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siRNA Binding Proteins of Microglial Cells: PKR is an Unanticipated Ligand

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Abstract Small interfering RNA (siRNA), double-stranded RNA (dsRNA) 21–23 nucleotides (nt) long with two nt 3′ overhangs, has been shown to mediate powerful sequence-specific gene silence in mammalian cells through RNA interference (RNAi). Due to its high efficiency and high specificity siRNA has been used as a powerful post genomic tool and a potent therapeutic candidate. However, there is still a lot to learn about the mobility of siRNA inside cells and the cellular factors that might interfere with the specificity and activity of siRNA. Microglia are the brain's effector cells of the innate immune system and suitable targets in the development of novel therapeutic strategies. Here, we show the cellular uptake and intracellular distribution of siRNA in murine microglial N9 cells. siRNA was internalized by microglial N9 cells without transfection reagent and mainly localized to the endosomes However, no significant gene silencing effects were observed. Its cellular uptake and cellular distribution pattern were similar with that of a same length single stranded DNA (ssDNA). Further, cellular binding proteins of siRNA were purified and identified by mass spectrometry. Negative control siRNA and siRNA targeted to β-actin were used in this part of experiment. Most of the siRNA binding proteins for negative control siRNA and siRNA targeted to β-actin were dsRNA-binding proteins, such as dsRNA-dependent protein kinase R (PKR). Furthermore, both control siRNA and siRNA targeted to β-actin activated PKR in N9 cells, which suggest that siRNA might cause off-target effects through activation of PKR. J. Cell. Biochem. 97: 1217–1229, 2006.

Key words: microglia; siRNA; RNAi; PKR; uptake; cellular distribution; unspecific effects

siRNA has been shown to mediate powerful sequence-specific gene silence in mammalian cells through RNAi. RNAi is an evolutionary conserved post-transcriptional gene silencing mechanism that proceeds through a two-step process. In the first step, long dsRNAs are recognized and digested by an RNase III enzyme, Dicer, to generate siRNA. Subsequently, these siRNAs, which act as a guide to ensure specific interaction with the target transcript, are incorporated into the RNA-induced silencing complex, which cleaves the corresponding transcript. This dsRNA mediated gene silencing was first named RNAi by Fire and co-workers. Since then RNAi has been discovered in many different biological systems, ranging from *Caenorhabditis elegans* to mammalian cells [Zamore, 2001; Hannon, 2002; Tijsterman et al., 2002].

In mammals, long dsRNA results into global non-sequence specific changes of gene expression through two major pathways. The first involves the activation of the PKR [Clemens and Elia, 1997] and the second involves the activation of a sequence-nonspecific RNase, RNaseL [Player and Torrence, 1998]. Activation of either of these pathways will result in global changes of gene expression, which might obscure any gene-specific knockdown. To specifically silence a target gene in mammalian cells, chemically synthesized or in vitro transcribed 21–23-nt-long siRNA, which were reported that they

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wouldn't cause any non-specific effects, were used [Caplen et al., 2001; Elbashir et al., 2001; Castanotto et al., 2002]. To obtain stable transfection in cells or animals, DNA expression vector-based siRNAs were developed [Brummelkamp et al., 2002; Sui et al., 2002]. Such specific gene silencing methods were considered to provide a powerful tool to elucidate gene function, to identify drug targets and to develop specific therapeutics.

Because of its powerful gene knockdown activity and high specificity, siRNA has been widely studied from different point of views in mammalian cells. A variety of cell lines from different species have been shown to be good recipients for siRNA [McManus and Sharp, 2002]. Several groups used siRNA as a tool for functional genomics [Fraser et al., 2000; Paddison and Hannon, 2002]. Also siRNA has been experimentally applied in therapies against cancer or virus infections and in treatment of some genetic diseases [Capodici et al., 2002; Xia et al., 2002; Scherr et al., 2003].

Despite the wide range of applications there is still a lot to learn about cellular uptake of siRNA, the distribution of siRNA inside cells and the cellular factors that can interfere with the specificity and action of siRNA.

A vital assumption for siRNA-mediated RNAi as genomic tool is that siRNA only specifically knocks-down target genes. siRNA-mediated RNAi was shown to have a high specificity and little attention has been given to its potential unspecific effects [Miller et al., 2003]. Lessons from antisense research showed that unspecific effects of oligonucleotide-based reagents might result from mismatched pairing, from specific nucleotide group such as the CpG motif, from cellular responses to foreign oligonucleotides and from unspecific binding to cellular proteins. Recently, several groups reported observations about unspecific effects of siRNA in mammalian cells. Unspecific effects due to mismatched pairing and cellular responses to siRNA were proven in mammalian cells [Saxena et al., 2003; Sledz et al., 2003; Scacheri et al., 2004] and studies of siRNA cellular binding proteins are urgently needed. Here, siRNA-mediated protein purification and identification by mass spectrometry were used to identify cellular siRNA binding proteins in vitro.

Microglial cells are the brain's effectors cells of the innate immune system and play a key role in all major central nervous system pathologies ranging from acute events such as bacterial infections to neuro-inflammatory and degenerative diseases such as Alzheimer's disease [Aldskogius, 2001]. siRNA mediated gene silencing was also applied in microglial cells [Gan et al., 2003; Giri et al., 2003].

Here, we compared the cellular uptake and cellular distribution of siRNA with a same length ssDNA. Cellular binding proteins for siRNA were purified and identified in microglial N9 cells.

MATERIALS AND METHODS

Synthetic siRNA and ssDNA

Sequences of negative control siRNA were as follows: 5′-UUCUCCGAACGUGUCACGUdTdT-3′ (sense strand), 5′-ACGUGACACGUUCGGAGAAdTdT-3′ (antisense strand). 5′ end fluorescein isothiocyanate (FITC) labeled, or Alexa Fluor 555 labeled and 5′ end biotin labeled negative control siRNA were used and were obtained from QIAGEN GmbH, Hilden, Germany. Sequences of siRNA targeted to β-actin were as follows: 5′-GAUGAGAUUGGCAUGGCUUdTdT-3′ (sense strand), 5′-AAGCCAUGCCAAUCUCAUCdTdT-3′ (antisense strand) and was obtained from Ambion (Europe) Ltd., Huntingdon, United Kingdom. 5′ end biotin labeled β-actin siRNA was from QIAGEN GmbH, Hilden, Germany. Sequences of applied ssDNA were 5′-TCCATGAGCTTCCTGATGCT-3′ and 5′ end FITC labeled ssDNA were synthesized by MWG-Biotech AG, Ebersberg, Germany.

Cell Cultures

Murine N9 microglial cells [Ferrari et al., 1996] and human cerebromicrovasular endothelial cells (HCEC) [Esco et al., 2002] were cultured in RPMI-1640 with 10% heat inactivated fetal calt serum (FCS) with penicillin and streptomycin at 100 U/ml (Gibco, Grand Island, NY) at 37°C in 5% CO_2.

Cytofluorometric Analysis of siRNA Uptake

10^6 microglial cells in 10 ml RPMI 1640 with 10% FCS were seeded into a 10 cm^2 Petri dish and cultured for 24 h. Afterwards, medium was removed and cells were washed twice with FCS-free RPMI 1640 and then incubated with FCS-free RPMI 1640 at 37°C for 1 h to remove residual FCS. After incubation, medium was replaced by serum-free RPMI 1640 containing

FITC-siRNA (10 μM) or FITC-ssDNA (10 μM). Cells were cultured in the dark for 1 h. Then, incubation was terminated and cells were incubated with PBA buffer (Phosphate-buffered saline (PBS), 2% bovine serum albumin (BSA), 0.1% sodium azide) at 4°C for 10 min to remove siRNA bound to the cell surface. Thereafter, cells were washed twice with PBS and detached from dishes with trypsin-EDTA. After neutralizing trypsin with medium containing FCS, cells were collected by centrifugation at 1,000 rpm for 5 min. After washing, cellular fluorescence intensity was quantified by flow cytometry.

Fluorescence Microscopy

After flow cytometric analysis, cells were collected by spinning at 1,000 rpm for 5 min. Thereafter, supernatant was removed and two drops of mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Labratories, Inc., Burlingame) were added. After vortexing, the cell suspension was dropped onto glass slides and cellular distribution of siRNA was observed by fluorescence microscopy.

To further define the sub-cellular distribution of siRNA co-localization of siRNA with endosomes was studied using immunocytochemistry. Briefly, 10^5 cells in 1 ml RPMI1640 were seeded onto four-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp. Naperville) and cultured at 37°C, 5% CO_2 for 24 h. Afterwards, cells were washed with FCS-free RPMI1640 and incubated with Alexa Fluor 555 labeled negative control siRNA (1 μM) for 3 days. After washing twice with PBS, cells were fixed with 4% formaldehyde (PFA) for 5 min at room temperature. Subsequently, cells were permeabilized and blocked by incubation in 0.1% Triton X-100 containing 3% BSA for 10 min. Early endosome marker Rab4 (Abcam Ltd., Cambridge, UK; 1:500 diluted with 1% BSA in PBS) was added and incubated with cells at room temperature for 90 min. After washing, cells were incubated with FITC labeled secondary antibody (Abcam Ltd., Cambridge, UK; 1:200 diluted with 1% BSA in PBS) at room temperature for 90 min. After washing, cells were mounted in a mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Inc., Burlingame), and then observed by fluorescence microscopy.

siRNA Mediated Gene Silencing

The transfection reagent mediated siRNA delivery was performed according to the Silencer™ siRNA Transfection Kit Instruction Manual (Ambion Europe Ltd., Huntingdon, United Kingdom). Briefly, 2×10^4 N9 cells were seeded into four-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp., Naperville) and cultured at 37°C, 5% CO_2 for 24 h. Then cells were washed with FCS and antibiotic-free medium and cultured in 200 μl FCS and antibiotic-free medium. siRNA targeted to β-actin or negative control (final concentration: 25 nM) and Ambion siPORT Lipid complex was added into medium and co-cultured with cells for 4 h. Afterwards 1 ml medium with 10% FCS was added and cells were cultured for further 72 h. Subsequently, cellular β-actin protein expression was analyzed using standard immunofluorescence methods.

For siRNA delivery without transfection reagent, most steps were performed as above described except that cells were cultured in six-well plates and siRNA was added directly to the cell suspension (final concentration: 500 nM). Cellular β-actin expression was analyzed using real-time PCR because of the relative low gene-silencing efficiency of naked siRNA. Total RNA from cultured cells was prepared using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's instruction. 1 μg RNA was reverse transcribed into cDNA using randomized primers. Subsequently mRNA expression of β-actin was quantified by real-time PCR using SYBR-Green as detection reagent and 18s rRNA as reference standard. Following primers were used: β-actin (sense, CCC TGT GCT GCT CAC CGA; antisense, ACA GTG TGG GTG ACC CCG TC), and 18s rRNA (sense, ACA TCC AAG GAA GGC AGC AG; antisense, TTT TCG TCA CTA CCT CCC CA).

siRNA-Mediated Protein Purification

Cells cultured in flasks were rinsed two times with ice-cold PBS and then 20 ml ice-cold PBS containing PMSF was added and cells were collected with a scraper. 1.5×10^8 cells were sonicated and incubated in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, Munich, Germany) for 2 h on ice. Lysates were cleared by centrifugation at

14,000*g* at 4°C for 5 min. An aliquot of the supernatants was used for protein purification.

Magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) were treated according to the handbook to deactivate RNase activity. One microgram (100 μl) of treated magnetic streptavidin microbeads were coated with 200 pmol of biotin labeled siRNA by incubation in 1 ml selection buffer with RNasin® Ribonuclease Inhibitor (Promega, Mannheim, Germany) (30 min, room temperature). The lysates were incubated with the siRNA coated magnetic microbeads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer containing RNasin® Ribonuclease Inhibitor and DDT (total volume 1.5 ml, 0°C, 15 min). Magnetic streptavidin microbeads without siRNA were also incubated with cell lysate and served as control. The protein-siRNA-magnetic bead-complex was recovered in a magnetic device and washed four times. Proteins were removed from siRNA coated beads by heating in loading buffer and analyzed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Blue [Zhang et al., 2005a]. Bands were analyzed by in-gel tryptic digestion and mass spectrometry.

Protein Identification

In-gel tryptic digestion was performed as described [Shevchenko et al., 1996] and modified as outlined below. Briefly, the protein band was excised from the gel, fully de-stained, and digested for 3 h with porcine trypsine (sequencing grade, modified; Promega, Mannheim, Germany) at a concentration of 67 ng/μl in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1–2 μl were left and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method [Arnott et al., 1998] was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337 nm laser, gridless pulsed ion extraction and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionization source. Gold/palladium-coated glass capillary nanoflow needles were obtained from PROXEO (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, nonredundant protein database) were done using the MASCOT software from Matrix Science [Perkins et al., 1999].

PKR Activation Assays

Negative control siRNA and siRNA targeted to β-actin, whose sequence were showed above, were used here. PKR activation was analyzed using Western blotting. Briefly, 3×10^5 N9 cells per well were seeded into 12-well cell culture plates and cultured overnight. Subsequently, negative control siRNA (50 nM), β-actin siRNA (50 nM), or poly rI:rC (Sigma, Munich, Germany) were transfected into cells, respectively, using Ambion siPORT Lipid. Transfection reagent alone was used as negative control and poly rI:rC (50 ng/ml) was served as positive control [Sledz et al., 2003]. The transfection process was performed according to the Silencer™ siRNA Transfection Kit Instruction Manual. Total protein lysates were collected 48 h post-transfection. Fifty micrograms of total protein were separated on 10% SDS–polyacrylamide gel and transferred to PVDF membrane. Immunostaining was performed using ECL (Amersham Pharmacia Biotech). The antibody against PKR (Cell Signaling Technology) and antibody against phospho-PKR (BioSource Europe) were used at a 1:1,000 dilution and peroxidase-linked anti-rabbit secondary antibody (Amersham Pharmacia Biotech) was used at a 1:3,000 dilution.

RESULTS

Uptake, Cellular Distribution, and Gene-Silencing Effects of Naked siRNA in Microglial Cells

To study the characteristics of siRNA uptake and cellular distribution independent of gene knockdown effects, we used a FITC-labeled

siRNA, which has no homologous sequence in mammals (negative control siRNA). In antisense technology, oligonucleotide uptake and cellular distribution have been well studied. So here siRNA uptake and cellular distribution were compared with a 20-nt long ssDNA whose uptake and cellular distribution has been described in N9 cells previously [Zhang et al., 2005b].

Without transfection reagent, negative control siRNA or ssDNA was incubated with N9 cells. As shown in Figure 1, after incubation with FITC-labeled siRNA the fluorescence intensity of N9 cells increased, which indicated that siRNA can be imported by N9 cells without transfection reagent. Similar phenomena were observed using the ssDNA and the fluorescence intensity enhancement for siRNA and ssDNA was comparable, which suggested that they have similar uptake efficiency in N9 cells.

Cellular distribution of FITC-labeled siRNA was studied using fluorescence microscopy. As shown in Figure 2A, after 1 h of incubation, siRNA was unequally distributed in the cytoplasm of unfixed N9 cells. Nuclear distribution was not observed at this point of time. For the ssDNA, after 1 h incubation, ssDNA also unevenly localized to the cytoplasm but not to the nucleus in N9 cells (Fig. 2A), which was similar to previous observation [Zhang et al., 2005b]. These observations further support that siRNA can be internalized by N9 cells without transfection reagent. As naked siRNA showed a spotted distribution in N9 cells, which indicated an endosomal localization, the sub-cellular distribution of siRNA was further characterized by double staining with the endosome marker Rab4. As shown in Figure 2B co-localization of siRNA (red) and early endosome (green) was observed. Much less siRNA fluorescence was seen in fixed cells (Fig. 2B) as compared to living cells (Fig. 2A), which may be due to the fixation, permeabilization, and washing processes.

To evaluate the gene-silencing effects of naked siRNA, siRNA targeted to β-actin were added directly to cell suspension at high concentrations. The gene silencing effects of this β-actin siRNA was verified in N9 cells using siPORT Lipid transfection (Fig. 3A). As shown in Figure 3A, at relative low concentrations (25 nM) β-actin siRNA can knockdown β-actin protein expression in N9 cells as the β-actin fluorescence intensity of N9 cells treated with

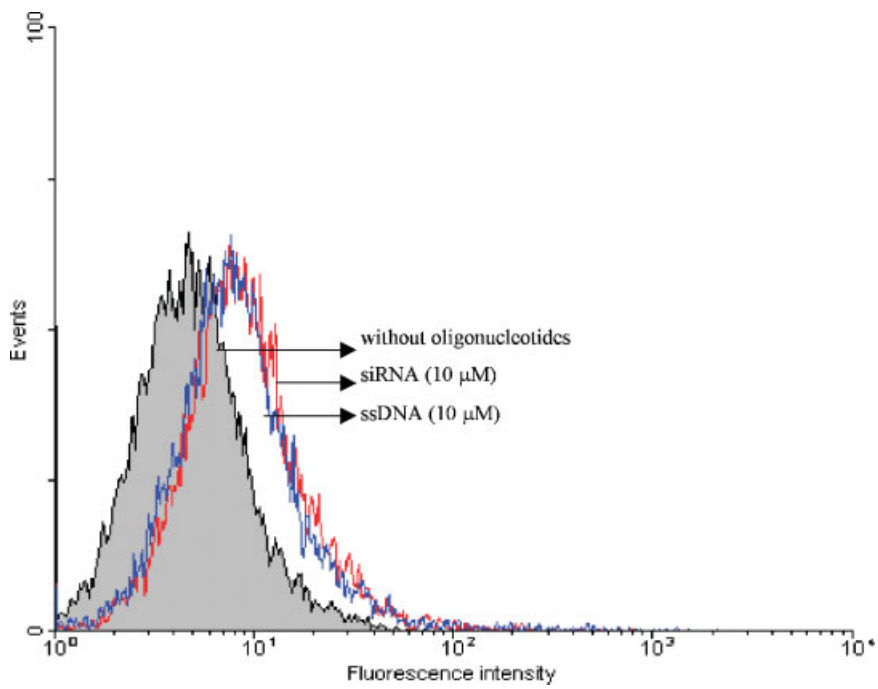

Fig. 1. siRNA and ssDNA uptake by microglial N9 cells. FITC-labeled negative control siRNA (10 μM) or ssDNA (10 μM) were incubated with N9 cells at 37°C for 1 h. The fluorescence intensity of N9 cells was measured by flow cytometry.

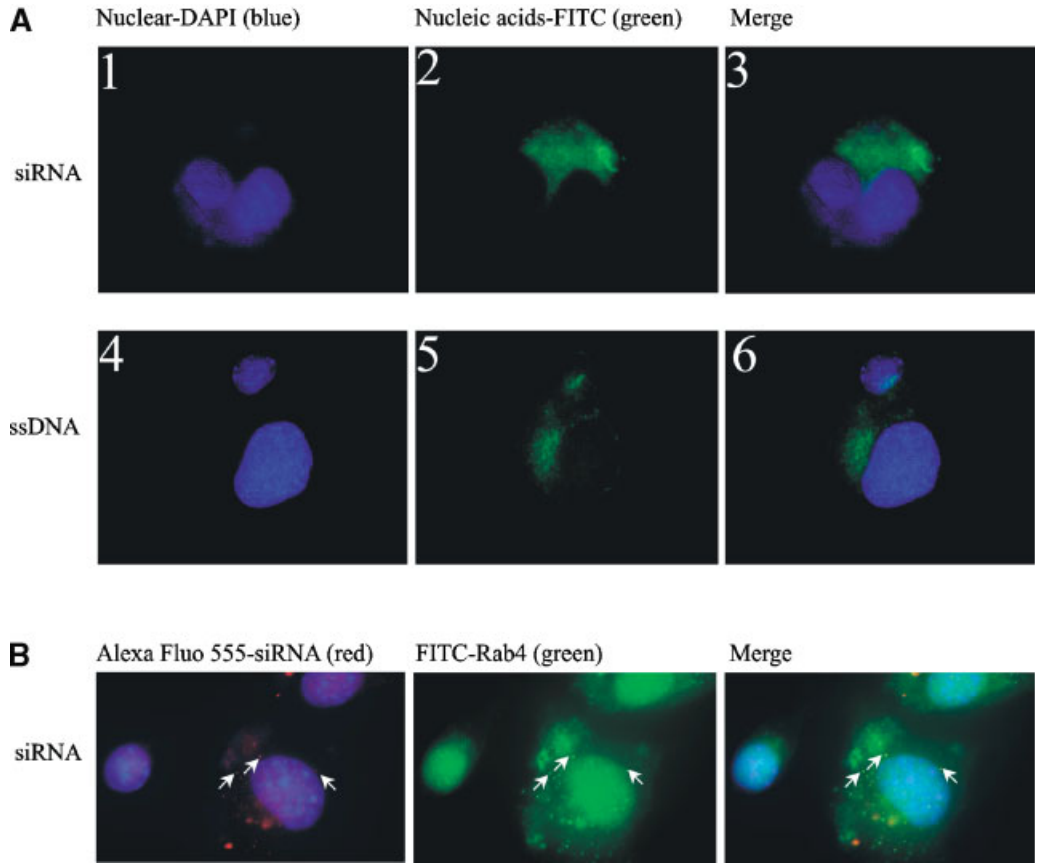

Fig. 2. Cellular distribution of naked negative control siRNA in microglial N9 cells. **A**: Cellular localization of naked siRNA and ssDNA in unfixed N9 cells. Negative control siRNA and ssDNA were FITC labeled. After FACS analysis described above N9 cells were observed by fluorescence microscopy. The upper panel showed the negative control siRNA cellular distribution and the lower panel showed the ssDNA cellular distribution. **B**: Co-localization of naked siRNA with endosome. Alexa Fluor 555 labeled negative control siRNA (1 μM) was incubated with cultured N9 cells for 3 days. After incubation, the endosomes were stained by early endosome marker Rab4, which was visualized with FITC labeled secondary antibody, and nucleus were stained by DAPI. Double-labeled siRNA are marked with arrows.

siRNA targeted to β-actin was much lower than that of N9 cells treated with negative control siRNA. However, without transfection reagent the same siRNA targeted to β-actin didn't significantly reduce β-actin RNA expression even at much higher concentrations (500 nM) (Fig. 3B).

Cellular Binding Proteins of siRNA in Microglial Cells

The same negative control siRNA was biotinylated and used to study N9 cellular proteins that bind to siRNA. Biotinylated siRNA was adsorbed to magnetic streptavidin microbeads and magnetic streptavidin microbeads alone served as control. After protein purification, siRNA was released from magnetic microbeads, analyzed on 4% low-melting gel to show integrity (data not shown).

As shown in Figure 4, siRNA bound to a number of proteins but magnetic streptavidin microbeads also bound to several proteins. Proteins bound to siRNA or magnetic streptavidin microbeads were identified by mass spectrometry. It is obvious, that siRNA binding proteins were different from that of magnetic microbeads binding proteins (Table I), which are biotinylated proteins binding to streptavidin.

As further control, we used a different cell line, HCEC cells, which were derived from human microvessel endothelial cells, to compare whether the same siRNA has different binding proteins in different cell types. As shown in Figure 4, the binding proteins of the negative control siRNA in N9 and HCEC cells were not completely identical.

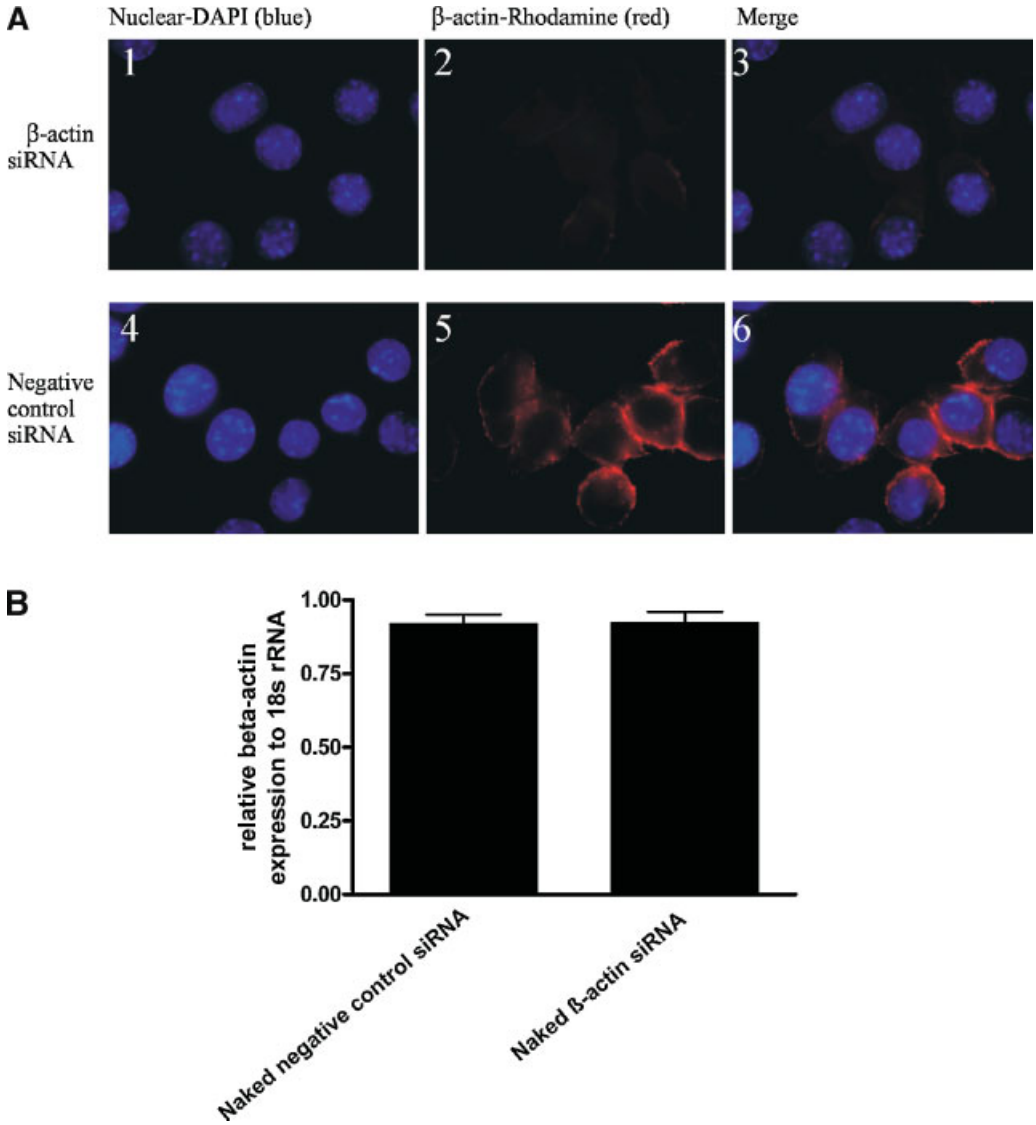

Fig. 3. Gene silencing effects of siRNA targeted to β-actin in microglial N9 cells with (**A**) or without (**B**) transfection reagents. A: N9 cells were transfected with β-actin siRNA (1, 2, and 3, 25 nM) or negative control siRNA (4, 5 and 6, 25 nM) according to the Silencer™ siRNA Transfection Kit Instruction Manual. β-actin expression was assayed using immunofluorescence methods with an anti-β-actin antibody. Result represented one of three separate experiments. B: β-actin siRNA (500 nM) or negative control siRNA (500 nM) were added directly to cell suspension. β-actin expression was assayed using real-time PCR. Data are expressed relative to the expression of intern reference 18s rRNA. Each bar represents the mean and SEM of triplicates.

To further compare whether different sequence siRNA have different binding protein in the same cell type, a siRNA targeted to the mammalian cellular β-actin transcript was used to purify binding proteins. The gene silencing effects of this β-actin siRNA was verified in N9 cells (Fig. 3A). Subsequently, cellular binding proteins of this β-actin siRNA in N9 cells were studied by the same method as described above. As shown in Figure 4, β-actin siRNA also has a number of cellular binding proteins in N9 cells and the binding proteins in the SDS–PAGE gel seemed to be not completely identical with that of the negative control siRNA in N9 cells (Fig. 4).

Part of the purified siRNA binding proteins was identified by mass spectrometry sequencing. Names and functions of the identified siRNA binding proteins are shown in Table I. Among those, DHX9, ADAR, PKR, and Stau1 belong to the dsRNA binding protein family, which share a common evolutionarily conserved motif specifically facilitating interaction with dsRNA. Protein Tial1 also has RNA binding

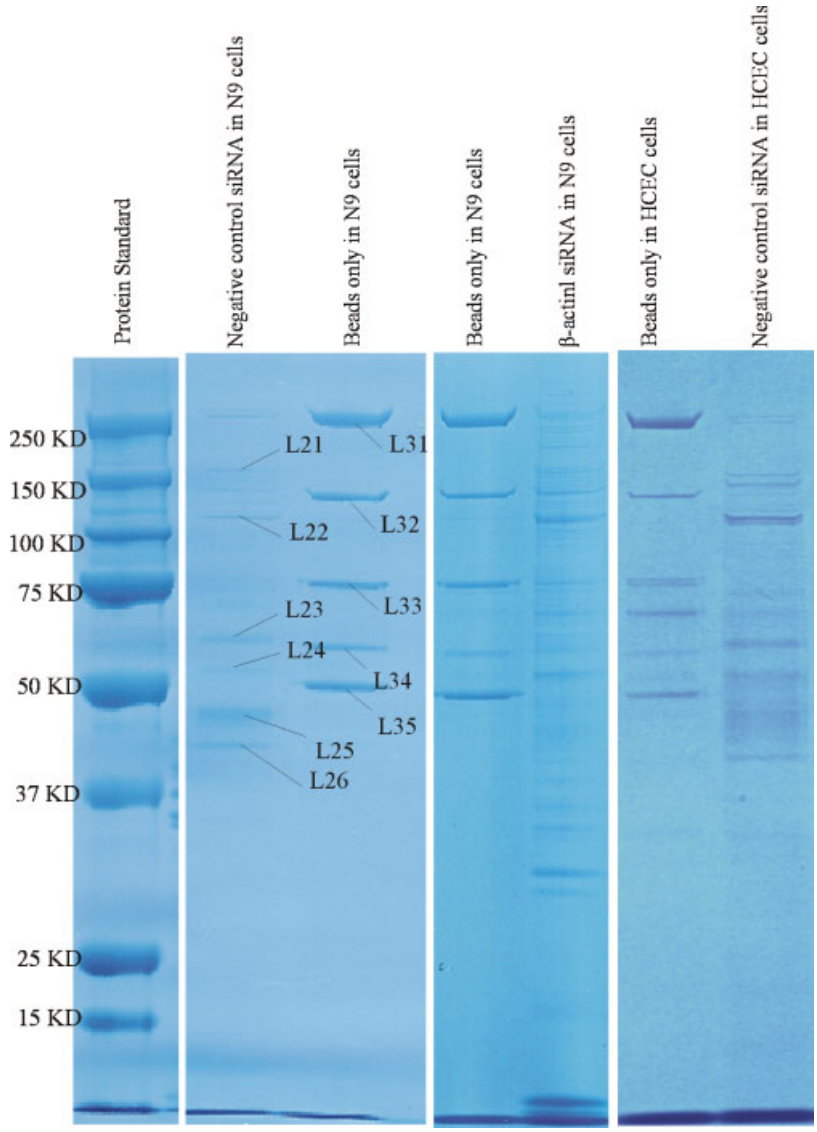

Fig. 4. Cellular binding proteins for siRNA. Biotin-labeled siRNAs were used for cellular binding protein purification. Negative control siRNA and β-actin siRNA were applied. Two cell lines, murine microglial N9 cells and human cerebromicrovasular endothelial HCEC cells, were used. Magnetic streptavidin microbeads alone was served as negative control. The indicated bands were identified as: L21: DHX9, L22: ADAR, L23: PKR and Staul, L24: unnamed protein product, L25: Rahl, L26: Tial1, L31: Acac, L32: Pcx, L33: Pcca and Mccc1, L34: Pccb and 4930552N12Rik and L35: Dbt.

activity. All these proteins have important physiological roles.

PKR Activation by siRNA in Microglial Cells

PKR is a regulator of global expression pattern and it is believed that siRNA did not trigger PKR activation. Interestingly, PKR was identified to bind siRNA in N9 cells. Further, we want to see whether siRNA can activate PKR. Activation of PKR results in PKR dimerization and autophosphorylation at threonine 446 and threonine 451 in the activation loop. Antibodies specific for PKR phosphorylated at threonine 451 were used to detect activated PKR and antibodies against total PKR were used to normalize protein loading. Both negative control siRNA and siRNA targeted to mammalian β-actin gene were used in this experiment. siRNA were introduced into cells by transfection reagent. Transfection reagent alone was used as negative control and poly rI:rC was used as positive control [Sledz et al., 2003]. Experiments were repeated three times and similar results were observed. Representative data are shown in Figure 5. Both, siRNA and poly rI:rC significantly induced PKR activation in N9 cells

TABLE I. siRNA Mediated Protein Purification in N9 Cells

Band	Symbol[a]	Name	Functions	Comments
Lane 3 (siRNA mediated protein purification)				
L21	Dhx9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	Double-stranded RNA binding; ATP binding; RNA dependent and ATP driven helicase activity; pre-mRNA conformational rearrangement, Persengiev et al. [2004]	
L22	Adar	Double-stranded RNA-specific adenosine deaminase	Double-stranded RNA binding; interferon-inducible RNA-editing enzyme implicated in the site-selective deamination of adenosine to insone in double-stranded RNA, Saunders et al. [2003]	
L23	Prkr	Protein kinase, interferon-inducible double stranded RNA dependent	Double-stranded RNA and structured single-stranded RNA binding protein; serine/threonine kinase activity; interferon induction activity and eIF2α inhibitory activity, Clemens et al. [1997]	Two proteins were identified in this band.
	Stau1	Staufen (RNA binding protein) homolog 1 (*Drosophila*)	Double-stranded RNA binding; tubulin binding; mRNA intracellular transport, Chi et al. [2003]	
L24	Ugt1a10	UDP glycosyltransferase 1 family, polypeptide A10		
L25	Rnh1	Ribonuclease/angiogenin inhibitor 1	Ribonucleolytic activity and angiogenic activity inhibition, Semizarov et al. [2003]	
L26	Tial1	Tial1 cytotoxic granule-associated RNA binding protein-like 1	RNA binding; nucleolytic activity; induction of apoptosis, Ferrari et al. [1996]	
Lane 4 (only streptavidin beads Without oligonucleotides)				
L31	Acac	Acetyl-Coenzyme A carboxylase		Biotinylated protein
L32	Pcx	Pyruvate carboxylase		Biotinylated protein
L33	Pcca	Propionyl-Coenzyme A carboxylase, alpha polypeptide		Biotinylated protein
	Mccc1	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)		Biotinylated protein
L34	Pccb	Propionyl Coenzyme A carboxylase, beta polypeptide		Biotinylated protein
	Mccc2	Methylcrotonyl-Coenzyme A carboxylase 2 (beta)		Biotinylated protein
L35	Dbt	Dihydrolipoamide branched chain transacylase E2		Biotinylated protein

[a]Official gene symbol according to LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/).

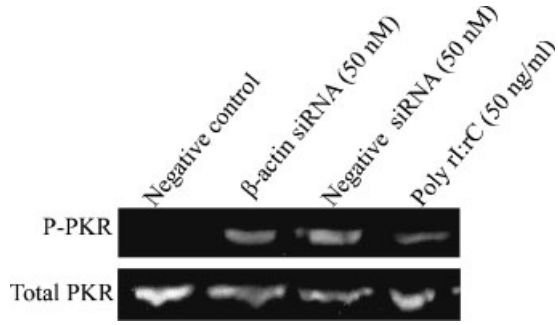

Fig. 5. Western analysis of PKR activation by siRNA in microglial N9 cells. N9 cells were transfected with negative control siRNA (50 nM), β-actin siRNA (50 nM) or poly rI:rC (50 ng/ml) using transfection reagent and transfection reagent alone was served as negative control. PKR activation was assayed using western analysis. The upper panel used an antibody specific to activated PKR and the lower panel used an antibody specific to whole PKR. Experiments were performed in triplicates.

but not the transfection reagent alone. Furthermore, siRNA targeted to β-actin decreased β-actin expression in N9 cells as well (Fig. 3A).

DISCUSSION

RNAi technology is an efficient high-throughput method to investigate gene function and is also a potential method to develop highly specific dsRNA based gene-silencing therapeutics. Due to these advantages, RNAi technology has been widely used to elucidate mammalian gene functions and to treat certain kind of experimental diseases [Dykxhoorn et al., 2003; Trulzsch and Wood, 2004]. In microglial cells, siRNA mediated RNAi is also used as a powerful tool to elucidate molecular functions [Gan et al., 2003; Giri et al., 2003]. But the cellular uptake efficiency and cellular localization of siRNA still hasn't been fully elucidated. Though the specificity of siRNA mediated RNAi has been reported by many groups, recently some studies gave clues to further non-specific effects caused by siRNA in mammalian cells [Jackson et al., 2003; Saxena et al., 2003; Sledz et al., 2003; Scacheri et al., 2004].

Here, our study showed that naked siRNA (without transfection reagent) could be internalized by microglial N9 cells, and unevenly localized to the cytoplasm but could not silence gene expression even at high concentrations (500 nM). Its cellular uptake and cellular distribution was similar to that of ssDNA. siRNA had a number of cellular binding proteins, most of which belong to the dsRNA binding protein family. siRNA can not only bind PKR in vitro but also activates PKR in vivo in N9 cells.

In vivo transfection efficiency is one of the major problems for oligonucleotide-based therapeutic strategies, including siRNA applications. In the brain, because of the blood–brain barrier (BBB), direct microinjection is the most widely used method for oligonucleotide-based therapeutic strategies. Naked oligonucleotide is mainly used for injection because of the neurotoxicity of transfection reagents. In vivo, viral vector based RNAi is used [Brummelkamp et al., 2002; Sui et al., 2002]. Naked siRNA can also be efficiently delivered to peripheral organs in adult animals and inhibit specific gene expression with the hydrodynamic transfection methods [Lewis et al., 2002; Makimura et al., 2002]. Though gene knockdown in brain using vector-mediated RNAi has been reported, one observation indicated that naked siRNA applied directly to rat brain might not induce RNAi because of low efficiency [Hommel et al., 2003; Isacson et al., 2003]. Therefore, it is important to investigate naked siRNA uptake and cellular distribution in brain-derived cells. Here, we studied the characteristics of naked siRNA uptake and cellular distribution in N9 microglial cells. Naked siRNA can be internalized by N9 cells and mainly localized to the cytoplasm after entry, which is similar with that of ssDNA. Further double-staining experiments showed that naked siRNA is mainly restricted to endosomes. Due to the development of antisense technology, ssDNA uptake and cellular distribution has been fully studied. The similar uptake efficiency and cellular distribution of siRNA with ssDNA indicated they might share similar uptake mechanism.

Although naked siRNA can be internalized by N9 cells, no significant gene silencing effects was detected after naked siRNA treatment. siRNA targeted to β-actin significantly reduced β-actin expression at low concentrations (25 nM) using transfection reagent, however, the same siRNA didn't silence β-actin expression even at high concentrations (500 nM) without transfection reagent. In accordance with our observations, Lingor et al., reported that naked siRNA (205 nM) could not silence endogenous or reporter genes expression [Lingor et al., 2004].

Interestingly, a number of cellular siRNA binding proteins were identified in vitro. Two

biotin labeled siRNAs, control siRNA and β-actin siRNA, were used for purification of binding proteins. Though sequences of these two siRNA are very different they still share a number of binding proteins. And the negative control siRNA also shares a number of binding proteins in murine microglia N9 cells and human endothelial HCEC cells.

Further, some binding proteins were identified by mass spectrometry sequencing. As shown in Table I, except protein Rnh1 and an unnamed protein, the other five proteins all have RNA binding activity. Among these five RNA-binding proteins, DHX9, ADAR, PKR, and Stau1 are members of the dsRNA binding protein family, which share a common evolutionarily conserved motif specifically facilitating interaction with dsRNA. This interaction is independent of nucleotide sequence arrangement. All these identified binding proteins play important physiological roles in vivo and their interaction with siRNA might interfere with their normal cellular functions. In particular, DHX9 has helicase and dsRNA binding activity. In theory, siRNA might bind to DHX9 within cells and might be resolved by the helicase activity through unwinding the two strands. However, the relevant enzyme for dsRNA degradation within cells is ADAR. ADAR catalyses the conversion of adenosines to inosines. The resulting dsRNAs containing I-U pairs are unstable and easy to unwind [Kumar and Carmichael, 1998; Wagner et al., 1998; Sui et al., 2002; Saunders and Barber, 2003]. DHX9 and ADAR might be involved in cellular siRNA degradation. Stau1 has dsRNA binding and mRNA intracellular transport activity [Belanger et al., 2003]. Within cells, siRNA might also be transported and Stau1 might play a role in siRNA cellular localization.

Notably, the direct interaction of siRNA with PKR was observed. PKR has two dsRNA binding domains (DRBDs) and a serine/threonine kinase domain. The DRBDs have been reported to interact with as little as 11 bp dsRNA and such interaction appears to be independent of any specific RNA nucleotide motif or sequence. PKR's interaction with dsRNA causes PKR to form homodimers and to autophosphorylate on serine/threonine residues. After autophosphorylation, PKR is able to catalyse the phosphorylation of target substrates, the most well characterized being the eIF2α subunit and IκB. Phosphorylated eIF2α sequesters eIF2B, a rate-limiting component of translation, leading to inhibition of protein synthesis. Phosphorylated IκB is released from NF-κB, which then translocates to the nucleus and activates transcription of genes containing NF-κB binding side, including the interferon-β (INF-β) gene. Subsequently, a specific class of genes, the IFN-stimulated genes, are activated for transcription [Kumar and Carmichael, 1998; Sui et al., 2002].

It has been reported, that activation of PKR is dependent on length of the dsRNA. And only dsRNA longer than 80 base pairs activate PKR [Manche et al., 1992], but that the smaller siRNA avoids this triggering of non-specific responses to dsRNA, such as PKR activation [Caplen et al., 2001; Elbashir et al., 2001]. But our data here demonstrate that siRNA can not only bind to PKR in vitro, but also causes activation of PKR in vivo. Our observations are in agreement with previous studies, which reported that siRNA globally upregulates interferon-stimulated genes through PKR activation [Sledz et al., 2003]. Further, expression profiling performed in cultured mammalian cells treated by siRNA also revealed non-specific, concentration-dependent global changes of gene expression [Persengiev et al., 2004]. However, our result should not rule out the many known selective effects of siRNA [Chi et al., 2003; Semizarov et al., 2003] as we also have demonstrated the selective effect of anti β-actin siRNA on the actin cytoskeltion of microglial cells.

Briefly data presented here demonstrate that naked siRNA can be directly taken up by N9 cells and mainly localized to the endosomes. The uptake efficiency and cellular distribution of siRNA is similar with that of ssDNA. siRNA has a number of cellular binding proteins. Most of these binding proteins belong to the dsRNA binding protein family. Most notable is the direct interaction of siRNA with PKR. Furthermore, siRNA can not only bind PKR in vitro but also activate PKR in vivo in N9 cells. siRNAs have, in addition to their specific effects, broad effects beyond the selective silencing of target genes. This is very important, as high specificity is of critical importance for siRNA either serving as a genomic tool or a therapeutic candidate.

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