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Overexpression of DRG2 Increases G_2/M Phase Cells and Decreases Sensitivity to Nocodazole-Induced Apoptosis

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DRG2, a member of the DRG subfamily in the GTP-binding protein superfamily, was identified as a repressed gene product in fibroblasts transformed by SV40. The significance of this down-regulation and the cellular role of DRG2 has not been understood in the past. To investigate the function of DRG2 we made a Jurkat cell line, Jurkat-LNCX2-DRG2, stably transfected with pLNCX2-DRG2 to overexpress human DRG2. Cell cycle distribution analysis revealed an increased accumulation of G_2/M phase cells in Jurkat-LNCX2-DRG2 cells, indicating a retardation of cell-cycle progression. In addition, an overexpression of DRG2 reduced the sensitivity of Jurkat cells to the mitotic poison nocodazole. Our data suggest that overexpression of DRG2 in Jurkat cells affects genes regulating cell-cycle arrest and apoptosis, and that these molecular changes may be important in the growth or differentiation of cells.

Key words: DRG2, delay in cell cycle, G_2/M phase, Jurkat cells.

A decade ago Noda and his colleagues identified that a number of genes that were differentially expressed in neurogenesis, and these were named NEDDs (*1*). One of the genes, NEDD3, had characteristic GTP-binding motifs and was later named DRG (developmentally regulated GTP-binding protein) (*2*, *3*). The murine DRG was then found as an ortholog of previously identified gene products of *Drosophila* and Halobacteria (*4*). Because of its interesting structure with the GTP-binding domain, expression patterns in embryogenesis, and high conservation from halobacteria to mammals, DRG may play an important role(s) in certain essential cellular and/or developmental functions.

There are two closely-related DRG genes, DRG1 and DRG2, from yeast to plant to human (*5*). Though the physiological function of DRG has not yet been delineated, there are several reports addressing the possible roles of DRG1: (i) modular structure such as GTP-binding motifs; (ii) tissue distribution and post-natal reduction of DRG1; (iii) factor(s) associated with DRG1. Both DRG1 and DRG2 have characteristic motifs for GTP binding (*5*). GTP-binding proteins are responsible for such diverse cellular functions as molecular switches that are active in GTP-bound form (*6–8*). The localization of AtDRG1 is limited in the growing shoot of a plant (*9*). DRG1 associates with SCL/TAL1, a transcription factor for hematopoietic development, *in vivo* and *in vitro* (*10*, *11*). Since SCL/TAL1 has a vital role in hematopoietic tissue development (*12*, *13*), the association of DRGs with SCL/TAL1 could be an important regulation during the development, but its significance has not been clarified. Interestingly, the adult tissue distributions of DRG1 and DRG2 were similar (*5*). Recently, Elsea and coworkers have also found that the message levels of human DRG2 in a fetus were similar in the brain, lung, liver and kidney (*14*). They also addressed the fact that the mRNA levels of DRG2 in various tissues of an adult and a fetus were not significantly different and that DRG2 may not be regulated developmentally. However, no definitive evidence for the functions of DRG1 and DRG2 has yet been reported. Mahajan *et al.* (*10*) have reported that an overexpression of DRG1 triggered cell transformation in fibroblasts with c-myc and ras. Whether or not DRG is oncogenic has yet to be answered, but, these results suggest that DRG could be involved in cell-cycle regulation, in either cell proliferation or apoptotic pathways depending on the cellular context.

To determine whether DRG2 has a role in cell-cycle regulation, we prepared a stable Jurkat cell line that overexpressed DRG2 using pLNCX2 retroviral expression vector. Here, we demonstrate that an overexpression of DRG2 in Jurkat T cells delays cell-cycle progression and that this delay slowed down the apoptosis induced by treatment of cells with nocodazole, a microtubule-disrupting compound.

MATERIALS AND METHODS

Materials—Carboxyfluorescein diacetate succinimidyl ester) (CFSE) and propidium iodide (PI) were obtained from Molecular Probes (Eugene, Oregon). Retroviral vector pLNCX2 was purchased from Clontech (Palo Alto, CA). pGEM-T vector system was obtained from Promega (Wisconsin, WI). Most chemical reagents were purchased from either Sigma (Saint Louise, MO) or Calbiochem (San Diego, CA).

Cell Culture and Maintenance—Human T cell line, Jurkat, was routinely cultured in a RPMI 1640 medium

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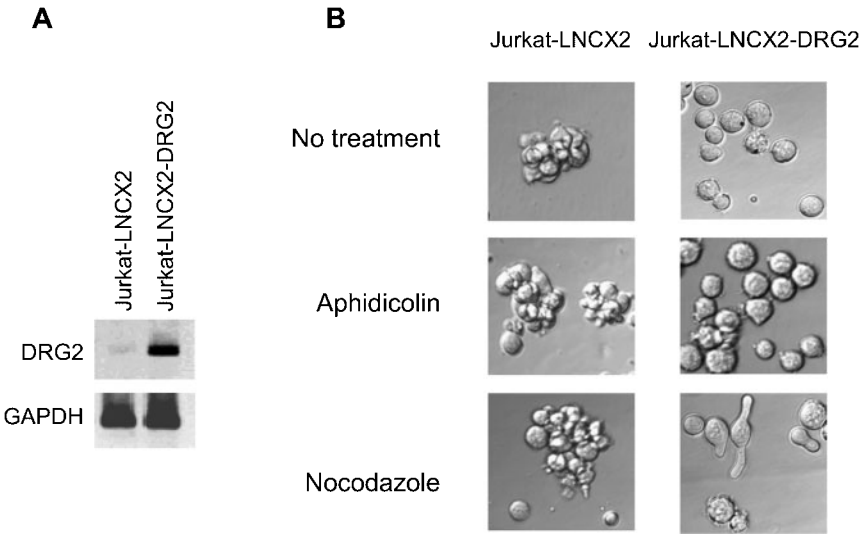

Fig. 1. **RT-PCR analysis and distinct morphological changes in Jurkat cells transformed to overexpress human DRG2.** Jurkat-LNCX2 and Jurkat-LNCX2-DRG2 cells were prepared from Jurkat cells by stable transfection with pLNCX2 retrovirus vector and pLNCX2-DRG2, respectively (see details in MATERIALS AND METHODS). (A) The expression level of DRG2 in these two clones was determined by using RT-PCR. (B) Morphological changes were observed under a microscope after treatment with either aphidicolin (1 μg/ml) or nocodazole (20 ng/ml) for 24 h.

in a humidified 37°C chamber under an atmosphere of 5% CO_2. The medium was supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin, and 100 U/ml of penicillin. Culture conditions were those recommended by ATCC (Rockville, MD).

Cloning of DRG2 and Establishing a Permanent Cell Line Overexpressing DRG2—Human DRG2 gene was cloned by RT-PCR from the RNA preparation of fibrosarcoma HT1080 cell lines using a forward primer CTCGAGCTGCTGCTACCATGGGGATCTTA and a reverse primer GTCGACTTACTTCTTCACGATCTGGATGA. The resulting PCR product was subcloned into the retroviral vector pLNCX2. Nonreplicating retrovirus was obtained by transfecting the RetroPack PT67 cell line with the pLNCX2-DRG2 construct according to the protocol supplied by the manufacturer (Clontech). Jurkat cells were infected with pLNCX2-DRG2 retrovirus, and stable clones, Jurkat-LNCX2-DRG2, that express DRG2 were prepared according to the manufacturer's protocol (Clontech). A control cell line, Jurkat-LNCX2 was generated by infecting Jurkat cells with pLNCX2 retrovirus.

RT-PCR—Five μg of DNase I-treated total RNA was reverse transcribed using random priming and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Then PCR was carried out using, as template, cDNAs from Jurkat-LNCX2 and Jurkat-LNCX2-DRG2. Primers were designed using the Primer3 software: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. PCR primer pair was as follows: forward, Cccaacagtgtggtcatcag; reverse, acaccaggcgtacttgaac. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 25 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. Amplified PCR products were analyzed by agarose gel electrophoresis.

Annexin V Staining—Annexin V staining was conducted using an Annexin-V-FLUOS staining kit, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) and resuspended with binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) containing Annexin V and propidium iodide. Cells were analyzed for fluorescence intensity using a FACS flow cytometer (Becton Dickinson).

Cell Cycle Analyses—Cells were harvested and washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) at room temperature and resuspended at 2×10^6 cells/ml in PBS. For propidium iodide staining, the washed cells were fixed in pure ethanol in –20°C overnight, then treated according to the manufacturer's procedure (Molecular Probes). Cells were washed twice in PBS, resuspended in a FACS buffer (PBS, 0.2% BSA and 1% sodium azide), and then were analyzed by using a FACS flow cytometer (Becton Dickinson, Inc., San Jose, CA).

CFSE Staining—The CFSE (Molecular Probes) stock solution (5 mM in DMSO) was diluted to 5 μM in PBS and mixed with prepared cells. The mix was incubated at room temperature for 10 min with occasional agitation. The labeling process was quenched by adding an equal volume of calf serum and incubating for 1 min. Cells were washed three times in DMEM. The CFSE stained cells were cultured in DMEM with 10% FCS. After washing twice with PBS, cells were analyzed by using a FACS flow cytometer (Becton Dickinson, San Jose, CA).

Cell Growth Curve—For measuring proliferation, cultured cells were monitored for cell volume and enumerated on a hematocytometer every 2 days. Cell concentration was maintained at 5×10^5/ml throughout the culture.

RESULTS AND DISCUSSION

Based on several reports, there seems to be a correlation between DRG expression and growth and proliferation. These suggest the possibility that DRG expression may cause changes in the cell cycle. To determine whether overexpression of DRG2 affects cell-cycle progression, we prepared Jurkat cell lines that overexpressed DRG2 using pLNCX2 retroviral expression vector. A stable Jurkat cell line transfected with pLNCX2-DRG2 was selected by screening for the overexpression of DRG2 by RT-PCR. As shown in Fig. 1A, the Jurkat-LNCX2-DRG2 cells overexpressed DRG2 compared with Jurkat-LNCX2 cells, a negative control. An interesting morphological feature was observed in Jurkat-LNCX2-DRG2 cells.

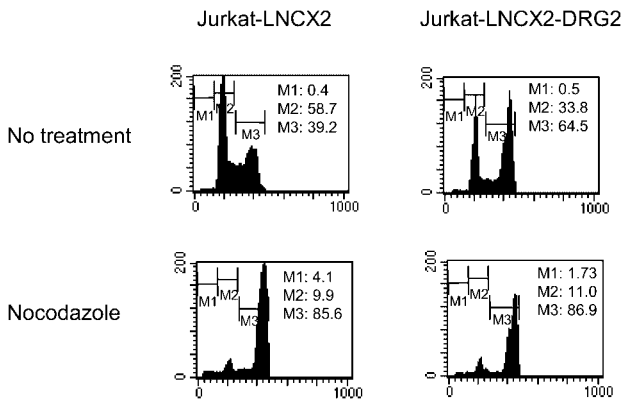

Fig. 2. **Cell cycle analyses of Jurkat cells.** Cells (2×10^5 cells/ml) were treated with nocodazole (20 ng/ml). After being cultured for 24 h, cells were fixed in absolute ethanol at –20°C, stained with propidium iodide according to the manufacturer's protocol (Molecular Probes Inc.), and the resulting cells were analyzed by using flow cytometer. M1 stands for sub-G1 population, M2 for cells in G_1 phase, and M3 for cells in G_2 and M phase.

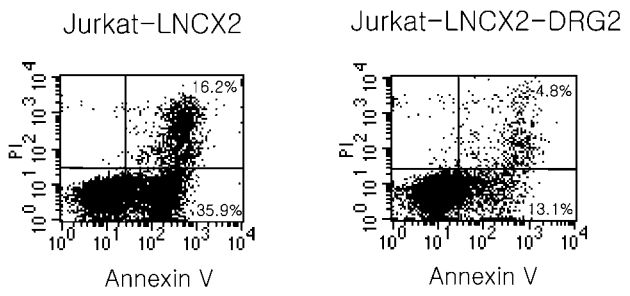

Fig. 3. **Effects of nocodazole on the death of Jurkat cells.** Cells (2×10^5 cells/ml) were treated with nocodazole (20 ng/ml). After being cultured for 24 h, cells were stained with Annexin V and propidium iodide, and the resulting cells were analyzed by flow cytometry.

While Jurkat-LNCX2 cells were mostly in an aggregated form, Jurkat-LNCX2-DRG2 cells were in disaggregated forms (Fig. 1B). Usually, suppression of cell adhesion results in a form of apoptosis often referred to as anoikis (*15*, *16*). However, despite the suppression of cell-to-cell adhesion, apoptosis was not induced in the Jurkat-LNCX2-DRG2 cells. This can be partly explained by our previous observation that DRG2 induced an expression of prosurvival genes CX3CR1, CXCR5, ACK1, and RUNX2 (*17*).

To determine whether the loss of dependency in intercellular interaction was related to actin filamentous or microtubule structure, we treated cells with cytoskeleton-disrupting drugs, cytochalasin D for actin filament and nocodazole for microtubule. On treatment with up to 10 μM cytochalasin D, Jurkat-LNCX2 and Jurkat-LNCX2-DRG2 showed similar morphological responses under a microscope (data not shown). In contrast, the morphology of cells treated with microtubule disrupting drugs (nocodazole or paclitaxel) showed significant differences between Jurkat-LNCX2 and Jurkat-LNCX2-DRG2 cells. The treatment of Jurkat-LNCX2-DRG2 with nocodazole (the same effect was observed with paclitaxel, data not shown) for as short as 2 h rendered a tubular and finger-like structure on the surface of the cells. Very few of the Jurkat-LNCX2 cells treated with the same drug showed similar responses (Fig. 1B). Next, the effect of aphidicolin on morphology was examined. Aphidicolin is reported to arrest the cell cycle by interfering with the activity of DNA polymerase-alpha (*18*). This compound did not show similar effects to nocodazole (Fig. 1B). Therefore, we could exclude the possibility that morphological changes are caused by cell-cycle arrest. These results show the possibility that the combined effects of DRG2 and nocodazole (or paclitaxel, but not cytochalasin D) may weaken the tensegrity forces, a term conceived by Ingber for the major forces maintaining cellular morphology (*19*). Tensegrity in Jurkat cells may be largely attributed to the microtubule network rather than microfilaments.

Previous attempts to find out whether DRG1 or DRG2 has a role in cell growth have not shown clear-cut answers (*2*, *3*, *20*). Since Jurkat-LNCX2-DRG2 cells showed significant differences in growth (*17*) and morphology, and in responses to nocodazole treatment compared to Jurkat-LNCX2, we tried to analyze the cell cycle through measuring DNA contents by using flow cytometric analysis. Interestingly, the number of cells in the G_2 and M phases of Jurkat-LNCX2-DRG2 was twice as much as that of Jurkat-LNCX2, implying that there could be a delay in passing through the G_2 or M phase in Jurkat-LNCX2-DRG2 (Fig. 2). This result indicates that DRG2 slows the proliferation of Jurkat cells when overexpressed. Investigation of the cause of this retarding effect on cell-cycle progress by DRG2 is underway.

In our previous experiment, nocodazole treatment affected cell morphology. Nocodazole, a microtubule-interfering agent, is known to arrest the cell cycle at G_2/M phase and to activate the JNK/SAPK signaling pathway and induce apoptosis in cells (*21*). In an attempt to investigate whether the drug also affects cell-cycle progress, we treated cells with nocodazole for 24 h. The nocodazole treatment delayed cell-cycle progress in both Jurkat-LNCX2 and Jurkat-LNCX2-DRG2 cells, and the percentages of cells of Jurkat-LNCX2-DRG2 and Jurkat-LNCX2 in the G_2/M phase were similar (86.9% vs. 85.6%) (Fig. 2). However, there was a significant difference in the change in the percentage of cells in the G_2/M phase between the two cell types. That in Jurkat-LNCX2-DRG2 cells, from 64.5% to 86.9%, was much smaller than that in Jurkat-LNCX2 cells, from 39.2% to 85.6%. In addition, there was a significant difference in cell death between the two cell types. While the percentage of sub-G1 apoptotic cells in Jurkat-LNCX2 was 4.1%, that in Jurkat-LNCX2-DRG2 was 1.73% following nocodazole treatment (Fig. 2). Annexin V staining also showed that nocodazole-induced cell death in Jurkat-LNCX2-DRG2 was significantly reduced compared with that in Jurkat-LNCX2; while the percentage of cell death in Jurkat-LNCX2 was 52.13%, that in Jurkat-LNCX2-DRG2 was 17.92% (Fig. 3). This suggests that Jurkat-LNCX2-DRG2 cells have already been adapted to a retardation of cell-cycle progress at the G_2/M phase and, thus, the delay of the cell cycle caused by nocodazole treatment is less stressful than in Jurkat-LNCX2 cells.

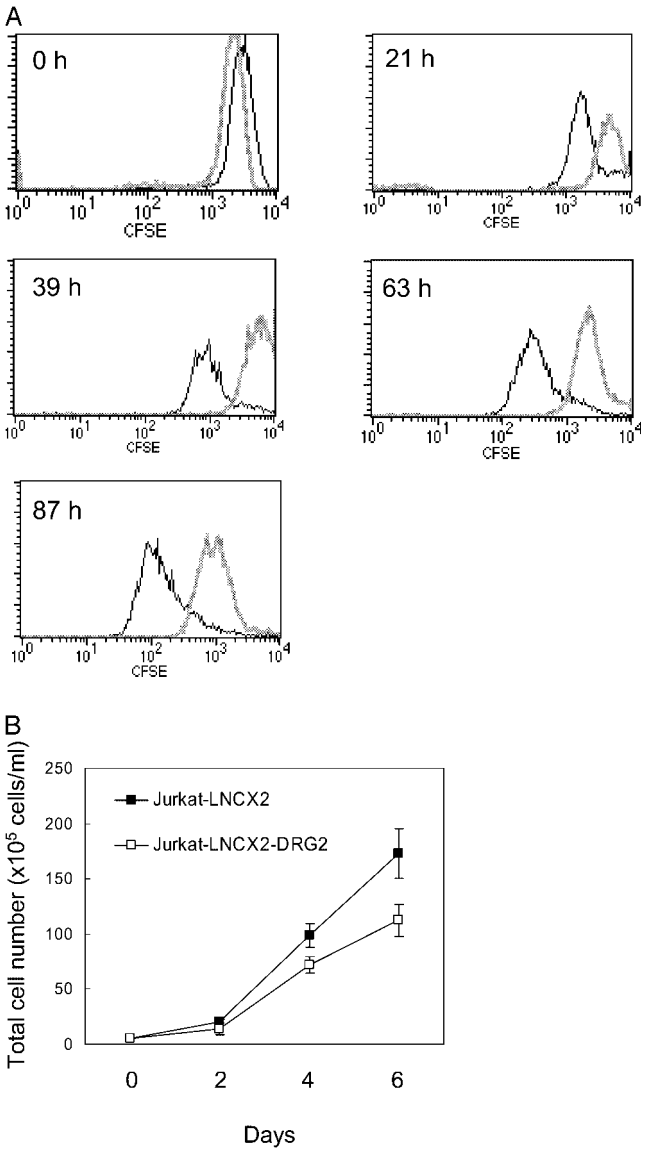

Fig. 4. **Effects of DRG2 on the cell-cycle progress of Jurkat cells.** (A) Cells were stained with CFSE, then were withdrawn at the indicated times. The fluorescence of the cells was analyzed by flow cytometry as described in "MATERIALS AND METHODS." Thin black line, Jurkat-LNCX2; pale grey line, Jurkat-LNCX2-DRG2. (B) Cultured cells were counted every 2 days. Results represented are based on triplicate samples.

Since there was a significant delay at the G_2/M phase in the Jurkat-LNCX2-DRG2, we compared the overall rate of cell cycle-proliferation by staining cells with CFSE, a nontoxic intracellular fluorescent dye which is applicable to track the division of individual cells (*22*). The fluorescence intensity is two-fold serially reduced following each cell division as the dye is equally portitioned to the two daughter cells. The dividing activity was noticed as early as the first sample withdrawn at 21 h, and the peak position of Jurkat-LNCX2 moved much faster towards the left compared to that of Jurkat-LNCX2-DRG2, suggesting that the overall cell-cycle progress of Jurkat-LNCX2-DRG2 is significantly slow compared to that of the Jurkat-LNCX2 cells (Fig. 4A). Next, we counted the cells number every 2 days and compared the growth of Jurkat-LNCX2-DRG2 cells with that of Jurkat-LNCX2 cells. As shown in Fig. 4B, the growth of Jurkat-LNCX2 cells was suppressed compared with that of Jurkat-LNCX2 cells. These findings may be summarized as two distinct points: 1) an overexpression of DRG2 causes morphological changes in Jurkat cells; 2) DRG2 delays passage through the G_2/M phase in Jurkat cells.

At present it is not certain how DRG2 regulates the progress of the cell cycle. The factor interacting with DRG2 may provide clues to the action mechanisms of DRG2. DRG1, a member of the DRG family, was found to interact with SCL, a basic helix-loop-helix (bHLH) transcription factor (*10*). Thus, it is possible that DRG2 interacts with bHLH transcription factors and may positively or negatively alter their functions. Mahajan *et al.* (*10*) demonstrated that the C terminus of the DRG1, which constitutes the outer boundary of the G1-G5 motifs for GTP binding, is responsible for the association with SCL. Interestingly, the C terminus 75 amino acids of both DRG1 and DRG2 possess a putative TGS domain according to the structural motif alignment program of Pfam alignment (http://pfam.wustl.edu). Thus, the TGS domain of DRG2 may be involved in interaction with other proteins such as bHLH transcription factors.

The well-classified GTP-binding proteins are known to possess GTPase activity, which allows them to function their roles. DRG2 has the characteristic GTP-binding motif, G1-G5, and, thus, it is highly possible that DRG2 possesses GTPase activity. If DRG2 possess GTPase activity, DRG2 may require additional factors to enhance this activity, as Ras family proteins do. These facts suggested that there may be a GTPase activity enhancing factor to regulate the GTPase activity of DRG and allow it to perform its biological functions. All these suggest that further studies on the proteins interacting with DRG2 could reveal how DRG2 regulates the cell cycle.

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