RESEARCH COMMUNICATION

Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA

Malgorzata KISIELOW[1,2], Sandra KLEINER[1], Michiaki NAGASAWA[3], Amir FAISAL and Yoshikuni NAGAMINE[4]
Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66 CH-4058 Basel, Switzerland

Many eukaryotic genes are expressed as multiple isoforms through the differential utilization of transcription/translation initiation sites or alternative splicing. The conventional approach for studying individual isoforms in a clean background (i.e. without the influence of other isoforms) has been to express them in cells or whole organisms in which the target gene has been deleted; this is time-consuming. Recently an efficient post-transcriptional gene-silencing method has been reported that employs a small interfering double-stranded RNA (siRNA). On the basis of this method we report a rapid alternative approach for isoform-specific gene expression. We show how the adaptor protein ShcA can be suppressed and expressed in an isoform-specific manner in a human cell line. ShcA exists in three isoforms, namely p66, p52 and p46, which differ only in their N-terminal regions and are derived from two different transcripts, namely p66 and p52/p46 mRNAs. An siRNA with a sequence shared by the two transcripts suppressed all of them. However, another siRNA whose sequence was present only in p66 mRNA suppressed only the p66 isoform, suggesting that the siRNA signal did not propagate to other regions of the target mRNA. The expression of individual isoforms was achieved by first down-regulating all isoforms by the common siRNA and then transfecting with an expression vector for each isoform that harboured silent mutations at the site corresponding to the siRNA. This allowed functional analysis of individual ShcA isoforms and may be more generally applicable for studying genes encoding multiple proteins.

Key words: post-transcriptional gene silencing, RNAi, transient knockdown-in.

INTRODUCTION

In eukaryotes, many genes encode multiple isoforms by way of differential transcription/translation initiation or alternative splicing, thus giving rise to related proteins with biochemically as well as biologically distinct features [1]. Although, in many cases, multiple isoforms are expressed in the same cell at the same time, the expression level and pattern of each isoform may vary with the cell type and its stage of development, making the study of each isoform confusing and difficult. Investigation of the function of individual isoforms ideally requires conditions where only one isoform is expressed or eliminated. While several protocols for tissue-specific expression or elimination of the gene of interest have been developed [2–4], reports of isoform-specific gene inactivation [5] or expression in a clean background are limited. Such protocols are usually lengthy, often requiring several months to establish the desired conditions. During this time, cells or organisms may adapt to the new conditions [6] and caution is required when interpreting the results.

The signalling adaptor/scaffold protein ShcA is a member of the Shc family, which consists of three genes, *ShcA*, *ShcB/Sli/Sck* and *ShcC/N-Shc/Rai* [7]. ShcA is ubiquitously expressed, whereas ShcB and ShcC are expressed specifically in the brain [8]. ShcA is recruited to, and phosphorylated by, activated receptor tyrosine kinases and, in turn, recruits the growth-factor-receptor-bound protein 2 (Grb2)–Son-of-sevenless ('Sos') complex via the Src homology 2 (SH2) domain of Grb2, thus relaying growth-factor-induced signals to the Ras/extracellular-signal-regulated protein kinase ('ERK') signalling pathway [9]. It is also involved in growth-factor-mediated activation of c-Jun N-terminal kinase ('JNK') and protein kinase B, but the precise mechanism is unknown [9]. There are three isoforms of ShcA, namely p66ShcA, p52ShcA and p46ShcA, derived from a single gene through differential usage of transcription initiation sites and translation start sites, which differ only in the N-terminal regions [7]. While all three isoforms contain three tyrosine residues in the phosphotyrosine-binding (PTB) domain that are phosphorylated by activated receptor tyrosine kinases, they differ in the pattern of serine/threonine phosphorylation induced by growth factors and tumour phorbol esters. This suggests a different cellular function for each isoform ([5,10]; A. F. El-Shemerly, A. Faisal and Y. Nagamine, results not shown). However, a systematic analysis of each isoform without the influence of other isoforms has not been reported.

Post-transcriptional gene silencing (PTGS) is a phenomenon originally reported in plants [11,12], where introduction of the transgene causes silencing of the endogenous homologous gene and itself. The mechanism of PTGS involves enhanced mRNA degradation with double-stranded (ds)RNA as the trigger [13,14]. A similar phenomenon (quelling) was observed in *Neurospora* [15]. In the Animal Kingdom, dsRNA-mediated gene silencing was first described in the nematode *Caenorhabditis elegans* [16] and was termed 'RNA interference' (RNAi). Subsequently, RNAi has been observed in a wide range of organisms, including flies, trypanosomes, *Hydra*, zebrafish (*Danio rerio*) and mice

Abbreviations used: CH1 and CH2, collagen homology 1 and 2; dsRNA, double-stranded RNA; Grb2, growth-factor-receptor-bound protein 2; HA, haemagglutinin; PTB, phosphotyrosine-binding; PTGS, post-transcriptional gene silencing; RNAi, RNA interference; SH2, Src homology 2; siRNA, small interfering RNA.

[1] These authors contributed equally to this work.
[2] Present address: Istituto di Ricerca in Biomedicina, Via Vela 6, 6500 Belinzona, Switzerland.
[3] Present address: Kyorin Pharmaceutical Central Research Laboratories, 2399-1 Mitarai, Nogi-machi, Shimotsuga-gun, Tochigi 329-0114, Japan.
[4] To whom correspondence should be addressed (e-mail nagamine@fmi.ch).

[13,17]. The mechanism underlying RNAi has been partially elucidated, and a 21–23-nt-long dsRNA was found to be the intermediate/mediator of mRNA decay [18,19]. Elbashir et al. [20] have shown recently that transfection of the 21-nt dsRNA, termed 'small interfering RNA' (siRNA), can trigger PTGS of both the co-transfected and the endogenous gene in cultured mammalian cells. In a cell-free system of dsRNA-mediated mRNA decay using *Drosophila* embryonal cell extracts, the mRNA was shown to be cleaved only within the region of identity with the dsRNA [18], suggesting that endonucleolytic cleavage induced by siRNA is very specific and that it is probably not propagated to other regions of mRNA. However, the interesting possibility of distinguishing closely related mRNAs by siRNA has not been addressed with mammalian cells.

In the present study, using transient transfection assays in HeLa cells, we established that the target of siRNA is restricted to mRNAs containing the identical sequence. This facilitated the isoform-specific knock-down of p66ShcA as well as isoform-specific expression of ShcA isoforms.

EXPERIMENTAL

Cells and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10 % (v/v) fetal-calf serum (AMIMED; BioConcept, Allschwil, Switzerland), 0.2 mg/ml streptomycin and 50 units/ml penicillin at 37 °C in a humidified 5 % CO_2 incubator. A day before transfection with siRNA, cells were plated in six-well plates in medium without antibiotics at 1.4×10^5 cells/well. The next morning, siRNAs were introduced into HeLa cells using the OLIGOFECTAMINE™ reagent (Life Technologies) according to the manufacturer's instructions, with 10 μl of 20 μM siRNA and 3 μl of transfection reagent/well. Transfection with expression vectors was carried out 2 days after the OLIGOFECTAMINE™ transfection using LIPOFECTAMINE™ 2000 (Life Technologies).

cDNA cloning of ShcA isoforms

The full-length mouse p46, p52 and p66ShcA cDNAs were isolated from NIH 3T3 cells by reverse transcriptase-PCR using the sense primers 5′-CGG AAT TCA TGG GAC CTG GGG TTT CCT ACT-3′, 5′-CGG AAT TCA TGA ACA AGC TGA GTG GAG GCG-3′ and 5′-CGG AAT TCA TGG ATC TTC TAC CCC CCA AGC CGA AGT A-3′ respectively and the common antisense primer 5′-CGG AAT TCA CAC TTT CCG ATC CAC GGG TTG C-3′. Full-length ShcA cDNAs were initially cloned into pBluescriptII KS$^+$ and nucleotide sequences verified by the dideoxynucleotide-chain-termination procedure.

Construction of expression vectors

The haemagglutinin (HA)-tagged expression vector pcDNA3HA was constructed by inserting the overlapping oligonucleotide pair 5′-CCC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT G-3′ and 5′-AAT TCA GCG AAT TCT GGA ACA TCG TAT GGG TAA GCC ATG GTG GGG TAC-3′ into the *Kpn*I–*Eco*RI site of pcDNA3 (Invitrogen). To construct expression vectors for HA-tagged ShcA, p46HA, p52HA and p66HA, the full-length cDNAs of p46, p52 and p66 were inserted into the *Eco*RI–*Eco*RV site of pcDNA3HA. ShcA mutants in which potential internal initiation methionine codons were converted into leucine codons, thus expressing only the p66ShcA or 52ShcA forms, were created using the QuickChange site-directed mutagenesis kit (Stratagene). The overlapping oligonucleotide pair 5′-CTC CTC CAG GAC CTG AAC AAG CTG AGT G-3′ and 5′-CAC TCA GCT TGT TCA GGT CCT GGA GGA G-3′ was used to mutate Met^{65} (start site for p52) to leucine in p66HA, resulting in p66HA-ml. Another overlapping oligonucleotide pair, 5′-CCA ACG ACA AAG TCC TGG GAC CCG GGG-3′ and 5′-CCC CGG GTC CCA GGA CTT TGT CGT TGG-3′, was used to mutate the initiation sites for p46 in both p66HA-ml and p52HA, resulting in p66HA-ML and p52HA-ML. Silent mutations were introduced into these vectors at the sites corresponding to h/m-shc siRNA as above using the overlapping oligonucleotide pair 5′-GGG GTT TCC TAC TTG GTC CGC TAC ATG GGT TGT C-3′ and 5′-CAC AAC CCA TGT AGC GGA CCA AGT AGG AAA CCC C-3′ (mutated nucleotides underlined) to give p46HA-sm, p52HA-ML-sm and p66HA-ML-sm (h/m-shc means that the sequence of siRNA is common to both human and mouse shc sequences). Note that proteins expressed from these vectors are identical with the parent proteins.

Oligoribonucleotides

The following 21-mer oligoribonucleotide pairs were used: h/m-shc siRNA from nt 677–697 (in the PTB domain), 5′-CUA CUU GGU UCG GUA CAU GGG-3′ and 5′-CAU GUA CCG AAC CAA GUA GGA-3′; and p66-shc siRNA from nt 236–256 [in the CH2 (collagen homology 2) domain], 5′-GAA UGA GUC UCU GUC AUC GUC-3′ and 5′-CGA UGA CAG AGA CUC AUU CCG-3′. Entire sequences were derived from the sequence of human p66ShcA mRNA (accession number HSU7377) and its complement and each pair has a 3′ overhang of 2 nt on each side. Designed RNA oligonucleotides were 'blasted' against the GenBank®/EMBL database to ensure gene specificity. The RNA oligonucleotides were obtained from Microsynth (Balgach, Switzerland). Annealing was performed as described by Elbashir et al. [20]. The complementary two strands (each at 20 μM) in 200 μl of annealing buffer [100 mM potassium acetate/30 mM Hepes/KOH (pH 7.4)/2 mM magnesium acetate] were heated for 1 min at 90 °C and then incubated for 1 h at 37 °C. An siRNA corresponding to nucleotides 753–773 of the firefly luciferase mRNA was used as a negative control.

Western-blot analysis

At the times indicated, cells were lysed in a buffer containing 120 mM NaCl, 50 mM Tris, pH 8.0, and 1 % Nonidet P40 plus Complete (Roche) protein inhibitor tablets. The whole-cell extracts (20 μg) were analysed by Western blotting using a polyclonal rabbit anti-Shc antibody (1:250; Transduction Laboratories), mouse monoclonal anti-Grb2 (1:1000; Transduction Laboratories) or a mouse monoclonal anti-β-tubulin antibody (1:1000; Sigma). We used anti-rabbit or anti-mouse horseradish peroxidase-linked antibodies from Amersham as secondary antibodies. An enhanced chemiluminescence (ECL®) detection method (Amersham) was employed, and the membrane was exposed to Kodak X-Omat LS film. Quantification of ShcA proteins was done using ImageQuant 5.0.

RESULTS

Efficient down-regulation of ShcA by siRNA

Three isoforms of ShcA are derived from a single gene through differential usage of transcriptional initiation sites (p66 versus p52/p46) and translational initiation sites (p52 versus p46) (Figure 1). The primary transcript of p52/p46 mRNA contains the entire sequence of p66 mRNA; however, the very-5′ region of p66 mRNA is present in the first intron of p52/p46 mRNA, but is absent in the latter mRNA, having been spliced out. The p46

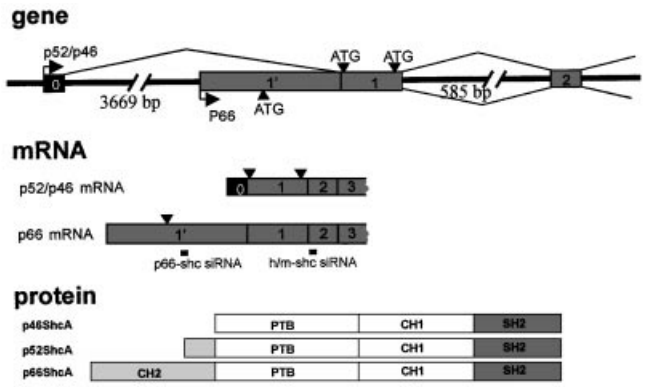

Figure 1 Relationship between the ShcA gene, mRNA and protein

The gene and mRNA are drawn to the same scale. Two transcription initiation sites are indicated by arrows. The boxes of the gene represent exons. Exon 0 that is under control of the p52/p46 promoter is ligated to exon 1 after splicing. Exons 1′ and 1 are transcribed contiguously under the p66 promoter. Translation initiations sites are indicated both on the gene and mRNAs by triangles. The protein domains (CH1 and 2, PTB and SH2) are demarcated and indicated. The two siRNAs used in the present study are indicated below p66 mRNA.

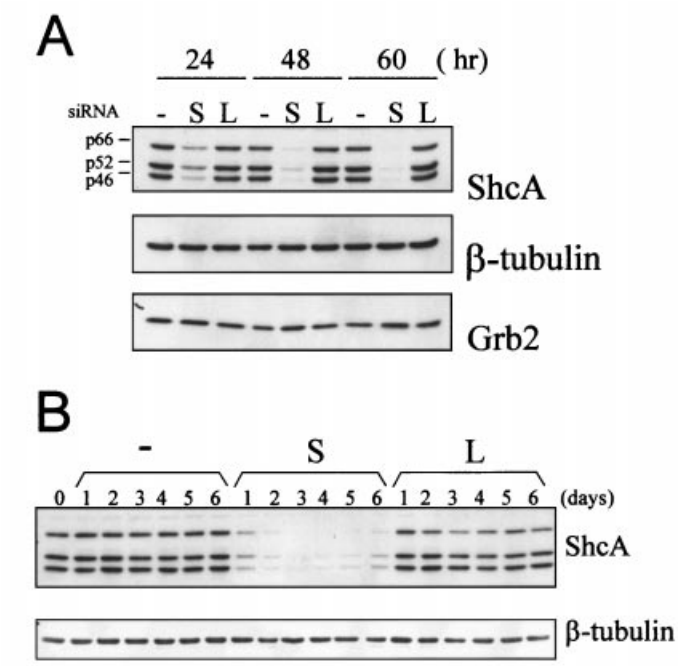

Figure 2 Knock-down of ShcA in HeLa cells

Cells were transfected using the OLIGOfectAMINE™ reagent without siRNA (-) or with h/m-shc siRNA (S) and firefly luciferase siRNA (L). At different times after transfection, whole-cell extracts were prepared and analysed by Western blotting as described in the Experimental section. Membranes were probed for Shc, β-tubulin and Grb2. (**A**) Specificity; (**B**) time course.

and p52 isoforms are derived from the same mRNA using different translation initiation sites. Target sites of two siRNAs used are shown in Figure 1 below the p66 ShcA mRNA. Note that the sequence of p66-shc siRNA is from a 5′ region of human p66ShcA mRNA and is absent in p52/46 ShcA mRNA.

When HeLa cells were transfected with the h/m-shc siRNA, the levels of all three ShcA isoforms were strongly decreased 24 h after transfection and reached less than 20% of the control after

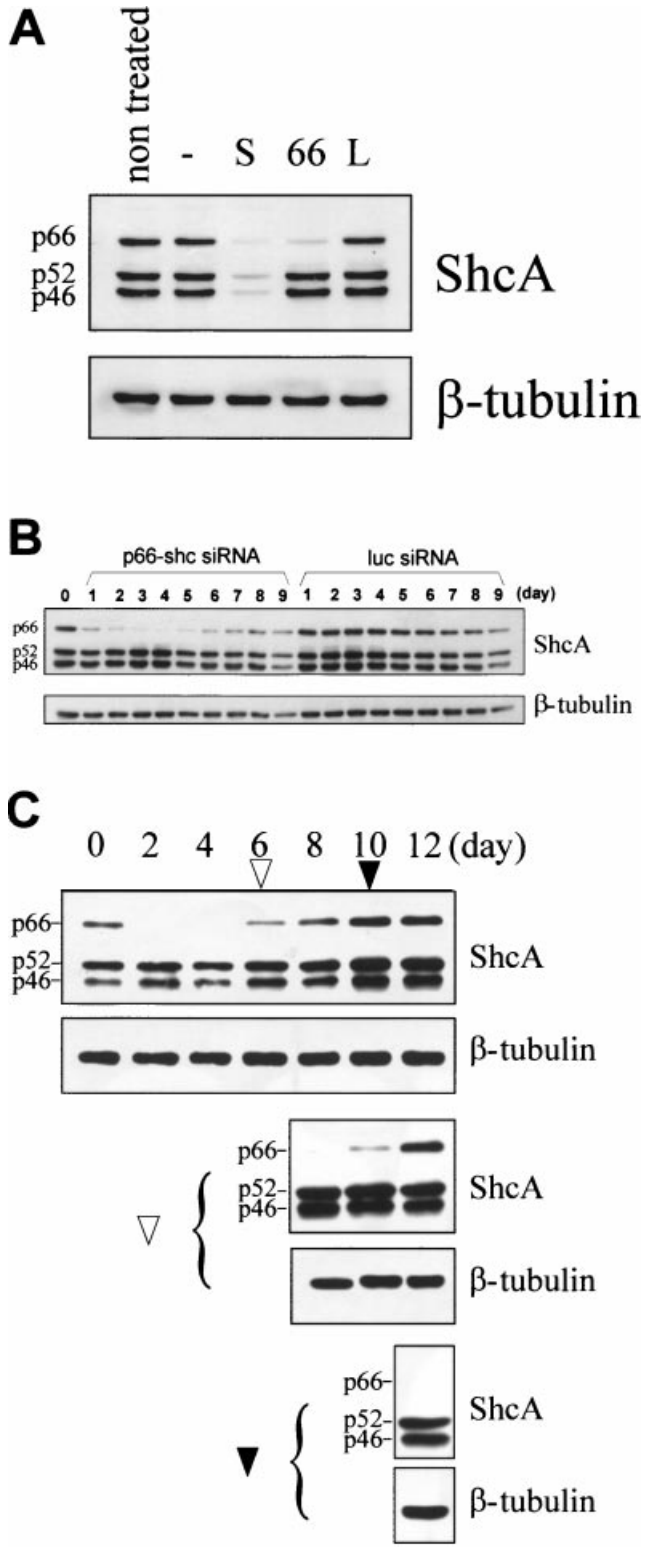

Figure 3 Isoform-specific knock-down

(**A**) p66-selective knock-down. Cells were untreated or transfected without siRNA (-) or with h/m-shc siRNA (S), p66-shc siRNA (66) and control firefly luciferase siRNA (L). Whole-cell extracts were prepared 48 h later and analysed for ShcA and β-tubulin levels as in Figure 2. (**B**) Time course. Cells were transfected with p66-shc siRNA or luc siRNA and the levels of ShcA proteins were analysed at different times as above. (**C**) Repeated transfection. On days 6 (▽) or 10 (▼) after the first transfection, cells were transfected again with the same p66-shc siRNA. The levels of ShcA and control β-tubulin proteins were analysed at different times as above. Abbreviation: luc siRNA, firefly luciferase mRNA.

48 h and 4 % after 60 h (Figure 2A). The level of control protein (β-tubulin) was not affected under the conditions employed, and this was also the case for Grb2, a protein that specifically interacts with ShcA upon activation of growth-factor signalling [7]. Time-course analysis showed that the levels of all ShcA isoforms remained low until the fifth day after transfection, but started to increase thereafter (Figure 2B). The decrease in the three isoforms was uniform, suggesting that both p52/p46 and p66 ShcA mRNAs were equally targeted by the siRNA.

Isoform-specific ShcA knockdown

When cells were transfected with p66-shc siRNA, only the p66ShcA isoform was decreased, with kinetics similar to that obtained with h/m-shc siRNA; the other two isoforms were not affected (Figures 3A and 3B). In another experiment, cells were challenged a second time with the same p66-shc siRNA 6 days after the initial transfection, when the level of p66ShcA was very low, but about to increase, and 10 days after the initial transfection, when the level of p66ShcA recovered substantially. As shown in Figure 3(C), the level of p66ShcA remained low and decreased markedly again after transfection at days 6 and 10 respectively.

Isoform-specific ShcA expression: transient knockdown-in (see below)

The target site of p66-shc siRNA is in the 5′ region of p66 ShcA mRNA that is derived from the exon 1′ and is absent in p52/p46 ShcA mRNA (see Figure 1). The results of the above experiments suggest that the effect of an siRNA is restricted to mRNAs containing a sequence identical with that of the siRNA used. Furthermore, there was no spreading effect of the siRNA signal, at least not towards the 3′ of the target site in the mRNA. Otherwise, the p46 and p52 isoforms would also have been down-regulated by p66-shc siRNA. We exploited this specificity to establish conditions under which ShcA would be expressed in an isoform-specific manner, which we call ‘transient knockdown-in’. We constructed expression vectors encoding mouse ShcA isoforms with or without silent mutations at the region corresponding to h/m-shc siRNA that left the protein sequences unchanged. Two point mutations were introduced into each expression vector so that it was not recognized by h/m-shc siRNA. Cells were first transfected with h/m-shc siRNA to knock-down all three isoforms and then 2 days later with an expression vector encoding each isoform of mouse ShcA. As shown in Figure 4, h/m-shc siRNA knocked down all three isoforms of endogenous ShcA almost completely. Transfection of these cells with mutant expression vectors for individual isoforms resulted in the expression of only the corresponding isoforms. As expected, almost no protein was detected in cells transfected with wild-type expression vectors. Cells which were not transfected with h/m-shc siRNA expressed elevated levels of ShcA isoforms, irrespective of the presence or absence of mutations in the expression vectors.

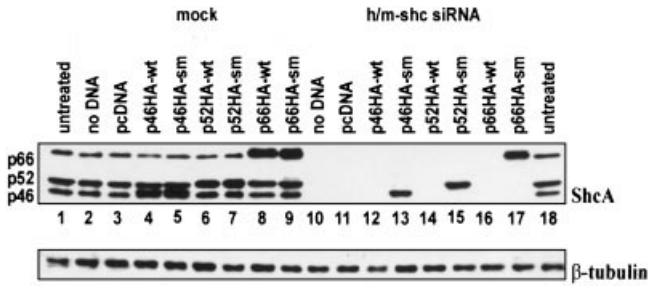

Figure 4 Isoform-specific expression of ShcA

Cells were first transfected with no siRNA (mock) or with h/m-shc siRNA to down-regulate endogenous ShcA proteins. After 2 days, the cells were transfected with an empty expression vector, pCDNA3, or an expression vector for each isoform of wild-type (-wt) and silent mutant (-sm) ShcA. A day later, whole-cell extracts were prepared and analysed by Western blotting for the ShcA expression level. Membranes were blotted with polyclonal anti-ShcA and anti-β-tubulin antibodies.

DISCUSSION

In the present study we have shown that siRNA can efficiently, specifically and rapidly down-regulate the level of an endogenous protein in mammalian cells. The effect of siRNA was restricted to mRNAs containing a sequence identical with that of the siRNA used. That the primary action of siRNA on mRNA, which is most likely an endonucleolytic attack, does not propagate to other regions of the target mRNA was inferred from the following observations: (1) the effect of p66-shc siRNA was restricted to p66ShcA (Figure 3) and (2) h/m-ShcA siRNA targeted wild-type ShcA, but not mutant, mRNAs (Figure 4). In the first observation, expression of p52/p46 ShcA was not affected, although p66 and p52/p46 mRNAs shared sequence identity in most of the region 3′ of the siRNA site (see Figure 1), indicating that the silencing signal does not propagate to regions of mRNA 3′ to the siRNA. The second observation was with ectopically expressed ShcA mRNAs. In this experiment, sequences of wild-type ShcA mRNA and mutant ShcA mRNA for each isoform were identical, except for two nucleotides at the siRNA recognition site located in the middle of the mRNA. If the silencing signal did spread either 5′ or 3′ of the siRNA, ShcA expression from both wild-type and mutant mRNAs would have been suppressed. That expression from wild-type mRNAs but not from mutant mRNAs was suppressed strongly argues for stringent specificity of siRNA-mediated mRNA decay. This possibility was already suggested indirectly by Zamore et al. [18]. Using cell-free decay reactions containing insect cell lysates and dsRNA, they showed cleavage of target mRNA only within the region corresponding to the dsRNA. However, this analysis was only on the primary action of dsRNA-mediated mRNA cleavage and did not consider the possibility of signal amplification taking place *in vivo*. Indeed, results of experiments in an insect cell-free system [21] and *C. elegans* [22] have led to the suggestion that long dsRNA molecules synthesized by RNA-dependent RNA polymerase are intermediates in RNAi that amplify and maintain the effect of siRNA. This implies 5′ spreading of the silencing signal from siRNA. Our finding in HeLa cells that siRNA-mediated RNAi does not propagate to homologous regions of the target RNA suggests that this may not be the case in mammalian cells. Moreover, no endogenous RNA-dependent RNA polymerase has been reported in mammalian cells, except after RNA virus infection [23]. This may also explain why there is no systemic PTGS in mammals, which requires the amplification of siRNA, as has been often observed in other Kingdoms [16,24]. Also, if amplification is involved in silencing in mammalian cells, antisense RNA oligonucleotides alone should serve as a primer for RNA-dependent RNA polymerase and thus be sufficient for gene down-regulation. We found that antisense RNA did not induce silencing (results not shown), in agreement with the previous report by Tuschl et al. [25]. Thus it may be a unique feature of mammalian cells that the silencing signal

spreads neither 5′ nor 3′ of the siRNA, restricting the action of siRNA to mRNAs that carry a sequence identical with that of siRNA.

An important implication of our results is that the combination of PTGS using siRNA and the isoform-specific expression of an homologous gene with silent mutations, which we call transient knockdown-in, causes the cell to express only one isoform, while keeping the levels of other isoforms very low. Using the transient knockdown-in method, it should be possible to examine the effect of various mutations of individual isoforms on cellular activity. The critical point in this method is that expression vectors should be designed so that they are not recognized by siRNA. Two mismatches in the mutated expression vectors were sufficient for them to be exempted from siRNA-mediated suppression (Figure 4). We used expression vectors for mouse ShcA in the present study because there is a high degree of sequence similarity between mouse and human ShcA (90% mRNA sequences and 93% amino acid sequences for p66 isoform). Thus, instead of introducing mutations into expression vectors, it would have been possible to design a different siRNA with a sequence matching perfectly the endogenous human ShcA gene, but not the transfected mouse gene, and achieve similar results. The advantage of our approach, however, is that we can obtain isoform-specific ShcA expression in both human and mouse cells using the same set of probes.

The sequence of p66-shc siRNA is present in the primary transcripts of p52/p46 ShcA mRNA, but is spliced out of the mature mRNA (see Figure 1). The fact that only the p66 ShcA isoform was down-regulated by p66-shc siRNA strongly suggests that the site of action of siRNA is confined to the cytoplasm. If siRNA acted in the nucleus and triggered the decay of transcripts containing the corresponding sequence, the p52ShcA and p46ShcA proteins should have been equally down-regulated by the same p66-shc siRNA. Whether siRNA can enter the nucleus is not known. Even if it does so, it would be able to access to mRNA only after completion of its processing to mature mRNA.

The response of cells to ShcA siRNA was rather fast and efficient; the target ShcA isoforms were already greatly decreased within 24 h of transfection ($> 60\%$), and the protein levels remained low until 5 days after transfection. After 5 days, the reduced ShcA isoforms started to increase. There are two possibilities to account for this reappearance of the down-regulated isoforms: (1) adaptation of the cells to the siRNA by establishment of a resistant mechanism; or (2) dilution or degradation of siRNA with time. Because repeated transfection maintained or re-established the low levels of target ShcA isoform (Figure 3B), the latter simple dilution/degradation mechanism seems to apply. While a period of 5 days of down-regulation by a single transfection is long enough for some biochemical analysis, repeated transfection should allow long-term experiments. Repeated transfection will be necessary where the target protein has a slow turnover.

In summary, we have shown in mammalian cells that the site of action of siRNA-mediated mRNA degradation is confined to the cytoplasm and that the target mRNA is restricted to those mRNAs containing a sequence identical with that of the siRNA used. These specific features of siRNA-mediated gene knock-down can be employed over a short time period in conjunction with specific expression vectors to establish conditions for expression of the ShcA gene in an isoform-specific manner. This knockdown-in method should be applicable and useful for the study of genes expressed as multiple isoforms.

Received 12 December 2001/31 January 2002; accepted 8 February 2002

We thank Dr Frederick Meins for very helpful discussion during the work and preparation of the manuscript and Dr Patrick King and Dr George Thomas for critical reading of the manuscript before submission. The Friedrich Miescher Institute is a part of the Novartis Research Foundation.

REFERENCES

1 Andreadis, A., Gallego, M. E. and Nadal-Ginard, B. (1987) Generation of protein isoform diversity by alternative splicing: mechanistic and biological implications. Annu. Rev. Cell Biol. **3**, 207–242

2 Johnson, J. E., Wold, B. J. and Hauschka, S. D. (1989) Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. Mol. Cell. Biol. **9**, 3393–3399

3 Tan, S. S. (1991) Liver-specific and position-effect expression of a retinol-binding protein-lacZ fusion gene (RBP-lacZ) in transgenic mice. Dev. Biol. **146**, 24–37

4 Gorman, C. and Bullock, C. (2000) Site-specific gene targeting for gene expression in eukaryotes. Curr. Opin. Biotechnol. **11**, 455–460

5 Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L. and Pelicci, P. G. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature (London) **402**, 309–313

6 Muller, U. (1999) Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech. Dev. **82**, 3–21

7 Luzi, L., Confalonieri, S., Di Fiore, P. P. and Pelicci, P. G. (2000) Evolution of Shc functions from nematode to human. Curr. Opin. Genet. Dev. **10**, 668–674

8 Nakamura, T., Muraoka, S., Sanokawa, R. and Mori, N. (1998) N-Shc and Sck, two neuronally expressed Shc adapter homologs. Their differential regional expression in the brain and roles in neurotrophin and Src signaling. J. Biol. Chem. **273**, 6960–6967

9 Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L. and Pelicci, P. G. (1996) Not all Shc's roads lead to Ras. Trends Biochem. Sci. **21**, 257–261

10 Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. M., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L. and Pelicci, P. G. (1997) Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J. **16**, 706–716

11 van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. and Stuitje, A. R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2**, 291–299

12 Napoli, C., Lemieux, C. and Jorgensen, R. A. (1990) Introduction of a chimeric chalcone synthase gene in petunia results in reversible cosuppression of homologous genes in trans. Plant Cell **2**, 931–943

13 Cogoni, C. and Macino, G. (2000) Post-transcriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. **10**, 638–643

14 Carthew, R. W. (2001) Gene silencing by double-stranded RNA. Curr. Opin. Cell Biol. **13**, 244–248

15 Cogoni, C., Romano, N. and Macino, G. (1994) Suppression of gene expression by homologous transgenes. Antonie Van Leeuwenhoek **65**, 205–209

16 Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature (London) **391**, 806–811

17 Bosher, J. M. and Labouesse, M. (2000) RNA interference: genetic wand and genetic watchdog. Nat. Cell Biol. **2**, E31–E36

18 Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**, 25–33

19 Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature (London) **409**, 363–366

20 Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature (London) **411**, 494–498

21 Lipardi, C., Wei, Q. and Paterson, B. M. (2001) RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107**, 297–307

22 Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. and Fire, A. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107**, 465–476

23 Cohen, S. S. (1968) Virus-induced Enzymes, Columbia University Press, New York

24 Fagard, M. and Vaucheret, H. (2000) Systemic silencing signal(s). Plant Mol. Biol. **43**, 285–293

25 Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. and Sharp, P. A. (1999) Targeted mRNA degradation by double-stranded RNA *in vitro*. Genes Dev. **13**, 3191–3197