Tumor-suppressive function of mutated gelsolin in ras-transformed cells

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Abstract

The flat revertant R1, isolated from human activated Ha-ras oncogene-transformed NIH3T3 fibroblasts (EJ-NIH3T3), expresses a variant form of the actin-regulatory protein gelsolin (p92-5.7). We have cloned CDNAS encoding p92-5.7 and identified as the cause of the expression of p92-5.7 a point mutation in codon 321, which results in an amino acid change from proline to histidine. In order to understand the role of p92-5.7 in reversion of ras-transformed cells, CDNAS encoding p92-5.7 or human authentic gelsolin as a control were transfected into EJ-NIH3T3 cells. All the transfectants that produced p92-5.7 and one of three transfectants that produced human authentic gelsolin either lost or reduced tumorigenicity in syngeneic mice. These results demonstrate that mutated gelsolin can suppress a ras tumor and suggest that authentic gelsolin, if expressed at increased levels, may have a similar suppressive potential. Our data propose an important role for gelsolin in cellular signal transduction pathways that involve the mammalian ras proto-oncogene.

Introduction

Gelsolin is representative of a class of actin-regulatory proteins, found in species from lower eucaryotes to mammals, which sever actin filaments (Stossel et al., 1985; Hartwig & Kwiatkowski, 1991). In contrast to the disruptive effect of gelsolin on actin filament formation in vitro, there is a positive correlation between the amount of gelsolin and the degree of microfilament organization demonstrated by a number of human somatic cell hybrids and their parental cell lines (Banyard et al., 1990). A diminished expression of
gelsolin has been shown to be characteristic of a variety of transformed cells (Chaponnier & Gabbiani, 1989; Van-dekerckhove et al., 1990). Recently, several reports have suggested that actin-regulatory proteins such as profilin, gelsolin, gCap39, and cofillin are involved in cellular signal transduction because of their specific binding to membrane polyphosphoinositides in vitro (Lassing & Lindberg, 1985; Janmey & Stossel, 1987; Yu et al., 1990; Yonezawa et al., 1991). In Saccharomyces cerevisiae, profilin may contribute to a signaling pathway that is regulated by the ras proto-oncogene (Vojtek et al., 1991). We previously reported the isolation of a flat revertant R1 from human activated Ha-ras oncogene-transformed NIH3T3 (EJ-NIH3T3) cells by treatment with the mutagen ethyl methanesulfonate (Kuzumaki et al., 1989). R1 cells express a variant form of gelsolin, designated p92-5.7, but NIH3T3 and EJ-NIH3T3 cells do not (Fujita et al., 1990). EJ-NIH3T3 cells show the typical refractile morphology of transformed cells, but R1 cells are flat and have an ordered growth pattern. In comparison with EJ-NIH3T3 cells the doubling time of R1 cells is markedly prolonged and they do not survive culture in low serum. Although R1 cells retain an unchanged human activated Ha-ras oncogene they are non-tumorigenic in syngenic mice and resistant to retransformation by various oncogenes (Kuzumaki et al., 1989). In the present study we isolated CDNAS encoding p92-5.7 and examined the role of p92-5.7 in the reversion process.

Results

Cloning of CDNAS encoding p92-5.7
To clone the CDNA encoding a variant form of gelsolin (p92-5.7) about 5 x 105 clones from an unamplified λgt10 CDNA library of R1 cells were screened with a human gelsolin CDNA probe. Four clones, designated λ 2, λ 6, λ 8 and λ 10, showed inserts of approximately 2.4 kb, which corresponds to the size of full-length murine gelsolin CDNA. As described previously (Fujita et al., 1990) authentic murine gelsolin protein of NIH3T3 and EJ-NIH3T3 cells is resolved into two isoelectric forms on two-dimensional gels. R1 cells produce these authentic gelsolins (spots 2 and 2* in Figure 1) and additionally p92-5.7 (spot 1 in Figure 1), as well as a more acidic variant (spot 1* in Figure 1) of low abundance. In order to assign the isolated CDNA clones to the specific
gelsolin form, RNAs Were transcribed from clones λ2, λ6, λ8, λ10 and authentic murine gelsolin clone 319 (Dieffenbach et al., 1989) as a control. The in vitro-transcribed RNAs Were translated into proteins and the 35S-labeled translation products were mixed with unlabeled R1 cell protein extracts and analysed by two-dimensional gel electrophoresis. The main in vitro translation products of clones λ2, λ6 and λ10 co-migrated with p92-5.7, whereas those of clones λ8 and 319 co-migrated with the major, basic form of authentic gelsolin (Figure 1). The minor translation products of clones λ2, λ6 and λ10 co-migrated with the R1 cell-specific low-abundant gelsolin variant (spot 1* in Figure 1). Likewise, the minor translation products of clones A8 and 319 migrated with the minor, acidic form of authentic gelsolin. Since a major and minor in vitro translation product were observed, it is likely that either one of them or both are post-translationally modified forms of gelsolin. Although the chemical nature of this putative post-translational modification is at present unknown, previous studies have excluded phosphorylation and dolichol-linked glycosylation of gelsolin (Wang et al., 1984; Yin et al., 1984). Taken together, these in vitro transcription-translation experiments suggested the existence of a nucleotide sequence difference between clones λ2, λ6 and λ10 on the one hand and clones A8 and 319 on the other hand. We next determined and compared the nucleotide sequences of clones 319, λ2 and λ8. Within the coding region, we detected only one difference between nucleotides. A cytosine in the second position of codon 321 in clone 319 was replaced by an adenine in clone λ2, thus causing an amino acid change from proline to histidine (Figure 2). Within the same region, clone λ8 showed the same sequence as clone 319 (Figure 2). Both of the CDNA clones λ6 and λ10 underwent the same nucleotide alterations as clone λ2 (data not shown). These sequencing results agree with the in vitro transcription-translation data. Further-more, the change from the neutral amino acid proline to the basic amino acid histidine could explain the altered mobility and the more basic isoelectric point (pI) of p92-5.7, as compared with the authentic gelsolin protein, in two-dimensional gels.

Suppression of ras-transformed cells by p92-5.7 cDNA
To examine the mutated gelsolin’s role in reversion of the ras transformant, we transfected the A2 cDNA into EJ-NIH3T3 cells and obtained stable transfectants (EJ/β
To distinguish effects between the mutated gelsolin p92-5.7 and increased unmutated gelsolin, we also established transfectants (EJ/LKCG-4, EJ/LKCG-12 and EJ/LKCG-19) that would produce a human authentic gelsolin with the expression vector LKCG (Cunningham et al., 1991). The high amino acid homology (94.6%) of human and mouse gelsolin proteins seems to make such a comparison valid. The expression of the transfected genes was analysed by two-dimensional gel electrophoresis (Figure 3). The human authentic gelsolin protein can be distinguished from the endogenous murine gelsolin protein of the transfectants, owing to its slightly lower molecular weight and more basic isoelectric point (pI) on two-dimensional gels. This was determined by co-electrophoresing a total cellular protein extract of human embryonic fibroblasts with murine authentic gelsolin protein produced in E. coli (H. Fujita et al., manuscript in preparation). Furthermore, three control clones (EJ/neo-8, EJ/neo-11 and EJ/neo-14), transfected with the expression vector alone, were isolated. The transfectants showed a refractile morphology similar to parental EJ-NIH3T3 cells, except for one clone, Rev-4, which was flat. The doubling time of the clones expressing p92-5.7 at a serum concentration of 10% was on average longer than that of the parental EJ-NIH3T3 cell line, of clones transfected with vector alone and of clones expressing human authentic gelsolin (Table 1). When cultivated in a medium containing 1% fetal calf serum (FCS), the clones that expressed the relatively highest amounts of p92-5.7 did not survive. In contrast, the clones transfected with vector alone and clones expressing human authentic gelsolin grew at rates similar to EJ-NIH3T3, except for one clone that expressed human authentic gelsolin (EJ/LKCG-4), which was less robust (Table 1). All five clones that expressed different amounts of p92-5.7 either lost or reduced tumorigenicity in 2-day-old syngeneic NIH/Swiss mice (Table 2). On the other hand, three vector control clones (EJ/neo-8, EJ/neo-11, EJ/neo-14) formed tumors that grew progressively. Similarly, two of the clones (EJ/LKCG-12 and EJ/LKCG-19) that expressed human authentic gelsolin developed rapidly growing tumors. However, one clone (EJ/LKCG-4) had a clearly reduced tumor frequency amongst 15 mice. The differences in growth and tumorigenicity among the transfected cell lines were not due to a loss of the ras oncogene, since the presence of human c-Ha-ras DNA sequences and RNA transcripts was confirmed by Southern and Northern blot hybridization (Figure 4).

Taken together, our results demonstrate that p92-5.7 functions as a growth and tumor
suppressor in human ras oncogene-transformed NIH3T3 cells and suggest that the authentic gelsolin gene may have suppressive effects similar to p92-5.7, although weaker, if expressed at increased levels.

**Discussion**

In this study, we describe the cloning of cDNAs encoding the gelsolin variant p92-5.7. These cDNAs contain a point mutation in codon 321, which results in an amino acid change from proline to histidine. The stable introduction of a p92-5.7 encoding cDNA into EJ-NIH3T3 cells caused a reduced growth rate in culture and a suppression of tumorigenicity in syngeneic mice. These results raise the question of how p92-5.7 becomes involved in suppressing the ras-transformed phenotype. The mutation identified in p92-5.7 is located in the third (S3) of six repeated sequence segments (S1-S6) in the gelsolin protein (Way & Weeds, 1988). CT28N fragment, which corresponds to S2-S3, specifically binds to actin filaments, and this binding is inhibited by such polyphosphoinositides as phosphatidylinositol 4,5-biphosphate (PtdInsP2) (Yin et al., 1988). Judging from the location of the mutation, we might speculate that it is the interaction of p92-5.7 with actin filaments and/or PtdInsP2 that is affected. By using deleted mutants of human plasma gelsolin, Kwiatkowski et al. (1989) localized the binding sites of gelsolin to actin filaments and PtdInsP2 within the amino terminus of S2. Such experimental results seem to exclude the possibility of their being an effect of the mutation of the interactions of p92-5.7 with actin filaments and/or PtdInsP2. Nevertheless, the characteristics of the heavily truncated mutant proteins employed by Kwiatkowski et al. (1989) may differ from the intact gelsolin protein. Furthermore, a recent report suggests that the actin filament binding site may involve the amino terminus of S2 as well as additional residues in segments 2 and 3, which are not yet identified (Yu et al., 1991). Therefore, an attractive working hypothesis is that, as in the case of profilin, p92-5.7 may bind PtdInsP2 with increased affinity by amino acid substitution from proline to histidine with a positive charge, thus blocking PtdInsP2 hydrolysis by phospholipase C (PLC) in response to growth factor stimulation (Goldschmidt-Clermont et al., 1991). In fact, the most basic profilin isoform found in the Acanthamoeba has a much higher affinity for PtdInsP2 and inhibits its hydrolysis by PLC.
more strongly than the acidic isoform (Machesky et al., 1990). Although a breakdown of polyphosphoinositides is not always associated with changes in the actin cytoskeleton (Dadabay et al., 1991), such a model is supported by the findings that (a) gelsolin can inhibit PtdInsP2 breakdown by phospholipase C in vitro (Banno et al., 1992) and (b) that suppression of PtdInsP2 turnover by microinjection of an anti-PtdInsP2 antibody into ras transformed cells cause a reduction in proliferation (Fukami et al., 1988). The authentic gelsolin protein may have transformation-suppressing activity by inhibiting PtdInsP2 hydrolysis, and the mutation in p92-5.7 may strongly enhance this activity. Alternatively, if the mutation impairs the severing activity of the protein, p92-5.7 might be able to compete with authentic gelsolin for binding to actin filaments and thereby exert a stabilizing effect on the cytoskeleton; this would lead to tumor suppression. In vitro studies with p92-5.7 protein produced in E. coli now in progress will provide for a better understanding of the mechanisms underlying tumor growth suppression by p92-5.7.

Materials and methods

Cell lines
The origin and properties of NIH3T3, EJ-NIH3T3 and R1 cells, as well as cell culture conditions, were reported previously (Kuzumaki et al., 1989; Fujita et al., 1990).

Cloning of gelsolin cDNAs
About 5 x 10^5 plaques of an unamplified R1 cell cDNA library (Mtillauer et al., 1991) were screened with an ECORI fragment of human plasma gelsolin cDNA (plasmid M1D; Kwiatkowski et al., 1988). A 500-ng aliquot of probe DNA was labeled with [α-32P]dCTP (3000 Ci mmol-1) to a specific activity of 2.8 x 10^8 c.p.m. per µg of DNA by using a nick-translation kit (Takara Shuzo, Tokyo, Japan). Hybridization was carried out at 37°C overnight in 400/ formamide, 10 x Denhardt's solution, 5 x SSPE, 0.3010 SDS and 250 µg ml^-1 denatured salmon sperm DNA. Filters were washed sequentially in (a) 500 ml of 2 x SSC/0.1% SDS at room temperature for 1.5 min, three times; (b) 500 ml of 2 x SSC/0.1% SDS at 40°C for 15 min, twice; and (c) 500 ml of 2 x SSC/0.1% SDS at room temperature for 10 min. Positive phage plaques were suspended in distilled water and phage DNAs were released by boiling for 10 min. Insert DNAs were amplified by
PCR (DNA Thermal Cycler, Perkin Elmer Cetus, Emeryville, USA) with forward and reverse primers (Takara Shuzo) flanking the ECORI cloning site in \(\lambda gt10\); the length of the cDNA inserts was determined by agarose gel electrophoresis.

**In vitro transcription-translation**

As murine gelsolin cDNA contains an internal EcoRI site, complete EcoRI digestion of phage clones deleted from the gelsolin cDNA inserts the 5' untranslated region and the first 18 nucleotides of the coding region. For in vitro transcription experiments, therefore, EcoRI-HindIII fragments of R1 gelsolin cDNAs which were joined to Sacl-EcoRI fragments (which contained the 5' untranslated region and the first 18 nucleotides of the coding region) of murine gelsolin clone 319 (Dieffenbach et al., 1989) were inserted into the Sacl-Hind-III site of pBluescript KS(-) (Stratagene, La Jolla, CA, USA). Furthermore, a Clal-HindIII fragment of clone 319 was subcloned into pBluescript KS(-) for in vitro transcription experiments. RNA was transcribed from each pBluescript KS(-)-gelsolin cDNA construct by using a commercially available in vitro transcription system (mCAP mRNA cap-ping kit, Stratagene). The in vitro-transcribed RNA was translated into protein with rabbit reticulocyte lysates (Amersham, Buckinghamshire, UK, and Wako Pure Chemical Industries, Osaka, Japan) and L-[\(^{35}\)S]methionine (1000 Ci mmol\(^{-1}\)). The synthesized polypeptides were mixed with unlabeled R1 cell protein extracts and analysed with O'Farrell's two-dimensional gel electrophoresis as previously described (Fujita et al., 1990). Proteins were visualized with a silver-staining kit (2D-Silver stain II, Daiichi Pure Chemicals, Tokyo, Japan) and the location of 35S-labeled proteins was determined by autoradiography.

**DNA sequencing**

DNA sequencing was carried out on a single- or double-stranded tinplates after subcloning the cDNAs into bacteriophages M13mp18/mp19 or plasmid pBluescript KS(-). The gelsolin cDNAs nucleotide sequences were determined for both strands with murine gelsolin cDNA-specific oligonucleotide primers, designed from the sequence of clone 319 CDNA. Dideoxy sequencing was performed with [\(\alpha\)-32P]dCTP (3000 Ci mmol\(^{-1}\)) and used a Sequenase Version 2.0 DNA sequencing kit (US Biochemicals, Cleveland, OH, USA) according to the manufacturer's instructions.
Expression vectors and DNA transfection

The expression vectors β 2 and RSV/2 were constructed by ligating the p92/5.7-encoding CDNA clone λ 2 into the Hind-III cloning sites of the eucaryotic expression vectors pH β APr-l-neo (Gunning et al., 1987) and pRc/RSV (Invitrogen, San Diego, CA, USA) respectively. The pH β APr-1-neo-derived human gelsolin expression vector LKCG was described previously by Cunningham et al. (1991). EJ-NIH3T3 cells were transfected with these expression vectors either by the calcium phosphate precipitation method (Graham & van der Eb, 1973) or by lipofection (Lipofectin, Bethesda Research Laboratories, Gaithersburg, MD, USA). Transfected cells were selected by cultivating them in a medium containing 400 μg ml-1 G418 (Southern & Berg, 1982; Geneticin, Sigma, St Louis, MO, USA). After growth for 7-10 days in the selective medium, individual colonies were picked out randomly, expanded and the expression of their transfected genes was analysed by two-dimensional gel electrophoresis as previously described (Fujita et al., 1990).

Growth rate and tumorigenicity assay

For the determination of doubling times and serum dependency, cells (1 x 10⁵) were plated in duplicate in 60-mm dishes and cultivated in 4 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% or 1% fetal calf serum (FCS) and 0.03% L-glutamine. At regular intervals, cells were collected by trypsinization and counted by hemocytometer. The medium was replaced every 2 days for 6 days. Data are representative of at least two independent experiments. For the determination of tumorigenicity, 2-day-old NIH/Swiss mice (Kuzumaki et al., 1989) were injected sub-cutaneously with 1 x 10⁶ cells. We have taken care of the mice according to the guidelines for experimental animals laid down by the Hokkaido University School of Medicine.

DNA and RNA extraction

Genomic DNA was prepared as described previously (Oikawa et al., 1988). Total cellular RNA was isolated with RNAzol (Cinna/Biotex Laboratories International, Friends-wood, USA) according to the manufacturer's instructions. Cells were harvested for RNA extraction at subconfluency, 48 h after the last medium change.
Southern and Northern analysis

Southern and Northern analyses were carried out as described previously (Oikawa et al., 1988). A 2.9-kb Sacl fragment of plasmid pEJ 6.6 (Shih & Weinberg, 1982) was used as a human Ha-ras-specific probe. Probe labeling was done with a random primer DNA labeling kit (Takara Shuzo) and [α-32P]-dCTP (3000 Ci mmol-1). Filters were washed in 2 x SSC and 1 x SSC containing 0.1(−) SDS at 25-55°C until the radioactive bands could be distinguished from the background noise with a Geiger counter.

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References

Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S. & Takenawa, T.


Figure legends:

Figure 1 Two-dimensional gel analysis of in vitro transcription-translation products. Unlabeled total cellular protein extracts from R1 cells were co-electrophoresed with 35S-labeled in vitro transcription-translation products of either murine authentic gelsolin clone 319 and R1 cell gelsolin cDNA clones 2, λ 6, λ 8 and λ 10. IEF, isoelectric focusing. R1, R1 cell proteins visualized by silver staining (only the area of the gel which contains gelsolin is shown). 1 = p92-5.7, 1* = minor R1 cell specific gelsolin variant; 2 = major, basic form of authentic gelsolin; 2* = minor, acidic form of authentic gelsolin. 319, λ 2, λ 6, λ 8 and λ 10, 35S-labeled in vitro translation products of the corresponding cDNA clones, visualized by autoradiography

Figure 2 DNA sequence analysis of murine authentic gelsolin cDNA clone 319 and R1 cell gelsolin CDNA clones λ 2 and λ 8. Nucleotide sequences of the region that contains codon 321 are shown

Figure 3 Expression of the transfected gelsolin gene in EJ-NIH3T3 cells. Total cellular protein extracts from the parental cell line EJ-NIH3T3, control clone EJ.neo-14 ( = expression vector pβ APr-1-neo-transfected clone), EJ/β 2-14 ( = p92-5.7 expression vector p2-transfected clone) and EJ/LKCG-4 ( = human authentic gelsolin expression vector LKCG-transfected clone) were analysed by two-dimensional gel electrophoresis and visualized by silver staining (only the areas of the gels which contain gelsolin are shown). 1, 1* = murine authentic gelsolin; 2 = p92-5.7; 3 = human authentic gelsolin

Figure 4 Detection of the human activated Ha-ras oncogene. (a) Southern blotting: each lane contains 5 μg of BamHl-digested DNA from the indicated cell line. Lanes: 1, NIH3T3; 2, EJ-NIH3T3; 3, EJ/β 2-12; 4, EJ/β 2-13; 5, EJ/β 2-14; 6, EJ/β 2-15; 7, Rev-4; 8, EJ/LKCG-4; 9, EJ/LKCG-12; 10, EJ/LKCG-19; 11, EJ.neo-8; 12, EJ.neo-11; 13, EJ.neo-14. (b) Northern blotting: each lane contains 30 μg of total cellular RNA from the indicated cell line. The order of samples is as described in a. Bottom: Ethidium bromide stained gel prior to blotting
Fig. 1
Fig. 2
## Table 1  Growth characteristics of EJ-NIH3T3 cells transfected with mutated or authentic gelsolin genes

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<th>Cell line</th>
<th>Transfected genes</th>
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<th>Authentic human gelsolin</th>
<th>Morphology</th>
<th>Doubling time (h) 10% FCS</th>
<th>Doubling time (h) 1% FCS</th>
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*Kuzumaki et al. (1989).*
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*In 2-day-old NIH/Swiss mice.