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RNAi-dependent and -independent antiviral phenotypes of chromosomally integrated shRNA clones: Role of VASP in respiratory syncytial virus growth

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Abstract

Stable RNA interference (RNAi) is commonly achieved by recombinant expression of short hairpin RNA (shRNA). To generate virus-resistant cell lines, we cloned a shRNA cassette against the phosphoprotein gene of respiratory syncytial virus (RSV) into a polIII-driven plasmid vector. Analysis of individual stable transfectants showed a spectrum of RSV resistance correlating with the levels of shRNA expressed from different chromosomal locations. Interestingly, resistance in a minority of clones was due to mono-allelic disruption of the cellular gene for vasodilator-stimulated phosphoprotein (VASP). Thus, pure clones of chromosomally integrated DNA-directed RNAi can exhibit gene disruption phenotypes resembling but unrelated to RNAi.

Keywords

Chromosomal; Antiviral; RNAi; Plasmid vector; Mutation; Infectious disease

Introduction

Due to its remarkable efficiency, RNA interference (RNAi) has gained rapid acclaim as a tool for post-transcriptional gene silencing [1–3]. In the execution phase of RNAi, the antisense strand of an appropriately designed short interfering RNA (siRNA) engages the RNA-induced silencing complex that degrades the target RNA. In basic research, RNAi is routinely used to knock down specific genes for phenotypic analysis. In clinical medicine, RNAi is a highly promising regimen for silencing aberrant or pathogenic transcripts in diseases as diverse as cancer and viral infections. We have shown that silencing of the phosphoprotein messenger RNA (mRNA) of respiratory syncytial virus (RSV) by chemically synthesized siRNA strongly inhibits viral replication and macromolecular synthesis *ex vivo* and *in vivo*, thus providing a potent antiviral regimen [2–5]. Although synthetic siRNA is suitable for acute infections, a sustained RNAi is desirable for chronic diseases, such as cancer, hepatitis, and AIDS. This can be achieved by recombinant expression of short hairpin RNA (shRNA) within the cell, which undergoes trimming by cytoplasmic Dicer to generate functional siRNA. A variety of viral and plasmid vectors have been employed to clone and express shRNA genes in a relatively stable manner [3, 6–11]. Nonintegrating cytoplasmic viral vectors derived from adenovirus, adeno-associated virus, or herpes simplex virus-1 have been used with success. More persistent expression,

however, is achieved by oncoretroviral vectors, such as lentiviral vectors, which integrate into nuclear chromosomal DNA, producing stable clones. Although such integrative events can potentially disrupt cellular genes and *cis*-acting sequences and the exact site of integration may also affect shRNA expression, there has been no detailed study of these outcomes. In this study, we map the shRNA integration sites in a number of individual cellular clones and provide an account of their diverse properties.

Materials and methods

Generation of shRNA clones

The two synthetic oligodeoxynucleotides corresponding to the shRNA sequence (Fig. 1) were annealed and cloned into the pSUPER.Retro.Puro vector (from OligoEngine, Seattle, WA) following the manufacturer's protocol. In brief, we restricted the vector plasmid with XhoI and BglII at the multi-cloning site and digested with calf intestinal phosphatase. We phosphorylated the double-stranded annealed DNA with T4 polynucleotide kinase before ligation to the linearized vector. After ligation, we digested the mixture with BglII to remove any uncut vector and reduce the background. We then introduced the DNA into competent *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, CA) and confirmed by sequencing. We transfected HeLa cell monolayer using Lipofectamine (Invitrogen) and a range of plasmid DNA and selected stable transfectants by 2 µg/ml puromycin dihydrochloride (Calbiochem), a concentration that produced the optimal killing curve on untransformed HeLa cells in prior test. After a week of selection (by which time untransfected cells in the control flask all died), we plated individual cells by serial dilution of the culture from the smallest amount of transfected DNA and grew further to obtain single clonal cultures.

RNAi and related assays

We transfected cultured cells with synthetic siRNA using the TransIT-TKO reagent (Mirus, Madison, WI) as described before. We designed two siRNAs against the following 19-mer sequences of the human vasodilator-stimulated phosphoprotein (VASP) gene (XM_009141) with dTdT extensions added to the 3' end of each strand: GGAGGTGGAGCAGCAGAAA(408–426); CGCCCA GCTCCAGTGATTA(1004–1022). We measured the silencing activity of the siRNAs at different concentrations by immunoblot analysis of VASP protein at 24 h posttransfection. The IC₅₀ of the two siRNAs, i.e., concentration required for 50% loss of VASP protein, was 8 and 22 nM, respectively (data not shown). The first siRNA was, therefore, used in the rest of the paper. The shRNA sequence against VASP was designed by the same principles used for P (Fig. 1) and cloned in the same pSUPER.Retro.Puro vector described above. We also optimized the siRNA against human vimentin sequence (NM_003380) (900)GGCTG CCAACCGGAACAAT(918). We have already described siRNA against RSV F, profilin, luciferase [3–5, 12]. To generate siRNA-mismatched recombinant VASP, we first cloned the wild-type VASP open reading frame into pcDNA3. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), we then changed the wild type sequence GAGGTGGAGCAGCA GAAA (409–426) into GAAGTAGAACAACAAAAG (with the changes underlined), such that the wobble base (third base) of each codon was changed to produce a synonymous codon. Where mentioned, the plasmid was transiently transfected into A549 cells using standard Lipofectamine-based procedure.

For viral growth assay, we infected monolayer cells with sucrose-purified RSV, measured intracellular RSV growth by quantitative immunoblot of the viral N protein, and determined progeny RSV titer by serial dilution and plaque assay as described [4, 12]. To determine shRNA expression, we performed Northern analysis of the antisense strand as described before [5].

DNA sequencing

We isolated chromosomal DNA of the shRNA clones by using the TRI Reagent[®] kit (Ambion, Austin, TX) and used it as template in multiple polymerase chain reactions (PCRs) with internal primers corresponding to various sequences within the plasmid and a 15-mer degenerate primer to prime within the chromosome. Elongation was limited to 4 min and amplification performed for 30 cycles. We resolved the products on 0.8% agarose gel, purified DNA within the 1- to 5-kb size range, and subjected them to a second round of amplification of 30 cycles. We purified the products again and cloned by TOPO-TA cloning (Invitrogen). Finally, we sequenced individual clones using vector-specific primers and examined the sequences for chromosomal homology using basic local alignment search tool search of human genome in National Center for Biotechnology Information GenBank.

Results and discussion

We designed the anti-P shRNA gene based on the sequence of RSV P siRNA [4, 5, 13, 14] and cloned it into pSUPER.Retro.Puro [9] such that the shRNA was transcribed from the constitutive H1 RNA promoter (a Pol III promoter) of the vector (Fig. 1). We transfected HeLa cells with relatively low amounts of the shRNA plasmid (to minimize multiple chromosomal integrations) and selected for stable transformants by growth in the presence of puromycin. Pure clonal cultures were grown from single cells obtained by serial dilution and results of 20 clones are presented here in detail.

We first measured the resistance of each clone to RSV growth by determining progeny RSV titer released after infection and numbered the clones from generally high to low resistance (Table 1). With four exceptions (clones #17–20), all failed to produce progeny RSV, releasing 3- to 4-log less extracellular infectious virus than the untransfected HeLa cells. The level of resistance showed a general correspondence to the amount of siRNA expressed by the clones (clones #12–20 presented in Fig. 2, and all clones presented in Table 1). For example, clones #15 and #16 expressed roughly about half as much siRNA than clone #1 and showed a 3-log drop in viral titer as opposed to a 4-log drop for clone #1. A nonspecific effect on viral morphogenesis was ruled out by a quantitative immunoblot of intracellularly synthesized viral N protein, the reduction of which paralleled extracellular virus (e.g., clones #12–20 presented in Fig. 2). The ‘control’ synthetic siRNA [4, 5], corresponding to the cloned shRNA, caused a similar reduction in intracellular as well as extracellular virus, confirming the efficacy of the sh/siRNA design. To sum up, stably expressed shRNA led to the establishment of virus-resistant state in the vast majority of cellular clones by intracellular inhibition of viral replication.

An interesting exception to the above generalization was clone #11, which showed a number of unexpected behaviors. First, it expressed extremely low amounts of shRNA, only about 5% of the optimal amount (Table 1, Fig. 2). Second, it supported robust intracellular virus growth in agreement with poor shRNA expression (Table 1, Fig. 2); nonetheless, extracellular progeny virus was strongly inhibited (about 3 log₁₀). It, therefore, appeared that this clone had a defect in a post-replication step of virus growth, perhaps in virion morphogenesis or maturation. This clone is pursued in detail later.

In an attempt to understand the mechanism of the clonal variation, we set out to determine the chromosomal locations of the integrated shRNA plasmids. We mapped the plasmid–chromosome junction using a combination of PCR amplification and sequencing. We point out that puromycin-resistant clones obtained with the lowest amount of plasmid DNA are presented in this communication because the goal was to obtain single integrated copies such that the effect of each event on shRNA expression can be studied. The presence of a single copy plasmid in all clones was indeed confirmed by Southern analysis (data not

shown). Our sequence analysis (Fig. 1 and Table 1) revealed that plasmid insertion occurred in a variety of chromosomes and most likely via homologous recombination (Table 2). We will briefly describe the major classes here, grouped according to the plasmid region through which integration occurred.

- a. H1 promoter: A significant percentage of recombinations (clones 2, 4, 5, 12, 14, 18) occurred within the H1 promoters of the plasmid and chromosome 14. As the integration regenerated the H1 promoter, these clones all expressed shRNA and were RSV resistant. The sole exception was clone #18 with tenfold lower expression and RSV sensitivity, the reason for which is unknown.
- b. 5'-LTR: In the other major class (clones 3, 8, 11, 13, 16, 17) integration occurred through the 5'-LTR of the plasmid and, most likely, through endogenous retroviral LTRs or sequences of close similarity in the various chromosomes of the HeLa genome (chromosomes 2, 4, 10, 19, 21).
- c. Phosphoglycerokinase (PGK): Two recombinations (clones 10, 15) occurred within the PGK promoters of the plasmid and the X chromosome, producing somewhat different levels of shRNA.
- d. VASP and others: A few clones were the result of integration through plasmid sequences of no known function (clones 1, 6, 19, 20) and fortuitous homologous sequences in various chromosomes. Although the shRNA gene and its promoter were intact in all these clones (data not shown), the effect on shRNA expression was variable (Fig. 2 and Table 1), ranging from full expression (clone 1) to only about a tenth of the maximal value (clone 19, 20).

Even within a class, the precise site of integration also differed from clone to clone at the nucleotide level (Fig. 1 and Table 2). Together, therefore, these results show that the shRNA levels may vary substantially depending on chromosomal location. Although we do not yet know the exact reason for this variability, it could be due to either short-range regulatory sequences or long-range chromatin interactions, both of which regulate gene expression by a variety of mechanisms including epigenetic ones such as DNA methylation [15, 16].

As noted above, the silencing efficiency and antiviral effect correlated with the shRNA levels with one exception—clone #11 (Fig. 1 and Table 1). In this clone, recombination occurred between the 5'-LTR sequence of the plasmid and a short homologous sequence on chromosome 19. This led to a drastic loss of shRNA expression for unknown reasons. Surprisingly, however, RSV growth was strongly inhibited (~3 log), pointing to an RNAi-independent mechanism. Further sequencing of chromosome 19 across the junction led to the finding that the plasmid had disrupted the coding sequence of the VASP gene. VASP, the founding member of the Ena/VASP family [17, 18], is a 380-amino acid protein with a central proline-rich core that interacts primarily with profilin, the major regulator of the cytoskeletal protein, actin. In addition, VASP also contains an Ena/VASP homology domain at each terminus, of which the C-terminal one directly interacts with filamentous actin. Thus, VASP plays a strategic role in regulating actin and profilin, and we have shown previously that the latter two proteins are important in RSV transcription and morphogenesis [12, 19, 20].

Direct measurement of VASP in clone #11 by immunoblot revealed about 40% of the normal level seen in standard HeLa cells (that did not receive the shRNA plasmid), consistent with the loss of one allele. Control clones, exemplified by #12, in contrast, had normal VASP levels. We made the provisional conclusion that VASP is important for productive RSV growth and that its full amount in HeLa cells is needed for this function. As RSV naturally infects lung epithelial cells, we proceeded to validate these findings in A549

alveolar carcinoma cells that retain features of type II lung epithelial cells, including synthesis of surfactants, and are preferred in studies of RSV infection. In these cells, synthetic siRNA corresponding to the anti-VASP shRNA caused strong silencing of VASP (Fig. 3a). siRNAs against viral fusion protein F, cellular profilin, or vimentin had no effect on VASP levels, showing specificity of silencing. To further rule out off-target effects, we generated a recombinant VASP with synonymous codons in the siRNA target region such that its mRNA is mismatched with the siRNA. This recombinant VASP, expressed by transient transfection of A549 cells, was resistant to the anti-VASP siRNA (Fig. 3a).

Having optimized the siRNA-mediated silencing of VASP, we tested its effect on RSV replication. Silencing of VASP in A549 cells indeed reduced the extracellular infectious progeny virus by about 3 log (Fig. 3b). Essentially, similar effect was seen when anti-VASP shRNA was produced by stable transfection (data not shown). In contrast, A549 cells expressing siRNA-resistant recombinant VASP supported productive RSV growth even when treated with anti-VASP siRNA. In positive control experiments, silencing of viral F protein or cellular profilin inhibited RSV progeny titer, confirming previous findings [4, 12]. Silencing of vimentin, another cytoskeletal protein, had no effect, serving as negative control.

The observed loss of infective viral titer in VASP-depleted cells could be due to either inhibition of viral morphogenesis or production of noninfective virus particles. To distinguish between these two, we conducted immunoblot analysis of growth media from these cells and detected little or no virion proteins even when large volumes of media were concentrated and analyzed (Fig. 3c). We conclude that VASP plays a critical role in RSV morphogenesis and/or budding, possibly through its actin/profilin-regulatory function. We note that the VASP-depleted cells grew normally, consistent with earlier findings that VASP is a nonessential protein in HeLa cells and that VASP knockout mice are normal except for a platelet defect [21–23]. This raises the interesting possibility that targeting of VASP by RNAi may be a part of the RSV therapeutic regimen.

In summary, we show that chromosomal location may affect the level of shRNA expressed from individual shRNA clones and that, occasionally, disruption of a cellular gene important for viral growth may occur, generating a shRNA-independent virus-resistant phenotype. Importantly, these results also led to the unexpected discovery of an important role of cellular VASP in the final steps of viral development, offering additional antiviral therapeutic prospects.

Acknowledgments

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Abbreviations

bp	base pair
RNAi	RNA interference
siRNA	small interfering RNA
RISC	RNAi-induced silencing complex
shRNA	short hairpin RNA
RSV	respiratory syncytial virus

VASP vasodilator-stimulated phosphoprotein

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Biographies

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Fig. 1.

a shRNA design. Two oligodeoxynucleotides were synthesized to correspond to the siRNA sequence optimized earlier against RSV P gene sequence. The stem regions of the prospective hairpin are *boxed*. The *four pairs in bold font* were originally A/T base pairs but were changed to G/C in the sense strand only. This reduced the similarity between the two direct repeats in the DNA clone, minimizing chances of deletion in *E. coli*. As G/U base pairing is allowed in RNA, formation of the RNA hairpin and the resultant siRNA were not perturbed. The function of the siRNA was also unaffected because the sequence of the antisense (guide) strand was not altered. The loop region of the hairpin contain a BamHI site (GGATCC; *underlined*) to facilitate screening of the plasmid clones. The cohesive ends correspond to XhoI and BglII half sites and respectively allowed ligation to XhoI and BamHI sites of the vector. This destroyed the BamHI site of the vector, making the BamHI site in the loop region (*underlined*) unique in the construct. The locations of the key features of the plasmid, e.g., H1 promoter (*black*), antibiotic resistance genes (*striped*), and the LTRs (*gray*) are shown. The sequences of the shRNA and siRNA are deduced from previous studies [13, 14] and have not been experimentally verified. **b** Recombination sites for the clones described in Table 1 are schematically indicated on the line drawing of the plasmid

Fig. 2.

RSV growth and shRNA expression in representative HeLa clones. **a** The clones were numbered from 12 to 20 in the order of decreasing amounts of siRNA content. The unique clone #11 contain disrupted VASP gene (see later). Untransfected cells and cells transfected with synthetic anti-RSV P siRNA are indicated as *C* and *siRNA*, respectively. *Lower panel* shows immunoblot for RSV N protein expressed in the infected cell. **b** Infectious progeny RSV liberated from the cells shown above. *Error bars* are from three independent experiments. Note the severe reduction of liberated viral titer in clone #11 in spite of normal intracellular replication in (a). In *siRNA lanes*, the cells were transfected with 20 nM anti-P siRNA [4, 5] 18 h before RSV challenge

Fig. 3.

Role of VASP in RSV morphogenesis. **a** VASP levels in the following cells were measured by immunoblot: VASP-disrupted heterozygous HeLa clone #11; control clone #12 containing wild-type VASP genes; A549 cells after 18 h of transfection with siRNA against RSV F, profilin, vimentin, or VASP (see “Materials and methods”). A549-R denotes A549 cells in which siRNA-resistant recombinant VASP was expressed from a transiently transfected pcDNA3 clone. Control GAPDH remained unchanged. **b** Extracellular infectious progeny virus released from A549 cells transfected with the indicated siRNA (10 or 20 nM) was determined by plaque assay. A549-R cells express siRNA-resistant recombinant VASP as in (a) above. The titers were average of three assays but we omitted the standard errors and rounded the coefficients for simplicity. **c** Extracellular viral particles, regardless of their infectivity, were concentrated from 2–20 ml growth media of A549 cells treated with the indicated siRNA and measured by immunoblot using anti-RSV antibody (Chemicon, Temecula, CA). The viral proteins are *marked at left*

Table 1

Summary of twenty single copy HeLa-shRNA clones

Clone #	RSV titer (Pfu/ml)	Intracellular RSV growth	Integration site in the plasmid	Chromosome #; gene disrupted	shRNA level
HeLa	3.2×10^7	100	–	–	–
HeLa+siRNA	5.1×10^3	4±2	–	–	–
1	2.1×10^3	4±2	3,299	16; None	100
2	2.1×10^3	5±3	H1 promoter	14; None	105±5
3	2.2×10^3	5±1	5'-LTR	21; None	100±4
4	2.6×10^3	4±3	H1 promoter	14; None	95±6
5	2.6×10^3	5±4	H1 promoter	14; None	91±4
6	3.1×10^3	6±3	5,924	14; IgG-γ	98±8
7	3.1×10^3	6±4	ND	ND	101±4
8	3.2×10^3	8±4	5'-LTR	2; None	92±11
9	3.2×10^3	8±2	3'-LTR	10; None	88±6
10	4.1×10^3	9±4	PGK promoter	X; None	85±5
11	6.6×10^4	85±8	5'-LTR	19; VASP (±)	5±3
12	4.2×10^3	6±2	H1 promoter	14; None	82±8
13	5.1×10^3	6±2	5'-LTR	4; None	78±5
14	5.3×10^3	8±4	H1 promoter	14; None	72±10
15	2.1×10^4	12±5	PGK promoter	X; None	55±6
16	4.3×10^4	15±4	5'-LTR	21; None	46±8
17	2.5×10^7	87±6	5'-LTR	10; None	11±2
18	2.6×10^7	94±5	H1 promoter	14; None	9±2
19	2.8×10^7	92±8	6,036	13; None	10±4
20	3.1×10^7	98±4	1,302	8; None	9±3

All assays were done as described in "Materials and methods." The top lines are untransfected HeLa cells and HeLa cells transfected with 20 nM of anti-P siRNA, respectively. All others are stable anti-P shRNA clones.

Table 2

Chromosome–plasmid junction sequences in recombinant clones

Clone #	Chromosome sequence	Plasmid sequence
1	56398239 (Chr16) CGCATATTAAGTAATCCTGACcCAA	3299 (Plasmid) TTAGCcaCTgTTTTGAATCCACATa
2	19881570 (Chr14) GGTGTTCCTCCCTAGTGACACTGGG	1629 (Plasmid) CCCgcGATTcCTTGGAGCGGGTTGA
3	23555718 (Chr21) TAAGTTTCTccttATAGAACCATCA	234 (Plasmid) GATGTTtccagggtgcccccaaggac
4	19881586 (Chr14) CCGCGATTcCTTGGAGCGGGTTGAT	1658 (Plasmid) GACAGCGTTCGAATTCTACCGGGTA
5	88553921 (Chr14) ACTCCCCTGTCCcGcCaAGaCATCT	1580 (Plasmid) TCCTGCCagggcgccagcgcgctgg
6	20524 (Chr14) GCGGGCTCTTCGCTATTACGCCAG	5924 (Plasmid) CTGGCGAAAGGGGGATGTGCTGCAA
8	182188066 (Chr2) taagttcctgCAAGAACAGATGGT	190 (Plasmid) CCCCAGatgcgggtcccgccctcagc
9	21228784 (Chr10) tttaatggatcgtttggcaagctag	2935 (Plasmid) AGAAcCATcagatgtttccagggtg
10	1444192 (ChrX) CGTGCCcGGACGTGACAAAcGGAAGc	1913 (Plasmid) aGCACGTCTCACTAGTctCgTgcag
11	50719212 (Chr19) tgggAgAAGAAcAgCACAAcCttgC	364 (Plasmid) actcGgcGcgccaGTcTCcgaTaG
12	19881518 (Chr14) ATAGCGACATGCAAAATATTGCAGGG	1526 (Plasmid) CGCCACTCCCCTGTCCCTCACAGCC
13	71833119 (Chr4) ctgctttaacaattAATAAGCCT	427 (Plasmid) CTTGCTGTTTgcacccgaatcgtgg
14	19881435 (Chr14) CTTATAAGATTCCCAAATCCAAAGA	1493 (Plasmid) ACATTTACAGTTTATGGTGATTTC
15	1444170 (ChrX) gaaggttccttgcggTTCGCGcCGT	1891 (Plasmid) GCaGGACGTGACAAAtGGAAGtaGC
16	23555714 (Chr21) ccagttaAGTTTCTccttAtAGAAC	229 (Plasmid) CATCAGATGTTtccAGggTgcccCA
17	54949804 (Chr10) GAtAaAgacaactTtAGGTTAGG	106 (Plasmid) AACAGAGAgacagcAgaaTaTGggc
18	19881414 (Chr14) AaaaaGTGGTCTCATACAGAACTTA	1473 (Plasmid) TTTGATTCCCAAATCCAAAGACATT
19	111736078 (Chr13) GaccaccTGCAcGCAAGGAGATGGC	6036 (Plasmid) GCCCCaagctCCcCcGgccacgggg
20	101831104 (Chr8) CcaggAtCtaccCgTtCtCCTCCT	1302 (Plasmid) CTTCTCCATccgCcCCgTctcTCc

The junction sequences were determined by a combination of degenerate and specific PCR amplification as described in “Materials and methods.” A stretch of 25 nucleotides is shown for chromosomal (bold) and plasmid (light) sequences. The numbers denote the starting nucleotide of

corresponding segment. The plasmid nucleotides are numbered as presented in the instruction manual of the OligoEngine web site (<http://www.oligoengine.com/>). Chromosome numbers were identified by “Human BLAT search” in the University of California, Santa Cruz server (<http://genome.ucsc.edu/cgi-bin/hgBlat>). Recombination occurred through regions of significant sequence similarity between the chromosome and the plasmid whereby the chromosomal nucleotides that were conserved in the plasmid, and vice versa, are shown in capital. For junctions that contain palindromes, extensive overlaps, or repeats, the exact line of demarcation between the chromosomal sequence and the plasmid sequence is uncertain by a few nucleotides (e.g., the CT dinucleotide repeat in the junction sequence of clone #20).