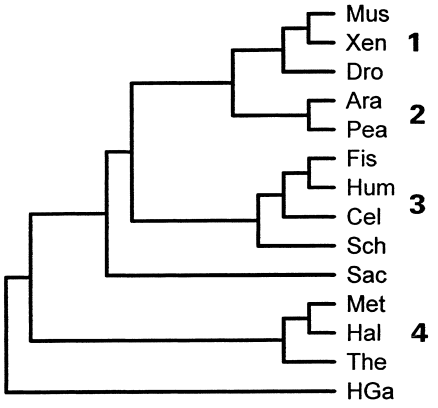

Fig. 2. Phylogenetic status of the novel GTP-binding protein in GTP-binding proteins. Amino acid sequences of 14 GTP-binding proteins were obtained from the GenBank database and their phylogenetic relationship was analyzed using TreeView. Human α subunit of heterotrimeric GTP-binding protein (HGa, X56009) was included in the analysis as an outgroup. The three-letter abbreviations for the organisms are described in the legend of Fig. 1.

GTP-binding protein is more closely related to human GTP-binding protein rather than to *Xenopus* GTP-binding protein.

Northern blot analyses of mock- and IHNV-infected CHSE-214 cells, using as probes the 413-bp fragment from the 5′ end of the fish novel GTP-binding protein gene, clearly reveal that the gene is regulated during the IHNV infection. In mock-infected normal cells, the transcripts of the GTP-binding protein expressed constitutively at a negligible level throughout the incubation period of 48 h. In IHNV-infected cells, the transcript was increased at 24 h p.i. and then decreased after 36 h (Fig. 3). The time of appearance of the transcript coincided with the release of infectious virions from the IHNV-infected cells. These results show that the transcript of the novel GTP-binding protein was induced at a late stage of IHNV infection.

Northern blot analysis with multiple fish tissues was performed using a 413-bp cDNA fragment as a probe. As is shown in Fig. 4, a transcript of 1.5 kb was identified. The transcript was detected in liver and heart at high levels but was not detected in other tissues such as egg, kidney, spleen, eye and brain. This pattern of tissue distribution of the transcript is similar to that of human novel GTP-binding protein [21] but somewhat different from that of mouse novel GTP-binding protein. In mouse, the transcript of this gene was detected in various tissues, especially in brain, but not in heart [20]. This suggests that the pattern of tissue distribution of the transcript is similar between the organisms of the same subgroup but different between the organisms of different subgroups.

The function of this GTP-binding protein has not been identified yet. However, the presence in a wide variety of organisms certainly represents an important role. The function may be different among different subgroups because the tissue distribution of the transcript is different between different subgroups. In subgroup 1, mouse and *Xenopus*, the novel GTP-binding proteins are detected in brain, show regulated expression patterns during embryonic development and may play a role in the development of the vertebrate central nervous system [18–20]. The fish is a member of subgroup 3 and, based on the sequence similarity and similar tissue distribution, it is possible that the fish GTP-binding protein plays a similar role as human GTP-binding protein. The human novel GTP-binding protein was produced in normal, but decreased in SV40-transformed cells [21]. The fish GTP-binding protein was expressed at extremely low levels in normal cells but increased in virus-infected cells at a late stage of infection when the virus-infected cells began to die. Thus, it is possible that it plays a role in the regulation of cell growth and/or death. Alternatively, it may function as a tumor suppressor gene. When the expression of the novel GTP-binding protein is increased, the cell undergoes death. In contrast, if the expression is decreased, cellular growth is increased.

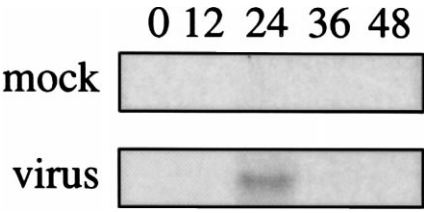

Fig. 3. Northern blot analysis of the expression of the novel GTP-binding protein. At 12-h intervals, total RNA was extracted from mock- and IHNV-infected CHSE-214 cells and hybridized with [α-^{32}P]CTP-labeled probes generated from a cDNA fragment of the GTP-binding protein clone.

If increased expression of the novel GTP-binding protein causes the death of a virus-infected cell, what is the function of the novel GTP-binding protein in fish rhabdovirus infection? It is not certain but the cell death caused by the novel GTP-binding protein may be apoptosis. A growing number of viruses are now known to induce cell death at a late stage of infection not by necrosis but by apoptosis. Virus-induced apoptosis may play a key role in the spread of progeny virus to neighboring cells while evading host immune inflammatory responses and protecting progeny virus from host enzymes and antibodies [28]. There is recent evidence that rhabdovirus may cause the death of cells by apoptosis [29–31]. Bjorklund et al. [32] reported that a fish rhabdovirus also induced apoptosis in a fish cell. DNA from virus-infected cell showed a 200-bp ladder pattern indicative of oligonucleosomal DNA fragmentation. The DNA fragmentation became evident simultaneously with the rise in virus titer (at about 24 h after infection), indicating that active virus replication and production of progeny virus is necessary to induce apoptosis in fish cell. Thus it is possible that fish rhabdovirus induces the expression of the novel GTP-binding protein at a late stage of infection and then this protein cause cell death by apoptosis. Further studies might therefore focus on the involvement of the novel GTP-binding protein in apoptotic cell death and dissemination of progeny virus by deletion or overexpression of the gene of the novel GTP-binding protein.

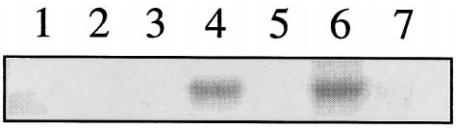

Fig. 4. Tissue distribution of novel GTP-binding protein mRNA. Total RNA from the indicated tissues was hybridized with [α-^{32}P]CTP-labeled probes generated from a cDNA fragment of the GTP-binding protein clone. Lane 1, kidney; lane 2, egg; lane 3, spleen; lane 4, liver; lane 5, eyeball; lane 6, heart; lane 7, brain.

Acknowledgements: This work was supported in part by grants from the Genetic Engineering Research Program.

References

[1] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) Nature 349, 117–127.
[2] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[3] Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P. and Paxton, W.A. (1996) Nature 381, 667–673.
[4] Feng, Y., Broder, C., Kennedy, P.E. and Berger, E.A. (1996) Science 272, 872–876.
[5] Choi, G.H., Chen, B. and Nuss, D.L. (1995) Proc. Natl. Acad. Sci. USA 92, 305–309.
[6] Wang, P. and Nuss, D.L. (1995) Proc. Natl. Acad. Sci. USA 92, 11529–11533.
[7] Cavender, J.F., Conn, A., Epler, M., Lacko, H. and Tevethia, M.J. (1995) J. Virol. 69, 923–934.
[8] Ray, R.B., Lagging, L.M., Meyer, K. and Ray, R. (1996) J. Virol. 70, 4438–4443.
[9] Joe, A.K., Ferrari, G., Jiang, H.H., Liang, X.H. and Levine, B. (1996) J. Virol. 70, 7744–7751.
[10] Folgueira, L., Algeciras, A., MacMorran, W.S., Bren, G.D. and Paya, C.V. (1996) J. Virol. 70, 2332–2338.
[11] Simon, J.P., Ivanov, I.E., Shopsin, B., Hersh, D., Adesnik, M. and Sabatini, D.D. (1996) J. Biol. Chem. 271, 16952–16961.
[12] Shimmin, L.C. and Dennis, P.P. (1989) EMBO J. 8, 1225–1235.
[13] Tamura, T., Tamura, N., Cejka, Z., Hegerl, R., Lottspeich, F. and Baumeister, W. (1996) Science 274, 1385–1389.
[14] Bult, C.J. et al. (1996) Science 273, 1058–1073.
[15] Wilson, R. et al. (1994) Nature 368, 32–38.
[16] Hudson, J.D. and Young, P.G. (1993) Gene 125, 191–193.
[17] Sommer, K.A., Petersen, G. and Bautz, E.K.F. (1994) Mol. Gen. Genet. 242, 391–398.
[18] Kumar, S., Iwao, M., Yamagishi, T., Noda, M. and Asashima, M. (1993) Int. J. Dev. Biol. 37, 539–546.
[19] Sazuka, T., Tomooka, Y., Ikawa, Y., Noda, M. and Kumar, S. (1992) Biochem. Biophys. Res. Commun. 189, 363–370.
[20] Sazuka, T., Kinoshita, M., Tomooka, Y., Noda, M. and Kumar, S. (1992) Biochem. Biophys. Res. Commun. 189, 371–377.
[21] Schenker, T., Lach, C., Kessler, B., Calderara, S. and Trueb, B. (1994) J. Biol. Chem. 269, 25447–25453.
[22] Park, M.A., Sohn, S.G., Lee, S.D., Jeon, S.K., Park, J.W., Fryer, J.L. and Hah, Y.C. (1993) J. Fish Dis. 16, 471–478.
[23] Hara, E., Kato, T., Nakada, S., Sekiya, S. and Oda, K. (1991) Nucleic Acids Res. 19, 7097–7104.
[24] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[25] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[26] Page, R.D.M. (1996) Comput. Applic. Biosci. 12, 357–358.
[27] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[28] Teodoro, J.G. and Branton, P.E. (1997) J. Virol. 71, 1739–1746.
[29] Marcovistz, R., Bertho, A.L. and Matos, D.C.S. (1994) Brazil. J. Med. Biol. Res. 27, 1599–1603.
[30] Koyama, A.H. (1995) Virus Res. 37, 285–290.
[31] Jackson, A.C. and Rossiter, J.P. (1997) J. Virol. 71, 5603–5607.
[32] Bjorklund, H.V., Johansson, T.R. and Rinne, A. (1997) J. Virol. 71, 5658–5662.