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Co-expression of anti-NFκB RNA aptamers and siRNAs leads to maximal suppression of NFκB activity in mammalian cells

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ABSTRACT

The specific down-regulation of gene expression in cells is a powerful method for elucidating a gene's function. A common method for suppressing gene expression is the elimination of mRNA by RNAi or antisense. Alternatively, oligonucleotide-derived aptamers have been used as protein-directed agents for the specific knock-down of both intracellular and extracellular protein activity. Protein-directed methods offer the advantage of more closely mimicking small molecule therapeutics' mechanism of activity. Furthermore, protein-directed methods may synergize with RNA-directed methods since the two methods attack gene expression at different levels. Here we have knocked down a well-characterized intracellular protein's activity, NFκB, by expressing either aptamers or small interfering RNAs (siRNAs). Both methods can diminish NFκB's activity to similar levels (from 29 to 64%). Interestingly, expression of both aptamers and siRNAs simultaneously, suppressed NFκB activity better than either method alone (up to 90%). These results demonstrate that the expression of intracellular aptamers is a viable alternative to siRNA knock-down. Furthermore, for the first time, we show that the use of aptamers and siRNA together can be the most effective way to achieve maximal knock-down of protein activity.

INTRODUCTION

Genome-wide sequencing projects have lead to the uncovering of thousands of new genes of unknown function. However, the role of well-studied genes in complex multi-gene dependent processes, such as disease pathology, remains hidden. Thus, there is a strong need for methods for the elucidation of gene function, particularly in relation to disease progression. The most effective methods for this purpose are those that suppress gene activity (1,2). Various methods target gene expression at the DNA, mRNA or protein level (2). Once a gene's activity is specifically suppressed the effects can be assessed in either mechanistic or disease models.

RNAi has become a widely used tool for the suppression of gene activity in invertebrates, plants, and, with the advent of small interfering RNA (siRNA) techniques, in mammalian cells (3–5). siRNAs can be introduced via direct transfection or by expression from various plasmids either transiently or stably (6–8). The siRNA molecules bind to a protein complex, called the RNA-induced silencing complex. This complex contains a helicase activity that unwinds the two strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule (9) and an endonuclease activity that hydrolyzes the target RNA at the site where the antisense strand is bound.

Although a powerful method, there are limits to siRNA techniques. Firstly, siRNAs don't always promote complete degradation of mRNA (10,11). If even a small amount of mRNA survives it may be able to produce sufficient amounts of protein for significant activity. Secondly, recent studies indicate that siRNAs can have adverse effects by activating sensors in the interferon response pathway (12,13) or other non-specific genes (14). Finally, siRNA (as well as antisense or gene knock-out strategies) may completely or severely deplete targeted protein levels. Since many proteins exist in multi-protein complexes that may be involved in multiple functional pathways, deleting the protein will likely have pleiotropic effects not specific to the relevant pathway.

Recently, nucleic acid-derived aptamers have been used to regulate intracellular protein activity (2,15). Aptamer

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'decoys', short RNAs containing the RNA-binding site for HIV-1 RNA-binding proteins, such as Rev and Tat, have been developed that inhibit HIV-1 replication when over-expressed (16,17). Aptamers specific to non-RNA-binding proteins, as well as RNA-binding proteins, can be generated by an *in vitro* process known as systematic evolution of ligands by exponential enrichment (SELEX). These non-decoy aptamers can bind with high specificity and affinity (2,18) and can knock-out intracellular protein activity (19). For instance, aptamers that recognize the cytoplasmic domain of the β2-integrin, leukocyte function-associated molecule-1 (LFA-1) have been isolated (19,20). LFA-1 mediates the adhesion of leukocytes in immune responses by binding to intercellular adhesion molecules (21). Intracellular expression of anti-LFA-1 aptamers inhibits LFA-1 activity, as measured by a decrease in cell adhesion (19). Mayer *et al.* (22) have extended these observations by blocking the intracellular function of cytohesin-1, a guanine-nucleotide-exchange factor that is thought to regulate the adhesion of LFA-1 to ICAM-1, with an anti-cytohesin 1 aptamer. Moreover, aptamer-based cytohesin-1 inhibition results in a decrease in cell adhesion and cytoskeletal rearrangement.

In this study, we have used an anti-NFκB aptamer, a-p50 (23), that has been shown previously to inhibit the DNA-binding activity the p50 subunit of the NFκB transcription factor in yeast (23,24), to, for the first time, knock-down NFκB activity in mammalian cells. Furthermore, we have knocked down NFκB activity with siRNA. We find that both aptamers and siRNAs when used alone knock-down the activity of NFκB to a similar extent and interestingly, when used in combination the two methods work better than either method alone, leading to essentially complete (90%) knock-down of NFκB activity.

MATERIALS AND METHODS

Plasmids

U6/TAR-a-p50 contains a U6 promoter followed by the TAR-a-p50 sequence. It was made by inserting the a-p50-TAR sequence, GGGTCTCTCTGGTTAGCATCCTGAAAC-TGTTTTAAGGTTGGCCGATGTAGCTAGGGAACCCACT (flanked by XhoI/BamHI sites and generated by PCR), into the XhoI/BamHI restriction sites of the plasmid MYHIV (kindly provided by Nouria Hernandez). The HIV-1 promoter sequence was then replaced by the pol III U6 promoter by inserting a PCR-generated fragment into EcoRI/XhoI linearized plasmid.

pAV7SL-a-p50 was made by inserting a PCR-generated fragment consisting of anti-NFκB aptamer a-p50 (23) (Figure 1) into the SalI–XbaI sites of pAV7SL (kindly provided by D. Engelke).

pSilencer-2.0-U6-siRNA2 was made by inserting the fragment encoding siRNA2: TATTAGAGCAACCTAAACA into the XbaI/BamHI sites of the vector pSilencer (Ambion).

Design of short interfering RNA (siRNA)

siRNA targeting NFκB p50 was designed according to Tuschl's method found online http://www.rockefeller.edu/labheads/tuschl/sirna.html. The sequence and its complement is BLAST searched against the human genome to ensure that only NFκB p50 was targeted. Six sequences were designed siRNA1, TGGCAGAAGATGATCCATATT; siRNA2, TATTAGAGCAACCTAAACATT; siRNA3, AGGTTATTG-TTCAGTTGGTTT; siRNA4, GTACAGGTCCAGGGTATA-GTT; siRNA5, AGCCCTAAAATTCACTGCGTT; siRNA6, TATTTAATCCAGAAGTATTTT.

The sequence that showed the greatest ability to reduce protein levels by western blot after *in vitro* transcription and transfection (see below) was sequence 2. Sequence 2 typically knocked down protein levels from 55 to 65% (Figure 4) while both sequence 4 and sequence 6 typically knocked down protein levels from 40 to 50% (data not shown). Sequences 1, 3 and 5 showed activity now.

Western blot analysis of cells transfected with *in vitro* transcribed siRNAs

HeLa cells were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS) in 12-well culture dishes at a density of 100 000 cells/well 24 h before transfection. siRNAs were *in vitro* transcribed using Ambion's (Austin, TX) Silencer siRNA Construction kit. siRNA was transfected into HeLa cells using siPORT Lipid (Ambion, Austin, TX) with a siRNA concentration of 40 and 50 nM. ssRNA control was simply the top strand only of the indicated sequence. After 48 h incubation in a 37°C incubator, the HeLa cells were stimulated with human TNFα at a concentration of 10 ng/ml for 4 h. The cells were extracted over ice in extraction buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.125% NP-40, 0.875% Brij 97, 1.5 mM Na-Vanadate, 1 mini-EDTA free protease inhibitor tablet Roche). Total protein concentration levels were determined using a BCA Protein Assay kit (Pierce). Total protein (2 μg) was loaded into a 15-well Invitrogen NuPAGE Novex 10% *Bis*–Tris gel (Carlsbad, CA) and run in 1× MES Buffer (20× MES: 1 M MES, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA, pH 7.3) for 35 min at a constant 200 V. Transfer of 1 h at a constant 30 V onto nitrocellulose film using 1× NuPAGE Transfer Buffer (20× Transfer Buffer: 500 mM Bicine, 500 mM *Bis*–Tris, 20.5 mM EDTA, 1 mM Chlorobutanol, pH 7.2).

Western blots used antibodies to NFκB-p50 (Upstate Cell Signaling Solutions, Charlottesville, VA) and TFIII-B (kindly provided by N. Hernandez) at a dilution of 1:5000. The TFIII-B antibody serves as an internal control to normalize for variations in protein loading. The nitrocellulose film was incubated overnight in primary antibody followed by 1 h secondary anti-rabbit antibody incubation. Imaging is done with ECL western blot kit (Amersham, Piscataway, NJ) and the fluorescence was read at on the STORM machine according to Amersham protocol.

Western blot analysis of cells transfected with siRNA-expressing constructs

HEK293 cells were plated in 12-well plates at 100 000 cells/well in 1 ml of DMEM supplemented with 10% FBS and P/S. siRNA plasmid (450 ng) and 50 ng of GFP plasmid were transfected by Fugene 6. TNFα (Calbiochem, San Diego, CA) was added at 10 ng/ml concentration 48 h later for 6 h. Cell extracts were generated by lysis in extraction buffer

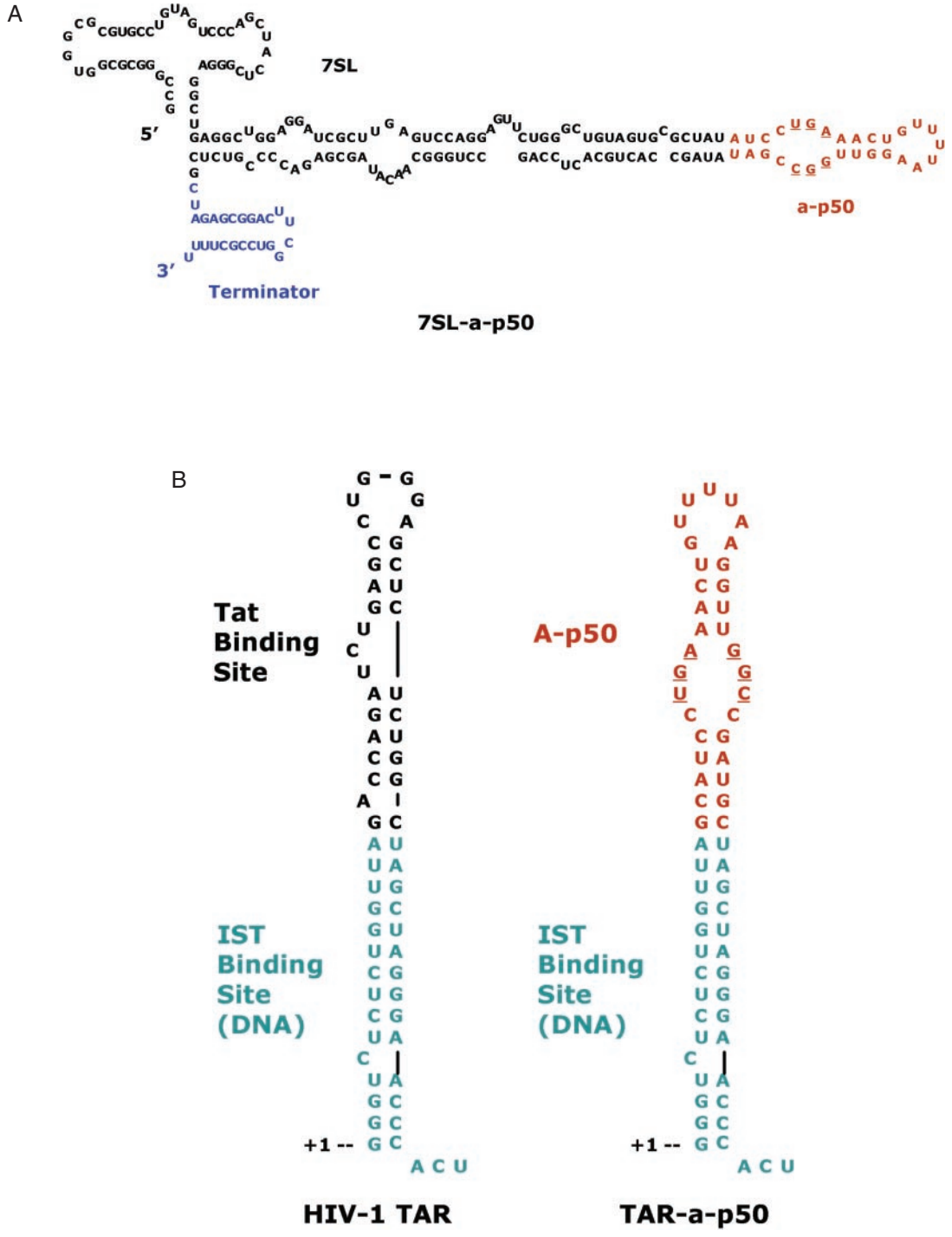

Figure 1. Diagram of theoretical transcript generated from aptamer expression constructs. (**A**) Transcript generated from 7SL-a-p50. 7SL sequences are in black, aptamer sequence in red and terminator sequence in blue. (**B**) The HIV-1 TAR transcript (left), a 59 nt stable stem and loop structure. The DNA encoding the stem also encodes the bipartite IST element [in cyan (25)]. The sequence most important for Tat binding is shown in black. To make TAR-a-p50 (right) the Tat binding site was replaced with the similarly shaped p50 aptamer (in red).

(10 mM Tris, pH 7.5, 150 mM NaCl, 0.125% NP-40, 0.875% Brij 97, 1.5 mM Na-Vanadate and 2 mM EDTA protease free inhibitor). Protein (7.5 μg) (as determined by Bradford assay) of each sample was run on a NuPAGE 10% *Bis*–Tris gel using the invitrogen gel running system. Western blot analysis was performed as above.

Electrophoretic mobility shift assay (EMSA)

A DNA probe containing the binding site for NFκB was constructed using the following primers: 5′-GCC ATG GGG GGA TCC CCG AAG TCC-3′ and the reverse primer 5′-GGA CTT CGG GGA TCC CCC CAT GGC-3′. PAGE purified primers

were obtained from Integrated DNA Technologies (Skokie, IL). Single-stranded oligonucleotides were annealed and end-labeled using Gibco (Carlsbad, CA) polynucleotide kinase and 50 μCi gamma p32 ATP. Label was ethanol precipitated and resuspended in TE buffer.

Lysates were prepared from HeLa or 293 cells grown to 60–90% confluency in 6-well dishes. Cells were grown in DMEM or RPMI plus 10% fetal calf serum. They were washed twice in phosphate-buffered saline (PBS) and then stimulated with TNFα in RPMI for 10 min at 37°C. After being washed once with PBS, lysates were made using 200 μL extraction buffer (as per western blot extraction buffer, described above). Lysates were cleared by centrifugation and stored at −80°C before use.

For the gel-shift, 1 μl of extract was incubated with 2 μl of aptamer to which 1 μl of polyDIDC, 1 μl of probe and 5 μl of binding buffer (20 mM Tris, pH 8, 200 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.1% NP40, 1 mM DTT, 0.4 mg/ml BSA) was added. Reactions occurred for 15 min at room temperature. Samples were loaded onto 6% DNA retardation gels (Invitrogen) and run in 0.5× TBE at 150 V for 1.5 h at room temperature. Dried gels were placed on phosphoimager plates and radioactive bands were visualized by analysis with the Storm 860 (Molecular Dynamics, Sunnyvale, CA).

NFκB luciferase assays

HEK293 cells were cultivated in DMEM supplemented in 10% FBS in 96-well white plates at a density of 10 000 cells/well 24 h before transfection. NFκB TA Luciferase plasmid (5 ng) (Clontech) along with 80 ng of siRNA expression plasmid and 7SL expression plasmid were introduced into HEK293 cells using fuGENE 6 (Roche). Cells were stimulated with human TNFα at a concentration of 10 ng/ml for 5 h at 37°C, 24 h later. Luminescence was measured using the Steady-Glo kit (Promega) and a TopCount Luminometer. In the combination experiments, 35 ng of the pAV7SL-derived plasmid, 55 ng of the pSilencer-derived plasmid and 5 ng of NFκB TA Luciferase plasmid were used per well.

RESULTS

Knock-down of *in vivo* NFκB activity with RNA aptamers

Using *in vitro* selection methods, Cassiday and Maher (24) identified a 31 nt RNA aptamer that binds to the p50 subunit of NFκB with high affinity. Furthermore, they showed that this aptamer can inhibit the p50/p65 heterodimer protein from binding to its cognate DNA-binding site *in vitro* and in a yeast three-hybrid assay. To knock-down intracellular NFκB activity we expressed this aptamer in mammalian cells with various expression constructs. To deliver a-p50 we used the vector pAV-7SL which places the aptamer within the natural cytoplasmic RNA 7SL and drives expression with the 7SL promoter (Figure 1A). We also developed a novel expression vector. The vector U6-TAR expresses the HIV-1 TAR construct to high levels driven by the pol III U6 promoter. This vector also contains the HIV-1 IST element which has been shown to dramatically increase production of short transcripts from variety of promoters including the U6

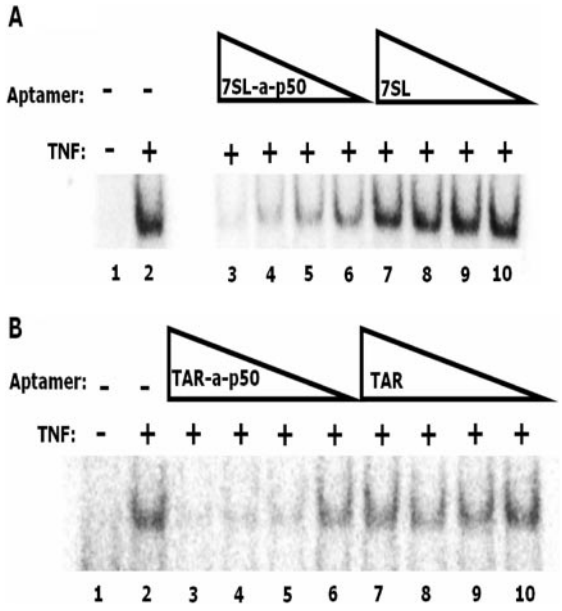

Figure 2. a-p50 retains NFκB-binding activity within the context of 7SL and TAR RNA vehicles. Lysates from TNFα stimulated 293 (**A**) or HeLa (**B**) cells were incubated with titrations of *in vitro* transcribed a-p50 or control aptamer in the presence of radiolabeled NFκB DNA probe. Samples were run on 6% DNA retardation gels and then visualized after being exposed to phosphoimager plates. Lane 1, no TNF A. Lanes 2–10, with TNFα at 10 ng/ml. Lane 2, no aptamer. Lane 3, 25 μg/ml 7SL a-p50. Lane 4, 12.5 μg/ml 7SL a-p50. Lane 5, 5 μg/ml 7SL a-p50. Lane 6, 2.5 μg/ml 7SL a-p50. Lane 7, 25 μg/ml 7SL. Lane 8, 12.5 μg/ml 7SL. Lane 9, 5 μg/ml 7SL. Lane 10, 2.5 μg/ml 7SL. Shown experiment is representative of multiple experiments. (B) Lanes 2–10, with TNFα at 30 ng/ml. Lane 2, TNFα alone. Lane 3, 90 μg/ml TAR-a-p50. Lane 4, 45 μg/ml TAR-a-p50. Lane 5, 30 μg/ml TAR-a-p50. Lane 6, 10 μg/ml TAR-a-p50. Lane 7, 90 μg/ml TAR transcript. Lane 8, 45 μg/ml TAR. Lane 9, 30 μg/ml TAR. Lane 10, 10 μg/ml TAR. Shown experiment is representative of multiple experiments.

promoter (25,26). We replaced the dispensable top of the TAR which contains a natural aptamer that binds the HIV-1 Tat protein with a-p50 (Figure 1B). EMSA studies using aptamers produced from these plasmids *in vitro* show that a-p50 is capable of inhibiting p50 binding within the context of 7SL or TAR (Figure 2A and B, respectively) and the 7SL or TAR alone cannot. RT–PCR of extracts from cells transfected with these vectors with probes specific for the predicted RNA aptamers verify that both vectors are capable of expressing the aptamers (data not shown).

To determine if expression of these aptamers can inhibit NFκB function in mammalian cells, either the parent constructs (7SL or U6-TAR) or the respective a-p50 aptamer expression vectors were co-transfected into 293 cells with a NFκB dependent luciferase reporter construct. Transfected cells were treated with TNFα to enhance NFκB activity and luciferase levels were measured 6 h later. The results in Figure 3A show that in untreated cells co-transfected with 50 ng of 7SL-a-p50 NFκB levels are reduced 39% compared with control plasmids and this reduction increases to 51% upon TNFα stimulation. Similarly, although less dramatically, in unstimulated cells co-transfected with 50 ng of TAR-a-p50 there is a small but reproducible down-regulation of NFκB activity (11%) compared with cells transfected with the same amount of control plasmid (Figure 3B). Stimulation of NFκB activity by TNF-α treatment increases

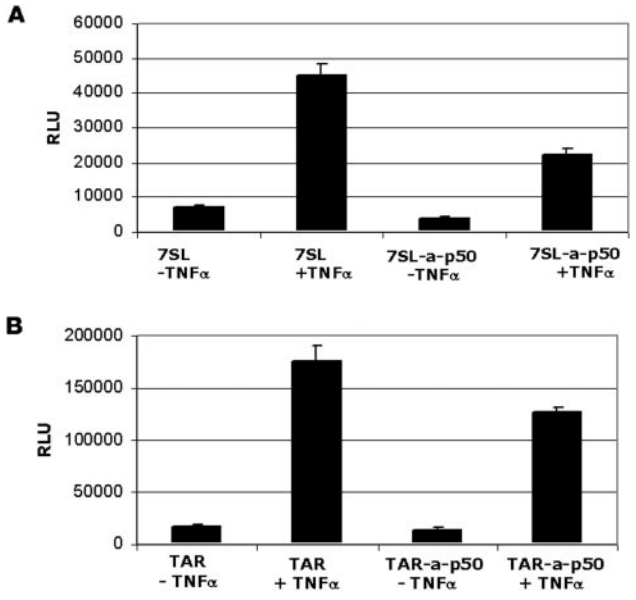

Figure 3. TNFα treated 7SL-a-p50 and TAR-a-p50 transfectants show reduced NFκB-luciferase activity compared with vehicle controls. The 293 cells were co-transfected with NFκB-luciferase reporter and 7SL or 7SL-a-p50 (**A**) or TAR or TAR-a-p50 (**B**). Transfectants were treated with TNFα at 20 ng/ml (plus) or medium alone (minus). Substrate and lysis solution was added to cells and fluorescence was measured and reported as relative luciferase units (RLU). Shown experiment is representative of multiple experiments. Error bars generated by Microsoft Excel.

down-regulation to 29%. For both plasmids, transfecting more plasmid (up to 80 ng) did not increase inhibition although transfecting smaller amounts showed dose responsive decreases in inhibition (data not shown).

To determine if siRNA could also inhibit NFκB activity, a series of siRNAs were designed by the method of Tuschl (see Materials and Methods), transcribed *in vitro*, and transfected into 293 cells. The amount of NFκB protein and a control were assayed by western blot and gel-shift analysis 48 h later. Of the sequences tested, siRNA2 yielded the best results, reducing protein levels by 61% (Figure 4).

Next we wanted to compare siRNA's and aptamer's ability to reduce NFκB activity in our NFκB dependent luciferase assay. To more easily compare the two methods we delivered siRNA2 into the cell by expression from a plasmid. Expression of siRNA2 resulted in significant reductions in protein levels by western blot (Figure 4B). Expression of siRNA2 also significantly reduced NF-κB activity (63%, Figure 4C).

Since aptamers and siRNA work at different levels of the gene expression pathway, we theorized that combining the two methods might lead to stronger inhibition. We tested this by transfecting different combinations of the siRNA-expressing plasmid and aptamer expressing plasmids. As shown in Figure 5, transfection of siRNA2 along with control 7SL plasmid results in 64% reduction in NFκB activity compared with the control. Furthermore, transfection of 7SL-a-p50 along with siRNA control results in 62% repression. Transfection of both 7SL-a-p50 and siRNA2, however, leads to 90% reduction of NFκB activity. Thus, the most effective way to knock-down NF-κB activity using the two methods is to use them in combination. Interestingly, the TAR-a-p50 also enhances siRNA2's ability to inhibit NFκB activity (Figure 5B).

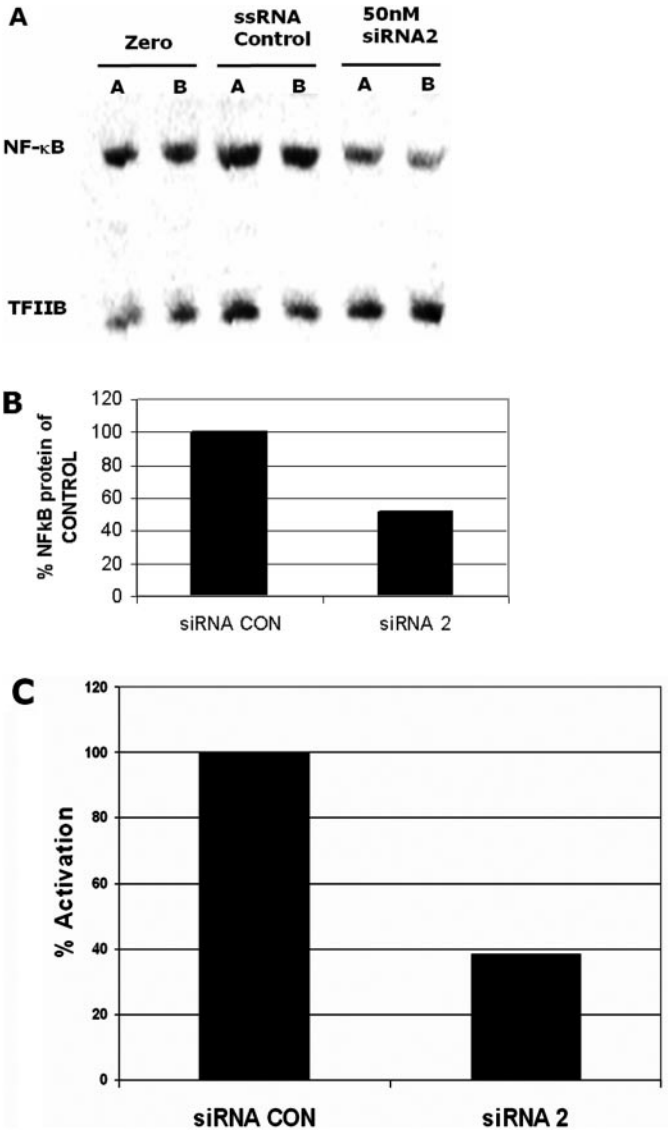

Figure 4. siRNA reduces NFκB protein levels and activity. (**A**) Western blot of extract from cells mock transfected (Zero A and B), with 50 nM *in vitro* transcribed single-strand control RNA using the top strand only (ssRNA Control A and B) or 50 nM siRNA sequence number 2 (siRNA2 A and B). A and B refer to extracts from identically treated cells. The blot was simultaneously treated with antibody to the p50 subunit of NFκB and the TFIIB transcription factor (that should not be affected by siRNA treatment). Shown experiment is representative of three experiments. (**B**) Bar graph of normalized results of western blot of cells transfected with control p-Silencer-2.0 (Ambion) plasmid (CONTROL) or pSilencer-2.0-U6-siRNA2 (SEQ 2). Shown experiment is representative of three experiments. (**C**) Bar graph of results of NFκB-dependent luciferase assay of cells transfected with NF-κB-dependent reporter plasmid and p-Silencer-2.0 (siRNA CON) or pSilencer-2.0-U6-siRNA2 (siRNA2). Shown experiment is representative of three experiments.

Predictably, the effect is smaller, demonstrating a type of dose dependence, in that the weaker construct enhances to a weaker extent.

DISCUSSION

We have significantly down-regulated activity of the intracellular protein, NFκB, in mammalian cells by expressing RNA

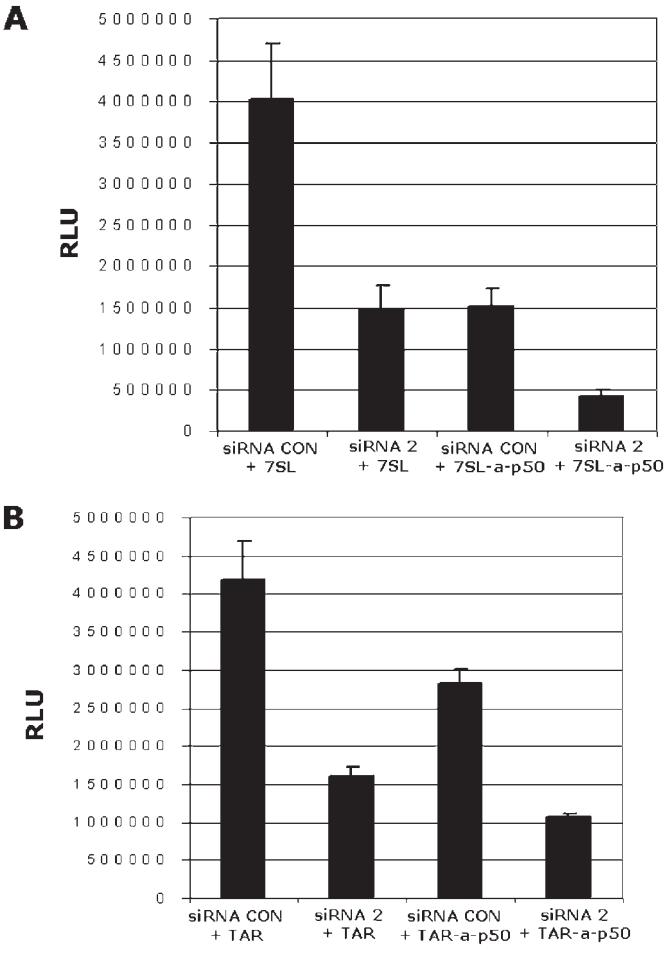

Figure 5. NFκB activity is most significantly inhibited in the presence of both p50-specific siRNA and p50-specific aptamer. (**A**) Bar graph of results of NFκB-dependent luciferase assay of cells transfected with NFκB-dependent reporter plasmid along with control plasmids for both siRNA and aptamer expressors (siRNA CON + 7SL), p-50 specific siRNA and aptamer control plasmid (siRNA2 + 7SL), p-50 specific aptamer plus siRNA control plasmid (siRNA CON + 7SL-a-p50), and plasmids expressing both p-50 specific siRNA and aptamer (siRNA 2 + 7SL-a-p50). (**B**) Bar graph of results of NFκB-dependent luciferase assay of cells transfected with NFκB-dependent reporter plasmid along with control plasmids for both siRNA and aptamer expressors (siRNA CON + TAR), p-50 specific siRNA and aptamer control plasmid (siRNA2 + TAR), p-50 specific aptamer + siRNA control plasmid (siRNA CON- + TAR-a-p50), and plasmids expressing both p-50 specific siRNA and aptamer (siRNA 2 + TAR-a-p50) Shown experiments are representative of multiple experiments. Error bars generated by Microsoft Excel.

aptamers specific for its p50 subunit. We have used two simple vectors. pAV7SL uses sequences derived from the natural 7SL siRNA to stabilize the transcript and direct it to the cytoplasm. It has been used previously to generate large numbers of small RNAs (D. Engelke, personal communication) but this is the first time it has been used to express an inhibitory aptamer. The second construct, U6/TAR, is novel and based on the construct U6-HIV/LTR (25) that expresses the natural, predominantly nuclear (27) HIV-1 TAR aptamer from the U6 promoter. This construct offers several advantages over other published nuclear-directing constructs. Firstly, the RNA vehicle generated is simple compared with tRNA-based constructs (28). Secondly, the TAR has been reported to be very stable, with a half-life of 2–3 h *in vivo* (29). Finally, the DNA encoding the bottom third of the TAR contains an IST (inducer of short transcripts) element. The IST element has been shown to dramatically increase the amount of constitutively produced short transcripts from a variety of promoters including the U6 promoter (25). Thus, this plasmid would be expected to produce significantly more aptamer than other vectors containing snRNA promoters (U6 or U1). 7SL-a-p50 is more effective than TAR-a-p50 at inhibiting NFκB activity (29% versus 51%). This could be due to differences in stability/folding of the RNA transcripts or to possible differences in the distribution of the different transcripts. It is also possible that the difference in the first 2 bp of the stem of the aptamer introduced during the making of the constructs affected binding *in vivo* although we believe this unlikely as two constructs inhibited NFkB binding similarly *in vitro*.

There are theoretical and practical reasons for combining siRNA and aptamers. They are both RNA molecules and can be introduced in similar ways (i.e. expressed from plasmids or *in vitro* transcribed and directly transfected). They work by inhibiting different levels of the protein expression pathway thus they are unlikely to interfere with each other and more likely to synergize. Here we demonstrated, for the first time, that the simultaneous expression of siRNA and RNA aptamers in cells inhibits a protein's expression better than either method alone. This technique will be useful in situations where a near complete knock-out is desired and neither technique is capable of doing the job alone.

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Conflict of interest statement. None declared.

REFERENCES

1. Lindsay,M.A. (2003) Target Discovery. *Nature Rev. Drug Discov.*, **2**, 831–838.
2. Epstein,D.M., Marsh,H.N. and Pendergrast,P.S. (2002) Target validation with nucleic acid aptamers. *Pharm. Discov. Dev.*, **3**, 24–27.
3. Hannon,G.J. (2002) RNA interference. *Nature*, **418**, 244–251.
4. Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K. and Tuschl,T. (2001) *Nature*, **411**, 494–498.
5. Caplen,N.J., Parrish,S., Imani,F., Fire,A. and Morgan,R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl Acad. Sci. USA*, **98**, 9742–9747.
6. Paul,C.P., Good,P.D., Winer,I. and Engelke,D.R. (2002) Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.*, **20**, 505–508.
7. Famulok,M. and Verma,S. (2002) *In vivo*-applied functional RNAs as tools in proteomics and genomics research. *Trends Biotechnol.*, **20**, 462–466.
8. Gupta,S., Schoer,R.A., Egan,J.E., Hannon,G.J. and Mittal,V. (2004) Inducible, reversible, and stable RNA interference in mammalian cells. *Proc. Natl Acad. Sci. USA*, **101**, 1927–1932.
9. Schwarz,D.S., Hutvagner,G., Haley,B. and Zamore,P.D. (2002) Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Mol. Cell*, **10**, 537–548.

10. Holen,T., Amarzguioui,M., Wiiger,M.T., Babaie,E. and Prydz,H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.*, **30**, 1757–1766.
11. 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. *J. Biol. Chem.*, **278**, 7108–7118.
12. Sledz,C.A., holko,M., de Veer,M.J., Silverman,R.H. and Williams,B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nature Cell Biol.*, **5**, 834–839.
13. Bridge,A.J., Pebernard,S., Ducraux,A., Nicoulaz,A.L. and Iggo,R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nature Genet.*, **34**, 263–264.
14. Persengiev,S.P., Zhu,X. and Green,M.R. (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene exptression by small interfering RNAs (siRNAs). *RNA*, **10**, 12–18.
15. Burgstaller,P., Girod,A. and Blind,M. (2002) Aptamers as tools for target prioritization and lead identification. *Drug Discov. Today*, **7**, 1221–1228.
16. Lee,S.W., Gallardo,H.F., Gaspar,O., Smith,C. and Gilboa,E. (1995) Inhibition of HIV-1 in CEM cells by a potent TAR decoy. *Gene Ther.*, **2**, 377–384.
17. Symensma,T.L., Giver,L., Zapp,M., Takle,G.B. and Ellington,A.D. (1996) RNA aptamers selected to bind human immunodeficiency virus type 1 Rev *in vitro* are Rev responsive *in vivo*. *J. Virol.*, **70**, 179–187.
18. Marshall,K.A. and Ellington,A.D. (2000) *In vitro* selection of RNA aptamers. *Methods Enzymol.*, **318**, 193–214.
19. Famulok,M., Blind,M. and Mayer,G. (2001) Intramers as promising new tools in functional proteomics. *Chem. Biol.*, **8**, 931–939.
20. Blind,M., Kolanus,W. and Famulok,M. (1999) Cytoplasmic RNA modulators of an inside-out signal-transduction cascade. *Proc. Natl Acad. Sci. USA*, **96**, 3606–3610.
21. Bleijs,D.A., Geijtenbeek,T.B., Figdor,C.G. and van Kooyk,Y. (2001) DC-SIGN and LFA-1: a battle for ligand. *Trends Immunol.*, **22**, 457–463.
22. Mayer,G., Blind,M., Nagel,W., Bohm,T., Knorr,T., Jackson,C.L., Kolanus,W. and Famulok,M. (2001) Controlling small guanine-nucleotide-exchange factor function through cytoplasmic RNA intramers. *Proc. Natl Acad. Sci. USA*, **98**, 4961–4965.
23. Lebruska,L.L. and Maher,L.J. (1999) Selection and characterization of an RNA decoy for transcription factor NF-kappa B. *Biochemistry*, **38**, 3168–3174.
24. Cassiday,L.A. and Maher,L.J. (2001) *In vivo* recognition of an RNA aptamer by its transcription factor target. *Biochemistry*, **40**, 2433–2438.
25. Ratnasbapathy,R., Sheldon,M., Johal,L. and Hernandez,N. (1990) The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAs from various mRNA and snRNA promoters. *Genes Dev.*, **4**, 2061–2074.
26. Sheldon,M., Ratnasbapathy,R. and Hernandez,N. (1993) Characterization of the inducer of short transcripts, a human immunodeficiency virus type 1 transcriptional element that activates the synthesis of short RNAs. *Mol. Cell. Biol.*, **13**, 1251–1263.
27. Good,P.D., Krikos,A.J., Li,S.X., Bertrand,E., Lee,N.S., Giver,L., Ellington,A., Zaia,J.A., Rossi,J.J. and Engelke,D.R. (1997) Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther.*, **4**, 45–54.
28. Bertrand,E., Castanotto,D., Zhou,C., Carbonnelle,C., Lee,N.S., Good,P., Chatterjee,S., Grange,T., Pictet,R., Kohn,D. *et al.* (1997) The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA*, **3**, 75–88.
29. Pfeifer,K., Bachmann,M., Schroder,H.C., Weiler,B.E., Ugarkovic,D., Okamoto,T. and Muller,W.E. (1991) Formation of a small ribonucleoprotein particle between Tat protein and trans-acting response element in human immunodeficiency virus-infected cells. *J. Biol. Chem.*, **266**, 14620–14626.