Suppression of v-src Transformation by the drs Gene

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Previously, we isolated a novel gene, drs, which was downregulated by retroviral oncoproteins such as v-src and v-K-ras, from a cDNA library of primary rat embryo fibroblasts. Experiments using a temperature-sensitive mutant of the v-src gene indicated that downregulation of drs mRNA was dependent on functional expression of v-Src. In addition, expression of drs mRNA was also reduced by serum stimulation of G0-arrested normal rat fibroblast cells. To clarify the function of the drs gene in cell transformation and proliferation, we introduced drs linked to a potent promoter into a normal rat cell line, F2408, and examined the effect of ectopic expression of exogenous drs on the transformation by the v-src gene and growth properties. Cells expressing exogenous drs gene showed significantly decreased efficiency of transformation by v-src irrespective of functional expression of v-Src kinase, while the growth rate and G0/S progression of the cells were not suppressed by expression of exogenous drs gene, indicating that drs has the ability to suppress v-src transformation without disturbing cell proliferation.

Transformation by viral oncoproteins induces a variety of cellular changes such as cell rounding, loss of contact inhibition, decrease of serum requirement for cell proliferation, and anchorage-independent growth. The v-src oncogene of Rous sarcoma virus (RSV) has been most intensively investigated (29). The product of v-src is a membrane-associated phosphoprotein, pp60V-src, which has tyrosine-specific protein kinase activity (9, 20, 32). The v-src gene also positively or negatively alters the expression of many cellular genes (1, 6, 12, 14, 16, 28, 33, 40, 44, 45, 49). A few of the genes whose expression is negatively regulated by v-src have been shown to function as tumor suppressor genes (11, 33, 41). We have reported that a suppressive factor(s) for v-src transformation is expressed in primary rat embryo fibroblasts (REF) (25, 51, 52). On searching for such transformation suppressor genes, we recently isolated a novel gene, drs (downregulated by v-src), which was expressed in normal rat fibroblast cells but completely suppressed in the cells transformed by the v-src gene, from a cDNA library of REF (42). The drs gene was also demonstrated to be downregulated by other retroviral oncoproteins such as v-fps, v-ras, v-mos, v-sis, and v-abl. The molecularly cloned cDNA of drs was about 1.8 kb in size, containing an open reading frame composed of 464 amino acid residues. This protein had one transmembrane domain in the C terminus and three consensus repeats conserved in various numbers in the extracellular domain among the selectin family of adhesion molecules and complement binding proteins (4, 30). Marked downregulation of drs mRNA in oncogene-transformed cells suggests that the drs gene plays a role in suppression of transformation.

To clarify the function of drs for cell transformation, we initially investigated the correlation between downregulation of drs mRNA and functional expression of the v-src gene. To perform this experiment, we used a rat F2408 cell clone (OS7-2) containing a temperature-sensitive mutant (OS122) of RSV (13, 38, 39, 54). As shown in Fig. 1, OS7-2 displays round refractile morphology typical of transformed cells when incubated at 35°C. When the temperature was shifted to 39°C from 35°C, the morphology of OS7-2 was gradually converted to a flat phenotype within 24 h. Tyrosine kinase activity of v-Src by in vitro kinase assay with anti-Src serum was also reduced by temperature shift from 35 to 39°C (Fig. 2A). The change of tyrosine kinase activity roughly paralleled the morphological change in OS7-2 cells. The levels of drs mRNA gradually increased after the temperature increase to 39°C in parallel with the decrease of the kinase activity in OS7-2 cells (Fig. 2B). In the F2408 cell clone S7-1 (23), containing wild-type RSV, the transformed morphology, tyrosine kinase activity, and level of drs mRNA were not changed by temperature shift (Fig. 1 and 2). The expression of drs mRNA in untransformed F2408 cells was also not affected by temperature shift (Fig. 2B). These results indicate that downregulation of drs mRNA depends on functional expression of v-Src tyrosine kinase and parallels the expression of transformed morphology.

To examine whether drs mRNA expression varies with cell cycle progression, F2408 cells arrested in G0 phase by serum starvation were stimulated by serum (10% fetal calf serum [FCS]) and the change in the amount of drs mRNA was examined (Fig. 3). Under these conditions, G0-arrested F2408 cells synchronously progressed to S phase 12 h after serum stimulation (26). Expression of drs mRNA was gradually reduced until 5 h after serum stimulation (Fig. 3). The level of drs mRNA was unchanged from 5 to 18 h (the peak of S phase) (Fig. 3). These results suggest that expression of the drs gene is also regulated during the cell cycle.

To assess the function of the drs gene in v-src transformation and cell proliferation, we constructed a recombinant plasmid, pSRvNeo/drs, that expresses the drs gene under the potent promoter-enhancer SRe (50), and transfected this plasmid into an F2408 cell line. After G418 selection, six independent G418-resistant clones (F-drs-2, -3, -4, -5, -7, and -10) were isolated, and expression of exogenous drs gene in these clones was examined by Northern blot hybridization. As shown in Fig. 4A, three clones (F-drs-2, -4, and -7) expressed high levels of exogenous drs mRNA (upper band) in addition to expressing endogenous drs mRNA (lower band), while another three clones (F-drs-3, -5, and -10) expressed only endogenous drs mRNA. The cell morphologies of F2408 and the six G418-
resistant clones were similar (data not shown). To clarify whether the drs gene has the activity to suppress v-src transformation, F2408 and these clones were infected with a high titer of a recombinant murine retrovirus (MRSV) containing the v-src gene (2), and the colony-forming abilities in soft agar and the focus-forming abilities in liquid culture of these cells were investigated. As shown in Fig. 5A, the colony-forming efficiencies of the clones expressing exogenous drs (F-drs-2, -4, and -7) were significantly lower than those of F2408 and the clones expressing only endogenous drs. Focus-forming efficiencies of F-drs-2, F-drs-4, and F-drs-7 by MRSV were also markedly decreased compared with those of F2408 and F-drs-10 (Fig. 5B). These results suggest that the drs gene acts to suppress v-src transformation. To exclude the possibility that expression of functional v-Src protein was inhibited in the clones expressing exogenous drs gene, the tyrosine kinase activity of v-Src protein in MRSV-infected cells was examined by in vitro protein kinase assay with anti-Src serum. Figure 6 shows the results. v-Src kinase activities of F-drs-2, -4, and -7 infected with MRSV were not reduced compared with those of MRSV-infected F2408, F-drs-3, -5, and -10, indicating that suppression of v-src transformation in the clones expressing exogenous drs gene is not due to the reduced expression of v-Src tyrosine kinase. We also examined the expression of exogenous and endogenous drs mRNA in mock- and MRSV-infected F-drs-7 cells. As shown in Fig. 4B, the level of endogenous drs mRNA was reduced by MRSV infection, whereas the expression of exogenous drs mRNA driven from the SRα promoter was not affected by MRSV, confirming that v-src certainly functions to downregulate endogenous drs in MRSV-infected F2408 cells expressing exogenous drs. However, the drs gene driven from the exogenous promoter was not downregulated by v-src. This
result further supports our conclusion that an ectopically expressed exogenous *drs* acts to suppress transformation by v-src.

To further confirm the correlation between expression of exogenous *drs* and suppression of transformation by v-src, we constructed a recombinant retrovirus containing the *drs* gene and the puromycin-resistant gene as a selective marker (pBabePuro-drs) and infected F2408 cells with the virus. After incubation in selection medium containing 1 μg of puromycin/ml, puromycin-resistant cells were pooled, infected with MRSV, and inoculated into soft agar. F2408 cells infected with pBabePuro-drs virus expressed a considerable amount of viral mRNA hybridized with *drs* cDNA (Fig. 4C). As shown in Fig. 5C, the colony-forming efficiency of F2408 cells containing pBabePuro-drs virus (F/pBP-drs) by MRSV was markedly decreased compared with that of F2408 cells containing vector virus (F/pBP). v-Src kinase activities in MRSV-infected F/pBP and F/pBP-drs cells were also almost similar (Fig. 6), confirming the suppression function of *drs* for v-src transformation. In addition, we also examined colony formation in soft agar and focus formation of F2408, F-drs-2, F-drs-4, F-drs-7, and F-drs-10 cells by a murine retrovirus containing v-K-ras (Ki-MSV). As shown in Fig. 5D (soft agar assay) and E (focus assay), transformation efficiencies of F-drs-2, F-drs-4 and F-drs-7 were also significantly decreased compared with those of F2408 and F-drs-10. These results, together with those of v-src transformation, indicate that ectopic expression of the *drs* gene suppresses transformation by viral oncogenes such as v-src and v-K-ras.

To investigate the effect of ectopic expression of the *drs* gene on growth properties of F2408 cells, we examined the growth rates of F2408, F-drs-2, F-drs-7, and F-drs-10 cells. As shown in Fig. 7A, expression of exogenous *drs* gene did not affect the growth rate of the cells. The growth rates of F/pBP and F/pBP-drs cells were also similar (data not shown). To examine whether overexpression of exogenous *drs* gene suppresses G1/S progression of the cell cycle, F2408, F-drs-2, F-drs-7, and F-drs-10 cells were arrested in G0 phase by serum starvation and stimulated with serum. Progression of the cell cycle of these cells was examined by flow cytometry-activated cell sorting (Fig. 7B). The entry into S phase at 18 h after serum stimulation was not reduced by expression of exogenous *drs* gene in F-drs-2 and F-drs-7 cells compared with that of F2408 and F-drs-10 cells, indicating that the exogenous *drs* gene does not suppress G1/S progression of the cell cycle. From these results, we concluded that the *drs* gene acts to suppress transformation induced by v-src and v-ras without affecting cell proliferation in F2408 cells.

Previously, we showed that *drs* mRNA was markedly reduced in rat cell lines transformed by v-src, v-fps, v-ras, v-mos, v-sis, v-abl, or middle T antigen of polyomavirus but not in cell lines transformed by large T antigen of simian virus 40 or the E6 and E7 genes of human papillomavirus type 16 (24, 42). As the oncogenes that reduced *drs* mRNA are considered to act upstream of the p42/p44 mitogen-activated protein (MAP) kinase pathway (19, 43), we speculated that expression of the *drs* gene is negatively regulated by mitogenic signals from growth factors downstream of the MAP kinase pathway but upstream of the cyclin/CDK-Rb pathway. In fact, as shown in Fig. 3, the level of *drs* mRNA was considerably reduced by serum stimulation of G0-arrested cells although the expression was not completely suppressed. This result suggested that downregulation of *drs* mRNA by mitogenic signals plays a role in the progression of the cell cycle. However, overexpression of the exogenous *drs* gene by the SRα promoter did not affect cell proliferation (Fig. 7). Although mitogenic factors in the serum act to modestly regulate the expression of *drs* gene, the downregulation might not be critical for G1/S progression of the cell.
cycle. However, we cannot completely rule out the possibility that the level of exogenous drs mRNA is not sufficient to affect cell proliferation in this cell line.

Figure 4B shows that introduction of the v-src gene reduced the level of endogenous drs mRNA but did not affect that of exogenous drs mRNA driven by the SRα promoter. This result also implies that downregulation of the drs gene by v-src is caused by transcriptional repression in the 5' regulatory region of the drs gene. Activated MAP kinase moves into the nucleus and acts to regulate gene transcription (19, 43). Viral onco- genes such as v-src and mitogenic factors in serum may repress transcription of the drs gene through the MAP kinase pathway. The mechanism of downregulation of the drs gene by onco- genes and mitogens still remains to be worked out.

Introduction of the v-src gene into normal cells results in multiple cellular events including morphological change, activation of the mitogenic pathway, and anchorage-independent growth. Overexpression of the drs gene with the SRα promoter significantly suppressed anchorage-independent growth and focus formation by v-src without disturbing usual cell proliferation. Expression of v-Src tyrosine kinase by MRSV infection was not affected by ectopic expression of the drs gene (Fig. 6), indicating that drs acts to suppress v-src transformation after function of v-Src kinase. The most prominent biochemical change induced by v-Src is an extensive tyrosine phosphorylation of cellular proteins (29). Most of these target proteins of v-Src kinase are localized in the focal adhesions linked to the plasma membrane. Deregulated phosphorylation of these focal adhesion proteins, such as paxillin, focal adhesion kinase, talin,
and tensin, is considered to be the cause of rounding and disordered proliferation of the cells. The gene structure of Drs implies the membrane-associated localization of Drs protein. It seems possible that the product of the Drs gene interacts with these focal adhesion proteins at the membrane and interferes with transformation by v-Src. The Drs gene also contains repeated motifs conserved in the extracellular domain among selectin family adhesion molecules (4, 30). Three selectins (L-selectin, E-selectin, and P-selectin) are included in this family. They share a similar molecular structure, consisting of an amnio-terminal C-type lectin domain, an epidermal growth factor-like domain, from two to nine short complement regulatory repeats, a transmembrane domain, and a short cytoplasmic tail (5, 27, 31, 48, 53). The complement regulatory repeats of the selectin family had homology with three consensus repeats of the Drs gene (42). The selectin family is considered to be crucial for the initial step of leukocyte-endothelial interaction in response to inflammatory stimuli such as injury and infection (4, 30). Recently, in addition to adhesion function, E-selectin was shown to associate with actin-associated proteins such as α-ac-tinin, vinculin, filamin, pavillin, and focal adhesion kinase (57). L-selectin has also been reported to act as a signaling molecule which activates MAP kinase and Ras pathways through tyrosine phosphorylation (7, 56). In this regard, selectins resemble another family of adhesion molecules, integrins. The integrin-mediated signaling pathway is thought to play an important role in adhesion-dependent cell cycle progression as well as regulation of the cytoskeleton (3, 21, 46, 47). Accumulating evidence indicates that some of the adhesion molecules localized in the plasma membrane are able to act as tumor suppressor genes (15, 18, 55). The Drs gene may also function as a receptor for adhesion signaling and be involved in the anchorage-dependent pathway. Further investigation of the Drs gene is necessary to clarify the mechanism of suppression of transformation. Recently, we found that the Drs gene is highly homologous (80% in nucleotide sequence) to a human gene which is deleted in patients with X-linked retinitis pigmentosa (10, 35), suggesting that Drs has significant physiological functions in a variety of cell types.

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REFERENCES