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Small Interfering RNAs Mediate Sequence-Independent Gene Suppression and Induce Immune Activation by Signaling through Toll-Like Receptor 3[1]

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Small interfering (si) and short hairpin (sh) RNAs induce robust degradation of homologous mRNAs, making them a potent tool to achieve gene silencing in mammalian cells. Silencing by siRNAs is used widely because it is considered highly specific for the targeted gene, although a recent report suggests that siRNA also induce signaling through the type I IFN system. When human embryonic kidney 293 (HEK293) or keratinocyte (HaCaT) cell lines or human primary dendritic cells or macrophages were transfected with siRNA or shRNAs, suppression of nontargeted mRNA expression was detected. Additionally, siRNA and shRNA, independent of their sequences, initiated immune activation, including IFN-α and TNF-α production and increased HLA-DR expression, in transfected macrophages and dendritic cells. The siRNAs induced low, but significant, levels of IFN-β in HEK293 and HaCaT cells. Secretion of these cytokines increased tremendously when HEK293 cells overexpressed Toll-like receptor 3 (TLR3), and the increased secretion of IFN-β was inhibited by coexpression of an inhibitor of TIR domain-containing adapter-inducing IFN-β, the TLR3 adaptor protein linked to IFN regulatory factor 3 signaling. Although siRNA and shRNA knockdown of genes represents a new and powerful tool, it is not without nonspecific effects, which we demonstrate are mediated in part by signaling through TLR3. ***The Journal of Immunology*****, 2004, 172: 6545–6549.**

Ribonucleic acid interference (RNAi)[3] has revolutionized the study of gene function by allowing sequence-specific gene silencing initiated by dsRNA that is homologous to the targeted transcript. The typical response to long dsRNA in mammalian cells is a global increase in RNA degradation and inhibition of protein synthesis. These are mediated by the activation of PKR, which phosphorylates elongation initiation factor-2α, and the generation of 2′-5′-oligoadenylate, which activates RNase L (1). The initial studies of RNAi in mammalian cells were complicated by the activation of not sequence specific suppression by the long dsRNA used to activate the system (2). A major breakthrough occurred when it was hypothesized that as at least 30 bp of dsRNA was required to activate these sequence-independent suppressive systems; thereby the use of 21-bp small interfering RNA (siRNA), similar in size to the products of DICER, would not produce activation (3). Two recent reports demonstrate that plasmid-delivered short hairpin RNA (shRNA) induces IFN-β (4), and extracellularly delivered siRNA induces a set of IFN-inducible genes that are dependent on PKR (5), suggesting that this hypothesis may be incorrect.

The main family of dsRNA binding domain (DRBD)-containing proteins, which exhibit an $\alpha\beta\beta\alpha$ structure and have high homology throughout evolution, is found in many proteins with diverse functions, including host defense; cellular stress; translation activation; mRNA localization, transportation, and processing; RNA editing; and endoribosomal activities (reviewed in Refs. 6 and 7). As few as 11 bp of dsRNA can bind a DRBD, and 17 bp of dsRNA efficiently activates PKR (8, 9), a DRBD-containing protein important in antiviral defense, cellular stress, and multiple signaling pathways. Other dsRNA binding motifs have been identified, including zinc finger-containing motifs and lysine-rich regions (reviewed in Refs. 6 and 7). Toll-like receptors (TLRs) are a germline-encoded family that recognizes pathogen-associated molecular patterns and host proteins associated with danger (reviewed in Refs. 10 and 11). TLR3 is a receptor of dsRNA (12) and cellular mRNA (13). It does not contain obvious dsRNA binding motifs, but shares leucine repeat protein binding motifs with other TLR, suggesting that a dsRNA binding protein may mediate the interaction. TLR3 signals through NF-κB, PKR, and IFN regulatory factor 3 (IRF-3) pathways (14–17) and is dependent on two adapter proteins for signaling; one, MyD88, is shared with all TLR, and the second, TIR domain-containing adapter-inducing IFN-β (TRIF), is used by TLR3 and TLR4 and mediates the induction of type 1 IFN through IRF-3 and activation of an antiviral response (15, 17).

The early studies of siRNA treatment of mammalian cells suggested that specific reduction of targeted mRNA was observed without nonspecific (sequence-independent) mRNA degradation or protein synthesis inhibition (3, 18). In this report we demonstrate that siRNA and shRNA signal through TLR3 and induce cellular activation, including the generation of type I IFN and TNF-α, and sequence-independent inhibition of gene expression.

Materials and Methods

Cell culture

HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2 mM glutamine (Invitrogen, Carlsbad, CA) and 10% FCS (HyClone, Ogden, UT) (complete medium). The

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[3] Abbreviations used in this paper: RNAi, RNA interference; DC, dendritic cell; DRBD, dsRNA binding domain; HEK, human embryonic kidney; IRF3, IFN regulatory factor 3; luc, luciferase; MDM, monocyte-derived macrophage; MR, mannose receptor; sh, short hairpin; si, small interfering; SL2, *Drosophila* Schneider line 2; TLR3, Toll-like receptor 3; PKR, RNA-dependent protein kinase; TRIF, TIR domain-containing adapter-inducing IFN-β.

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TLR3–293 cell line, derived from 293 cells after transformation with pELAM-luciferase (luc) and pCMV6-XL5 containing human TLR3 cDNA (Origen Technologies, Rockville, MD), were grown in complete medium with zeocin (125 μg/ml) and G418 (400 μg/ml; Invitrogen). *Drosophila* Schneider line 2 (SL2; American Type Culture Collection) was grown in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FCS. The immortalized human keratinocyte cell line, HaCaT, obtained from Prof. N. Fusenig (German Cancer Research Center, Heidelberg, Germany) (19) was cultured in complete medium containing 15 mM HEPES, 50 μg/ml gentamicin, and 2.5 mg/l fungizone (Invitrogen).

Leukopheresis samples were obtained from HIV-uninfected volunteers through an institutional review board-approved protocol. PBMC were purified by Ficoll-Hypaque density gradient purification. Monocytes were produced as described previously (20) and were cultured in AIM V serum-free medium (Life Technologies, Gaithersburg, MD) supplemented with GM-CSF (50 ng/ml) and IL-4 (100 ng/ml; R&D Systems, Minneapolis, MN) to generate immature dendritic cells (DCs). Fresh medium containing cytokines was added to the cells on days 2 and 5. The resulting DCs were used between 6 and 9 days after initial culture of monocytes.

Monocyte-derived macrophages (MDMs) were obtained by culturing monocytes in complete medium supplemented with 100 U/ml M-CSF (R&D Systems). Medium was changed every 3 days, and macrophages were used on day 6 or 7 of culture.

SiRNA and shRNA

SiRNAs and shRNAs were by made by several different methods. The same sequence for each gene target was used when methods of synthesis differed to avoid additional variance. A suffix of 1 or 2 was added to the name of the RNAs to denote their chemical or enzymatic origin, respectively. All siRNA and shRNA contained two additional nucleotides (UU) at their 3′ ends. Using chemical synthesis (Dharmacon, Lafayette, CO), gE1, gag1, and luc1 siRNA, homologous to mRNAs encoding gE protein of HSV, HIV gag and firefly luciferase were generated. The sense strands of the gE1, gag1, and luc1 siRNAs correspond to (pAAUAUACGAA UCGUGUCUGUA), (pGAUGGUGCUUCAAGCUAGUAC), and (pGA AACGAUAUGGGCUGAAUAC), respectively (21). In the chemically synthesized luc1 shRNA, a UUAA loop joined the luc-specific complementary sequences. Using the T7 RNA polymerase-mediated transcription method (Silencer siRNA Construction kit; Ambion, Austin, TX), gE2, luc2, and mannose receptor (MR) 2 (MR-specific) (PCCTAATAATTAT CAAAATGT) siRNAs were synthesized. When MR2 shRNA and CD4-2 shRNA (UGAAGUGGAGGACCAGAAG), which targeted to human CD4 mRNA, were transcribed, the complementary sequences were linked by an AAUU loop. SiRNAs were also generated by digesting long dsRNA, corresponding to gag and luciferase-encoding sequences, with RNase III-specific enzyme (DICER, Silencer siRNA Cocktail kit; Ambion). RNase-resistant shRNAs targeted to cleave luciferase and gag mRNAs were synthesized as previously described (21) using a Dura-Script kit (Epicentre, Madison, WI). All siRNA and shRNA, analyzed by PAGE under denaturing conditions as described previously (21), demonstrated to be of the correct length and without contamination. Following annealing, the siRNAs were reanalyzed by PAGE under nondenaturing condition and found to have the length expected if they form dsRNA.

Treatment of cells

TLR3–293 (also called 293), SL2, and HaCaT cells were seeded into 96-well plates 1 day before stimulation and were cultured without antibiotics. MDM and DC were cultured in 48-well plates. Cells were stimulated with medium, lipofectin alone, ssRNA, poly(I):poly(C) dsRNA (Sigma-Aldrich, St. Louis, MO), and siRNA and shRNA (5 μg/ml; ~330 nM of siRNA or shRNA depending on sequence) complexed with lipofectin (22). In certain experiments F-siRNAs were added at 50 μg/ml without lipofectin complexing. Eight or 24 h later supernatants were collected for cytokine measurement, and where indicated, cells were lysed with luciferase lysis buffer (Promega, Madison, WI) and analyzed with luciferase substrate (Promega) in an MLX luminometer (Dynatech Laboratories, Chantilly, VA).

Transient transfections

293 cells seeded into 96-well plates at 60–80% confluence were transfected with pUnoTLR3 (Invivogen, San Diego, CA) and pCMV-luc (22) and pEF-BOS TRIF dominant negative (TRIFdn) (17) or pEF-BOS control expression plasmids using the FuGENE 6 reagent (Roche, Indianapolis, IN). Twenty-four hours later cells were stimulated with the indicated ligands, and supernatants were collected 8 h later for cytokine measurement.

Analysis of MDM cell surface markers

MDM were stained with MR-PE mAb (Research Diagnostics, Flanders, NJ) or HLA-DR-PE mAb and analyzed on a FACScan flow cytometer using CellQuest Pro software (BD Biosciences, Mountain View, CA).

ELISAs for cytokines

Supernatants from treated macrophages, DC, HaCaT, TLR3–293, and 293 cells were collected at 8 or 24 h for cytokine measurement. IFN-α, IFN-β, TNF-α, and IL-8 (BioSource International, Camarillo, CA) were measured by sandwich ELISA. Cultures were performed in duplicate to quadruplicate and were measured in duplicate.

Results

The original description of siRNA treatment of mammalian cells suggested that the 21-bp-long siRNA would not activate sequence-independent protein synthesis inhibition or degradation of mRNA (3) based on the assumption that at least 30-bp-long dsRNA is required to activate PKR or 2′,5′-oligoadenylate synthetase (3, 18). The treatment of 293 cells with sequence-specific and nonspecific siRNA demonstrated that, in fact, some sequence-independent inhibition occurred in a concentration-dependent manner after control sequence siRNA treatment (Fig. 1*A*). As the suppressed gene (luc) was delivered by the expression plasmid, we determined whether a similar sequence-independent inhibition occurred when an endogenous gene was targeted. MDM were treated with siRNA and shRNA targeting MR mRNA or with control siRNA with sequence unrelated to MR. Sixteen hours after treatment, a modest decrease in cell surface MR was observed in control siRNA-treated cells compared with a more robust reduction

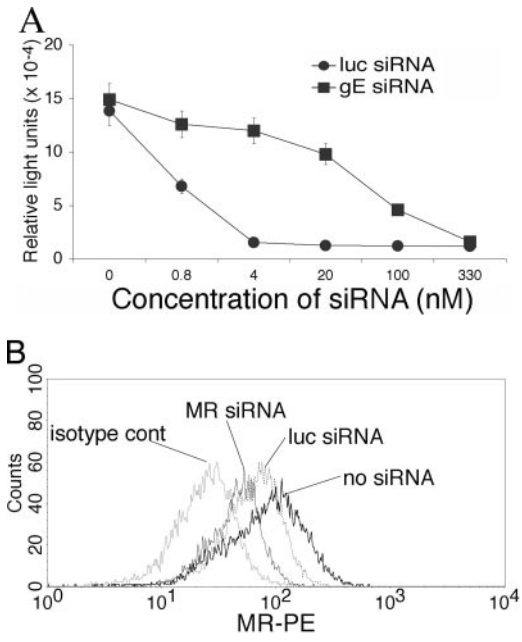

FIGURE 1. SiRNA and shRNA induce sequence-dependent and -independent suppression of gene expression from both plasmid vector and an endogenous gene. *A*, HEK293 cells were transfected with a CMV-luciferase expression plasmid. Twenty-four hours later they were transfected with lipofectin-complexed luc-specific siRNA or gE-specific control siRNA, both synthesized chemically. Luc activity was measured 8 h later. This experiment was performed in quadruplicate, and the SEM is shown. *B*, Human MDM were incubated with lipofectin-complexed MR-specific siRNA or luc-specific control siRNA. Both constructs were generated by the Silencer siRNA construction kit. Data obtained with cells exposed to only lipofectin and isotype control mAb are shown. Surface expression of MR was measured by flow cytometry 16 h after transfection. Experiments were repeated six (*A*) and five (*B*) times.

with MR-specific siRNA and shRNA (Fig. 1*B* and data not shown). We observed that siRNA not specific for MR led to a decrease in MR expression, and MR-specific siRNA reduced its expression to a greater degree. We believe that siRNA activates the cells leading to proinflammatory cytokine production, and this leads to a reduction in MR expression. An additional decrease in the expression of MR is observed when MR-specific siRNA is used. We believe that this additional decrease in MR expression is due to specific suppression through RNAi. All studied siRNA and shRNA, generated either by chemical or enzymatic syntheses or from long dsRNA by RNase III digestion, induced some level of sequence-independent suppression of gene expression. In addition, siRNA were made with 2′-deoxy-2′-fluoro-substituted C and U nucleotides, which makes the resulting RNA resistant to RNase A. These siRNA were delivered to 293, TLR3–293, and DC without lipofectin complexing, and sequence-independent suppression and type I IFN production were observed.

To address whether siRNA and shRNA were acting through known mechanisms of sequence-independent suppression, type I IFN levels were measured. Supernatants from siRNA- and shRNA-treated human primary DC, MDM, HEK293, and HaCaT cell lines were analyzed for IFN-α or IFN-β. A significant increase in type I IFN was observed in all cell types (Fig. 2 and data not shown). The induction of IFN and sequence-independent suppression of both endogenous and exogenous gene expression suggested that an innate immune response was induced by externally added siRNA and shRNA, which is in agreement with a recently published paper demonstrating activation of IFN signaling pathways (5).

The known mechanisms of dsRNA induction of sequence-independent suppressive responses include the direct binding to proteins encoding DRBD, such as PKR and 2′5′-oligoadenylate synthetase, and to long intracellular dsRNA and through signaling by TLR3 (12), which induces NF-κB, IRF-3, and PKR signaling pathways (14, 15, 17, 23–25). HEK293 cells stably overexpressing TLR3 were treated with siRNA. Signaling through this receptor was documented by measuring luc production from an NF-κB reporter plasmid or the induction of endogenous IL-8 (26) (Fig. 3*A* and data not shown), which demonstrated comparable signaling by siRNA compared with poly(I):poly(C). In comparison with 293 cells (Fig. 2*A*), TLR3–293 cells produced much more IFN-β in response to siRNA and shRNA and poly(I):poly(C) (Fig. 3*B*). HEK293 cells expressed low levels of TLR3 mRNA by Northern blot (data not shown), which probably accounts for their ability to respond to extracellular siRNA. Thus, siRNA induced sequence independent suppression of endogenous and exogenous gene expression probably by signaling through TLR3.

In cells of the innate immune system, TLRs act to signal cells to the presence of foreign or dangerous entities. In APCs, this signaling results in cellular activation, as measured by increased expression of MHC and costimulatory molecules and the release of proinflammatory cytokines. Macrophages and DCs treated with siRNA and shRNA made by any method demonstrated an increase in expression of HLA-DR (Fig. 4*A* and data not shown) and release of TNF-α similar to those observed for poly(I):poly(C) (Fig. 4*B*). No activation was observed with the addition of poly(C) RNA homopolymers or sense or antisense 21-bp ssRNAs used to make the siRNAs (data not shown).

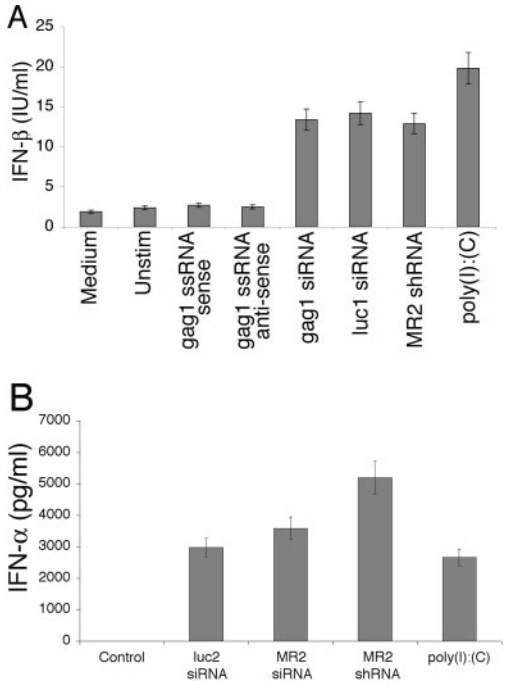

FIGURE 2. Treatment with siRNA and shRNA induces type I IFN secretion in HEK293 cells and MDM. *A*, HEK293 cells were transfected with medium, lipofectin alone (unstim), or lipofectin-complexed ssRNA, siRNA (generated by chemical synthesis) and shRNA (generated by Silencer construction kit), or poly(I):poly(C). Eight hours later supernatants were analyzed for IFN-β by ELISA. *B*, MDM were transfected with lipofectin alone (control), lipofectin-complexed siRNA and shRNA, or poly(I):poly(C). Luc siRNA and MR siRNA and shRNA were enzymatically synthesized using the Silencer siRNA construction kit. Eight hours later supernatants were analyzed for IFN-α by ELISA. Medium without lipofectin or ssRNA used to make the siRNA complexed to lipofectin gave values similar to unstim. Samples were run in triplicate to quadruplicate, and experiments were repeated three times. Error bars show the SEM.

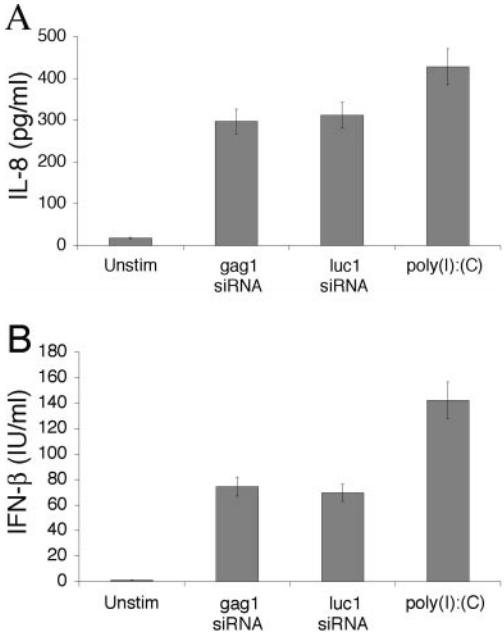

FIGURE 3. SiRNA induce IL-8 and IFN-β in TLR3–293 cells. HEK293 cells overexpressing TLR3 were transfected with lipofectin alone (unstim) or with lipofectin-complexed siRNA and poly(I):poly(C). Eight hours later supernatants were analyzed for IL-8 (*A*) or IFN-β (*B*) by ELISA. SiRNA were chemically synthesized. Medium without lipofectin or ssRNA used to make the siRNA complexed to lipofectin gave values similar to unstim. Samples were run in triplicate, and experiments were repeated three times. Error bars show the SEM.

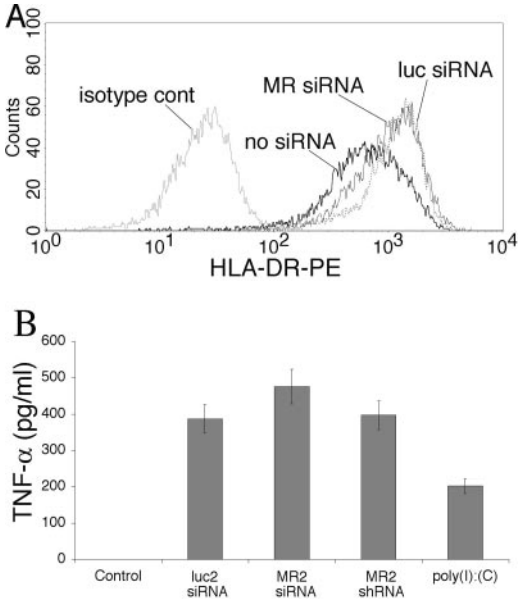

FIGURE 4. SiRNA and shRNA activate MDM, as measured by HLA-DR expression and TNF-α secretion. *A*, Human MDM were incubated with lipofectin-complexed MR-specific siRNA or luc-specific siRNA. Both were generated by the Silencer siRNA construction kit. Data obtained with cells exposed to only lipofectin and isotype control mAb are shown as positive and negative controls. Sixteen hours later cells were analyzed for HLA-DR expression by flow cytometry. *B*, Supernatants from MDM, treated as described in *A*, were analyzed for TNF-α by ELISA. Samples were run in triplicate, and experiment were repeated three times. Error bars show the SEM.

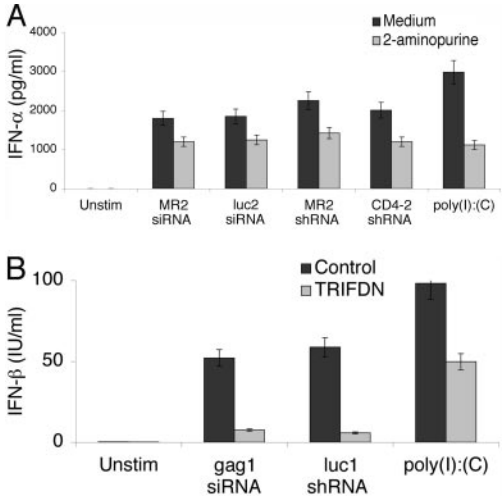

FIGURE 5. Inhibition of PKR partially, but incompletely, inhibits siRNA-induced IFN-β secretion from TLR3–293 cells, whereas dominant negative TRIF almost completely inhibits siRNA-induced IFN-β. *A*, TLR3–293 cells treated, or not, with 2-aminopurine (10 mM) were transfected with lipofectin alone (unstim), siRNA and shRNA, or poly(I):poly(C) complexed to lipofectin; 8 h later cells were analyzed for IFN-β. *B*, HEK293 cells were transiently transfected with a TLR3 expression plasmid (pUnoTLR3) and either a control vector or a TRIF dominant negative expression vector (17). Twenty-four hours later cells were stimulated with siRNA, shRNA, or poly(I):poly(C), and supernatant-associated IFN-β was measured by ELISA. Samples were run in triplicate, and experiments were repeated three times.

DsRNA can signal by multiple mechanisms depending on where the interaction takes place. It was recently demonstrated that extracellular dsRNA acted through TLR3, whereas intracellular dsRNA acted independently of TLR3 through PKR (7). To distinguish among siRNA/shRNA signaling through intracellular pathways, PKR, and extracellular receptors, TLR3, a specific inhibitor of PKR, 2-aminopurine, was used. This experiment is complicated by the fact that TLR3 also activates PKR directly (23). SiRNA, shRNA, and poly(I):poly(C)-stimulated MDM were pretreated, or not, with 2-aminopurine (10 mM), and significant, but incomplete, inhibition of TNF-α, IFN-α, and HLA-DR up-regulation was observed (Fig. 5*A* and data not shown). Interestingly, poly(I):poly(C) stimulation was the most inhibited by 2-aminopurine. We hypothesize that whereas siRNA and shRNA and poly(I):poly(C) signal through TLR3, siRNA and shRNA either have a decreased ability to enter and remain active in the cell or are less efficient activators of PKR. This demonstrates that in TLR3-expressing cells, the majority of activation by siRNA and shRNA occurs independently of PKR.

Our data demonstrate that in TLR3-expressing cells, signaling by externally administered siRNA and shRNA is probably mediated by TLR3. TLR requires adaptor molecules for signaling. To date, five such molecules have been identified (24). MyD88 is used by all TLR, whereas TRIF is only used by TLR3 and TLR4 and mediates type I IFN responses through induction of IRF-3 (15, 17). TRIF knockout mice have a severe defect in type I IFN production in response to dsRNA (27). HEK293 cells were cotransfected with TLR3 and control or TRIF dominant negative expression plasmids and were stimulated with siRNA or poly(I):poly(C). The dominant negative TRIF blocked siRNA induced IFN-β (Fig. 5*B*), demonstrating that TLR3 signaling or another TRIF-using signaling system was responsible for extracellular siRNA and shRNA activation of the IFN signaling pathway.

Having identified TLR3 as a likely mediator of siRNA and shRNA activation, we next ask what role TLR3 played in the ability to specifically suppress gene expression by siRNA. SL2 insect cells, which lack TLR3 and PKR, and TLR3–293 cells were treated with increasing concentrations of control and luc-specific siRNAs similar to the experiment shown in Fig. 1*A*. Total RNA transfected was kept constant by adding poly(C) homopolymer. SL2 cells demonstrated no suppression of luc expression by the control siRNA and a concentration-dependent silencing of luc expression by the luc2 siRNA (Fig. 6*A*). HEK293 cells demonstrated concentration-dependent inhibition by both control and luc-specific siRNA. The IC_{50} of each siRNA for each cell line was calculated. For HEK293 cells, the IC_{50} differed by $\sim$2 logs between control and luc-specific siRNA (Fig. 1). In TLR3–293 cells, the IC_{50} differed by $<$0.5 logs (Fig. 6*B*).

Discussion

These data demonstrate that externally delivered siRNA and shRNA signal cells through TLR3 and activate sequence-independent inhibition of gene expression. They are in agreement with two recent reports demonstrating that plasmid-expressed shRNA induce type I IFN (4) and that siRNA treatment of the human glioblastoma cell line T98G induced sequence-independent suppression through PKR (5). In this second report, the authors found that PKR was necessary for the sequence-independent activation, which differs from our finding that PKR is part of the pathway, but its inhibition by 2-aminopurine does not completely inhibit the siRNA-induced production of IFN-β. The likeliest explanation for the difference in results is that the T98G cell line does not express or has very low level expression of TLR3.

For the most part, TLR do not directly bind ligands, but, instead, contain protein interaction domains that bind other molecules that

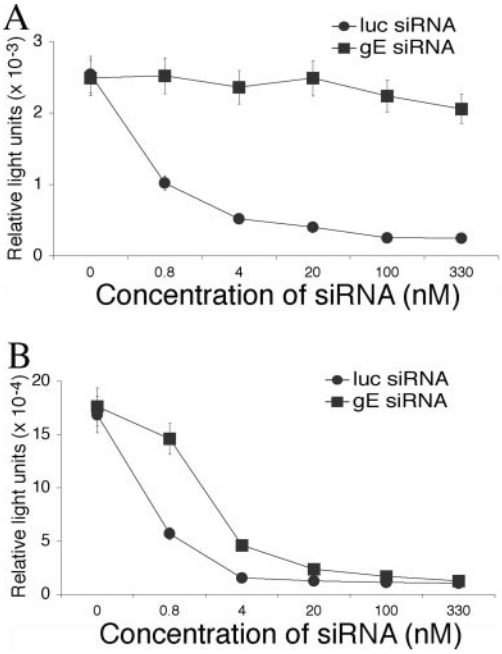

FIGURE 6. Overexpression of TLR3 increases sequence-independent suppression of gene expression by siRNA. SL2 (*A*) and TLR3–293 (*B*) cells were transfected first with a CMV-luciferase expression plasmid and then 24 h later with lipofectin-complexed control siRNA (gE) or luc siRNA, both synthesized chemically, in increasing concentrations. The total RNA delivered was kept constant by adding poly(C) homopolymer. Luc activity in cell lysate was measured 8 h later. Samples were run in triplicate to quadruplicate, and experiments were repeated three times. Error bars show the SEM.

bind the ligand. For most TLR, these accessory molecules have not been identified. Alexopoulou et al. (12) were the first to report that dsRNA is the specific ligand for TLR3 by demonstrating that TLR3 recognizes and responds to viral or synthetic dsRNA, but not to homopolymer ssRNA. Previous studies suggested that the dsRNA binding proteins, PKR and 2′,5′-oligoadenylate synthetase, required at least 30 bp of dsRNA for efficient activation, whereas other studies suggested that as little as 17 bp of dsRNA could signal (8, 9), In this study we present evidence that TLR3 responds to siRNA and shRNA with the production of type I IFN, IL-8, and TNF-α; activation of NF-κB promoters; and induction of sequence-independent gene suppression. It is conceivable that the first component of the TLR3 signaling cascade, probably a dsRNA-binding protein that has yet to be characterized, might efficiently interact with one helical turn of dsRNA within siRNA and shRNA (~11 bp) (7, 28), as has been suggested for other dsRNA-binding proteins (7), thus allowing short segments of dsRNA to signal.

The use of siRNA and shRNA as a tool in mammalian systems is commonplace. Our results demonstrate that these reagents may have unwanted effects; induction of a sequence-independent gene suppression, cellular activation, and type I IFN and proinflammatory cytokine production need to be considered in the analysis of results. These events appear to be dependent on the level of expression of TLR3, such that nonimmune cells, such as HaCaT and 293, with low levels of TLR3 have minimal type I IFN production, but still significant sequence-independent suppression, whereas DC and macrophages, with high TLR3 expression, yield high level cytokine production, sequence-independent gene suppression, and cellular activation. The use of siRNA, delivered by expression constructs or directly as a treatment for numerous diseases, has been proposed. The observation that these therapeutics may have sequence-independent suppressive properties as well as cellular activation potentials will need to be considered in their development.

References

1. Samuel, C. E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev. 14:778.*
2. Yang, S., S. Tutton, E. Pierce, and K. Yoon. 2001. Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol. Cell. Biol. 21:7807.*
3. Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411:494.*
4. Bridge, A. J., S. Pebernard, A. Ducraux, A. L. Nicoulaz, and R. Iggo. 2003. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet. 8:8.*
5. Sledz, C. A., M. Holko, M. J. de Veer, R. H. Silverman, and B. R. G. Williams. 2003. Activation of the interferon system by short-interfering RNAs. *Nat. Cell. Biol. 5:834.*
6. Fierro-Monti, I., and M. B. Mathews. 2000. Proteins binding to duplexed RNA: one motif, multiple functions. *Trends Biochem. Sci. 25:241.*
7. Saunders, L. R., and G. N. Barber. 2003. The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J. 17:961.*
8. Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews. 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol. 12:5238.*
9. Nanduri, S., B. W. Carpick, Y. Yang, B. R. Williams, and J. Qin. 1998. Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation. *EMBO J. 17:5458.*
10. Werling, D., and T. W. Jungi. 2003. Toll-like receptors linking innate and adaptive immune response. *Vet. Immunol. Immunopathol. 91:1.*
11. Armant, M. A., and M. J. Fenton. 2002. Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol. 3:3011.*
12. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature 413:732.*
13. Kariko, K., H. Ni, J. Capodici, M. Lamphier, and D. Weissman. mRNA is an endogenous ligand for Toll-like receptor 3. *J. Biol. Chem. 279:12542.*
14. Doyle, S. E., R. O'Connell, S. A. Vaidya, E. K. Chow, K. Yee, and G. Cheng. 2003. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. *J. Immunol. 170:3565.*
15. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immunol. 4:161.*
16. Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, et al. 2002. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature 420:324.*
17. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol. 169:6668.*
18. Jacque, J. M., K. Triques, and M. Stevenson. 2002. Modulation of HIV-1 replication by RNA interference. *Nature 418:435.*
19. Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol. 106:761.*
20. Weissman, D., H. Ni, D. Scales, A. Dude, J. Capodici, K. McGibney, A. Abdool, S. N. Isaacs, G. Cannon, and K. Kariko. 2000. HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. *J. Immunol. 165:4710.*
21. Capodici, J., K. Kariko, and D. Weissman. 2002. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol. 169:5196.*
22. Kariko, K., A. Kuo, E. S. Barnathan, and D. J. Langer. 1998. Phosphate-enhanced transfection of cationic lipid-complexed mRNA and plasmid DNA. *Biochim. Biophys. Acta 1369:320.*
23. Jiang, Z., M. Zamanian-Daryoush, H. Nie, A. M. Silva, B. R. Williams, and X. Li. 2003. Poly I:C-induced TLR3-mediated activation of NFκB and MAP kinases is through an IRAK-independent pathway employing signaling components TLR3-TRAF6-TAK1-TAB2-PKR. *J. Biol. Chem. 278:16713.*
24. O'Neill, L. A., K. A. Fitzgerald, and A. G. Bowie. 2003. The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol. 24:286.*
25. Sarkar, S. N., H. L. Smith, T. M. Rowe, and G. C. Sen. 2003. Double-stranded RNA signaling by Toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. *J. Biol. Chem. 278:4393.*
26. Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem. 274:10689.*
27. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, et al. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science 301:640.*
28. Williams, B. R. 2001. Signal integration via PKR. *Science STKE 2001:RE2.*