FEBS Letters 580 (2006) 2046–2052

Suppression of inducible nitric oxide synthase by 10–23 DNAzymes in murine macrophage

Indrajit Chaudhury, Sunil K. Raghav, Hemant K. Gautam, Hasi R. Das, Rakha H. Das*

Comparative Genomics Unit, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi 110 007, India

Received 28 January 2006; revised 23 February 2006; accepted 2 March 2006

Available online 10 March 2006

Edited by Vladimir Skulachev

Abstract iNOS mRNA of J774 murine macrophage cells was cleaved by 10–23 DNAzymes. DNAzyme target site I or translation initiation site and site II have computer predicted (MFOLD) secondary structures but site III has no secondary structure. All the three DNAzymes cleaved the short transcripts generated from cloned DNA almost with equal efficiency while cleavage efficiency is higher at site III than the other two sites on isolated iNOS mRNA. Interestingly, at intracellular level, DNAzyme targeted at translation initiation codon (site I) having secondary structure cleaved iNOS mRNA, and suppressed its activity and protein expression more efficiently than that targeted at sites II and III.

Keywords: DNAzyme; Nitric oxide; Macrophage

1. Introduction

Nitric oxide, a by-product of the oxidative reaction catalyzed by nitric oxide synthase (NOS) that converts L-arginine to citrulline [1], acts as a pleiotropic mediator in various physiological and pathophysiological conditions [2,3]. Nitric oxide synthase has three major isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS) being constitutive, inducible NOS (iNOS) is expressed only following the induction by inflammatory mediators such as lipopolysaccharides (LPS), interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα). Nitric oxide produced by constitutively expressed NOSes (cNOS) is mainly involved in cell signaling and the major purpose of iNOS seems to be in host-defense through the cytotoxic effects of high NO levels [4]. The increased production of NO by iNOS is also associated with various inflammatory diseases [5–7]. The selective inhibition of iNOS, without affecting cNOS that produces very low level of NO necessary for the signaling purposes, is of primary importance in ameliorating inflammatory conditions [8,9].

The 10–23 DNAzyme is a general purpose RNA phosphodiesterase, which works efficiently at simulated physiological conditions [10]. It contains a 15 nucleotide conserved catalytic domain flanked by two 6–9 nucleotide substrate recognition domains. The 10–23 DNAzyme cleaves RNA in presence of divalent cation in between any purine and pyrimidine (R-Y) junction, more efficiently between RU than RC [11,12]. It can be end modified to overcome the host exonuclease activity without reducing its efficiency. The modification of its one or two binding arm nucleotides with the locked nucleic acids (LNA) enhances its substrate binding affinity and subsequently its catalytic activity [13,14].

Despite its cleavable junctions being ubiquitous, secondary structure of the native RNA poses a challenge to select potential target site that can be available for DNAzyme binding. Experimental approaches to choose best cleavable site seems to be more reliable, as presently our ability to rationally select target site is limited [11,15,16]. As a first resort to gain insight into the secondary structure of nucleic acids, generally software based DNA/RNA folding predictions are considered. But the actual hybridization efficiency in in vivo varies from the predicted results because of unpredictable steric and topological constraints in the intracellular environment of the specific tissues. In several cases, AU junctions of initiation codon were selected as a target site [17–19] to stop translation process in the very beginning, presuming that it must be open at the time of translation initiation and hence will be free for DNAzyme binding. With an aim to avoid laborious experiment based target site selection and to find out a safe target site, we have done a comparative analysis of DNAzymes activity targeting initiation codon and MFOLD (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) predicted primary and secondary sites of iNOS mRNA of murine macrophage cells under intracellular and extracellular conditions.

2. Materials and methods

2.1. Oligonucleotides

Three pairs of PCR primers for making cDNA clones to generate short iNOS mRNA fragments were SetI: 5′-CTCCTCACGCTTGGGTCTTGTTC-3′ (FP), 5′-CCGATGTG GCCTTGTGGTGA-3′ (RP); SetII: 5′-CAGCGCTACAACATCCTGGAG-3′ (FP), 5′-CCTGGGCCTCAGCTTCTCATT-3′ (RP); SetIII : 5′-CAGCCCAACAATACAAGATGAC-3′ (FP), 5′-AGTGATGGCCGACCTGATG-3′ (RP); and two DNA sequencing primers 5′-GTCGTTAGAACGCGGCTAC-3′ (FP) and 5′-GGTTTTCACCGTCATCACCG-3′ (RP) for sequencing the inserts of Pspt18 clones were synthesized from TCGA (New Delhi, India). DNAzymes, 5′-ggcaagccaGGC TAGCTACAACGAgtctgagac-3′ (DzI targeting site I), 5′-ccagccaaaGGCTAGCTACAACGAccagtctgc-3′ (DzII targeting site II), 5′-ttgt ttctaGGCTAGCTACAACGAttcctttgt-3′ (DzIII targeting site III) and their corresponding mutant DNAzymes (MDzI, MDzII and MDzIII) were made from Sigma, UK. Mutant DNAzymes contain TG in place of GC (underlined) in the catalytic domain of DNAzymes.

*Corresponding author. Fax: +91 11 27667471.
E-mail address: rakhahdas@yahoo.com (R.H. Das).

Abbreviation: iNOS, inducible nitric oxide synthase

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.004

The capitalized 15 nucleotides within DNAzyme sequences represent the catalytic domain flanked by 9 nucleotides substrate binding domains (written in small letters) at the ends. All the DNAzymes and their mutants have 5′ phosphorothioate linkage and 3′ CPG-amine C7 cap. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-CAGTGCCAGCCTCGTCCCGTAGA-3′ (FP) and 5′-CACCCTGTTGCTGTAGCCGTATTC-3′ (RP) were synthesized from TCGA (New Delhi, India). Random hexamers for the first strand cDNA synthesis were part of the cDNA synthesis kit (Clontech, USA).

2.2. Cell culture

Murine macrophage cell line J774 was obtained from International Centre for Genetic Engineering and Biotechnology (N. Delhi, India). Cells were propagated in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B), and 10% heat inactivated fetal calf serum, and maintained at 37 °C in a humidified incubator containing 5% CO_2. The culture was allowed to grow to confluence and used for further experiment. The cells were resuspended in RPMI-1640 at a density of 2×10^6 cells/ml. Cell viability was determined by trypan blue exclusion. Lipopolysaccharide (LPS) from *E. coli* (strain 055:B8, Sigma) was used to induce iNOS in murine macrophage cells.

2.3. Isolation of mRNA

Total RNA from LPS induced and uninduced cells were isolated using EZ-RNA isolation kit (Biological Industries, Israel). Poly A + RNA was isolated from total RNA using oligotex mRNA midi kit (Qiagen) and quantitated by spectrophotometry (Perkin–Elmer Lambda 35 UV/VIS spectrophotometer).

2.4. In vitro transcription and DNAzyme cleavage assays in cell free system

Three Pspt18 clones having the insert sizes 400, 368 and 610 bp were made by RT-PCR of iNOS mRNA using the primer sets I, II and III, respectively. The inserts in Pspt18 clones were sequenced by an automated capillary DNA sequencer, ABI 3370, Prism (Applied Biosystems, Foster City, CA). The [α-^{32}P]UTP labeled transcripts of sizes 400 nts (SI, contains DzI site), 368 nts (SII, contains DzII site) and 610 nts (SIII, contains DzIII site) were generated by using SP6 RNA polymerase (Roche Applied Science) from their corresponding linearized Pspt18 clones.

Cell free DNAzyme cleavage assays of the in vitro transcripts and poly A^+ iNOS RNA from LPS induced or uninduced macrophage cells were carried out in a 20 μl reaction volume containing substrate RNA, DNAzyme, 5 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, and 150 mM NaCl at 37 °C for 2 h [18]. In the case of in vitro transcripts, reaction was stopped by transferring aliquots of the reaction mixture into formamide loading buffer (90% formamide and 20 mM EDTA). The cleaved and uncleaved RNAs were then separated by gel electrophoresis in a 5% urea denaturing polyacrylamide gel and autoradiographed. In the case of polyA^+ RNA substrate cleavage activities were ascertained by RT-PCR as described below.

2.5. DNAzyme cleavage assays in transfected cells

Semi confluent (60–80%) macrophage cells were transfected with DNAzymes and their mutants using lipofectamine reagent (GIBCO BRL) following the supplier's protocol in a 24 well plate. Initially 5×10^5 cells were seeded in each well and incubated for 24 h at 37 °C in complete medium. Transfections were carried out in Opti-MEM I reduced serum medium (GIBCO BRL) for 5 h followed by LPS (1 μg/ml) challenge for 4 h in serum containing medium. Total RNA from the cells from each experiment was isolated and quantitated.

DNAzyme activities on isolated mRNA and in DNAzyme transfected cells were assessed by semi quantitative RT-PCR using the primer sets I, II and III for DNAzymes (Dz and MDz) I, II and III, respectively. RT-PCR products were separated in 1.5% agarose gel. The differences in integrated density values (IDV) of RT-PCR products obtained with or without DNAzyme treatment (Alpha DigiDoc 1201, USA) were used as a measure of cleavage activity. GAPDH (993 bp) was kept as a house keeping gene for all the RT-PCR reactions.

2.6. Griess nitrite assay and Western blot analysis

Griess nitrite assays for the cell-free supernatants and Western blot analysis of the cell extracts were carried out after 16 h of LPS induction in DNAzyme transfected and untransfected cells. By Griess reaction, the NO level was measured as its stable oxidative product nitrites as described earlier [20]. Briefly, 100 μl of cell free supernatant was incubated for 10 min at room temperature with an equal volume of Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% phosphoric acid) and absorbance was measured at 546 nm using sodium nitrite as standard.

For Western blot analysis cell extracts were prepared by the method of Korhonen et al. [21]. Cell pellets were lysed in ice-cold extraction buffer (10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM *N*-octyl-β-D-glucopyranoside) and incubated on ice for 15 min for extraction and then centrifuged. Supernatants were boiled for 5 min in the sample buffer containing 62.5 mM Tris–HCl, pH 6.7, 20% glycerol, 2% SDS, and 10 mM 2-mercaptoethanol. Protein estimation of the supernatants was done by Bradford Coomassie brilliant blue method (Bio-Rad, Richmond, CA) using bovine serum albumin as standard. Cell extracts were fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis [22] by loading 10 μg of protein in each lane and blotted onto a nitrocellulose membrane. The blot was probed with 1:1000 diluted mouse monoclonal anti-iNOS antibody (Sigma, UK) and developed with secondary antibody (peroxidase conjugated goat anti-mouse IgG, Sigma, UK) as described by Sambrook et al. [23]. The housekeeping gene GAPDH was probed with the mouse monoclonal anti GAPDH antibody (Santa Cruz Biotechnology, CA).

3. Results

3.1. Secondary structure determination of iNOS mRNA

MFOLD generated partial secondary structure of 3991 nts iNOS mRNA of *Mus musculus* (NCBI Accession No. NM_010927) around selected DNAzyme target sites I, II and III are shown in the Fig. 1. Intramolecular hydrogen bonding or secondary structure formations are seen in the DNAzyme binding sites of site I and II but no such structures are observed at siteIII.

3.2. Cleavage of short in vitro transcripts

In vitro transcripts of sizes 400 nts (SI), 368 nts (SII) and 610 nts (SIII) contained the DNAzyme target sites I, II and III, respectively. Generation of RNA fragments of 300 and 100 nts by DzI, 225 and 143 nts by DzII, and 392 nts and 218 nts by DzIII indicated specificity of the designed DNAzymes (Fig. 2). Appreciable RNA cleavage activity of none of the mutant DNAzymes was observed at any one of the target sites. Cleavage activities of all the three DNAzymes increased with their increasing concentrations.

3.3. In vitro cleavage of iNOS mRNA

The cleavage activities of the three DNAzymes on three target sites in iNOS mRNA substrate (Fig. 3) differ from that on its short fragments (Fig. 2), however, in both the cases higher activities were realized with their increasing concentrations, and less than 10% activities were observed with their mutant DNAzymes.

3.4. Intracellular RNA cleavage activity

The increased RNA cleavage activities of all the three designed DNAzymes were also observed when macrophage cells were transfected with increasing concentration of DNAzymes.