Isolation of Transformation Suppressor Genes by cDNA Subtraction: Lumican Suppresses Transformation Induced by v-src and v-K-ras

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Received 21 June 1999/Accepted 5 October 1999

We have reported that suppressive factors for transformation by viral oncogenes are expressed in primary rat embryo fibroblasts (REFs). To identify such transformation suppressor genes, we prepared a subtracted cDNA library by using REFs and a rat normal fibroblast cell line, F2408, and isolated 30 different cDNA clones whose mRNA expression was markedly reduced in F2408 cells relative to that in REFs. We referred to these as TRIF (transcript reduced in F2408) clones. Among these genes, we initially tested the suppressor activity for transformation on three TRIF genes, TRIF1 (neuronatin), TRIF2 (heparin-binding growth-associated molecule), and TRIF3 (lumican) by focus formation assay and found that lumican inhibited focus formation induced by activated H-ras in F2408 cells. Colony formation in soft agar induced by v-K-ras or v-src was also suppressed in F2408 clones stably expressing exogenous lumican without disturbing cell proliferation. Tumorigenicity in nude mice induced by these oncogenes was also suppressed in these lumican-expressing clones. These results indicate that lumican has the ability to suppress transformation by v-src and v-K-ras.

Introduction of a single oncogene into primary cells is not sufficient to produce full transformation (21, 22, 27, 35). For tumorigenic transformation of primary cells, either a collaboration between two oncogenes such as v- myc and oncogenic ras or a combination of oncogenic ras and loss of function of one or more tumor suppressor genes is required (15, 17, 21, 22, 37). In contrast, established cell lines such as 3Y1 and F2408, which were established from rat embryo fibroblasts (REFs), can be transformed by single oncogenes such as v-src or v-K-ras (7, 9, 16). This indicates that cell sensitivity to transformation by viral oncogenes differs between REFs and established cell lines and that alteration of cellular factors is required for the oncogenic transformation of REFs (8). In hybrid cells formed from REFs and F2408 transfected by viral oncogenes, the REF phenotype was dominant and transformation of the hybrid cells was suppressed. In contrast, transformation was not suppressed in hybrid cells formed from F2408 transfected by viral oncogenes and untransformed F2408 cells (10, 28). From these results, it was surmised that REFs expressed genes that were able to suppress the transformation, whereas F2408 had lost or down-regulated such genes during the process of immortalization. Several genes such as DAX (NO3), 322, dram, and drs (10, 25, 29, 31, 38) whose expression has been found to be down-regulated in transformed cells relative to normal cells were isolated. Among these genes, expression of DAX and drs genes was shown to suppress transformation by v-src (11, 30). However, many genes whose expression may suppress the transformation remain to be identified.

As a first step toward isolating potential transformation suppressor genes, we carried out cDNA subtraction between REFs and F2408 cells. Using a protocol we recently developed to perform efficient cDNA subtraction (19), we prepared a subtracted cDNA library highly enriched in REF-specific genes. By Northern blot analysis with cDNA inserts prepared from a subtracted cDNA library as probes, we have isolated 30 clones whose expression was reduced in F2408 cells. We refer to these as TRIF (transcript reduced in F2408) clones. Homology searching with the use of BLASTN to search the DDBJ-GenBank-EMBL databases with the DNA sequences of TRIF clones indicated that 18 TRIF clones were identical or highly homologous to the published genes.

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a Particularly interesting new Cys-His protein.
b Cysteine-rich intestinal protein.
c Insulin-like growth factor binding protein 5.
d Osteoblast-specific factor 2.
e Fibroblast-inducible secreted protein.
f Insulin-like growth factor binding protein 3.
g Microtubule-associated protein 1A.
h Dimethylglycine dehydrogenase-like protein.
i Sterol regulatory element binding protein 2.
homologous to registered genes (Table 1). The remaining TRIF sequences were novel except for some homologies with expressed sequence tags (data not shown).

To examine whether reduced expression of TRIF genes in F2408 is common to other rat established cell lines, we carried out Northern blot analysis of two additional rat normal cell lines, 67I and 3Y1, and a transformed cell line, 67T. The 67I cell line was established from REFs by introducing human papillomavirus type 16 E6E7 oncogenes, and 67T was a spontaneously transformed cell line derived from 67I cells after long-term cultivation (8). All 12 TRIF genes examined showed reduced expression in 3Y1 and/or 67I relative to REFs (Fig. 1 and data not shown for novel genes).

To examine whether any of the candidate genes actually FIG. 1. Northern blot analysis using cDNA inserts of TRIF genes as probes. Poly(A)⁺ RNA purified from REFs and F2408, 3Y1, 67I, and 67T cells was separated on 1% agarose gels, transferred to nylon membranes, and hybridized to radiolabeled cDNAs. Type I clones were almost exclusively expressed in REFs, and type II clones showed stronger expression in REFs than in F2408 cells. A duplicate blot was hybridized with β-actin probe as a loading control. The signals were detected with a Bioimaging analyzer or by exposure to X-ray film. See Table 1 for definitions of abbreviations.

FIG. 2. Ectopic expression of lumican suppressed focus formation induced by the activated H-ras oncogene. F2408 cells were cotransfected with 1 µg of pHyg/ras and 10 µg of pSRSeoNeo/Lum or 10 µg of pSRSeoNeo/Lum or 10 µg of pSRSeoNeo vector. (A) Foci formed by cotransfection of pHyg/ras and pSRSeoNeo into F2408 and inhibition of focus formation by cotransfection of pHyg/ras and pSRSeoNeo/Lum into F2408. (B) Numbers of foci induced by activated H-ras. Numbers show the mean values obtained from two independent experiments, and the bars represent the standard deviations from the mean values. (C) Northern blot analysis of lumican and activated H-ras gene expression. Total RNA (20 µg) from each F2408 transfectant was separated by electrophoresis and transferred to a nylon membrane. Hybridizations were carried out by using the full-length lumican cDNA insert or the BamHI genomic fragment (6.6 kb) of the bladder carcinoma oncogene (32). (D) Activity of p42/p44 MAP kinases. The MAP kinase activity was assessed by Western blotting with polyclonal phosphospecific anti-p42/p44 MAP kinase (Thr-202/Tyr-204; New England Biolabs). The activities were normalized according to the amount of p42 MAP kinase.
suppress transformation, we selected three TRIF clones for further analysis. We selected TRIF1, TRIF2, and TRIF3 because their expression levels were extremely reduced in F2408, 3Y1, 67I, and 67T cells relative to REFs. DNA sequencing showed that TRIF1, TRIF2, and TRIF3 were identical to neuronatin, heparin-binding growth-associated molecule (HB-GAM), and lumican, respectively (Table 1) (12–14, 24, 34). There has been no report to date of the effect of expression of these genes on transformation. Since the original TRIF1, TRIF2, and TRIF3 clones were partial cDNAs, we isolated full-length clones of these transcripts from a REF cDNA library by colony hybridization and constructed expression plasmids containing the neuronatin (pSRαNeo/Neuro), HB-GAM (pSRαNeo/HB-GAM), and lumican (pSRαNeo/Lum) coding sequences in the expression vector pAP3neo (19). To examine whether ectopic expression of these genes suppresses transformation, we performed a focus formation assay with the activated H-ras gene. F2408 cells were transfected with pSRαNeo/Neuro, pSRαNeo/HB-GAM, or pSRαNeo/Lum together with a plasmid carrying the activated H-ras gene (pHyg/ras). After G418 and hygromycin B selection, cells were pooled and numbers of transformed foci were scored. As shown in Fig. 2A and B, ectopic expression of lumican inhibited the focus formation induced by activated H-ras, but expression of neuronatin or HB-GAM did not (data not shown). A high level of lumican expression was confirmed by Northern analysis (Fig. 2C, upper panel). The ectopic expression of activated H-ras mRNA between these two transfectants was found to be the same, and this served as a loading control (Fig. 2C, lower panel). Furthermore, mitogen-activated protein (MAP) kinase was similarly activated in both cell populations compared with F2408 cells (Fig. 2D). These results indicate that suppression of focus formation in the cells expressing the exogenous lumican gene is not due to the reduced expression of the activated H-ras gene.

To analyze the effect of lumican more directly, we isolated four F2408 clones that stably expressed lumican, Lu1, Lu2, Lu3, and Lu4, by transfection of a lumican plasmid construct (pSRαNeo/Lum) (Fig. 3B). The cells expressing lumican showed no significant changes in cell morphology (data not shown) or growth rate (Fig. 3A). However, cell density at confluence was relatively lower in lumican-expressing cells than in control cells (Fig. 3A).

To investigate whether expression of lumican suppresses transformation induced by the v-K-ras oncogene, lumican-expressing clones, vector clones (V1 and V2), and F2408 cells were infected with a high titer of Kirsten murine sarcoma virus (Ki-MSV) containing the v-K-ras oncogene (6). After infection, cells (10^5 cells/60-mm dish) were seeded into soft agar (0.4% Noble agar) and the efficiency of colony formation was investigated. The colony formation abilities of Lu1, Lu2, Lu3, and Lu4 were significantly lower than those of F2408 cells and the vector clones (Fig. 4A and C). The levels of v-K-ras expression were determined by Northern blot analysis with a v-K-ras-specific probe (6, 18). A v-K-ras transcript of 6.6 kb was detected in all infected cells but not in uninfected cells (Fig. 4D). The level of the v-K-ras transcript was somewhat different between the cells, but the variations in v-K-ras message levels did not correlate with differences in the efficiency of colony formation. These results indicate that lumican suppresses colony formation in soft agar induced by the v-K-ras oncogene.

We also examined the effect of ectopic expression of lumican on v-src transformation. Cells were infected with a high titer of a murine retrovirus (murine Rous sarcoma virus [MRSV]) containing the v-src oncogene (1), and the efficiency of colony formation in soft agar was investigated. As shown in Fig. 4B and E, colony formation was significantly lower in Lu1, Lu2, Lu3, and Lu4 than in V1, V2, and F2408 cells. The level of infection by the v-src gene was estimated by measuring the tyrosine kinase activity of v-Src protein. As shown in Fig. 4F, increased v-Src kinase activity was observed in all v-src-infected cells compared with uninfected cells. From these results, we conclude that exogenously expressed lumican suppresses colony formation in soft agar induced by v-src and v-K-ras oncoproteins.

Tumorigenicity is one of the hallmarks of transformation induced by v-src and v-K-ras oncoproteins. Therefore, we also examined the effect of lumican expression on tumorigenicity induced by these viral oncoproteins. Lumican-expressing clones, vector clones, and F2408 cells were infected with MRSV containing the v-src gene. After 3 days of infection, cells (1 x 10^5 to 2 x 10^5) were injected into BALB/c nude (nu/nu) mice. Tumorigenic potential was assessed by measuring the size of the resulting tumors, and the results are summarized in Table 2. F2408 cells and vector clones infected with v-src formed large tumors after 15 days, whereas most of the lumican-expressing clones did not form tumors after the same period, except for Lu3, which formed a small tumor. At 22 days, the other lumican-expressing clones had also formed tumors, but the tumor volumes of Lu1 and Lu2 clones were significantly reduced relative to controls, and tumors induced by Lu3 and Lu4 clones grew more slowly than those caused by F2408 and the vector clones. Similar results were obtained when lumican-expressing clones, vector clones, and F2408 cells were infected with MRSV containing v-K-ras, although the suppression of tumorigenicity was weak (tumor volume of lumican-expressing clones, 205.45 mm^3 ± 99.53 mm^3; tumor volume of control...
cells, 557.3 mm$^3$ ± 227.7 mm$^3$). These results indicate that ectopic expression of lumican also suppresses tumorigenicity induced by v-src and v-K-ras oncogenes.

To examine whether down-regulation of lumican is also correlated with expression of malignant phenotypes in human cancers, we examined expression of lumican mRNA in a variety of human cancer cell lines. As shown in Fig. 5A and B, expression of lumican was detected in most of the normal tissue, although expression patterns of rat and human lumican were somewhat different. That is, expression of lumican was detected in rat brain and spleen, whereas it was not detected in human brain and spleen. On the other hand, expression of lumican was not detected in most of the human cancer cell lines examined, such as CaSkI, SiHa, C33A, S3, G401, SW837, T24, HT1080, MIAPaCa-2, and RERF-LC-MS cells (Fig. 5C, lanes 3 to 10, 15, and 16), and was decreased in HeLa and A172 cells (Fig. 5C, lanes 2 and 11) relative to human embryo fibroblasts (Fig. 5C, lane 1).

In this study, we found that expression of lumican in F2408 suppressed the transformation induced by v-src and v-ras oncogenes without affecting the growth rate. We also showed reduced tumorigenicity in nude mice induced by these oncogenes. Lumican belongs to the family of small leucine-rich proteoglycans (SLRPs) that includes decorin, biglycan, fibromodulin, keratocan, epiphycan, and osteoglycin (12). Lumican colocalizes with fibrillar collagens in the connective tissues (2, 3) and inhibits the rate of collagen fibrillogenesis in vitro (33). In a recent study, a knockout mouse strain lacking the intact lumican gene was generated by gene targeting (4). Lumican deficiency was not lethal but caused skin laxity and fragility resembling certain types of Ehlers-Danlos syndrome. The growth of collagen fibrils in the skin and cornea was deregulated in the mutant mice, although there was no report of tumor formation. Decorin also is a member of the SLRP protein family, and the phenotypes of decorin-deficient mice were very similar to those of lumican-targeted mice (5). In previous reports, overexpression of decorin inhibited cell proliferation in Chinese hamster ovary cells (39) and de novo expression of decorin suppressed the growth and tumorigenicity of colon cancer cells by up-regulation of p21$^\text{WAF1}$, an inhibitor of cyclin-dependent kinases (26, 36). Suppression of decorin expression was found to be related to the induction of anchorage-independent growth caused by v-src in human fetal lung fibroblasts (20). Lumican therefore appears to be involved in the suppression of the transformed phenotype and to reduce tumorigenicity in a manner similar to the activity of decorin. As preliminary data, increased (up to fourfold) expression of p21$^\text{WAF1}$ protein was observed in lumican-transfected cells compared with control cells when a human lung cancer cell line, A549, was transfected with a lumican-expressing vector. Reduced expression of SLRPs such as decorin and lumican may result in abnor-

### TABLE 2. Tumorigenicities of F2408 cells and clones (V1 and V2 and Lu1 to Lu4) infected with MRSV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tumor vol (mm$^3$)</th>
<th>(no. of nude mice with tumor/total no.)</th>
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<tr>
<td></td>
<td>15 days</td>
<td>22 days</td>
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<tr>
<td>F2408$^{\text{ve}}$</td>
<td>282.9 ± 94.4 (4/4)</td>
<td>ND</td>
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<tr>
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<tr>
<td>V2$^{\text{ve}}$</td>
<td>107.0 ± 19.0 (2/2)</td>
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<tr>
<td>Lu1$^{\text{ve}}$</td>
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<td>Lu2$^{\text{ve}}$</td>
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<tr>
<td>Lu3$^{\text{ve}}$</td>
<td>48.0 ± 9.8 (4/5)</td>
<td>339.0 ± 214.3 (3/3)</td>
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<td>Lu4$^{\text{ve}}$</td>
<td>0 (0/3)</td>
<td>161.3 ± 28.6 (3/3)</td>
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*The cells were infected with MRSV containing the v-src gene. Three days after infection, about 1 × 10$^5$ to 2 × 10$^5$ cells were injected into nude mice. Tumor volumes were determined from the following equation: $V = \frac{(L \times W)^2}{2} \times 0.5$, where $L$ is length and $W$ is width. Values represent the means of tumor volumes, and estimates (± standard errors) represent the standard deviations. ND, not determined.
mality of the extracellular matrix engaged in collagen fibrillogenesis and/or of up-regulation of p21WAF1. This may influence sensitivity to transformation induced by viral oncogenes. However, tumors did not appear in either the lumican or the decorin knockout mouse model. One possible explanation for this is that lumican and decorin may carry out complementary functions in suppression of tumorigenesis. If this is the case, a double knockout mouse with deletion of both decorin and lumican may display an increased incidence of spontaneous tumor formation. Further studies are necessary to clarify the mechanism of suppression of transformation by lumican.

We also found that expression of lumican mRNA was down-regulated in a variety of human cancer cell lines. It has been reported elsewhere that lumican was expressed at a high level in human breast carcinoma (23). However, expression of lumican was restricted to stromal cells adjacent to tumor cells, and lumican mRNA was not detected in several epithelial breast cancer cell lines (23). In our Northern blot analysis, lumican was expressed in normal human embryo fibroblasts (Fig. 5C) and normal human lung cells, TIG-1 (data not shown), but expression of lumican mRNA was reduced in various human cancer cell lines (Fig. 5C) including seven lung cancer cell lines (data not shown). However, expression of lumican was detected at a high level in NB-1, AZ521, and MeWo cells (Fig. 5C, lanes 12 to 14). These results suggest that down-regulation of lumican expression may play a role in development of human cancers. It is interesting to examine the correlation between lumican expression and development of some human cancers.

We wish to thank Shigeo Fuji for excellent technical assistance. This work was supported by grants for Cancer Research and Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES


