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Overexpression of Fish DRG2 Induces Cell Rounding

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Previously, we reported induced expression of developmentally regulated GTP-binding protein 2 (DRG2) in fish cells at the late stage of rhabdovirus infection. To investigate the biological role of fish DRG2 (fDRG2), we transfected CHSE-214 cells with an expression vector containing complete fDRG2 fused to the N-terminal end of an enhanced green fluorescent protein (EGFP). Low level expression of fDRG2-EGFP did not induce morphological change or cell death. However, a high level expression of fDRG2-EGFP induced cell rounding and caused depletion of the cell population in FACS analysis. Several truncated fragments were fused to EGFP. FACS analysis was conducted to determine the presence of cells expressing high levels of the resulting chimera. While cells expressing a high level of N-terminus were detected, those expressing high levels of the C-terminal fragment 243-290 containing the G4 motif were absent in FACS analysis. Based on these observations, we propose that overexpression of fDRG2 may induce cell rounding, a representative cytopathic effect of virus-infected cells in the late stage of infection and the C-terminus of the fDRG2 is essential for this function.

***Key words*:** CHSE-214, cell rounding, fish DRG2, overexpression, rhabdovirus

Developmentally regulated GTP-binding protein (DRG) is an evolutionally conserved novel GTP-binding protein. DRG was first isolated from mice (Kumar *et al*., 1992) and has homologues in distantly related species including humans (Schenker *et al*., 1994), Xenopus (Kumar *et al*., 1993), fish (Lee *et al*., 1998), Drosophila (Sommer *et al*., 1994), *Caenorhabditis elegans* (Wilson *et al*., 1994), fission yeast (Hudson and Young, 1993), and prokaryotic cells (Shimmin and Dennis, 1989; Bult *et al*., 1996; Tamura *et al*., 1996). These proteins harbor the five characteristic motifs, G1-G5, that are believed to interact with GTP. Apart from these motifs, they do not display significant similarity with the well-characterized G-proteins and, therefore, they constitute a new subfamily within the superfamily of GTP-binding proteins (Schenker *et al*., 1994). A search of the EST databank revealed at least two distinct members, DRG1 and DRG2 (Li and Trueb, 2000). DRG1 and DRG2 are widely expressed in human and mouse tissues and show a very similar distribution pattern (Li and Trueb, 2000), which suggests that these two DRGs have similar functions.

The high degree of conservation of the DRGs suggests that they play an essential role in the control of cell growth and differentiation. Even though there is no clear evidence for a function of DRG, there are several reports supporting the possible role of it in the control of cell growth and differentiation. Mouse DRG1 was cloned based on its relatively high level of expression during embryonic development and its down-regulation after birth (Kumar *et al*., 1992). Human DRG2 was identified due to its down-regulation in fibroblasts transformed by SV40 (Schenker *et al*., 1994). The plant *drg* genes were expressed primarily in growing, as compared to non-growing, organs (Devitt *et al*., 1999). DRG1 from mice (Mahajan *et al*., 1996) and from humans (Zhao and Aplan, 1998) was found to interact specifically with the SCL oncogene product. SCL is a basic helix-loop-helix transcription factor involved in cell growth and differentiation. It is possible for the DRG1 to have some role in cell growth and differentiation by interaction with SCL. Taken all together, all these observations point to a role of DRGs in cell proliferation, differentiation and death.

We previously reported that the expression of fish DRG2 (fDRG2) was increased at the late stage of fish rhabdovirus infection (Lee *et al*., 1998) when the virus mediates cell rounding and detachment from the extracellular matrix. Detachment of cells from the extracellular matrix results in a form of apoptosis often referred to as anoikis (Meredith

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et al., 1993; Frisch and Francis, 1994; Frisch and Rouslahti, 1997). Recent reports showed that apoptotic cell death occurred in cells infected by fish rhabdovirus (Bjorklund *et al*., 1997; Chiou *et al*., 2000). Based on these reports, it is possible to speculate that fDRG2 plays some role in cell death at the late stage of rhabdovirus infection. In this report, we examine the possible role of fDRG2 in fish cells and present evidence that overexpression of DRG2 can cause cell rounding and that region 243-290 containing the G4 motif is important for this function.

Materials and Methods

Construction of fDRG2 fragments

The wild-type and the fragments of fDRG2 shown in Fig. 4 were constructed using polymerase chain reaction to generate DNA fragments encoding the appropriate N- and C-terminal protein sequences. A cDNA clone encoding full-length fDRG2 (Lee *et al*., 1998) was used as the template. The wild-type and fragments of fDRG2 were amplified with primers that incorporated a *Hind*III recognition sequence at the 5' end and a *Sal*I sequence at the 3' end. The stop codon of fDRG2 was deleted to make a continuous open reading frame with the N-terminus of enhanced green fluorescent protein (EGFP). The amplified polymerase chain reaction products were ligated into the *Hind*III and *Sal*I sites of the pEGFP-N1 mammalian expression vector (CloneTech) in-frame with the EGFP. The sequences of the final plasmids were confirmed by automated DNA sequencing.

Transfection

A fish cell line, CHSE-214 (Chinook salmon embryo), used for the transfection of the fDRG2 expression constructs, were cultured on a 25 cm^2 culture flask in Eagles minimum essential medium (EMEM) at 18°C supplemented with 10% fetal bovine serum (Gibco-BRL) and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively, Gibco-BRL). DNA transfection was performed by a liposome method using FuGENE 6 (Roche Molecular Biochemicals) with a total 5 μg DNA purified with Qiagen column, according to the protocol supplied by the manufacturer. The cells were plated on a 25 cm^2 flask a day before the transfection. The cells were 70% confluent on the day of the transfection. 200 μl of serum-free EMEM was mixed with 6 μl of FuGENE6. After 5 min, the solution was added to a tube containing the plasmid DNA, then the mixture was gently tapped. After incubation for 15 min at room temperature, FuGENE6:DNA solution was added to the cells. Over the next 2 days, the transfected cells were observed under fluorescent microscope (Nikon) and used for the FACS analysis.

TUNEL staining

TUNEL staining was conducted using an *in situ* cell death detection kit, TMR Red, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were fixed with 2% paraformaldehyde solution and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing twice with PBS, cells were incubated in TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and tetramethylrhodamine-dUTP. Cells were analyzed by flow cytometry.

FACS analysis

Forty-eight hours after transfection, the cells were collected by trypsin treatment, and 5 x 10^5 cells were washed and resuspended in 500 μl in FACS buffer (HBSS, 2% FCS, 0.1% NaN_3). The cell suspensions were then analyzed for fluorescence intensity using a FACS flow cytometer (Becton Dickinson, Inc.).

Results and Discussion

Previous study has demonstrated the induction of fish DRG2 (fDRG2) in fish cells at the late stage of infectious hematopoietic necrosis virus (IHNV) infection, ca 24 h post infection (Lee *et al*., 1998). This is the time for the exponential growth of IHNV and initiation of cell rounding, detachment, and apoptosis of IHNV-infected cells. Thus it was logical to examine the influence of the overexpression of the fDRG2 on cell death. In order to determine the effect of fDRG2 expression on cells, full-length-fDRG2 was subcloned as a fusion protein to the N-terminus of EGFP and we monitored the expression of fDRG2-EGFP in cells by flow cytometry.

EGFP-transfected cells exhibited a low to high level expression of fluorescence (Fig. 1A). This indicates that high level expression of EGFP did not have a toxic effect on cells. However, in fDRG2-EGFP-transfected cells, cells expressing high levels of fluorescence were depleted (Fig. 1A). As the high level expression of EGFP does not affect the viability of the transfected cells, the absence of cells with a high level expression of fDRG2-EGFP is highly significant. There are three possible explanations for this phenomenon. First, high level expression of fDRG2 may cause cell death, apoptosis. Second, fDRG2 is a metabolically unstable protein and the fDRG2 causes rapid degradation of the fusion protein, fDRG2-EGFP. Finally, transfection efficiencies are different between EGFP and fDRG2-EGFP.

The absence of cells with high levels of expression of fDRG2-EGFP did not seem to be caused by rapid degradation of the fDRG2-EGFP or low transfection efficiency. In either case, there should be an even reduction in the frequency of cells expressing low to high levels of fDRG2-EGFP. However, our data showed that, while the frequency of cells expressing a low level of fDRG2-EGFP was almost the same as that of EGFP: the fre-

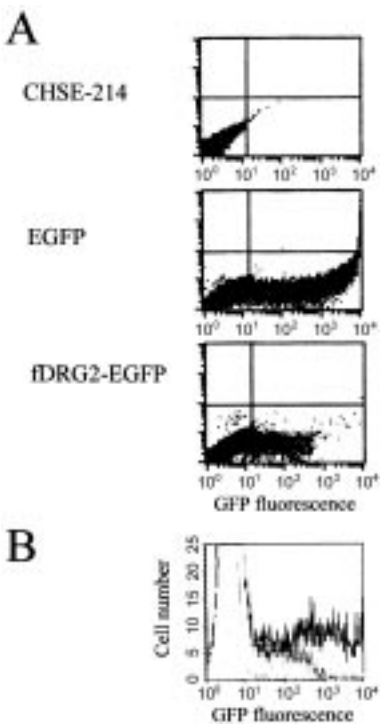

Fig. 1. (A) Flow cytometric analysis of the fluorescence of EGFP and fDRG2-EGFP. CHSE-214 cells were transfected with the vectors expressing these two proteins. The cells were collected with trypsin 48 h after transfection, and subjected to FACS analysis. (B) Depicted is a histogram showing the relative cell number vs fluorescent intensity on a logarithmic scale. Thin line, untransfected CHSE-214 cells; grey line, wt-fDRG2-EGFP; bold line, EGFP.

quency of cells expressing high level of fDRG2-EGFP was significantly reduced (Fig. 1B). This suggests the possibility that the absence of cells expressing high levels of fDRG2-EGFP is due to the death of these kinds of cells.

In order to determine whether the expression of fDRG2 can induce cell death, free DNA ends of fDRG2-EGFP transfected cells were labeled with TMR by the TUNEL technique and cell death in fDRG2-EGFP transfected cells was quantitated by flow cytometry. Similar to cells expressing EGFP alone, cells expressing low levels of fDRG2-EGFP showed no induction of cell death (Fig. 2). This suggests that, even though we could not obtain any information about the cells expressing high levels of fDRG2-EGFP, low level expression of fDRG2-EGFP does not induce cell death.

If the low level expression of fDRG2-EGFP does not induce cellular apoptosis, what is the reason for the absence of cells expressing high level of fDRG2-EGFP? In order to address this question, the transfected cells were examined by fluorescence microscopy. As shown in Fig. 3, the expression of low and high levels of EGFP did not exhibit any change in the morphology of the cells. Low level expression of fDRG2-EGFP also did not induce morphological change. However, high level expression of

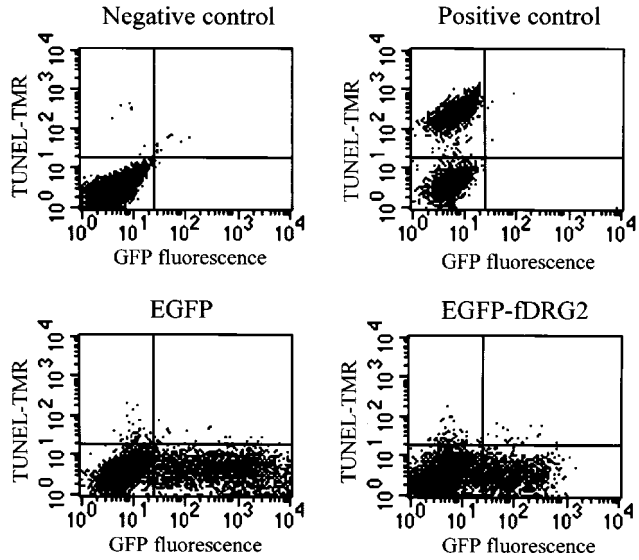

Fig. 2. Effect of expression of EGFP and fDRG2-EGFP on cell death. CHSE-214 cells were transfected with the vectors expressing these two proteins. 48 h after transfection, the cells were collected and free DNA ends were labeled with TMR by TUNEL technique as described in Materials & Methods. Cells were subjected to FACS analysis for quantitating apoptotic DNA fragmentation. Negative control, untransfected cells were incubated in TUNEL reaction mixture without terminal transferase. Positive control, untransfected cells were pretreated with DNase I (10 μg/ml, 10 min) and incubated in TUNEL reaction mixture with terminal transferase.

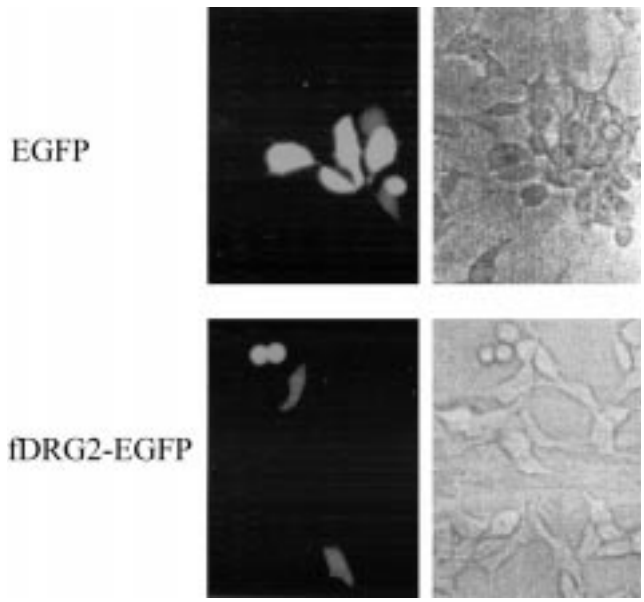

Fig. 3. Effect of expression of EGFP and fDRG2-EGFP on cell morphology. CHSE-214 cells were transfected with the vectors expressing these two proteins. The cells were examined at 48 h after transfection under fluorescence microscope.

fDRG2-EGFP induced cell rounding. The cell rounding is usually followed by cell detachment and upon detachment from the extracellular matrix, and cells enter into pro-

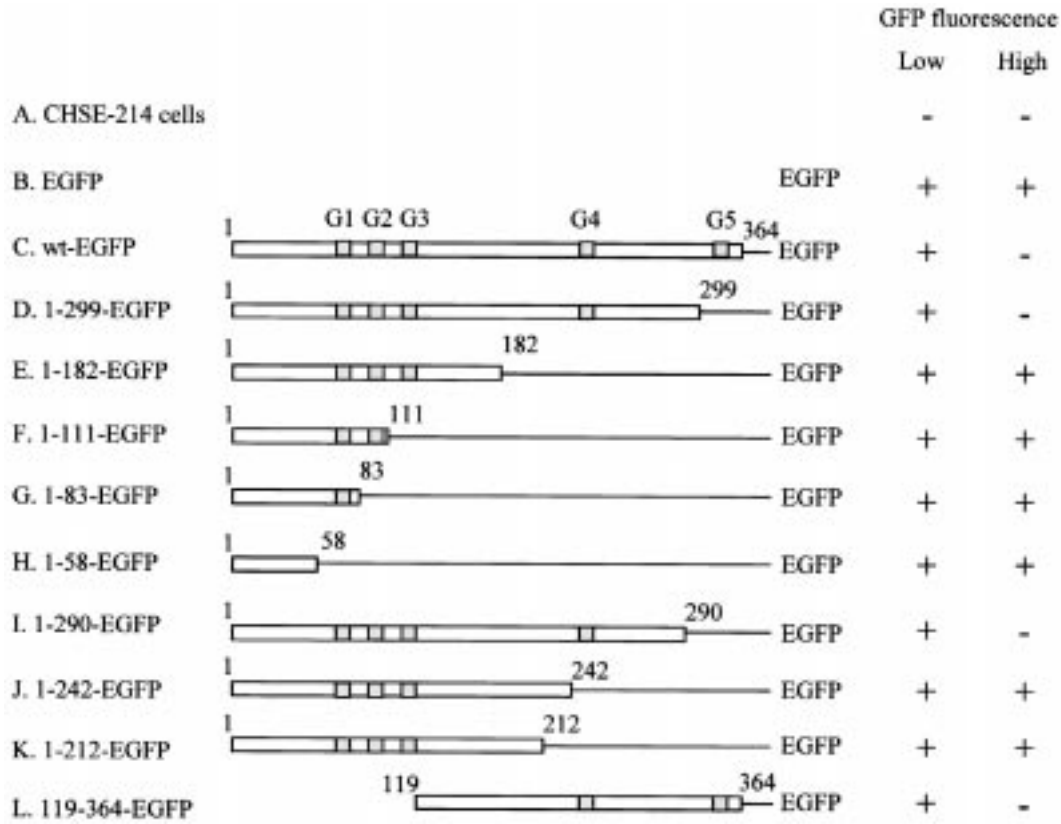

Fig. 4. Constructs of fDRG2 used in this study and summary of results. This illustration shows fragments used in this study. Five conserved motifs, G1-G5, for GTP binding of fDRG2 are indicated as grey boxes where present; G1 (GFPSVGKS), 69-76; G2 (YEFTTL), 92-97; G3 (DLPG), 115-118; G4 (NKVD), 246-249; G5 (GTSTK), 337-341. Boundaries are shown by the residue numbers above the white bars for fDRG2 residues. To the right of each construct is shown the results from flow cytometric analysis. Cell population expressing a high or low level of fluorescence was categorized as present (+), or absent (-).

grammed cell death, a phenomenon known as anoikis (Meredith *et al*., 1993; Frisch and Francis, 1994; Frisch and Rouslahti, 1997). This can possibly be the reason for the absence of cells expressing high levels of fDRG2-EGFP in FACS analysis because the detached cells can be washed out during the preparation process for FACS analysis. This phenomenon is also consistent with the cytopathic effect of virus infected cells, rounding, detachment, and cell death at the late stage of infection. However, we can not rule out the possibility that fDRG2-induced cell rounding does not cause cell death. There are reports that cell rounding and detachment do not induce cell death. For example, Ebola virus GP induces cell rounding and detachment but does not cause an increase in cell death (Simmons *et al*., 2002). Ras activation prevents down regulation of Bcl-X_L and induces resistance to anoikis (Rosen *et al*., 2000). Further studies should be done to identify whether cell rounding and detachment caused by high level expression of fDRG2-EGFP is followed by cell death.

If high level expression of the fDRG2 causes cell rounding and detachment, is GTP binding necessary for this function? The fDRG2 possesses five conserved motifs, G1-G5, for GTP binding. We used fragments of fDRG2 fused to the enhanced green fluorescent protein (EGFP) to confirm any role of these motifs for the detachment function of fDRG2. The fragments of fDRG2 used in this study are shown in Fig. 4. In cells transfected with truncated fragments fDRG2-EGFP, such as 1-58 (ΔG1-G5), 1-83 (ΔG2-G5), 1-111 (ΔG3-G5), and 1-182 (ΔG4-G5), both low and high level expressions of fluorescence were detected (Fig. 4E-4H). However, in cells transfected with truncated fragment 1-299 (ΔG5) (Fig. 4D), cells expressing high levels of fluorescence were absent. This suggests that region 183-299, including G4, is important for the detachment function of fDRG2. This region was studied by means of three further deletions. Additional deletion experiments indicated that high level expression of fluorescence was detected in cells transfected with fragments 1-212 (ΔG4-G5) and 1-242 (ΔG4-G5) (Fig. 4J and 4K) but was absent in cells transfected with fragment 1-290 (ΔG5) (Fig. 4I). This result indicated that the region 243-290 including G4 is critical for the detachment function of fDRG2.

These results suggest two possibilities. First, the GTP/

GDP binding is essential for the detachment function of the fDRG2 and the GTP-interacting motifs, especially G1-G4, are necessary for the detachment function. Even though the binding of GTP to DRG2 has not been demonstrated, DRG2 contains all the conserved residues of the GTP-interacting motif and DRG1, a member of the DRG family, bind GTP *in vitro* as demonstrated with bacterially expressed fusion proteins (Sazuka *et al*., 1992; Sommer *et al.*, 1994). Thus it is possible that the activity of DRG2 is regulated by GTP binding. Second, the G1-G3 motifs are not necessary, but region 243-290 including G4 motif is enough for the detachment function of fDRG2.

In order to confirm which one is true, a fragment from the C-terminus was constructed and tested for the cell detachment activity. As shown in Fig. 4L, high level expression of fluorescence was not detected in cells transfected with fragment 119-364(ΔG1-G3). Thus it is likely that G1-G3 motifs are not essential, but the C-terminus containing G4 and G5 motif is enough for the detachment function of fDRG2. All theses results suggest that the detachment function of fDRG2 is independent of the binding of GTP and requires only region 243-290. Even though region 243-290 includes G4 motif, it is not certain if the G4 motif plays a critical role in the function of fDRG2.

If the high expression of the fDRG2 causes cell rounding, what is the function of the fDRG2 in fish rhabdovirus infection? It is not certain, but the cell rounding caused by fDRG2 may lead to apoptosis, named anoikis. 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 (Teodoro and Branton, 1997). There is recent evidence that fish rhabdovirus induces apoptosis in fish cells (Bjorklund *et al*., 1997; Chiou *et al*., 2000). Thus it is likely that fish rhabdovirus induces the expression of the fDRG2 at the late stage of infection and then this protein causes cell death by anoikis.

If the overexpression of fDRG2 induces cell rounding and detachment, what is the mechanism for this phenomenon? The best way to understand the mechanism for the detachment activity of fDRG2 is to find any proteins interacting with fDRG2. Even though there are several reports that the expression of the DRG2 is induced or decreased depending on cell type and conditions, so far, there is no reported protein interacting with DRG2. However, DRG1, another member of the DRG family, was found to interact specifically with the SCL oncogene product in mouse (Mahajan *et al*., 1996) and human (Zhao and Aplan, 1998) cells. SCL is a basic helix-loop-helix transcription factor involved in cell growth and differentiation (Porcher *et al.*, 1996). Thus it is possible that DRG2 regulates the expression of surface adhesive molecules via interaction with helix-loop-helix transcription factors such as SCL.

In this report, we presented evidence that overexpression of fDRG2 can cause cell rounding in fish cells and region 243-290 including the G4 motif is important for this function. Further studies might therefore focus on identifying whether this cell rounding results in cell death and the finding of specific motifs or residues responsible for this function. Moreover, the finding of any regulatory protein or effectors which interact with this region of fDRG2 will reveal more exact mechanisms for the function of fDRG2, especially in fish rhabdovirus infection.

Acknowledgment

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