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## In vitro selection of specific RNA inhibitors of NFATc

Soo-Jin Bae,<sup>a</sup> Ji-Hyun Oum,<sup>b</sup> Surendra Sharma,<sup>c</sup> Jungchan Park,<sup>b</sup> and Seong-Wook Lee<sup>a,\*</sup>

<sup>a</sup> Department of Molecular Biology, Dankook University, San 8, Hannam-Dong, Yongsan-Gu, Seoul 140-714, Republic of Korea

<sup>b</sup> Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yong-in 449-791, Republic of Korea

<sup>c</sup> Department of Pediatrics and Pathology, Brown University, Women and Infants' Hospital of Rhode Island, Providence, RI 02905, USA

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### Abstract

NFAT (nuclear factor of activated T cells) plays a pivotal role in inducible gene transcription during the immune response and functions as a major target for immunosuppressive drugs such as cyclosporin A and FK-506. However, due to toxic effects of these drugs, which arise from their ability to inhibit calcineurin in non-immune cells, development of agents that directly target NFAT without toxic effects is warranted. Here, we present an in vitro selection of RNA aptamer to NFATc DNA binding domain (DBD) from a combinatorial RNA library with 41 nucleotide-long random sequences using the SELEX technique. The selected (SE) RNA was found to specifically and avidly bind NFATc DBD based on immunoprecipitation and competitive gel retardation assay. SE RNA also efficiently and specifically inhibited DNA binding capacity of NFATc, but not NFATp. Furthermore, transient RNA transfection studies show that only SE RNA can selectively and efficiently inhibit the NFATc- but neither the NFκB- nor NFATp-driven promoter activity in cells. These results suggest that SE RNA identified in this study is a specific inhibitor of NFATc activation, and hence, can be used not only for the study of NFAT functions but for the development of potent immune modulating agents.

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Nuclear factor of activated T cells (NFAT) is a multicomponent transcription factor which is expressed in most immune cells and plays a central role mainly in the expression of many cytokines and cell surface ligands critical for the regulation of the immune response [1,2]. The NFAT proteins are activated by stimulation of receptors coupled to calcium mobilization, such as antigen receptors on immune cells [1]. The receptor stimulation and calcium mobilization generate the activated form of phosphatase calcineurin, which dephosphorylates the NFAT proteins and activates them by promoting their translocation into the nucleus [3–5]. Four of five NFAT members (NFAT1/p, NFAT2/c, NFAT3, and NFAT4/x) are regulated by calcineurin and their functions have been characterized in respective knockout mice models [6].

The immunosuppressive drugs cyclosporin A (CsA) and FK-506, currently used for impeding the organ rejection during transplantation surgery, inhibit the phosphatase activity of calcineurin, thus preventing the subsequent dephosphorylation and translocation of NFAT proteins to the nucleus [7]. However, the side effects of these compounds, notably nephrotoxicity, neurotoxicity, diabetogenicity, gastrointestinal toxicity, and high incidence of malignancy, have markedly limited their utility in other clinical circumstances [8–10]. These toxic effects are probably due to the pleiotropic metabolic effects of these agents in other organs and cell types through inactivation of calcineurin [1,6].

Identification of small compounds that target NFAT directly, without affecting the phosphatase activity of calcineurin, could be an approach to the development of less toxic immunosuppressive therapy. Recently, a high-affinity calcineurin-binding peptide was isolated to selectively inhibit NFAT activation by interfering with the calcineurin-NFAT interaction without reducing calci-

\* Corresponding author. Fax: +82-2-798-4733.

E-mail address: [SWL0208@unitel.co.kr](mailto:SWL0208@unitel.co.kr) (S.-W. Lee).

neurin phosphatase activity [11,12]. Small molecules have also been identified to inhibit NFAT activation by mechanisms other than those described for CsA or FK506 [13,14]. However, targeting individual NFAT proteins could be a more selective immunomodulatory approach, although with the potential problem of redirecting immune response [6].

Isolation of small agents such as short peptides that bind with affinity to target proteins has been difficult mainly due to their broad specificity and unstable folded structure [15]. By contrast, short RNA molecules can form a fairly stable and unique tertiary structure via intramolecular base-pairing [15,16]. Moreover, by using in vitro selection techniques, called systematic evolution of ligands by exponential enrichment (SELEX), short RNA aptamer molecules have been isolated from random RNA libraries to bind several proteins including non-RNA binding proteins with high affinity and specificity [17,18]. We have recently shown that in vitro selected RNA aptamers to autoantibodies could be utilized as potent agents against autoimmune diseases by blocking the binding of antibodies to their self-antigens [19–21]. Other groups have also reported that certain RNA molecules selected in vitro can efficiently inhibit the physiological functions known to be associated with the target proteins in animal models [22]. Therefore, RNA molecules directed against NFAT could be potential candidates to act as specific and effective immunosuppressors.

In this study, in order to identify selective immunosuppressors, we employed an RNA combinatorial library and isolated an RNA aptamer against DNA binding domain (DBD) of NFATc which appears to be involved in Th2 type immune reactions [23]. This RNA was shown to bind NFATc with high specificity and affinity. In addition, the RNA aptamer specifically inhibited DNA binding activity of NFATc, but not that of NFATp. Furthermore, selected RNA (SE RNA) could act as a decoy and efficiently and selectively inhibit the reporter gene expression induced by NFATc, but not by either NF $\kappa$ B or NFATp, in human cells.

## Materials and methods

**Recombinant proteins.** A recombinant fragment of human NFATc DBD (encompassing amino acids 392–699), human NFATp DBD (amino acids 390–694), or a chimeric NFAT protein (Nc–Np) comprising N-terminal two-thirds of NFATc DBD (amino acids 392–583) plus C-terminal one-third of NFATp DBD (amino acids 581–694) was cloned into pET21 expression vector (Novagen), which expresses recombinant proteins tagged with a hexahistidine at C-terminus. Proteins were overexpressed in *E. coli* BL21DE3 strain and purified with nickel-chelate resin (Ni-NTA-agarose, Qiagen) as described [24].

**Selection procedure.** In vitro selection was carried out essentially as described [19–21], with a few modifications. A random pool of RNA oligonucleotides of sequence 5'-GGGAGAGCGGAAGCGUG CUGGGCCN<sub>41</sub>CAUAACCCAGAGGUCGAUGGAUCCCCC-3'

(where N<sub>41</sub> represents 41 nucleotide (nt) with equimolar incorporation of A, G, C, and U at each position) was generated by in vitro transcription of synthetic DNA templates with NTPs and T7 RNA polymerase. To remove nonspecific RNAs with binding activity to agarose beads, 10  $\mu$ g RNA library was preincubated with 20  $\mu$ l Ni-NTA-agarose beads in 100  $\mu$ l binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking in each cycle, precipitated, and discarded. The precleared supernatant was then transferred to a new tube and further incubated with 2  $\mu$ g his-tagged NFATc DBD for 30 min at room temperature. RNA–NFATc complexes were precipitated with beads and pellets were washed three times with 0.5 ml binding buffer. RNAs were recovered, amplified with RT-PCR and in vitro transcription, and used for next rounds of selection. From round 8, NFATc DBD concentration was reduced fivefold and RNA–NFATc pellets were washed five or more times. After 10 rounds of selection, amplified DNA was cloned and several clones were sequenced.

**Analysis of selected (SE) RNAs.** SE RNAs were internally radiolabeled and isolated as described [19–21]. Purified RNAs were incubated with proteins as described above. RNA–NFAT complexes were precipitated with Ni-NTA-agarose beads and bound RNAs were eluted from the pellets. RNAs were then analyzed on a 6% polyacrylamide gel with urea. Alternatively, purified RNAs were incubated with NFATc DBD in 40  $\mu$ l binding buffer with 1  $\mu$ g tRNA. The RNA–NFAT complexes were then analyzed on a 6% nondenaturing polyacrylamide gel containing 2% glycerol for gel shift analysis.

**Electrophoretic mobility shift assay (EMSA) of NFAT.** An oligonucleotide probe corresponding to the distal NFAT binding site derived from the murine IL-2 promoter is a 33-mer and its sequence is 5'-GATGCCCAAAGAGGAAAATTTGTTTCATACAG-3' [25]. Double-stranded oligonucleotides were prepared by annealing of synthetic single-stranded oligonucleotide and its complement and labeled with [ $\gamma$ -<sup>32</sup>P]dATP (3000 Ci/mmol, Amersham) using T4 DNA kinase. Binding reactions for EMSA assay were performed as previously described [26] with modifications. Briefly, NFAT was incubated at room temperature in a binding buffer containing 30 mM Tris, pH 7.5, 50 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1% BSA, and 1  $\mu$ g tRNA along with cold RNA competitors, if any. After a 10-min incubation, radioactive NFAT oligonucleotide probe was added and the incubation was continued for additional 20 min. The DNA–NFAT complexes were then analyzed on a 6% nondenaturing polyacrylamide gel.

**Cells and RNA transfection.** HeLa cells were maintained at 37 °C in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The NFATc expression vector, in which the full-length coding region of NFATc.  $\beta$  is expressed under the control of the SR $\alpha$  promoter, was previously described [24]. The NFATp expression vector constructed in pLGP3 basic plasmid was provided by Dr. A. Rao, Harvard Medical School [27]. The reporter plasmid, pXIL2-Luc, a gift from Dr. T. Hoey (Tularik), expresses firefly luciferase driven by three NFAT sites located at –45 of the murine IL-2 promoter [28]. The NF $\kappa$ B promoter-luciferase reporter plasmid, p3X $\kappa$ B-Luc, which expresses firefly luciferase gene under control of a minimal fos promoter with three copies of major histocompatibility complex (MHC) class I  $\kappa$ B element, was provided by Dr. B. Sugden, University of Wisconsin [29]. The pRLTK plasmid, which expresses renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter, served as an internal control for the efficiency of transfection. Exponentially growing HeLa cells (1  $\times$  10<sup>6</sup> cells) were transfected with 1  $\mu$ g NFAT expression vector, 2  $\mu$ g pXIL2-Luc, and 0.2  $\mu$ g pRLTK with or without RNA by lipotransfection with 6  $\mu$ l DMRIE-C (Gibco-BRL). In the NF $\kappa$ B experiment, 2  $\mu$ g p3X $\kappa$ B-Luc and 0.2  $\mu$ g pRLTK were lipo-transfected along with or without RNA. At 24 h after transfection, the cells were stimulated with 25 ng/ml PMA and 2  $\mu$ M ionomycin. After 8 h of treatment, the cells were harvested and reporter gene expression activities were

determined by measuring relative light units using a luminometer TD-20/20 (Turner Designs Instrument) and dual-luciferase reporter assay system (Promega).

## Results

### *In vitro* selection of NFATc-specific RNAs

Because NFATc transcription factor is thought to be essential for the inducible expression of Th2-type cytokines [23], we decided to isolate RNA aptamers against NFATc DBD for the development of type-specific immunomodulating agents. NFATc DBD, which lies between amino acids 392 and 699, was reported to be highly conserved within the NFAT family [1]. An RNA library of approximately  $10^{14}$  different molecules was generated with each molecule including 41-nucleotide (nt) long random sequences flanked by defined sequences as described in Materials and methods. After 10 cycles of selection, the amplified cDNAs were cloned and 20 different clones were sequenced.

RNAs from seven clones were found to nonspecifically bind beads used for precipitation (data not shown). The other 13 clones were found to contain six different but very similar RNA sequences (Fig. 1A). As a working model, the most stable secondary structure of selected (SE) RNA #1, as shown in Fig. 1B, was predicted using MULFOLD program [30]. In this folded structure, the 41-nt-long sequence, selected from the RNA library, is predicted to form two long and internal loop-embedded stem loop structures by itself or by base-pairing with flanked sequences. Other selected RNA clones showed similar pattern of predicted secondary structure to clone #1 (data not shown). To evaluate binding specificity of this SE RNA, a precipitation experiment was performed with internally radiolabeled RNAs (Fig. 1C). Both RNA pools from 10th selection cycle and SE RNA #1 were shown to bind strongly and specifically to NFATc DBD, but not to the agarose beads. In contrast, original library RNA hardly exhibited any binding to the protein. Other selected RNA clones also showed strong and specific binding to the NFATc DBD protein, but not to the beads (data not shown). The possibility of nonspe-

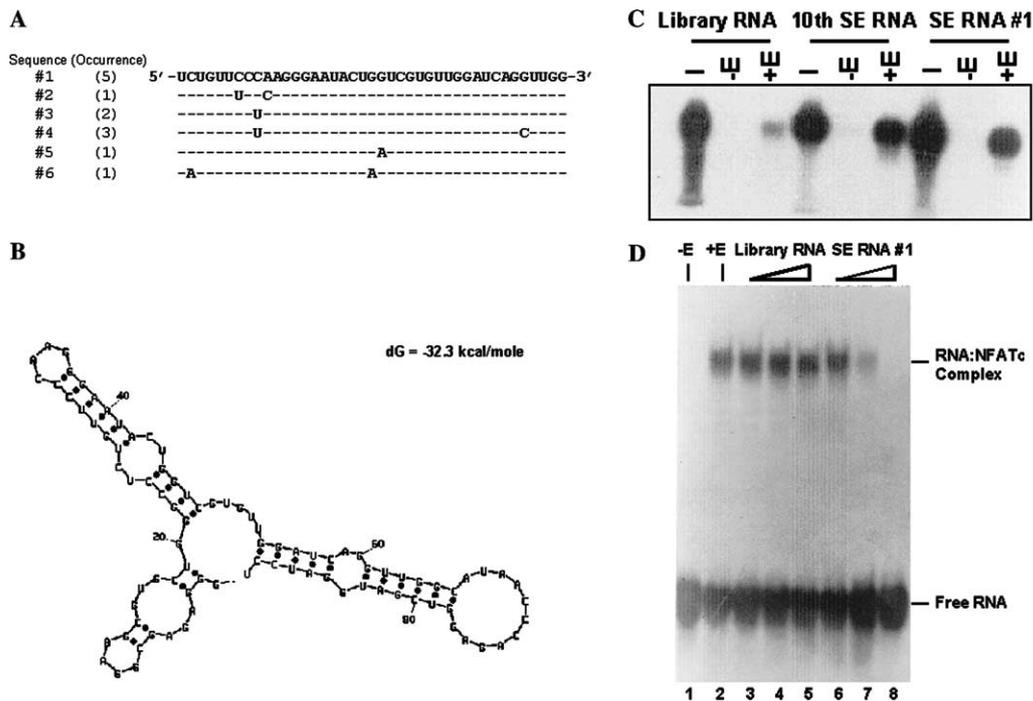


Fig. 1. Sequences, predicted structure, and binding specificity of selected (SE) RNAs. (A) Selected sequences of SE RNAs. After 10 rounds of *in vitro* selection, the sequences of 13 selected RNAs were determined. Six different but very similar RNA sequences were found in these clones and each was present in multiple times (number in parentheses). The line drawn for sequences indicates that nucleotides found at these positions are identical to those shown for sequences #1. (B) Predicted model of secondary structure of SE RNA #1. The most stable secondary RNA structure was determined by using the MULFOLD program [30]. Nucleotides 25–65 represented the sequences selected from randomized region of RNA library. (C) Specific binding of SE RNA #1 to NFATc DBD. One nM internally radiolabeled original library RNA, pooled RNA after 10 cycles of selection (10th SE RNA), or SE RNA #1 was incubated with (+E) or without (–E) NFATc DBD (100 nM) and RNA–protein complexes were precipitated with Ni-NTA beads. Bound RNAs were extracted and analyzed on a 6% polyacrylamide gel with urea. Lane 1 contains 20% of each input-labeled RNA. (D) Specific binding of NFATc DBD to SE RNA #1. Radiolabeled SE RNA #1 (50 pM) was incubated with (+E) or without (–E) NFATc DBD (50 nM). RNA–NFATc complexes were separated from unbound RNA in a 6% nondenaturing polyacrylamide gel. To determine the binding specificity of NFATc DBD to SE RNA, increasing amounts of unlabeled library RNA or SE RNA #1 (lanes 3 and 6, 5 nM; lanes 4 and 7, 50 nM; lanes 5 and 8, 500 nM) were added to the binding reaction.

cific binding to either DNA binding domain conserved within other NFAT members or tagged histidine moieties could be excluded on the basis of the observation that SE RNA did not bind to his-tagged NFATp DBD proteins (data not shown). Interestingly, NFATp is reported to be involved in both activation of Th1-type immune reaction and down-regulation of the late phase transcription of Th2-type cytokines [6]. SE RNA #1 was chosen for further characterization because it appeared most frequently in the selected clones.

To confirm that SE RNA specifically binds the NFATc DBD protein, a gel retardation assay was employed (Fig. 1D). SE RNA #1 efficiently formed a shifted nucleoprotein complex with NFATc DBD. Formation of this nucleoprotein complex can be competitively prohibited by addition of excess amount of unlabeled SE RNA #1 in a dose-dependent manner, but not by a nonspecific competitor, such as the original RNA library.

#### High-affinity binding of the selected RNA to NFATc

The binding affinity of the SE RNA #1 to NFATc DBD was determined by a precipitation experiment with trace amounts of radiolabeled RNAs and increasing amounts of the protein (Fig. 2). RNA library containing 41 nt-long random sequences used for this experiment was shown to have little affinity to NFATc DBD even at the highest concentration of the protein. However, SE RNA showed high affinity with about 30 nM of the equilibrium dissociation constant ( $K_d$ ); hence, SE RNA binds tightly to the NFATc DBD protein.

#### Selected RNA inhibits the DNA binding activity of NFATc but not NFATp

Given the observation that the SE RNA #1 selectively and avidly binds NFATc DBD, we next determined if the binding of the selected RNA affected the DNA binding capacity of NFATc DBD. DNA binding was measured in a gel retardation assay, using a labeled oligonucleotide element corresponding to the distal NFAT binding site found in the murine IL-2 promoter [25] (Fig. 3). Since NFAT is able to bind directly to the murine IL-2 NFAT motif in the absence of Fos and Jun proteins [31], we can test whether the effect of SE RNA on NFAT binding activity is mediated at the level of NFAT–DNA interaction. The selected RNA was effective at inhibiting DNA binding by the NFATc DBD protein in a concentration-dependent manner, whereas the control tRNA showed little inhibition even at the highest concentration tested in this study (Fig. 3A).

SE RNA-mediated inhibition of DNA binding activity of NFATc DBD was shown to be very specific from the EMSA experiments with NFATp DBD in the presence of excess amount of cold competitive RNAs (Fig. 3B). Neither SE RNA nor tRNA could prevent NFATp DBD from the binding to the NFAT site. This could be due to the specific binding of SE RNA to NFATc DBD, but not to NFATp DBD, as described in Fig. 1. In contrast, SE RNA efficiently blocked DNA binding capacity of a chimeric NFAT protein encompassing N-terminal two-third of NFATc DBD (amino acids 392–583) plus C-terminal one-third of NFATp DBD (amino acids 581–694) (Fig. 3C). These results

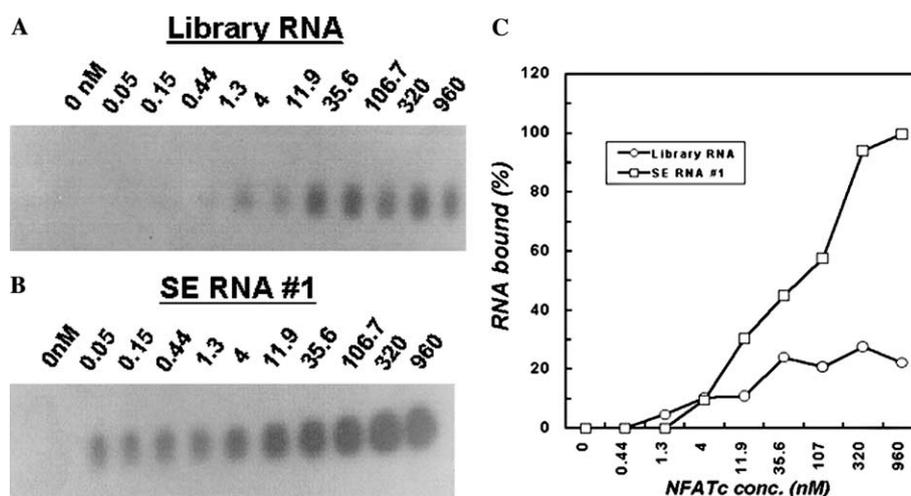


Fig. 2. The binding affinity between SE RNA and NFATc DBD. (A and B) Radiolabeled library RNA (A, 200 pM) or SE RNA #1 (B, 200 pM) was incubated with increasing amounts of NFATc DBD (0–960 nM). The RNA–NFATc complexes were precipitated with Ni-NTA beads. Bound RNAs were extracted and electrophoresed on a 6% polyacrylamide gel with urea. (C) The percentage of RNA to NFATc DBD was calculated by determining the fraction of radioactivity present in the RNA–NFATc complexes. A maximum binding percentage of RNA to NFATc was seen on 960 nM concentration of the protein. The plotted numbers have been normalized to that amount and values shown are averages of measurements performed in triplicate.

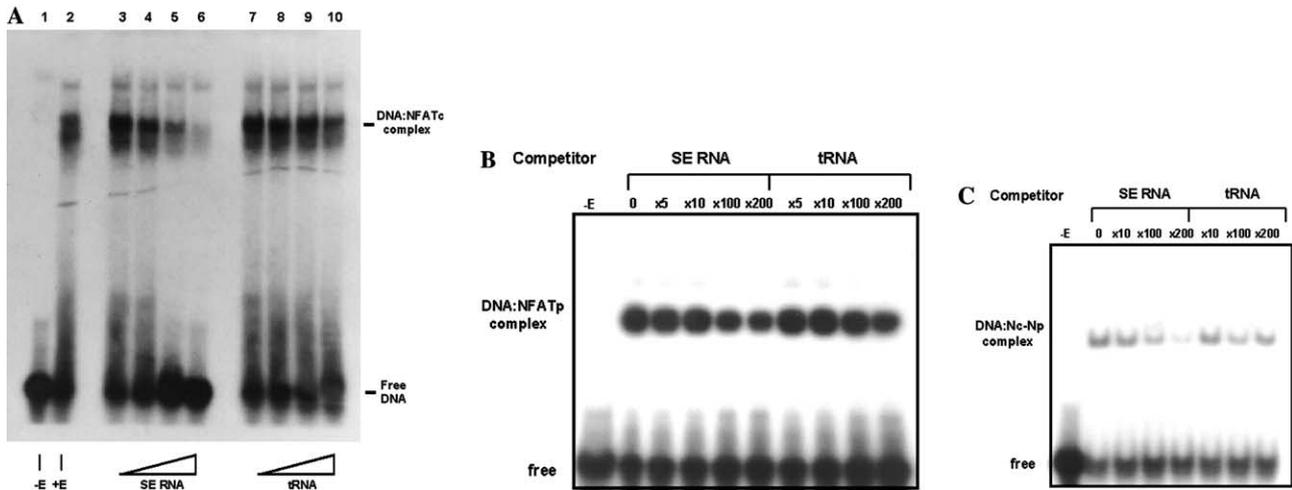


Fig. 3. SE RNA inhibits the DNA binding activity of NFATc but not NFATp. (A)  $^{32}$ P-labeled oligonucleotide containing the distal NFAT binding site derived from the murine IL-2 promoter (300 pM) was incubated with (+E) or without (–E) NFATc DBD (800 nM). DNA–NFATc complexes were separated from unbound DNA in a 5% nondenaturing polyacrylamide gel. To estimate the inhibitory function of SE RNA, increasing amounts of cold SE RNA #1 or tRNA (lanes 3 and 7, 3 nM; lanes 4 and 8, 30 nM; lanes 5 and 9, 300 nM; lanes 6 and 10, 3  $\mu$ M) were added to the binding reaction. (B) EMSA was performed with 20 ng purified recombinant NFATp DBD. To assess the inhibitory function of SE RNA, 5-, 10-, 100-, and 200-fold molar excess of cold SE RNA #1 or tRNA was added to the reaction mixture. (C) Binding reaction was carried out with 10 ng chimeric NFAT protein (Nc-Np) comprising N-terminal two-thirds of NFATc DBD plus C-terminal one-third of NFATp DBD. To assess the inhibitory effect of SE RNA, indicated amounts of molar excess of cold SE RNA #1 or tRNA were added to the reaction mixture.

suggest that SE RNA bind specifically to the N-terminal two-third region of NFATc DBD encompassing amino acid residues 392 and 583.

#### *Selected RNA specifically inhibits NFATc activity in human cells*

Once we had ascertained that SE RNA avidly and specifically bound to NFATc DBD and that it could interfere with complexing of NFATc and its cognate site, we wanted to determine if SE RNA could also inhibit induction of gene expression mediated by NFATc transcription factor. To this effect, transient expression assays were carried out in HeLa cells using NFATc expression plasmid along with NFAT promoter-luciferase reporter construct (Fig. 4). The reporter gene activity was hardly triggered when stimulated with PMA and ionomycin in HeLa cells transfected with only the reporter construct in the absence of NFAT vector (data not shown). By contrast, in control cells cotransfected with NFAT expression vector in the absence of any RNA, luciferase activity was induced by about 2-fold upon stimulation with PMA and ionomycin (Fig. 4). These indicate that HeLa cells have little endogenous NFAT, and hence, the cells could be utilized to assess the inhibitory effect of SE RNA on a specific type of NFAT. While cotransfection of a nonspecific RNA competitor, such as tRNA, could not inhibit the induction of reporter gene expression, SE RNA impeded NFATc and efficiently inhibited the NFATc-dependent gene expression to the background level observed in unstimulated HeLa cells (Fig. 4A). To determine if the

inhibition of NFATc-mediated gene expression by SE RNA was specific, transient transfection experiment was also employed using a reporter plasmid driven by NF $\kappa$ B that has distant sequence similarity of the Rel homology region to NFAT [32] (Fig. 4B). In a sharp contrast to NFATc experiment, both control tRNA and SE RNA poorly inhibited the induction of NF $\kappa$ B-mediated reporter gene expression in stimulated cells, suggesting that SE RNA specifically blocked NFATc-mediated gene expression in human cells. In concordance, induction of NFAT-luciferase reporter mediated by NFATp was not affected by the SE RNA in stimulated cells (Fig. 4C). Furthermore, SE RNA-mediated inhibition of NFATc-driven gene activation was dose-dependent with an  $IC_{50}$  of about 4 nM (Fig. 4D).

#### **Discussion**

Immune responses could be manipulated with immunosuppressive agents that target NFAT. To test this idea, here we isolated RNA decoy aptamers to NFATc with high affinity and specificity using in vitro selection methods. The SE RNA could block DNA binding activity of NFATc, but not of NFATp. Especially, SE RNA inhibited DNA binding capacity of a chimeric NFAT protein comprising of N-terminal two-thirds of NFATc DBD plus C-terminal one-third of NFATp DBD, which suggests that SE RNA bind specifically to the N-terminal two-third region of NFATc DBD. This observation of specific binding to NFATc part of the hybrid is not unexpected because the NFATc N-termi-

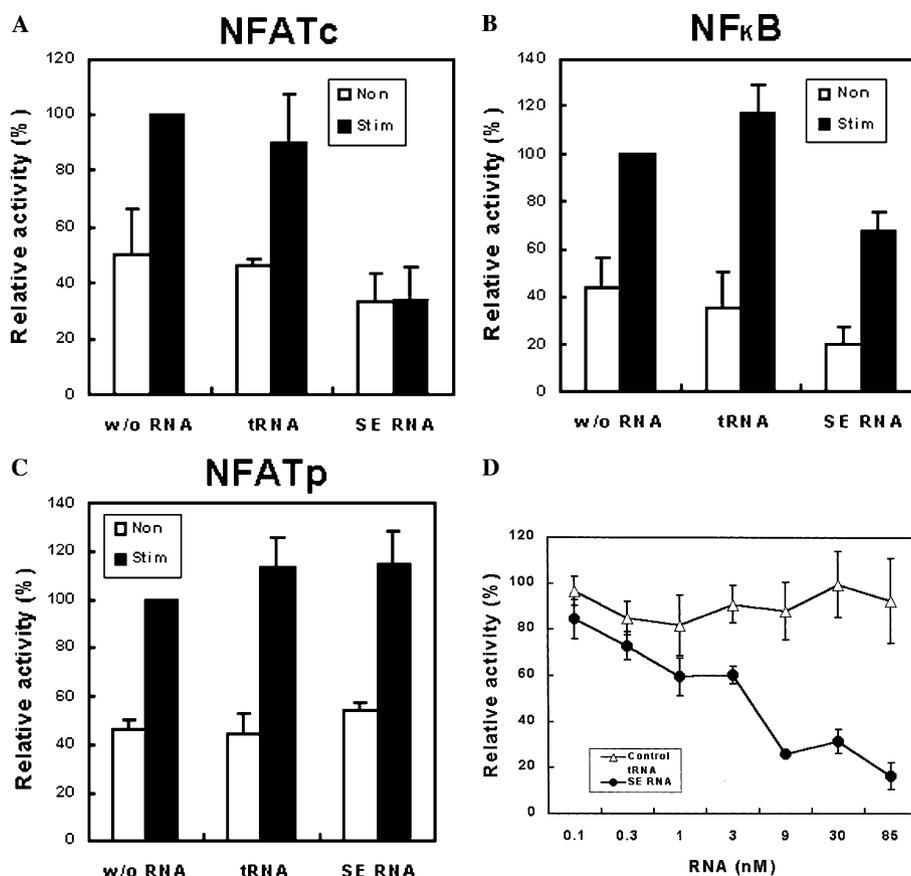


Fig. 4. Specific inhibition of NFATc activity with SE RNA on HeLa cells in culture. HeLa cells were transfected with NFATc expression vector plus pXIL2-Luc reporter construct (A), p3XκB-Luc reporter construct (B), or NFATp expression vector plus pXIL2-Luc vector (C). To measure the inhibitory function of SE RNA, cells were cotransfected also without RNA (w/o RNA), with tRNA (50 nM), or with SE RNA #1 (50 nM). After 24 h of transient transfection, cells were incubated with (black bar) or without (white bar) PMA and ionomycin for 8 h and luciferase activities were then determined. Values were expressed as percentage of the luciferase activity in PMA plus ionomycin-treated cells which were transfected without any RNA. Values shown represent means plus and minus standard deviation of three separate determinants. (D) SE RNA-mediated inhibition of NFATc activity in cells is dose-dependent. HeLa cells were cotransfected with NFATc expression vector, pXIL2-Luc reporter plasmid, and increasing amounts of control tRNA or SE RNA #1. After cells were stimulated with PMA plus ionomycin, luciferase activities were assayed as above.

nal DBD contains most DNA contact sites; however, it is also surprising in a way because this region is known to harbor the most conserved sequences within the NFAT protein family [32]. Thus, it is tempting to speculate that SE RNA could bind N-terminus region of NFATc DBD near and/or at the DNA contact sites which might be structurally different from that of NFATp. If it holds true, such structural differences among the NFAT members may provide a basis for the development of class-specific inhibitors of NFAT.

Importantly, SE RNA inhibits NFATc- but neither NFκB- nor NFATp-mediated gene expression. Even though inhibition of NFAT driven expression of Th2 type specific cytokines needs to be shown in primary human immune cells, our results suggest that decoy RNAs selected *in vitro* here could be useful reagents to specifically inhibit NFATc in cells. To our knowledge, this is the first report on the selection of RNA aptamers as small decoy ligands to a specific member of the NFAT proteins.

Inhibitors against a specific member of the NFAT family such as NFATc may be more consequential than inhibitors against a common target leading to activation of all NFAT members. Its significance lies in the fact that gene disruption of an individual NFAT protein in knockout mice has been shown to impart significantly different biological bias in the immune response [6]. Furthermore, individual NFAT members have unique roles in both immune and nonimmune cells. Thus, targeting individual NFAT proteins could be a more attractive approach for the development of selective immunomodulators. Our data presented here suggest that RNA aptamer against NFATc might be an attractive lead compound for the development of nontoxic immunosuppressive drugs. The selective inhibition of NFATc functions implies that application of small molecules such as the decoy RNA described here may provide a means of modulating the asthmatic response. These experimental drugs are also likely to aid in delineation of the NFATc function during the T cell

differentiation and development of a specific immune response.

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