

# Cellular Uptake and Localization of a Cy3-Labeled siRNA Specific for the Serine/Threonine Kinase Pim-1

ARNOLD GRÜNWELLER,<sup>1</sup> CLEMENS GILLEN,<sup>2</sup> VOLKER A. ERDMANN,<sup>1</sup>  
and JENS KURRECK<sup>1</sup>

## ABSTRACT

**A highly efficient and specific small interfering (siRNA) (PsiR4) for the serine/threonine kinase Pim-1 has been generated that silences the expression of a Pim1-green fluorescent protein (GFP) fusion gene at low nanomolar concentrations (~5 nM). Only one of four siRNAs tested against Pim-1 had high potency, whereas the three other siRNAs were completely inefficient up to a concentration of 100 nM. PsiR4 was labeled with Cy3 at the 5'-end of the sense strand to investigate cellular uptake and localization in living COS-7 and F-11 cells. This modification has only minor effects on the potency of PsiR4 to inhibit Pim1-GFP. Cellular uptake of the Cy3-labeled siRNA by lipofection was observed in more than 90% of the cells and reaches a plateau 4–6 hours after transfection. Cotransfection studies with low PsiR4-Cy3 concentrations demonstrated that most cells that still expressed Pim1-GFP did not show siRNA uptake. Localization studies with PsiR4-Cy3 in the neuronal hybridoma cell line F-11 displayed a dotted, perinuclear accumulation of siRNAs. Moreover, cells with neuritelike structures contain PsiR4 in this cellular compartment.**

## INTRODUCTION

**V**ARIOUS MEANS OF REDUCING THE EXPRESSION of potentially interesting genes, for example, the application of antisense oligonucleotides or small interfering RNAs (siRNAs), are currently in use for the purpose of validating candidate genes for drug development or to study their functional importance (for reviews, see Crooke, 2000; Eckstein, 2000; Kurreck, 2003; Dykxhoorn et al., 2003). RNA interference (RNAi) is an endogenous cellular mechanism, conserved from plants to mammals (Fire et al., 1998; Hamilton and Baulcombe, 1999; Elbashir et al., 2001), which is thought to be involved in virus defense, retrotransposon silencing, and heterochromatinization (Waterhouse, 2001; Hu et al., 2002; Schramke and Allshire, 2003; Volpe et al., 2002; Reinhart and Bartel, 2002; Hall et al., 2002). Cellular double-stranded RNA (dsRNA) serves as a target for the RNase III-like enzyme Dicer, which cuts dsRNA into 21–23 nt siRNAs, containing 2-nt 3'-overhangs (Zamore

et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001). These siRNAs are subsequently incorporated into the enzyme complex, RNA-induced silencing complex (RISC), wherein the siRNA is unwound in an ATP-dependent step (Hammond et al., 2000; Nykänen et al., 2001). The antisense strand of the siRNA guides the resulting ribonucleoprotein complex to complementary single-stranded RNA (ssRNA), which is subsequently degraded. In mammalian cells, the use of long dsRNA induces an unspecific interferon (IFN) response (Stark et al., 1998; He and Katze, 2002), whereas siRNAs were shown to be target specific (Harborth et al., 2001). Nevertheless, a recent microarray study demonstrated off-target activation of stress-inducible and apoptotic genes when siRNAs were introduced in cells at concentrations higher than 20 nM (Semizarov et al., 2003). From this study, it is obvious that there is a need to generate siRNAs with high potency for therapeutic applications or for functional analysis of gene knockdowns that show specific target silencing at low or subnanomolar concen-

<sup>1</sup>Freie Universität Berlin, Institut für Chemie-Biochemie, D-14195 Berlin, Germany.

<sup>2</sup>Grünenthal GmbH, Molecular Pharmacology, 52099 Aachen, Germany.

trations. Moreover, it is still under debate whether the efficiency of siRNAs exceeds that of single-stranded antisense oligonucleotides (Grünweller et al., 2003; Miyagishi et al., 2003) or both types of molecules have similar potencies (Vickers et al., 2003).

Here, we generated a highly efficient Pim-1-specific siRNA that silences its target at low nanomolar concentrations of 5–10 nM. Pim-1, a serine/threonine kinase that phosphorylates heterochromatin protein HP1 $\gamma$  and several other targets, for example, cdc25 and c-Myb (Koike et al., 2000; Mochizuki et al., 1999; Winn et al., 2003), is involved in the induction of B cell lymphoma when co-expressed together with c-Myb (van Lohuizen et al., 1989). In addition, Pim-1 seems to play a role in long-term potentiation, cell survival, differentiation, and proliferation (Konietzko et al., 1999; Wang et al., 2001). The Pim-1-specific siRNA was Cy3(Amersham Biosciences, Freiburg, Germany)-labeled at the 5'-end of the sense strand, with only minor effects on efficiency. We localized the siRNA in the neuronal hybridoma cell line F-11 and found a dotted, perinuclear, and neurite staining in these cells.

## MATERIALS AND METHODS

### siRNAs

siRNAs were purchased from Dharmacon (Boulder, CO) or IBA-NAPS (Göttingen, Germany) as deprotected duplexes. Sequences and some features of the siRNAs used in this study are listed in Table 1.

### Cell culture

COS-7 cells (monkey african green kidney fibroblasts) were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM) (PAA Laboratories, Coelbe, Germany), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories), 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin (Invitrogen, Karlsruhe, Germany). Cells were passaged by diluting them 1:10 before they reached confluency to maintain exponential growth.

F-11 cells (hybridoma cell line derived from mouse neuroblastoma and rat dorsal root ganglia [DRG]) were maintained in Ham's F-12 medium with glutamax-I (Invitrogen) supplemented with 20% FBS, 100  $\mu$ g/ml penicillin, and 100 mg/ml streptomycin. Cells were passaged by diluting them 1:4 after 3 days.

### Transfection and cotransfection conditions

The day before transfection, COS-7 cells were trypsinized, resuspended in medium without antibiotics, and transferred to 24-well plates at a density of  $8 \times 10^4$

cells per well in a volume of 500  $\mu$ l. Transfection and cotransfection experiments were carried out with Lipofectamine 2000 (Invitrogen).

For transfection, 1  $\mu$ g of a Pim1-green fluorescent protein (GFP)-containing plasmid, which was obtained by introducing the full-length rat cDNA of Pim-1 into pcDNA3.1/CT-GFP-TOPO (Invitrogen), and the respective amount of siRNA were mixed with 50  $\mu$ l OPTIMEM (Invitrogen). In a separate tube, 2.5  $\mu$ l Lipofectamine 2000 per reaction was added to 50  $\mu$ l OPTIMEM and incubated for 5 minutes at ambient temperature. Both solutions were combined and incubated for an additional 20 minutes at ambient temperature to allow complex formation. The solutions were then added to the cells in the 24-well plate, resulting in the indicated final concentrations of the siRNAs. Cells were incubated at 37°C in the presence of the transfection solution for 24 hours.

### Fluorescence microscopy

Transfection efficiency, siRNA effects, and cellular uptake were analyzed by fluorescence microscopy in living COS-7 cells, and localization of Cy3-labeled siRNA was performed in living F-11 cells. Therefore, the medium in the 24-well plates was aspirated from the cells, 200  $\mu$ l phosphate-buffered saline (PBS) was added, and fluorescence images were taken using an Olympus IX 50 fluorescence microscope and analysis 3.1 software (Soft Imaging Systems, Muenster, Germany).

### Northern blot

For Northern blot experiments, a digoxigenin-labeled antisense RNA was used. The vector pCRII TOPO containing the PIM-1 cDNA was linearized with *EcoRV*, and subsequently, an *in vitro* transcription and labeling reaction was performed (Roche, Mannheim, Germany), resulting in a 900-bp RNA probe. Total RNA was prepared from transfected COS-7 cells (RNAeasy) (Qiagen, Hilden, Germany) and separated on 1.2% formaldehyde gels. Northern blotting was carried out with the NorthernMax kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Hybridization and washing steps were performed at 68°C. For detection of the hybridized probe, the digoxigenin detection system was employed (Roche) using a digoxigenin-specific antibody conjugated with alkaline phosphatase. CDP-Star (Roche) was used as a substrate for alkaline phosphatase, and chemiluminescence was detected with x-ray films.

### Detection of protein knockdown by Western blotting

For Western blotting, cells were lysed directly in 24-well plates with lysis buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 1.4 M  $\beta$ -mercaptoethanol, 25% glycerol, and

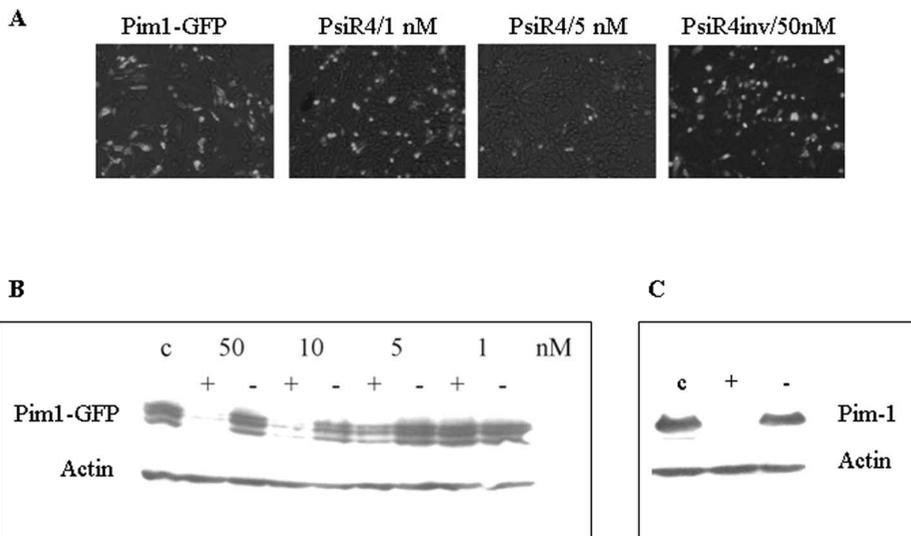
TABLE 1. CHARACTERISTICS OF siRNAS AGAINST PIM-1<sup>a</sup>

| siRNA    | Sequence   | Target region | Silencing    | GC content (%) |
|----------|--|---------------|--------------|----------------|
| PsiR1    | gcacguggagaaggaccggdTdT<br>dTdTcgugcaccucuccuggcc  | 199–219       | No silencing | 62             |
| PsiR2    | gugguccugcugaagaaggdTdT<br>dTdTcaccaggacgacuucc    | 266–286       | No silencing | 48             |
| PsiR3    | cccgaguguacaguccuccdTdT<br>dTdTgggcucacaugucaggagg | 609–629       | No silencing | 57             |
| PsiR4    | guguacuuuaggcaaaggdTdT<br>dTdTcacaugaaauccguuccc   | 755–775       | ≥5 nM        | 43             |
| PsiR4inv | ggaagaagucguccuggugdTdT<br>dTdTccuucucagcaggaccac  |               | No silencing | 43             |

<sup>a</sup>Targeted regions in the coding sequence of Pim-1 (accession number NM\_017034.1) are given.

0.05% bromphenol blue). The complete lysate was boiled for 5 minutes at 95°C, and equal amounts of protein were separated on 10% polyacrylamide gels. Transfer of separated proteins to PVDF membranes (Amersham, Freiburg, Germany) was performed with a semidry blotter (Bio-Rad, Munich, Germany). For immunostaining, membranes were incubated with a GFP antiserum

([Invitrogen] diluted 1:5000 in dry milk). Secondary antibodies were conjugated with alkaline phosphatase (Chemicon, Hampshire, UK) and diluted 1:5000. As a chemiluminescence substrate, we used CDP-Star. To confirm equal loading of the samples, membranes were reprobbed with a monoclonal mouse antibody specific for actin (Chemicon).



**FIG. 1.** Characterization of the Pim-1-specific siRNA, PsiR4. (A) Pim1-GFP was cotransfected in COS-7 cells with Lipofectamine 2000 at the indicated concentrations of PsiR4 or the inverted control sequence PsiR4inv. Cells were analyzed by fluorescence microscopy 24 hours posttransfection. COS-7 cells with a green fluorescent phenotype show Pim1-GFP expression. The first image shows the control of Pim1-GFP transfection in the absence of any additional siRNA. The subsequent images show cotransfection experiments with Pim1-GFP and PsiR4 at 1 nM and 5 nM and PsiR4inv at 50 nM, respectively. (B) Western blot analysis from cotransfection experiments of Pim1-GFP together with PsiR4 (+) or PsiR4inv (-) at the indicated concentrations. Cells were harvested after 24 hours, and Western blots were performed with an anti-GFP antibody. An anti-actin antibody serves as a control for equal loading. c, Pim1-GFP control. (C) Northern blot analysis demonstrating the specific degradation of Pim-1 mRNA in cotransfection experiments. As a control for loading equal amounts of RNA, an actin-specific probe was used. c, Pim1-GFP control; +, effect of 50 nM PsiR4 on Pim-1 mRNA; -, effect of 50 nM PsiR4inv on Pim-1 mRNA.

## RESULTS AND DISCUSSION

### Generation of *Pim-1*-specific siRNA

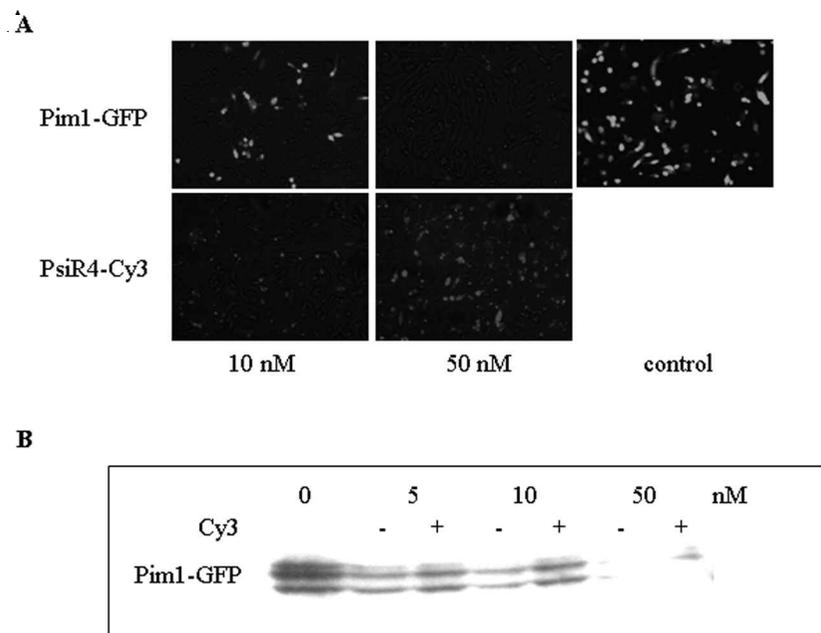
We designed a set of four siRNAs according to the criteria on Dharmacon's homepage ([www.dharmacon.com](http://www.dharmacon.com)). Screening of their silencing efficiency was performed according to a recently developed method based on a GFP assay (t Hoen et al., 2002). The siRNAs were transfected at various concentrations in COS-7 cells, and the effect on Pim1-GFP expression was monitored by fluorescence microscopy and Western blotting. The siRNAs PsiR1, PsiR2, and PsiR3 did not show significant downregulation of the Pim1-GFP reporter gene at concentrations up to 100 nM. Only PsiR4 led to strong Pim-1 silencing at lower concentrations, and, therefore, we characterized PsiR4 in more detail. We analyzed the silencing efficiency of PsiR4 compared with a control siRNA consisting of the inverted PsiR4 sequence in a concentration range between 1 and 50 nM. At the fluorescence level, we observed a reduction of cells with a green fluorescent phenotype at a concentration of 5 nM, whereas the inverted control had no detectable silencing effect up to a concentration of 50 nM (Fig. 1A).

Western blot experiments were performed to analyze the results obtained by fluorescence imaging with an independent method. Pim-1 is known to undergo autophos-

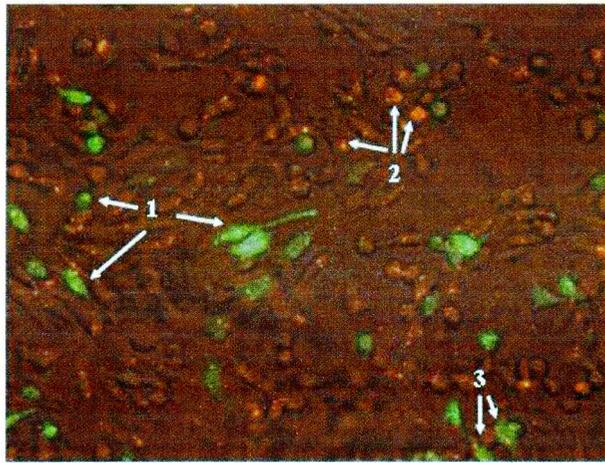
phorylation (Saris et al., 1991), and it is, therefore, likely that the observed double bands (Fig. 1B) result from different phosphorylation states of the protein. Regardless of the origin of the double band, which must be clarified in further experiments, it is obvious that the Western blot experiments exactly confirmed the results obtained by fluorescence microscopy. At a concentration of 1 nM, PsiR4 has no effect, but Pim1-GFP expression was clearly reduced at a PsiR4 concentration of 5 nM. Higher siRNA concentrations led to more than 90% knockdown of Pim1-GFP, whereas the control siRNA did not have any significant effect.

In order to prove that the observed siRNA knockdown phenotypes were due to degradation of the targeted Pim-1 mRNA, we performed Northern blot analysis with a Pim-1-specific probe. Pim-1 mRNA was degraded in the presence of PsiR4 but was unaffected in the presence of the inverted control siRNA at concentrations of 50 nM, respectively (Fig. 1C).

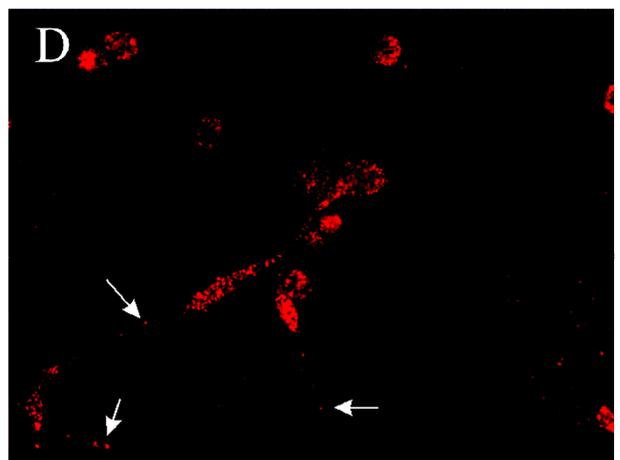
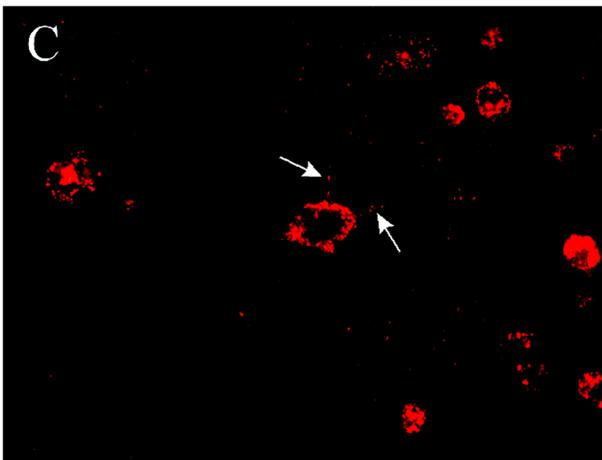
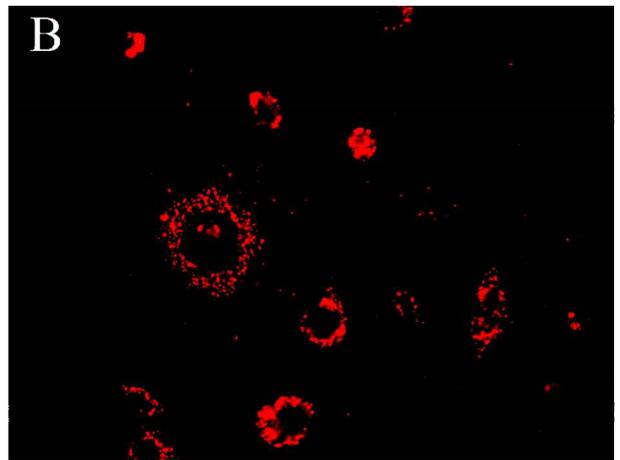
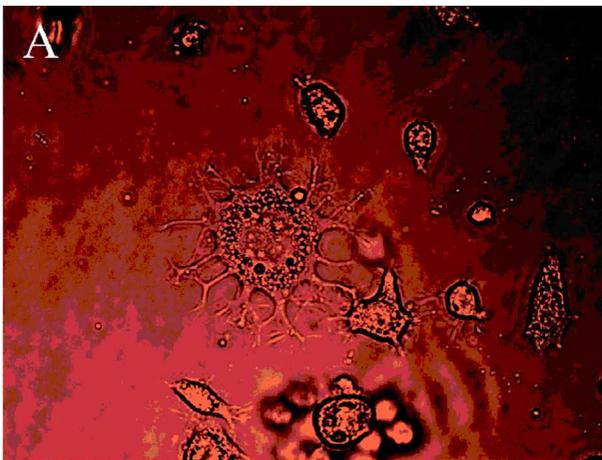
With our approach, we have generated a highly efficient siRNA specific for Pim-1, which shows potent knockdown (~90%) of Pim1-GFP at concentrations <20 nM. This is an important characteristic of PsiR4, as in a recent study (Semizarov et al., 2003), a significant number of genes involved in apoptosis and stress response were observed to be nonspecifically induced at siRNA concentrations >20 nM by using a genomewide gene expression approach.



**FIG. 2.** Effect of 5'-sense Cy3 labeling of PsiR4 on Pim1-GFP expression. (A, top) GFP fluorescence of COS-7 cells. (A, bottom) Cy3 fluorescence of COS-7 cells. Left and middle images of both rows reflect cotransfection experiments of Pim1-GFP and 10 or 50 nM PsiR4-Cy3, as indicated. Control cells were transfected with Pim1-GFP only. (B) Western blot analysis comparing the downregulation of Pim1-GFP with PsiR4 (-) and PsiR4-Cy3 (+) at the indicated concentrations.



**FIG. 3.** Colocalization of Pim1-GFP and PsiR4-Cy3 (10 nM) 24 hours after cotransfection of COS-7 cells. Arrows labeled 1 indicate cells that express Pim1-GFP in the absence of detectable amounts of siRNA, arrows labeled 2 indicated cells that do not express Pim 1-GFP but contain a visible quantity of siRNA, and arrows labeled 3 indicate cells that express Pim1-GFP and contain detectable amounts of siRNA. (Overlays were done with Adobe Photoshop 7.0).



**FIG. 4.** Localization of PsiR4-Cy3 in living F-11 cell 24 h after lipofection. **A:** F-11 cells in phase contrast; **B:** The same cells containing PsiR4-Cy3; **C:** and **D:** Two examples of F-11 cells with neuritelike structures that were transfected with PsiR4-Cy3. Localization of the siRNA in the neuritelike structures is indicated by arrows.

### Potency of Cy3-labeled *Pim1*-siRNA

Labeling of siRNAs, for example, with a fluorescence dye, is possible at four different positions: at the 5'-end and 3'-end of the sense or antisense strand, respectively. For our experiments, we chose the 5'-end of the sense strand for labeling PsiR4 with Cy3 because there are several lines of evidence that modifications at this position do not influence the activity of a given siRNA (Hamada et al., 2002; Amarzguioui et al., 2003; Harborth et al., 2003). To study cellular uptake, distribution, and localization, we first investigated the effect of PsiR4-Cy3 labeling on siRNA function. We cotransfected our reporter *Pim1*-GFP with various concentrations of PsiR4-Cy3 and compared the efficiencies of the labeled and unlabeled siRNA on *Pim1* expression. Cellular uptake of PsiR4-Cy3 was observable at low nanomolar concentrations, and this uptake increased constantly up to a concentration of 50 nM (data not shown). Fluorescence imaging revealed strong inhibition of *Pim1*-GFP expression at a concentration of 10 nM, with nearly complete *Pim1* silencing at a concentration of 50 nM (Fig. 2A). Western blot analysis confirmed the knockdown of *Pim1* with PsiR4-Cy3 at the protein level (Fig. 2B). Compared with unlabeled PsiR4, we found only a weak but reproducible negative influence of the dye label on siRNA efficiency. At low nanomolar concentrations, the unlabeled siRNA was somewhat more potent, but the silencing effect of both starts at a concentration of 5 nM. From these results, we conclude that PsiR4-Cy3 is a useful molecule to study siRNA uptake and localization in living cells because binding of the endogenous components of the RNAi machinery should not be influenced by the Cy3 labeling at the 5'-end of the sense strand.

### Cellular uptake of PsiR4

Cellular uptake of PsiR4-Cy3 in COS-7 cells was analyzed using lipofection. Cells with Cy3 fluorescence were observed as early as 15 minutes after transfection. The number of transfected cells increased constantly up to 4 hours after lipofection and reached a plateau thereafter (data not shown). During this period, >95% of the cells took up PsiR4-Cy3.

In the next set of experiments, we cotransfected *Pim1*-GFP and a low concentration of PsiR4-Cy3 (10 nM) to ensure that a significant number of cells still express *Pim1*-GFP in the presence of siRNA. Under these conditions, we found that most cells that contained PsiR4-Cy3 did not show any GFP phenotype (Fig. 3, arrows labeled 2), and most cells that still had a strong GFP phenotype did not show PsiR4-Cy3 fluorescence (Fig. 3, arrows labeled 1). The result suggests that inhibition of gene expression by siRNA correlates with localization of fluorescently labeled siRNA in the cell. Nevertheless, a significant number of cells contained neither a Cy3 label nor a GFP phenotype,

which might be because of silencing of the target gene at a nondetectable concentration of the labeled siRNA. It cannot be excluded, however, that these cells might not have expressed *Pim1*-GFP before siRNA was present. The number of cells with a GFP phenotype were greatly reduced to a level of ~20%, and only 50% of all cells took up detectable amounts of PsiR4-Cy3. Furthermore, in a previous study (Grünweller et al., 2003), we described an siRNA directed against the vanilloid receptor 1 (VR1) with an  $IC_{50}$  value of 60 pM. We could not observe detectable Cy3 siRNA uptake at concentrations of 0.5 nM (data not shown). Interestingly, some cells contain both, Cy3-labeled siRNA and a GFP phenotype (Fig. 3, arrows labeled 3). In this case, stability of the fusion protein *Pim1*-GFP might be the reason for this observation.

### Localization of PsiR4 in F-11 cells

To analyze the intracellular localization of PsiR4-Cy3 in more detail, we transfected F-11 cells. These cells were produced by fusion of embryonic rat DRG neurons with mouse neuroblastoma cells (Platika et al., 1985). F-11 cells are much larger than COS-7 cells and allow localization of the siRNA by fluorescence microscopy in living cells. In this context, it was shown recently that fixation of cells, which is necessary for confocal fluorescence microscopy, might produce artifacts (Richard et al., 2003).

We observed a dotted, perinuclear localization of PsiR4-Cy3 after lipofection (Figs. 4A, 4B). This staining pattern is likely to be due to endosomal uptake of the siRNA by F-11. The accumulation around the nucleus could be a hint that siRNAs can be imported into the nucleus to fulfill functions in heterochromatin formation. Another possible explanation for perinuclear localization of siRNAs is the following scenario. The activated RISC that contains the unwound antisense strand of the siRNA duplex is localized at nuclear pores for scanning mRNAs that are exported from the nucleus into the cytoplasm for possible binding sites to cut mRNAs efficiently when they are transported. This postulated nuclear pore scanning mechanism (Scherr et al., 2003) may account for the efficient targeting of siRNA to the mRNA.

F-11 cells have been shown to possess neuronal properties and are, therefore, useful models for authentic neurons (Platika et al., 1985). These cells were demonstrated to be labeled with markers for DRG neurons, to generate action potentials similar to DRG neurons, and to exhibit extensive neuritelike structures. A closer inspection of siRNA-transfected F-11 cells revealed that PsiR4-Cy3 not only was localized in the somatic compartment but also could be found in the neuritelike structures (Figs. 4C, 4D). Previously, specific transport of mRNAs along dendrites and axons was shown (Olink-Coux and Hollenbeck, 1996). It is, therefore, likely that gene expression

can be silenced by RNA interference in these cellular compartments.

In summary, we have identified a highly efficient siRNA targeting the serine/threonine kinase Pim-1. Labeling of the siRNA with the fluorescent dye Cy3 was found to have only minor effects on the silencing potency of the siRNA. This finding enabled us to perform localization studies of the Cy3-labeled siRNA in COS-7 cells as well as in the neuronal cell line F-11. The siRNAs are rapidly taken up by the cells after lipofection and are distributed throughout the cells even into the neuritelike structures of the neuronal cells. PsiR4 will be used in animal models to analyze the functional role of Pim-1 *in vivo*. In addition, an siRNA specific for the human Pim-1 will be generated to be used to investigate downstream targets of Pim-1, which is endogenously expressed in HeLa cells.

## ACKNOWLEDGMENTS

We thank B. Bieber for technical assistance and Ö. Altan for construction of the Pim1-GFP plasmid. In addition, we thank R. Jahnel, Dr. O. Bender, Dr. C. Weise, and Dr. E. Wade for helpful discussions and support. This work was financed by the Bundesministerium für Bildung und Forschung (grant No. 01G-G9818/0) and the Fonds der Chemischen Industrie.

## REFERENCES

- AMARZGUIOUI, M., HOLEN, T., BABAIE, E., and PRYDZ, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* **31**, 589–595.
- 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* **409**, 363–366.
- CROOKE, S.T. (2000). Progress in antisense technology: The end of the beginning. *Methods Enzymol.* **313**, 3–45.
- DYKXHOORN, D.M., NOVINA, C.D., and SHARP, P.A. (2003). Killing the messenger: Short RNAs that silence gene expression. *Nat. Rev. Mol. Cell Biol.* **4**, 457–467.
- ECKSTEIN, F. (2000). Phosphorothioate oligodeoxynucleotides: What is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **10**, 117–121.
- ELBASHIR, S.M., HORBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.
- 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* **391**, 806–811.
- GRÜNWELLER, A., WYSZKO, E., BIEBER, B., JAHNEL, R., ERDMANN, V.A., and KURRECK, J. (2003). Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-O-methyl RNA, phosphorothioates and small interfering RNA. *Nucleic Acids Res.* **31**, 3185–3193.
- HALL, I.M., SHANKARANARAYANA, G.D., NOMA, K., AYOUB, N., COHEN, A., and GREWAL, S.I. (2002). Establishment and maintenance of a heterochromatin domain. *Science* **297**, 2232–2237.
- HAMADA, M., OHTSUKA, T., KAWAIDA, R., KOIZUMI, M., MORITA, K., FURUKAWA, H., IMANISHI, T., MIYAGISHI, M., and TAIRA, K. (2002). Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of siRNAs. *Antisense Nucleic Acid Drug Dev.* **12**, 301–309.
- HAMILTON, A.J., and BAULCOMBE, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952.
- HAMMOND, S.M., BERNSTEIN, E., BEACH, D., and HANNON, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296.
- HARBORTH, J., ELBASHIR, S.M., BECHERT, K., TUSCHL, T., and WEBER, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.* **114**, 4557–4565.
- HARBORTH, J., ELBASHIR, S.M., VANDENBRUCH, K., MANNINGA, H., SCARINGE, S.M., WEBER, K., and TUSCHL, T. (2003). Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* **13**, 83–105.
- HE, Y., and KATZE, M.G. (2002). To interfere and to anti-interfere: The interplay between hepatitis C virus and interferon. *Viral Immunol.* **15**, 95–119.
- HU, W.Y., MYERS, C.P., KILZER, J.M., PFAFF, S.L., and BUSHMAN, F.D. (2002). Inhibition of retroviral pathogenesis by RNA interference. *Curr. Biol.* **12**, 1301–1311.
- KOIKE, N., MAITA, H., TAIRA, T., ARIGA, H., and IGUCHI-ARIGA, S.M. (2000). Identification of heterochromatin protein 1 (HP1) as a phosphorylation target by Pim-1 kinase and the effect of phosphorylation on the transcriptional repression function of HP1. *FEBS Lett.* **467**, 17–21.
- KONIETZKO, U., KAUSELMANN, G., SCAFIDI, J., STAUBLI, U., MIKKERS, H., BERNS, A., SCHWEIZER, M., WALTEREIT, R., and KUHLE, D. (1999). Pim kinase expression is induced by LTP stimulation and required for the consolidation of enduring LTP. *EMBO J.* **18**, 3359–3369.
- KURRECK, J. (2003). Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.* **270**, 1628–1644.
- MIYAGISHI, M., HAYASHI, M., and TAIRA, K. (2003). Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* **13**, 1–7.
- MOCHIZUKI, T., KITANAKA, C., NOGUCHI, K., MURAMATSU, T., ASAI, A., and KUCHINO, Y. (1999). Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. *J. Biol. Chem.* **274**, 18659–18666.

- NYKÄNEN, A., HALEY, B., and ZAMORE, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321.
- OLINK-COUX, M., and HOLLENBECK, P.J. (1996). Localization and active transport of mRNA in axons of sympathetic neurons in culture. *J. Neurosci.* **16**, 1346–1358.
- PLATIKA, D., BOULOS, M.H., BAIZER, L., and FISHMAN, M.C. (1985). Neuronal traits of clonal cell lines derived by fusion of dorsal root ganglia neurons with neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* **82**, 3499–3503.
- REINHART, B.J., and BARTEL, D.P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**, 1831.
- RICHARD, J.P., MELIKOV, K., VIVES, E., RAMOS, C., VERBEURE, B., GAIT, M.J., CHERNOMORDIK, L.V., and LEBLEU, B. (2003). Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **278**, 585–590.
- SARIS, C.J., DOMEN, J., and BERNS, A. (1991). The pim-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO J.* **10**, 655–664.
- SCHERR, M., MORGAN, M.A., and EDER, M. (2003). Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr. Med. Chem.* **10**, 245–256.
- SCHRAMKE, V., and ALLSHIRE, R. (2003). Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* **301**, 1069–1074.
- SEMIZAROV, D., FROST, L., SARTHY, A., KROEGER, P., HALBERT, D.N., and FESIK, S.W. (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. USA* **100**, 6347–6352.
- STARK, G.R., KERR, I.M., WILLIAMS, B.R., SILVERMAN, R.H., and SCHREIBER, R.D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.
- T HOEN, P.A.C., ROSEMA, B.-S., COMMANDEUR, J.N.M., VERMEULEN, N.P.E., MANOHARAN, M., VAN BERKEL, T.J.C., BIESSEN, E.A.L., and BIJSTERBOSCH, M.K. (2002). Selection of effective antisense oligodeoxynucleotides with a green fluorescent protein-based assay. *Eur. J. Biochem.* **269**, 2574–2583.
- VAN LOHUIZEN, M., VERBEEK, S., KRIMPENFORT, P., DOMEN, J., SARIS, C., RADASZKIEWICZ, T., and BERNS, A. (1989). Predisposition to lymphomagenesis in *pim-1* transgenic mice: Cooperation with *c-myc* and *N-myc* in murine leukemia virus-induced tumors. *Cell* **56**, 673–682.
- VICKERS, T.A., KOO, S., BENNETT, C.F., CROOKE, S.T., DEAN, N.M., and BAKER, B.F. (2003). Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J. Biol. Chem.* **278**, 7108–7118.
- VOLPE, T.A., KIDNER, C., HALL, I.M., TENG, G., GREWAL, S.I., and MARTIENSSEN, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837.
- WANG, Z., BHATTACHARYA, N., WEAVER, M., PETERSEN, K., MEYER, M., GAPTER, L., and MAGNUSON, N.S. (2001). Pim-1: A serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis. **2**, 167–179.
- WATERHOUSE, P.M., WANG, M.B., and LOUGH, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**, 834–842.
- WINN, L.M., LEI, W., and NESS, S.A. (2003). Pim-1 phosphorylates the DNA binding domain of c-Myb. *Cell Cycle* **2**, 258–262.
- 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.

Address reprint requests to:

Dr. Jens Kurreck  
Institute für Chemie-Biochemie  
Freie Universität Berlin  
Thielallee 63  
14195 Berlin, Germany

E-mail: jkurreck@chemie.fu-berlin.de

Received August 13, 2003; accepted in revised form  
September 15, 2003.