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Introduction of small interfering RNAs (siRNAs) into a cell can cause a specific interference of gene expression known as RNA interference (RNAi). However, RNAi activity in lymphocytes and in normal primary mammalian cells has not been thoroughly demonstrated. In this report, we show that siRNAs complementary to CD4 and CD8α specifically reduce surface expression of these coreceptors and their respective mRNA in a thymoma cell line model. We show that RNAi activity is only caused by a subset of siRNAs complementary to the mRNA target and that ineffective siRNAs can compete with effective siRNAs. Using primary differentiated T lymphocytes, we provide the first evidence of siRNA-mediated RNAi gene silencing in normal nontransformed somatic mammalian lymphocytes. The Journal of Immunology, 2002, 169: 5754–5760.

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2 Abbreviations used in this paper: RNAi, RNA interference; siRNA, small interfering RNA; PI, propidium iodide; GFP, green fluorescent protein; ORF, open reading frame; UTR, untranslated region; siRNA, small temporal RNA.

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of RPMI supplemented with 1 ng/ml IL-2 (BioSource International, Camarillo, CA). siRNA oligos (Dharmacon, Lafayette, CO) used were as follows (sense strand is given): effective CD4 siRNA, CD4 no. 4, (sense) gcagaccaucucucaucuagadtdt; effective CD8 siRNA, CD8 no. 4, (sense) cguacacauacucuacuagadtdt; ineffective siRNAs, CD8 no. 1, (sense) gcacuuggacgccuaucugadtdt; CD8 no. 3 (sense) aauuuguggacuaccaucucudtdt, (antisense) ggcccggucauuuacacudtdt; CD8 no. 5 (sense) cccacugccguccucucudtdt, (antisense) gccgagacacacagcagacadtdt. The use of siRNAs indicated that all of the cells expressing GFP also received an adenovirus containing a MIGW green fluorescent protein (GFP) retrovirus that was included when GFP expression was analyzed. Cell data were collected on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and four-color analyses (GFP, PE, PI, and allophycocyanin) were done with CellQuest software (BD Biosciences). All data were collected by analyses performed on 1 x 10^4 PI-negative events (viable cells). For the primary T cell studies, activated cells were analyzed as above, except that allophycocyanin-conjugated anti-CD4 and PE-conjugated anti-CD8 were used, and 5 x 10^4 PI-negative events were analyzed.

Northern blot analysis of mRNA

Cells were lysed in TRIzol reagent (Life Technologies, Grand Island, NY) and total cellular RNA was purified according to manufacturer's instructions. RNA (10 μg) was fractionated on a denaturing 1% formaldehyde/agarose gel and transferred to a nitrocellulose membrane. Blots were hybridized overnight with 32P-labeled CD4 (818 bp) or CD8 (1075 bp) cDNA fragments. After washes, blots were analyzed by a PhosphorImager (Molecular Dynamics).

Results

siRNAs transiently induce silencing in murine thymocyte cell lines

To study RNAi, siRNAs are typically delivered into cells by carrier-mediated transfection reagents. We developed an experimental system using a thymocyte-derived cell line, E10 (23), wherein we studied the use of siRNAs to silence either CD4 or CD8, using the other marker as an internal specificity control. However, typical of lymphocytes, E10 is insensitive to several different cationic and noncationic transfection reagents and thus electroporation was used to introduce siRNAs. Using this method, ~20% of the cell population expressed GFP from a transfected reporter vector. When CD4 or CD8α siRNAs were electroporated into E10, a marked reduction in surface CD4 or CD8α expression, respectively, occurred 36 h later. Flow cytometry analysis showed that most of the cells were transfected and expression levels were reduced >5-fold below wild-type expression levels (Fig. 1A). The degree of reduction of CD8α was frequently more pronounced than that of CD4 and, in both cases, a small population of cells appeared to be either untransfected or not responsive to the siRNA treatment. In repeated experiments, typically 70–95% of the cells exhibited a >5-fold reduction in CD8α expression, although sometimes a smaller fraction of cells down-regulated CD8α to a greater degree (Fig. 1A).

Elbashir et al. (13) reported that RNAi-induced silencing could be maintained for ~2 wk in HeLa cells, although neither the extent of silencing nor the number of cell divisions was reported. A time-course assay was performed in CD8α siRNA-transfected E10 cells. GFP was included in these transfections to investigate the relationship between the uptake and expression of plasmid DNA and siRNAs. Because these experiments were transient transfections, cell doubling results in a decrease in GFP fluorescence intensity and number of GFP-positive cells (Fig. 1B, NS RNA). When CD8α siRNAs were cotransfected with the GFP reporter vector, CD8α expression, but not GFP expression, was markedly reduced (see Fig. 1B, 24 h). Several cell populations were evident, with the major CD8α silenced population displaying >5-fold reduced CD8α expression. The majority of cells within this population did not express GFP. However, cells that did express GFP also silenced CD8α. This corresponded to ~20% of the total cells, similar to the control GFP alone (Fig. 1B, NS siRNA, 24 h). This indicates that all of the cells expressing GFP also received an adequate level of siRNAs to silence CD8α. In addition, a large fraction of cells incorporated biologically active levels of siRNAs and

FIGURE 1. CD4 and CD8α siRNAs transiently silence gene expression. A, Histogram showing the typical reduction of CD4 and CD8α gene expression seen 36 h post siRNA transfection in E10 T cells. Solid and dashed lines indicate untransfected and siRNA-transfected E10 cells. Approximately 85% of CD4+ cells and 94% of CD8+ cells down-regulated surface expression. B, Flow cytometry analysis of transiently transfected GFP and either CD8α or luciferase siRNAs (NS siRNA) in the E10 T cell line. At 24 h, ~60% of the CD8 siRNA-transfected cells down-regulated surface CD8 expression. Gated live cells are shown, and the numbers at the left correspond to the times in hours after electroporation transfection.
yet did not express plasmid DNA. In this experiment, time points were taken over a period of 6 days. At each time point, one-half of the cells were removed from the dish and replaced with fresh medium. The collected cells were stained for CD8α and analyzed by flow cytometry (Fig. 1). A decrease in CD8α surface expression was detectable at 12 h posttransfection, with maximal silencing at 36 h. By 96 h, nearly all of the cells expressed wild-type levels of CD8α. Thus, the RNAi effect in these T cells is a transient phenomena.

In these experiments, there was a dramatic decrease in GFP expression over time, which was likely a result of dilution of the plasmid or potentially due to toxicity of high GFP expression. Because 100% of the GFP-expressing cells exhibited CD8α silencing, it was possible to monitor the “fate” of this subset of silenced cells. The T cells that actively underwent CD8α silencing continued to express GFP over the time course, to the same level as the control population of cells that were not transfected with siRNAs (compare nonspecific RNA to CD8 siRNA). At 96 h, <5% of the total cells were GFP-positive in cells treated with nonspecific siRNAs and in CD8α siRNA-treated samples. These few remaining GFP-positive cells exhibited normal levels of CD8α expression. This suggests that the cells did not specifically undergo apoptosis as a result of siRNA transfection and subsequent CD8α silencing.

Specificity of siRNA-mediated silencing

Although the GFP transgene expression was not affected during CD8α silencing, the expression of endogenous genes might have been nonspecifically affected. To address this question, the expression levels of CD4 and Thy1.2 T cell markers were examined in cells actively undergoing CD8α silencing. Examination of these markers revealed that there was no reduction of nontargeted gene expression when compared with the control nontransfected cells (Fig. 2A), even over extended times (not shown). Although unlikely for this cell line, an additional analysis confirmed that the T cells did not become activated, as they do not up-regulate CD69 (Fig. 2A). Together, these experiments confirm the specificity of siRNA-mediated CD8α silencing.

Stability of targeted CD8α mRNA

Short temporal RNAs such as lin-4 and let-7 mediate silencing by binding to the 3′-untranslated region (UTR), thus suppressing translation (24–26). This is in marked contrast to the posttranscriptional mRNA degradation effected by siRNAs. To distinguish between these two potential mechanisms for CD8α silencing, a time course Northern blot analysis of CD8α mRNA was performed. The process of silencing did not appreciably affect the growth rate, as compared with control nonspecific siRNA transfections performed in parallel (not shown). Flow cytometry analysis indicated that the RNAi response in these cells lasted 3–4 days (8–10 cell doublings), which corresponds to an ~100-fold increase in cell mass (Fig. 2B). Time course analysis was performed in four independent experiments and expression of CD8α was typically suppressed ~5-fold or greater.

At various time points, a fraction of the cells was used to isolate total RNA for Northern blot analysis (Fig. 2C). The CD8α mRNA was resolved into two bands, due to alternative splicing (27, 28). Levels of CD8α mRNA decreased during the course of CD8α silencing. Densitometric analysis of the CD8α mRNA bands was performed and normalized to the internal control CD4 band. At the point of maximal silencing, mRNA levels decrease only 2.5-fold. This value is not commensurate with the ~5-fold decrease in protein expression determined by the flow cytometric analysis. However, this RNA was prepared from total cells in which 30% of the cells did not exhibit any silencing. When corrected for this reduction, CD8α mRNA was nearly proportionate to levels in reduction of CD8α protein. These Northern blots were performed multiple times with similar results. Thus, although it is clear that CD8α mRNA decreases, we cannot rule out additional silencing phenomena such as cotranslational repression.

Regional sensitivity of an mRNA to silencing by a siRNA

A major outstanding question is whether any region of a mRNA can serve as an effective target for siRNA-directed silencing. Several different siRNAs that targeted different regions of the CD8α
mRNA were tested. Of the first two CD8α siRNAs that were transfected, only one was active. To more quantitatively examine this difference, cells were transfected with varying amounts of siRNAs and CD8α expression was measured by flow cytometry. Cells undergoing silencing were quantified and compared with control nonspecific siRNA treatment (Fig. 3A). For the effective CD8α siRNA, picomolar amounts were sufficient to induce some silencing, and higher amounts produced a graded response. For the non-effective CD8α siRNA, even at the highest concentration tested, there was no activity.

As these studies progressed, we observed that the majority of the synthetic CD4 and CD8α siRNAs were noneffective at silencing. For CD8α, four different siRNAs were synthesized and tested in the flow cytometry assay; one overlapped the start codon, one which targeted the open reading frame (ORF), one which overlapped the stop codon, and one which targeted the 3′-UTR 15 nt after the stop codon. Only the siRNA which targeted the 3′-UTR ~15 nt after the stop codon effectively silenced CD8α expression. For CD4, five siRNAs were synthesized which targeted corresponding regions to those for the CD8α mRNA (Fig. 3B). In this case, only the siRNA that targeted the stop codon was effective at reducing CD4 expression levels. An examination of the nucleotide sequences did not reveal any obvious differences between the effective and ineffective siRNAs.

For each of the above siRNAs, the silencing assay was performed at different siRNA concentrations. None of the inactive siRNAs generated detectable silencing at five times the highest concentration of the active siRNAs (Fig. 3A and data not shown). However, these inactive siRNAs were able to compete with the silencing of the active siRNAs. In these competition experiments, inactive CD8α siRNAs were added into the cuvettes containing the active CD8α siRNA, so that both could be electroporated into the cells simultaneously. Varying concentrations were tested, and cells were monitored for CD8α silencing at 36 h (Fig. 4). It was found that when the total siRNA pool contained an inactive CD4 or CD8α siRNA, then silencing mediated by an active siRNA was markedly reduced (Fig. 4, A and B). These results mirror the ability for active siRNAs to compete for other active siRNAs, a response that we observed for attempting silencing of both CD4 and CD8α simultaneously (Fig. 4, C and D). The inability to silence both CD4 and CD8α simultaneously in the same cell might suggest that siRNA-mediated RNAi is titratable, as has been described for silencing using long dsRNAs in C. elegans (29).

To test whether the above siRNAs were also inactive in other cell types, the CD4 and CD8α genes were expressed from CMV-driven promoters in HeLa cells. The CD8α expression construct contained two regions that corresponded to target sites for effective and ineffective siRNAs in E10. In this assay, cationic lipid co-transfection of the mouse CD4 and CD8α plasmid vectors was performed with either the effective or noneffective CD8α siRNA. When compared with the nonspecific siRNA control, CD8α-specific RNAi silencing was recapitulated in HeLa cells, and the ORF-targeted siRNA was still ineffective at silencing (Fig. 5A). These results suggested that the noneffective siRNA phenomenon is not unique to the T cell line, but is likely a feature of either the siRNA sequence, or more likely the mRNA. The concentration dependence of the effective and ineffective siRNA was evaluated in the HeLa cell assay. In this experiment, cationic lipid:sRNA complexes were preformed and added to the cells as previously described (13). The effective siRNA exhibited a concentration dependence; however, the ineffective siRNAs remained inactive even at the highest concentrations (Fig. 5B).

**siRNA-mediated silencing in primary mouse T cells**

To test whether primary cells are sensitive to siRNA-mediated silencing, the CD4/CD8α siRNAs characterized above were used to silence in primary mouse T cells taken from spleen. In these

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3.** A restrictive number of designed siRNA sequences are effective at silencing. A. Effect of three different siRNAs targeting CD8α. Effective concentrations of three different CD8α siRNAs were evaluated by titrating increasing amounts of the siRNAs into the cuvettes before electroporation. CD8α expression was evaluated 36 h posttransfection, and is plotted as a percentage of wild-type levels. Effective siRNA (●); two ineffective siRNAs (▲, □). B. Relative target locations of the siRNAs used in these studies. Schematic of CD4 and CD8α mRNAs (not to scale), showing ORF and 5′- and 3′-UTR. +, Indicates siRNAs that are effective.

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** Effective and ineffective siRNAs can compete for silencing. Effective CD8α (A) and CD4 (B) siRNA-mediated silencing is competed by increasing concentration of cotransfected ineffective CD4 and CD8α siRNAs. Effective CD8α (C) or CD4 (D) siRNA-mediated silencing is competed by increasing concentrations of cotransfected ineffective CD8α or CD4 siRNA. Red indicates E10 cells transfected with 2.5 μmol effective siRNA and black is the nontransfected control. Green and blue indicate the addition of half the amount of an ineffective siRNA.
studies DO11.10 mice, which express a transgenic TCR that recognizes OVA peptide in the context of MHC class II were isolated from these mice are predominantly CD4⁺/H11001; however, a small number (~15%) of CD8⁺ cells exist in these mice. Efforts to transfect and silence naive T cells were unsuccessful, but if the cells were stimulated to divide by the cognate OVA peptide, CD4 and CD8⁺ silencing could be accomplished similar to the E10 thymoma cell line. Electroporation of CD4 siRNAs into activated primary T cells resulted in an approximate 5-fold decrease in CD4 surface expression compared with an unrelated siRNA control (Fig. 6). Costaining for CD8α on the same cells demonstrated that the down-regulation of CD4 was specific. The maximal degree of silencing was reached at 48 h posttransfection. Later time points could not be collected because of reduced cell viability after 72 h in culture. Similarly, the subset of CD8⁺-positive T cells electroporated with CD8 siRNA exhibited a maximal 3.3-fold decrease in CD8α levels. Furthermore, the degree of silencing in the sample population with the alternate coreceptor (i.e., CD4 in a CD8α siRNA-treated sample) verified that the RNAi response was specific (data not shown). These results demonstrate that primary, mature T cells are able to perform RNAi. The overall degree, kinetics, and specificity of silencing of CD4 or CD8α in primary T cells was comparable to that of the E10 cell line, further supporting the validity of using this line to characterize T cell RNAi.

FIGURE 5. Restrictive siRNA usage is not a T cell specific phenomenon. A, CD4 silencing in HeLa cells. CD4 and CD8α expression vectors (with or without CD4 siRNAs) were transiently transfected into HeLa cells. The histogram at the top shows the distribution of transfected cells (counts) expressing CD4 (green), and cells that were cotransfected with effective (dotted pink) or ineffective CD4 siRNAs (black). The dotted blue line indicates nontransfected HeLa cells. The bottom two density plots show the specificity of CD4 silencing. CD4-silenced cells were stained for CD4 and CD8α markers. The lower left density plot depicts a typical expression profile of an ineffective siRNA, which is identical to nonspecific siRNA control (not shown). The lower right density plot shows typical results of CD4 silencing using the effective siRNA, which does not affect CD8α expression. B, Titration of CD4 or CD8α siRNAs into the HeLa cell system. Effective concentrations of two different CD4 and two different CD8α siRNAs were evaluated by titrating increasing amounts of the siRNAs during cationic lipid cotransfection. CD4 and CD8α expression was evaluated 36 h posttransfection. Effective CD8α siRNAs (green) and CD4 siRNAs (black) reduce CD4 and CD8α expression, while ineffective CD4 (blue) and CD8α (red) maintain high expression levels. Ordinate shows the expression of either CD4 or CD8, which is normalized to 100%. Abscissa depicts the amount of the siRNAs added during transfection.

FIGURE 6. Time course of CD4 and CD8α suppression by siRNAs in primary T cells. A, Activated DO11 T cells were transfected with siRNAs and cultured for 3 days. Cells at each time point were analyzed by flow cytometry. The histograms are gated on viable cells that express either CD4 or CD8 respectively. The overlays (gray lines) in the histograms represent cells transfected with siRNAs specific for either CD4 or CD8; whereas the underlying histograms (filled) represent controls transfected with a nonspecific siRNA control. B, Time course of CD4 and CD8α silencing. The maximal level of suppression was determined by finding the peak fluorescence level of the suppressed curve and expressing it as a percent of the peak fluorescence level of nonspecific siRNA transfection control. Values are expressed as percent silencing and are plotted against time (hours).
Discussion

The CD4 and CD8α T cell surface glycoproteins are of central importance to immune function and disease. We have quantitatively tested the efficacy of a variety of siRNAs to suppress the expression of these glycoproteins. Targeting the CD4 and CD8α markers was attractive since turnover of coreceptor message is fairly rapid (~12 h for CD8α), and changes in surface expression can be rapidly and easily assayed by flow cytometry. In this analysis of two different genes, we observed that T cells and thymic cell lines are amenable to siRNA-mediated silencing. These studies revealed that siRNA-mediated RNAi is transient, lasting approximately eight cell doublings. Not every siRNA was able to induce silencing, and the RNAs which targeted the 3′-UTR were effective for both genes. Although small temporal RNAs (sRNs) mediated translational repression at the mRNA 3′-UTR (for recent reviews, see Refs. 30–34), Northern blot analysis of CD4 and CD8α mRNA indicated posttranscriptional degradation of the mRNA, consistent with a RNAi-type mechanism of silencing. Finally, in primary T cells, the overall penetrance and kinetics of CD4 and CD8α siRNA-mediated RNAi was found to be similar to that observed in the E10 thymoma cell line.

In several experiments, and using electroporation, we found efficient uptake and silencing of >90% of the cells. However, this required the addition of a relatively high amount of siRNA (2.5 μM/1.5 × 10⁶ cells); Northern blot analysis indicates that only a fraction of the siRNAs (~3 × 10⁴ siRNAs/cell) become associated with the cells (data not shown). Only a fraction of the siRNAs that become associated with cells probably are functional in silencing gene expression. At lower concentrations of siRNAs, a similar fraction (70–95%) of cells exhibit a reduction in CD8 expression, albeit at reduced efficiency. Using either electroporation for T cells or Lipofectamine 2000 for HeLa cells, we found that 100% of the cells that take up and express a cotransfected GFP marker also perform RNAi. Based on this fact, it should be possible to design gene function experiments which enrich the pool of silenced cells by selecting for the activity of a transfected plasmid reporter.

Time course analysis of CD8α silencing in the E10 cell line indicated that the silencing was transient in nature, lasting ~3–4 days. As this cell line doubles rapidly, this value corresponds to approximately eight cell doublings. Northern blots indicated that silencing corresponded to a reduction in mRNA levels, commensurate with the predicted model for RNAi. A translational repression mechanism has been suggested for silencing mediated by siRNAs via the 3′ untranslated region of developmentally important genes. Although the reduction in mRNA level approximated that of CD8α expression, we cannot rule out the possibility of additional translational repression mechanisms.

Only a limited number of the siRNA sequences tested could induce RNAi. For the silencing of most genes, on average one of two candidate siRNAs designed is active in contrast to the one of four and one in five siRNAs tested in targeting CD4 and CD8α (6). It is interesting to note that the siRNAs that were active in silencing targeted the 3′-UTR and stop codon. The restrictive utilization of the 3′-UTR siRNAs did not appear to be cell-type specific, as active and inactive siRNAs gave similar results in HeLa cells. It is unclear why targeting the mouse CD4 and CD8 mRNA 3′-UTRs were effective for performing siRNA-mediated RNAi, while other sites were not. One possibility is that further testing of other mRNA regions would result in productive silencing (35). Alternatively, perhaps the 3′-UTR of these genes is particularly accessible for targeting. Silencing of developmentally timed genes in the endogenous stRNA pathway is specific for the 3′-UTR (25, 36). This could be a common feature of developmentally timed genes, because both CD4 and CD8 are also expressed in a developmentally timed manner.

Attempting to silence both CD4 and CD8α simultaneously resulted in lower levels of silencing of each gene. These results supports a previously recognized observation that the RNAi response is titratable (29). Surprisingly, several of the siRNAs that were inactive competed for silencing when coelectroporated with active siRNAs. While this manuscript was in preparation, another group reported similar findings for the silencing of human coagulation factor X (37). However, another group has reported success in dual gene targeting of Lamin A/C and NuMA proteins in HeLa cells (38). The data presented in this study indicate that the inactive siRNAs are recognized by cellular processes but either cannot be converted to an active structure for gene silencing or cannot gain access to their complementary sequences on the target mRNA.

This work presents the first evidence for silencing by siRNA in primary somatic mammalian lymphocytes. In these studies, the degree and kinetics of CD4 and CD8α silencing in the activated primary cells was similar to that of the E10 cell line. In both the primary cells and E10 cells the onset of maximal silencing appeared around three to four cell doublings, which corresponded to 36–48 h posttransfection. In the E10 cells, 100% of the cells had reduced normal CD8α expression by 96 h. Because the viability of the primary cells began to diminish at around 60 h, it was difficult to determine how long the RNAi response would last past 72 h. It is interesting to note that the cells needed to be activated in order for silencing to be accomplished. This could be due to the inability to take up the siRNAs after electroporation, as primary T cells are known to be difficult to transfect with nucleic acids. It is unknown whether mammalian cells must be in a dividing, or “competent”, state to perform RNAi. Future studies of siRNA-mediated RNAi in primary cells are required to distinguish between these two possibilities. Nevertheless, these findings provide a precedent upon which future studies of T lymphocyte biology can be designed to validate function by siRNA-mediated silencing.

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