Antisense RNA-mediated Deficiency of the Calpain Protease, nCL-4, in NIH3T3 Cells Is Associated with Neoplastic Transformation and Tumorigenesis

Keyi Liu‡‡, Limin Li‡‡, and Stanley N. Cohen‡‡

From the ‡Department of Genetics and ¶Department of Medicine, Stanford University School of Medicine, Stanford, California 94305-5120

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We previously have described the use of an antisense RNA strategy termed random homozygous knock-out (RHKO) to identify negative regulators of cell proliferation. Here we report the discovery that RHKO-mediated deficiency of the nCL-4 calpain protease results in cellular transformation of and tumorigenesis by murine NIH3T3 fibroblasts. We isolated cell clones able to form colonies on 0.5% soft agar and found that these cells generated tumors when injected subcutaneously into nude mice. The gene inactivated by RHKO was identified as nCL-4 by genomic library screening, transcript analysis, and DNA sequencing. Anchorage-independent growth, as indicated by colony formation on soft agar, was reversed by reversal of antisense-mediated homozygous inactivation, but continued haplo-insufficiency of nCL-4 resulting from insertional mutagenesis of one nCL-4 allele was associated with persistent tumorigenesis. nCL-4 cDNA expressed in naive 3T3 cells in the antisense, but not sense, direction under control of the cytomegalovirus early promoter reproduced the anchoragel-independent growth effects of RHKO. Our results implicate deficiency of the nCL-4 calpain protease in neoplastic transformation.

Calpains are a large family of calcium-activated cysteine proteases that constitute one of several key cellular proteolytic systems. Calpains are widely prevalent from mammals to invertebrates and fungi (1). On the basis of the domain structure, the typical calpain large subunit comprises four domains (I, II, III, IV), whereas molecules that lack one or more of these domains are referred to as atypical calpains (2). Two ubiquitous isozymes are well characterized (µ- and m-calpain), and several tissue-specific isozymes (muscle-specific p94, stomach-specific nCL-2 and 2', and digestive tract-specific nCL-4) have also been reported (3-7). The activities of calpains are regulated by a variety of factors, including a 30-kDa small subunit, calcium, phospholipids, a calpain-specific inhibitor (calpastatin), and a limited auto-digestion process (1, 8-10). A protein activator may also exist (1).

Calpains have been implicated in tissue differentiation and development (6, 11-16), cell cycle progression (17), and apoptosis (18-22). Additionally, involvement of calpains in various pathological states such as Alzheimer's disease (23-25), muscular dystrophy (26, 27), and ischemia (6) has been suggested. Mutations in p94, a skeletal muscle-specific calpain, were suggested to be responsible for limb-girdle muscular dystrophy type 2A, which is linked to the deficiency of p94 (26, 28, 29). Calpains process, rather than completely digest, target proteins, frequently leading to their modification, inactivation, or activation through the removal of auto-inhibitory domains (1, 9). Several tumor suppressor genes including p53, neurofibromatosis type 2 (or merlin), and the retinoblastoma gene family member p107 have been reported to be regulated by calpains (30-36), and calpain-dependent proteolysis of neurofibromatosis type 2 has been linked to tumorigenesis (34, 37). Additionally, recent data indicate that the nCL-4 calpain gene is down-regulated in human gastric cancer tissue and certain gastric cancer cell lines (38) and that calpain-3 gene expression is decreased during experimental cancer cachexia (39). Random homozygous knock-out (RHKO), a novel strategy for analysis of gene function, can identify genes whose functional inactivation in murine fibroblasts leads to reversible cellular transformation (40). This approach uses a regulated promoter within a randomly inserted chromosomally integrated gene search vector (GSV) to produce antisense transcripts complementary to those originating in the chromosomal gene containing the GSV and, consequently, also complementary to transcripts from the other copy of that gene. Use of a β-geo reporter gene within the GSV allows the selection of integration events in transcriptionally active genes and also enables the monitoring of antisense effects. Reversal of antisense inhibition is accomplished by Cre/lox-mediated deletion of a gene encoding an activator of the antisense promoter (40).

Here we report investigations indicating that antisense RNA-mediated deficiency of the calcium-activated neutral protease nCL-4 (7), which recently has been implicated in gastric carcinoma (38), leads to neoplastic transformation of NIH 3T3 cells. Our results suggest that proteolytic processing by this calpain may have a role in the suppression of tumorigenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—NIH3T3 cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum, and 500 µg/ml G418. NIH3T3 cells were transfected by using the calcium phosphate precipitation technique (12). In some experiments, the antisense target was cotransfected in the vector pCT100 (13) as a plasmid rescue. The plasmid pCT100 contains a 3.8-kb HindIII fragment of the γ-geo gene under control of the human cytomegalovirus early promoter..neo}
and 10% calf serum (Life Technologies, Inc.). RHKO was done as described previously (40). Briefly, pLLGSV, a Moloney murine leukemia virus-derived retroviral gene search vector containing the β-geo reporter gene, was introduced into NIH3T3 cells, where it integrated at multiple chromosomal sites. Infected cells were selected using 15 μg/ml gancyclovir for 2-3 weeks. Resistant clones were isolated using 0.1 M EDTA using an SW41 rotor. The cosmid vector SuperCos1 was used to clone DNA fragments of size ranging in size from 40 to 50 kb were isolated by sedimentation on 10–40% sucrose gradients in 1.0 M NaCl, 20 mM Tris-HCl (pH 8.0), and 0.15 M NaCl containing 0.1% SDS at 65 °C for 30 min and twice in 0.1X SSC containing 0.1% SDS at 65 °C for 30 min. Positive clones were purified, and cosmid DNA from these clones were mini-prepared using a QIAGEN gel extraction kit. The sequences of the purified DNA were determined using an Applied Biosystems Model 310 genetic analyzer.

Identification of Fusion Transcripts—To identify fusion transcripts containing both nCL-4 and the gene vector sequences, RT-PCR was done using a specific forward primer for a mouse nCL-4 sequence (5'-ACCTTCCTAGCCAGCTTGATGTC-3') and a specific reverse primer for the gene vector sequence (5'-ATGCCTCGATGCTACTACCAGTC-3'). The RT-PCR was conducted against total RNA isolated from cells from which the transactivator gene had been removed. The sequences of the purified PCR DNA were determined as described above.

Isolation of cDNA Clones for Mouse nCL-4—The cDNAs for mouse nCL-4 were isolated by RT-PCR and analyzed using the Quality One program (pdi).

**RESULTS**

**Homologous Inactivation of nCL-4 Gene Produces Colony Formation on 0.5% Agar and Tumor Formation in Nude Mice**—We employed the RHKO procedure as described previously (40) to isolate clones of G418-resistant NIH3T3 cells able to form colonies on 0.5% agar, which has commonly been used to identify neoplastically transformed cells that have high metastatic potential (41–43). One of these clones (Fig. 1A) was expanded into the cell line designated CK; Southern blot analysis showed that CK cells contain the provirus form of the pLLGSV-derived retroviral gene vector sequence integrated as a head-to-tail tandem repeat at a single chromosomal site (Fig. 2, A and B).

To learn whether the anchorage-independent growth phenotype observed for CK cells is dependent on expression of antisense RNA from the pLLGSV-derived chromosomally integrated provirus, the antisense promoter in the provirus was turned off. This was accomplished by transfecting CK cells with pRSV-Cre, which deletes the LAP 348 (44) transactivator of the antisense promoter by site-specific recombination at lox sites bracketing a segment containing the transactivator and an adjacent Herpes simplex virus thymidine kinase gene (40). Cells deleting this segment were identified by their consequent resistance to gancyclovir (40), and loss of the transactivator was confirmed by Southern blotting (data not shown). All of 20 individually tested clones of CK cells had the ability to generate colonies in agar. However, 8 of 18 cell clones from a population that had deleted the transactivator had lost the ability to form colonies on agar, whereas 10 retained this capability; one clone of each type (designated CKΔT2 and CKΔT1, respectively) was chosen for further study, as described later.

**Identification and Characterization of Genomic Clones Containing the GSV**—Whereas 5'-rapid amplification of cDNA ends preparation of a cDNA library and cDNA capture methods previously have been used to identify other genes inactivated by RHKO (40, 45), our initial efforts at using these procedures to identify sequences fuses to the GSV reporter in CK cells or their derivatives were not successful. Accordingly, we constructed a cosmid DNA library using genomic DNA isolated in CK cells. From about 5 × 10⁸ clones of the genomic DNA library, 2 positive clones hybridized to an end-labeled oligo complementary to a β-geo sequence were identified. DNA cloning and sequencing confirmed that these two genomic DNA clones were identical and that they contained the GSV. A data base search using the BLAST program showed that 5' to the β-geo gene of the GSV was a 59-bp segment corresponding to 1983 bp of the previously described mouse nCL-4 gene (Gen...
Bank® accession number U89513). The boundaries of the 59-bp segment exhibit show probable 5' and 3' splice site consensus sequences (47), implying the cloned region is a nCL-4 exon. The nucleotide sequences at the junction point between the genomic DNA segment of nCL-4 and 5'-defective long term repeat (dLTR) of the GSV provirus are shown in Fig. 3A. Further evidence that insertion of the GSV had occurred within the nCL-4 gene was obtained by Southern blot studies in which DNAs from CK and parental NIH3T3 cells were cut with BamHI and hybridized with a 1.7-kb nCL-4 genomic DNA fragment probe (see below). A difference in band pattern between CK and NIH3T3 cells confirmed that the GSV insertion
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Fusion Transcripts—To verify that the chromosomal gene initiating transcripts fused to the β-geo reporter gene in CK cells is nCL-4, primers corresponding to chromosomal and vector sequences (see “Experimental Procedures”) were used together with RT-PCR to prepare cDNA corresponding to poly(A) RNA obtained from CKΔT2. This PCR amplification yielded a cDNA fragment corresponding in length to the transcript predicted for the nCL-4-β-geo fusion segment, as demonstrated by agarose gel electrophoresis (Fig. 3B). Sequencing of the PCR product isolated from gels confirmed that fusion of nCL-4 and β-geo sequences had occurred at the predicted splice acceptor site (Fig. 3C). Additionally, a 38-bp segment of the virus-packaging sequence was present between nCL-4 and β-geo (Fig. 3C).

Tumorigenic Effects of nCL-4 Inactivation in NIH3T3 Fibroblasts—Subcutaneous injection of CK cells or CKΔT1 cells, both of which formed colonies in agar, into nude mice resulted in tumors at the site of injection in all four animals receiving aliquots of each cell line (Fig. 1B and Table I). Although CKΔT2 cells had undergone reversal of anchorage-independent growth following excision of the transactivator, these cells nevertheless retained tumorigenic capabilities and produced tumors in three of four nude mice following injection into (Fig. 1, A and B). However, the tumors appeared later and grew more slowly than the tumors that developed in mice receiving CK or CKΔT1 cells.

Western blot analysis indicated that the nCL-4 protein was reduced to about 40% that of the wild-type level in CK cells (Fig. 4 and Table II). Reversal of antisense inhibition increased this to about 60% that of the wild-type level, consistent with persistent haploid insufficiency of nCL-4 as expected from the presence of a GSV insert in one allele of the gene (Fig. 4 and Table II). Reversal of antisense inhibition increased the presence of a GSV insert in one allele of the gene (Fig. 4 and Table II). This finding, i.e. that reduction of the nCL-4 protein to approximately half of the wild-type level in the absence of antisense inhibition is associated with persistent tumorigenic abilities, suggests either that non-reversible secondary changes characteristic of neoplastic progression (40, 45, 48–51) had occurred during RHKO of nCL-4 or, alternatively, that haplo-insufficiency of this gene product can result in tumorigenesis. Consistent with the latter notion is our observation that reduction of nCL-4 protein by only slightly more than 50% was sufficient to yield both anchorage-dependent growth of cells in vitro and tumor formation in nude mice, indicating that total ablation of nCL-4 production is not required for neoplastic

| Cell lines | TA | Colonies | Tumor formation |
|------------|----|---------|----------------|
| NIH 3T3    | −  | 0/10⁴   | ND             |
| CK         | +  | 719/10⁴ | 4/4            |
| CKΔT1      | −  | 462/10⁴ | 4/4            |
| CKΔT2      | −  | 0/10⁴   | 3/4            |

the sequence derived from 2020 through 2040 bp, which is within the exon we identified. This PCR amplification yielded a single band about 1.7 kb in length, which when isolated from gels and sequenced was found to contain a sequence identical to the sequence of the genomic DNA clone we identified by screening the genomic library for segments that contain the GSV. The additional finding that the 59-bp nCL-4 segment adjacent to the GSV is only 24 bp from the stop codon of the gene suggests that the GSV had inserted near the 3′ end of the gene.

cDNA Cloning and Identification and Characterization of Fusion Transcripts—To verify that the chromosomal gene initiating transcripts fused to the β-geo reporter gene in CK cells is nCL-4, primers corresponding to chromosomal and vector sequences (see “Experimental Procedures”) were used together with RT-PCR to prepare cDNA corresponding to poly(A) RNA obtained from CKΔT2. This PCR amplification yielded a cDNA fragment corresponding in length to the transcript predicted for the nCL-4-β-geo fusion segment, as demonstrated by agarose gel electrophoresis (Fig. 3B). Sequencing of the PCR product isolated from gels confirmed that fusion of nCL-4 and β-geo sequences had occurred at the predicted splice acceptor site (Fig. 3C). Additionally, a 38-bp segment of the virus-packaging sequence was present between nCL-4 and β-geo (Fig. 3C).

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The presence or absence of LAP 348 transactivator (TA) gene in these cell lines is indicated as + or −, respectively. For soft agar assay, colonies were counted 3 weeks after seeding cells in 0.5% agar. The colonies were derived from 10⁴ cells plated. For tumorigenicity assays, fractions of animals showing tumors were counted 32 days after subcutaneous injection of 10⁴ cells. ND, not done.

| Cell lines | TA | Colonies | Tumor formation |
|------------|----|---------|----------------|
| NIH 3T3    | −  | 0/10⁴   | ND             |
| CK         | +  | 719/10⁴ | 4/4            |
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transformation of NIH3T3 cells.

Antisense RNA to nCL-4 Transforms Naive NIH3T3 Fibroblasts—Using primers from the 5′ and 3′ ends of the nCL-4 cDNA sequence, we synthesized full-length nCL-4 cDNA by RT-PCR using as template total RNA obtained from NIH3T3 cells. To determine whether antisense expression of nCL-4 in naive 3T3 cells can lead to growth on 0.5% agar, as was observed during RHKO, we generated stably transfected cells that produced nCL-4 cDNA in the antisense or as a control sense orientation. Transcription of chromosomally integrated nCL-4 cDNA in an antisense orientation in stably transfected naive NIH3T3 cells under control of the cytomegalovirus early promoter resulted in colony formation in soft agar (frequency, 0.38% of 106 plated cells; Fig. 5). Parental NIH3T3 cells, cells containing the chromosomally integrated vector lacking a nCL-4 insert, or cells expressing chromosomally integrated nCL-4 cDNA in the sense direction all showed no evidence of cellular transformation (Fig. 5).

DISCUSSION

This investigation has identified nCL-4, a tissue-specific calpain normally expressed in the digestive tract (7) and recently found to be deficient in human gastric cancers and gastric cancer cell lines (38), as an inhibitor of neoplastic cell growth. Analysis of genomic DNA clones derived from CK cells, which unlike the parental NIH3T3 cell line form colonies on 0.5% agar and tumors in nude mice, identified the nCL-4 sequence 5′ to the GSV used for RHKO. The identity of the gene containing the GSV was confirmed by sequencing of the genomic PCR product amplified from NIH3T3 cells using primers corresponding to the exon sequence we isolated and the 3′ end of the previously reported protein-coding sequence of nCL-4 (GenBank® accession number U89513). Additionally, fusion transcripts containing both the nCL-4 sequence and the β-geo reporter gene sequence of the GSV were identified by RT-PCR and sequencing. The nCL-4 level was reduced in CK cells compared with wild-type cells, and antisense expression of nCL-4 cDNA resulted in cellular transformation of naive NIH3T3 cells.

CK-derived cells retained the ability to form tumors after the transactivator was removed from these cells, but some clones lacking the transactivator lost the capacity for anchorage-independent cell growth. Although an anchorage-independent growth phenotype commonly accompanies tumorigenic ability, the capacity to form tumors in nude mice but not colonies on soft agar has been observed previously in a cell line that over-expresses the oncogene MDM2 (52). We showed earlier that reversal of antisense inactivation of genes inactivated by RHKO can result in the loss of tumorigenic capability by some cells, but not by others (40, 45, 48), consistent with the occurrence of secondary genetic changes common to tumor progression. The inability of Rb, p53, and other tumor suppressor genes to reverse neoplasia associated with mutation of these genes (49–51) is also believed to result from such secondary changes occurring during the progression of cancer.

Calpains have been reported to cleave tumor suppressor gene products (30–37). The ability of nCL-4 deficiency to produce tumorigenesis may result from defective nCL-4-dependent processing of a tumor suppressor gene target into a form required for activation of tumor suppression, as calpains cleave target proteins in a restricted manner to modify their properties rather than degrade the proteins (1, 9). Reduced function of nCL-4 potentially could also lead to the activation of proteases including other calpain species (53) responsible for tumorigenesis. Experiments are currently under way to identify specific nCL-4 target(s) whose processing may be required for normal control of cell proliferation.

Tumor suppressor genes are generally viewed as being recessive at the cellular level, so that mutation or loss of both tumor suppressor alleles is commonly seen as a prerequisite for tumor formation (54–57). However, there is mounting evidence of the existence of a group of genes that exhibit dose-dependent suppression of tumor growth or progression including p53 (58), transforming growth factor β-1 (59), p27kip1 (60), Bax (61), and neurofibromatosis type 2 (34). Very recent data indicate that loss of one copy of sno increases susceptibility to tumor growth in mice (46). Similar findings are also observed for tsg101 (40) and vasp (45), whose deficiency in murine fibroblasts as a result of RHKO can lead to tumorigenesis.

**Table II**

| Cell lines | nCL-4 (mean ± S.E.) | α-tubulin (mean ± S.E.) | nCL-4/α-tubulin | Calculated Normalized |
|------------|---------------------|------------------------|----------------|-------------------|
| NIH3T3     | 1.41 ± 0.22         | 0.67 ± 0.19            | 2.10           | 1.00              |
| CK         | 1.01 ± 0.17         | 1.14 ± 0.24            | 0.89           | 0.42              |
| CKAT1      | 1.58 ± 0.21         | 1.27 ± 0.15            | 1.24           | 0.59              |
| CKAT2      | 1.47 ± 0.22         | 1.13 ± 0.14            | 1.30           | 0.62              |

* Relative density units.

**Fig. 5.** Colony growth in 0.5% agar by NIH3T3 cells transfected with nCL-4 cDNA and control constructs. Representative results are shown for soft agar assays measuring anchorage-independent growth of NIH3T3 fibroblasts only (O) or NIH3T3 cells expressing vector control lacking nCL-4 cDNA (V) and vectors containing full-length nCL-4 cDNA in antisense (AS) or sense (S) orientations, respectively. Transfected cells were selected for growth in 800 μg/ml G418 for 18 days, and 106 resistant cells were plated in 0.5% agar and incubated for 3 weeks.
