Quantitative detection of siRNA and single-stranded oligonucleotides: relationship between uptake and biological activity of siRNA

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ABSTRACT

The quantitative detection of oligomeric nucleic acids including short double-stranded RNA in cells and tissues becomes increasingly important. Here, we describe a method for the detection of siRNA in extracts prepared from mammalian cells, which is based on liquid hybridization with a 32P-labelled probe followed by a nuclease protection step. The limit of detection of absolute amounts of siRNA is in the order of 10–100 amol. This methodology is suited to quantitatively follow the spontaneous uptake of siRNA by mammalian cells, i.e. without the use of carrier substances. This protocol may also be used to detect extremely low amounts of other kinds of short nucleic acids, including antisense oligonucleotides.

INTRODUCTION

The application of biologically active oligonucleotides in molecular biology and molecular medicine includes the successful delivery to target cells and tissues, as well as the monitoring of therein. In particular, the application of oligonucleotides in vivo is hampered by difficulties to use carrier substances and transfectants (1), resulting in very low apparent uptake in most tissues (2–4). Thus, the robust and quantitative detection of oligomeric nucleic acid tools and drugs is a critical technical issue to be addressed.

Short double-stranded RNA (siRNA) has been shown to effectively suppress the expression of target genes in mammalian cells (5,6). The application of chemically synthesized siRNA in vivo is of particular interest, since it is conceivable and feasible to apply siRNA, according to the concepts along which the application of other more established classes of oligomeric nucleic acids drugs such as, for example, antisense oligonucleotides (7,8), CpG-oligonucleotides (9), or catalytic RNA and DNA (10–12) are being tested in vivo.

The application of siRNA to living mammalian cells, and attempts to increase their cellular uptake require robust methods, for their quantitative and sensitive detection in cellular preparations. Here, a simple protocol is described, which is suitable to detect and to quantify reliably, siRNA and other oligonucleotides in extracts prepared from mammalian cells that have been exposed to such nucleic acids.

MATERIALS AND METHODS

Oligonucleotides

All chemically synthesized oligonucleotides were purchased from a commercial supplier (IBA, Göttingen, Germany), purified by high-performance liquid chromatography (HPLC), and checked for purity, concentration and integrity using UV spectroscopy, as well as by PAGE (20%) under denaturing conditions followed by staining with ‘stains all’ (Sigma, Deisenhofen, Germany). The sequences used in this study are derived from the ICAM-1-directed siRNA ‘si2B’ (13). The nucleotide sequences are as follows: s2B, 5'-GCCUCAGACCUACCUCUAAtt-3'; as2B, 5'-UGAGGUACGUGCUGAGG-GCtt-3'; as-scr, 5'-CAACUCUAGGUGACCCt-3'; and s-scr, 5'-GCUAGACCUAGGAGGCGt-3'. The nucleotide sequence of the 3'-extension of the sense strand of derivatives of si2B are: s2B-b, 5'-GCCUCAGACCUACCUCUAAtt-3'; s2B-c, -gattcgtgtc-3'; s2B-d, -aaatcgtgtc-3'; s2B-e, -gattcgtgtc-3'; s2B-f, -aaaaggttc-3'.

Formation of double-stranded siRNA

Complementary RNA strands at 10 μM concentration were incubated at 95°C for 3 min in 100 mM NaCl and 20 mM Tris–HCl, pH 7.4 and annealed by slowly decreasing the temperature to 22°C. The formation of double-stranded RNA was confirmed by semi-denaturing (4 M urea, 15% polyacrylamide) gel electrophoresis and visualization of bands by staining with ‘stains-all’ (Sigma).

Cell lines and cell culture

The ECV304 cell line was described as an endothelial cell line, however, DNA fingerprinting of this cell line showed that it is a derivative of the cell line T-24, which is a human urinary bladder carcinoma cell line (14). ECV304 cells were maintained in medium 199 (Gibco-Invitrogen, Karlsruhe, Germany) buffered with 25 mM HEPES, pH 7.4 and supplemented with 0.68 mM L-glutamine and 10% foetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany). Cells were split three times a week.

Transfection of cells

For transfection, a number of 1.5 × 10^5 ECV304 cells was incubated overnight in 12-well culture plates; cells were washed twice with PBS, and siRNA was transfected by...
incubating cells in 0.4 ml of OptiMEM (Invitrogen) containing siRNA (0.2 pM to 20 nM) and 4 mg of LipofectamineTM 2000 (Invitrogen) for 4 h at 37°C. Thereafter, cells were incubated in medium 199 containing 10% FCS for further 2 h at 37°C; expression of ICAM-1 was stimulated by IL-1β (0.02 ng/ml) overnight, cells were harvested by trypsin-treatment, and the extract was isolated according to the method depicted in Figure 1.

Cellular uptake of oligonucleotides and siRNA

As schematically depicted in Figure 1, ECV304 cells were seeded in 6-well culture plates at a density of 2 × 10^5 cells/well 16 h prior to the uptake experiment. Subsequently, cells were washed twice with pre-warmed PBS and incubated with 200 nM siRNA in a volume of 1 ml of OptiMEM (Gibco-Invitrogen). After incubation for 14 h at 37°C in 5% CO2, cells were washed five times with 1.5 ml of pre-warmed PBS and trypsinized with 250 µl of trypsin/EDTA solution for 3 min at 37°C. The trypsin treatment was stopped by the addition of 750 µl of medium 199 (Gibco-Invitrogen) with 10% FCS. After centrifugation (800 g, 3 min) the cell pellet was resuspended in 200 µl of TE buffer, pH 7.4 containing 1% NP-40 followed by incubation on ice for 10 min. Subsequently, samples were extracted carefully once with 200 µl of phenol (pH4.5–5.0; Roth, Karlsruhe, Germany) and twice with 200 µl of chloroform/isoamylalcohol (24:1, v/v) followed by precipitation with 2.5 × vol ethanol (100%), 0.1 × vol 3 M sodium acetate pH 5.2, and 20 µg of glycogen. RNA pellets were resuspended in 30 µl of hybridization buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.4).

Detection of siRNA in intracellular extracts

Following the scheme in Figure 1 for the detection of siRNA, 10 µl samples were hybridized with 40 fmol of the corresponding 32P-labelled sense strand of siRNA for 10 min at 95°C and for 1 h at 37°C. Subsequently, RNase treatment was performed with varying amounts of enzymes (Figure 2). Samples were analysed by PAGE (15%, 4 M urea) followed by blotting onto nylon membrane (Hybond N+; Amersham, Freiburg, Germany) for 10 min at 3 mA/cm². To visualize and to quantify the signals on blots we used a PhosphorImager (Amersham Pharmacia Biotech, Freiburg, Germany). It turned out that the treatment of samples with 40 ng of RNase A and 0.1 U RNase T1 for 10 min at 30°C was suitable under those conditions to completely degrade the probe and conserve the signal monitoring si2B. Smaller amounts of both RNase, which were not sufficient to completely degrade the radio-labelled probe and, which gave rise to two si2B-specific bands (labelled ‘1’ and ‘2’ in Figure 2), were still suitable to quantify the autoradiograph of the gel since the sum of both band intensities (‘1’ and ‘2’) remained constant.

RESULTS AND DISCUSSION

Monitoring the carrier-free cellular uptake of naked nucleic acids requires a sensitive, robust and quantitative methodology. It might be obvious to make use of a 5'-32P-end label to follow and to detect intracellular oligonucleotides. As a prerequisite for this possibility, processes not related to cellular uptake but affecting the readout have to be excluded. For example, one has to consider a possible influence of the 5'-terminal phosphate of the 32P-labelled oligonucleotide versus the majority of species in the same experiment not carrying a 5'-terminal phosphate. If the 5' end positioned phosphate influences apparent uptake, then the 32P-end labelled population does not represent the total amount of
oligonucleotides in the experiment. Further, a sequence-dependent dephosphorylation of $^{32}$P-end labelled species has to be excluded. To investigate this possibility for siRNA, we tested the stability in serum-free culture supernatant of six siRNA species that contain the same $5'$ end labelled antisense strand of si2B and a sense strand that differs by the sequence of additional deoxyribonucleotides within a $3'$-extension (Figure 3A). This experiment shows a different apparent stability of the $^{32}$P-labelled $5'$-phosphate (Figure 3B) and, thus, indicates that it is not possible to directly relate the quantity of internalized $^{32}$P-labelled strands to the real amount that has been taken up by cells.

Detection of siRNA by liquid hybridization

First, the siRNA si2B (13), which follows the usual design for siRNA and does not contain any fused sequences, was used in a cell culture experiment, in order to investigate the sensitivity of the method and the dose–signal relationship after transfection. ECV304 cells were transfected with si2B at various concentrations and detected by the method described here. When sensitivity marker, i.e. si2B was used to spike cellular extracts prior to the liquid hybridization step, the apparent sensitivity is in the order of 10 amol (Figure 4, left panel). When analysing the signal of samples from the transfection experiments, dilutions had to be applied to gels (indicated in black numbers in lanes on the right panel in Figure 4), in order to achieve similar band intensity, and in order to keep the ratio between radiolabelled probe and siRNA target strands constant. Here, siRNA transfected at concentrations of $<$20 pM could be unequivocally detected (Figure 4, right panel). When ECV304 wells were simply exposed to si2B without the presence of any carrier or transfectant, a signal was measured at a medium concentration of si2B of 200 nM (Figure 4, middle panel).

Since the source for the preparation of siRNA might influence the sensitivity of detection, we tested this protocol by either adding si2B-f (Figure 3) to buffer or to a cellular extract prepared from ECV304 cells (Figure 5A and B). As a probe in the liquid hybridization assay, we used the $^{32}$P-labelled sense strand of si2B-f (Figure 3) termed s2B-f. The protected hybrid between the probe and the antisense strand of the original siRNA construct migrates as si2B-f and indicates its amount in the reaction. When added to buffer, the apparent detection limit for si2B-f is in the order of 10–100 amol, whereas the sensitivity was decreased to the order of 700 amol, when siRNA was added to cellular extracts.

Relationship between apparent uptake and biological activity of si2B

Since this detection protocol allowed one to determine the number of siRNAs per transfected cell, we attempted to relate the absolute number of siRNA molecules per cell to the extent of siRNA-mediated target suppression. ECV304 were used to transfect the ICAM-1-directed siRNA si2B and to measure target suppression as well as endogenous siRNA (Figure 6). It is interesting to note that in this system and averaged over all cells treated with siRNA a mean number between $10^4$ and $10^5$ siRNA duplexes per cell is related to half-maximal target suppression.
Discrimination between related sequences

Since this method is potentially suited to detect small cellular RNAs, some of which comprise large families of species with related but not identical nucleotide sequences, we tested the apparent ability of this methodology to discriminate between sequences that share varying sequence homology (Figure 7A). We used one strand of si-1 as a probe. After the binding step of the radiolabelled probe with the respective siRNA species, samples were analysed by gel electrophoresis under semi-denaturing conditions directly (Figure 7B) or after nuclease treatment (Figure 7C). As can be seen in Figure 7B and C, treatment by nucleases completely degrades the probe and produces shortened versions of the hybridization product between the probe and si-2, which contains two mismatches when compared with the completely matching si-1. A number of four mismatches (si-3) does not give rise to a detectable protected band as do all other siRNA that share even less

sequence homology with the probe, i.e. the upper strand of si-1 as depicted in Figure 7. It should be noted that this finding can be seen with or even without nuclease treatment (Figure 7B and C).

Detecting different classes of oligonucleotides in extracts of mammalian cells

Next, we investigated the sensitivity for oligomeric single-strands in cellular extract. The sequence of the antisense
A strand of si2B was synthesized as ribo-, deoxyribo- or phosphorothioate deoxyribo oligonucleotides and added to a cellular extract prepared from ECV304 cells. The detection was performed as described in Materials and Methods and in Figure 1, and representative autoradiographs of blots are shown in Figure 8. We used s-2B as a radioactive probe in all cases. The threshold of detection is in the order of absolute amounts of 100–500 amol for the single-stranded ribonucleic acid and deoxyribonucleic acid derivative, and in the order of 500 amol to 1 fmol for the single-stranded phosphorothioate deoxyribo derivative. In all cases, there is a clear band with no signal in its vicinity, i.e. the quantification of the signal can be performed at satisfying accuracy.

Perspectives

The detection protocol described here allows one to quantitatively determine the copy number of siRNA and oligomeric nucleic acid single strands contained in cellular extracts. By applying this method, and when one is able to measure the percentage of transfected cells in a given experiment, it is possible to measure apparent mean copy numbers of siRNAs per cell, which can be related to biological activity. This experiment indicates a surprisingly small mean copy number in case of the ICAM-1-directed siRNA si2B (Figure 6). On a more technical level, it is important to note that some systems consisting of probe and target may allow one to delete the nuclease treatment, thereby further simplifying this method.

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REFERENCES


