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Suppression of viral replication by *drs* tumor suppressor via mTOR dependent pathway

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ABSTRACT

The *drs* gene is an apoptosis-inducing tumor suppressor. By using *drs*-knockout (KO) mouse embryonic fibroblasts (MEFs), we showed that *drs* is involved in the host defense against viral infection. In *drs*-KO MEFs infected with vesicular stomatitis virus, the viral replication and protein synthesis were markedly enhanced without the upregulation of the cellular protein synthesis. Phosphorylation of S6K, S6, 4EBP1 and TSC2 proteins was closely correlated with the enhanced viral replication in *drs*-KO MEFs. *Drs* protein could associate with stress-inducible GADD34 to form a complex with TSC1/2, which suppresses mTOR activity. These findings indicate that *Drs* suppresses viral replication via mTOR-dependent pathway.

1. Introduction

The *drs* (down-regulated by v-*src*) gene was originally isolated as a transformation suppressor for the v-*src* oncogene [1,2]. The expression of *drs* mRNA has been shown to be markedly downregulated in a variety of human cancer cell lines and malignant tumor tissues [3–8]. Malignant tumors including lymphomas, lung adenocarcinomas and hepatomas were generated in about 30% of *drs*-knockout (KO) mice [9]. These findings indicate that *drs* contributes to the suppression of malignant tumor formation. Furthermore, we have shown that *drs* is involved in the processes of apoptosis and autophagy under environmental stressors such as tumor formation and low serum culture conditions. Ectopic expression of the *Drs* protein induced apoptosis in human cancer cell lines via a novel pathway initiated from the endoplasmic reticulum (ER) and involving the binding to ASY/Nogo-B/RTN-x_S, apoptosis-inducing proteins localized in the ER, and activation of caspase-12, -9, and -3 [10]. By using *drs*-KO mouse embryonic fibroblast (MEF) cells, we also found that *drs* is involved in the regulation of the maturation process of autophagy induced by low serum stress, and that this regulation also involves the binding to Rab24, a member of the Rab GTPase protein family, which plays a role in autophagy [11]. These findings suggest that *drs* is involved in the determination of cell fate under environmental stress via apoptosis and/or autophagy and plays protective roles against malignant tumor formation. Close relationships have been reported among the environmental stress responses, tumorigenesis, and the host defense against viral infection. Apoptosis has been shown to act as an innate immunity response against both viral infection and tumorigenesis [12–14]. Autophagy is also involved in the cellular response to infection by several viruses, although its function is double-edged and dependent on the type of the pathogens and the host cells [15–17]. The inactivation of tumor suppressor genes such as p53 and

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the induction of hTERT enhance the sensitivity to viral infection [18]. Previously, we have also reported that GADD34, a protein that is induced by various stressors, including DNA damage, heat shock, nutrient deprivation, energy depletion, and endoplasmic reticulum stress, is induced by viral infection and inhibits viral replication by suppressing the viral protein synthesis via the mTOR signaling pathway, which is known to regulate tumorigenesis, apoptosis, and autophagy [19]. Based on these findings, we attempted to investigate the role of the *drs* tumor suppressor on the host defense against viral infection by using *drs*-KO MEFs, and found that *drs* is involved in the suppression of viral replication via mTOR-dependent pathway.

2. Materials and methods

2.1. Viruses and cells

Vesicular stomatitis virus (VSV) (New Jersey strain), herpes simplex virus type 1 (HSV-1) (KOS strain), and murine Rous sarcoma virus (MRSV), a murine recombinant retrovirus containing the v-*src* oncogene which contains murine leukemia virus as a helper [20], were used in this study. MEFs deficient in *drs* (*drs*-KO MEFs) or wild-type MEFs (WT MEFs) were prepared from male embryos resulting from the mating of *drs* heterozygous (+/−) female and wild-type (+/Y) male mice, as the *drs* gene is located in chromosome X [9]. The immortalized *drs*-KO and WT MEF cell lines, KO-LT and WT-LT, were established by expressing the large T antigen of SV40 in *drs*-KO and WT MEFs, respectively. WT-LT and KO-LT MEFs were used in this study as WT MEFs and *drs*-KO MEFs. Vero was used for the plaque formation assay to titrate VSV and HSV-1. 3Y1 was used for the focus assay to titrate MRSV. 293T, a human embryonic kidney cell line expressing the E1 gene of adenovirus type 5 and the large T antigen of SV40, was used for the preparation of the recombinant retrovirus and the immunoprecipitation assays. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

2.2. Antibodies and inhibitors

Anti-S6, anti-phospho-S6 (Ser240/244), anti-S6K (49D7), anti-phospho-S6K (Thr421/Ser424), anti-4EBP1 (53H11), anti-phospho-4EBP1 (Thr37/46, 236B4), anti-phospho-TSC2 (Thr1462, 5B12), anti-Akt and anti-phospho-Akt (Thr308, C31E5E) antibodies were purchased from Cell Signaling Technology. Anti-phospho-eIF2α (Ser51) antibody was purchased from Biosource International. Anti-eIF2α (FL-315), anti-TSC2 (C-20) and anti-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology. Anti-ß-actin and anti-Flag M2 (F3165) antibodies were purchased from Sigma. Anti-GFP antibody (Living Colors™) was obtained from Takara Bio. Anti-HA (3F9) rat high-affinity antibody was obtained from Roche Applied Science. Anti-VSV antibody was provided by Bin Gotoh (Shiga University of Medical Science). Rapamycin was purchased from the LC Laboratory.

2.3. Plaque assay for VSV and HSV-1

Inoculation of 5×10^5 MEF cells was carried out using 60-mm dishes containing DMEM supplemented with 10% FBS. After 24 h of incubation, the virus was added to the culture at a multiplicity of infection (MOI) of 0.1 or 10 plaque forming units (PFU) per cell. After incubation for 1 h at 37 °C, the cells were washed once with phosphate-buffered saline (PBS), and 5 ml of DMEM containing 2% FBS was added to the cultures. Virus-infected cultures were then further incubated for 18 h (MOI, 0.1) or 6 h (MOI, 10). The cells, together with the culture medium, were harvested, and the virus stock was prepared by freeze–thawing and centrifuging the lysate at 500*g* for 20 min to eliminate the cell debris. The titer of the virus stock was assayed for infectivity by the plaque method using Vero cells. Monolayered Vero cells were infected with several dilutions of the viral stock and were incubated for 1 h at 37 °C. Unadsorbed viruses were then removed by washing, and an overlay of DMEM supplemented with 2% FBS and 0.4% methylcellulose was added to the cultures. The cultures were incubated until plaques were readily visible (~24 h) and were then fixed in formaldehyde and stained with 1% crystal violet in 20% ethanol.

2.4. Focus assay for MRSV

Inoculation of 2×10^5 of MEF cells was carried out in 60-mm dishes containing DMEM supplemented with 10% FBS. After 16 h of incubation, the cells were treated with Polybrene (2 μg/ml) for 30 min and infected with MRSV [20]. Five days after infection, the culture medium was recovered from the MRSV-infected MEFs and was filtrated; the titer was then assayed in 3Y1 cells. Ten days after infection, the number of transformed foci was counted.

2.5. ^{35}S metabolic labeling

Virus- (MOI, 10) and mock-infected cell cultures were incubated for 6 h in DMEM supplemented with dialyzed 2% FBS, and newly synthesized proteins were labeled with Tran^{35}S-label (100 μCi/dish) (MP Biomedical Inc.) in methionine- and cysteine-free DMEM supplemented with dialyzed 2% FBS for 30 min. The labeled cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% protease inhibitor cocktail (Nacalai Tesque) and were centrifuged for 20 min at 10,000*g* at 4 °C. Twenty microliters of the supernatant was precipitated with 1 ml of trichloroacetic acid, and the radioactivity of the labeled proteins was measured using a liquid scintillation counter. ^{35}S incorporation was indicated as counts per minute (CPM) per 10^5 cells.

2.6. Immunoprecipitation

The cells were lysed in RIPA buffer containing 20 mM Tris–HCl (pH7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% protease inhibitor cocktail (Nacalai Tesque) and 1% protein phosphatase inhibitor cocktail (SIGMA), and was centrifuged for 30 min