RNA Aptamers for Targeting Mitochondria Using a Mitochondria-Based SELEX Method

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The use of mitochondria-based systematic evolution of ligands by exponential enrichment (SELEX) was explored. Mitochondria were isolated from rat liver and confirmed intact by respiratory control index. Isolated mitochondria and a 2'-F RNA random library were mixed and the bound RNAs collected. The counter selection was applied with nucleus and unbound RNAs were collected. After 7 rounds of selection, two sequences (Mitomer1 and Mitomer2) were verified to bind to mitochondria and the truncated Mitomer2 (short Mitomer2) showed better binding to isolated mitochondria than Mitomer1.

Key words RNA aptamer; selection; mitochondria

After mitochondria were incorporated into eukaryotic cells, they became dependent on the many functions of mitochondria, with the subsequent development of a type of symbiotic relationship.10) Mitochondria, which host the citric acid cycle and oxidative phosphorylation supply major supplies of energy to eukaryotic cells for their life. Mitochondria also have their own DNA called mitochondrial DNA (mtDNA) and prepare some types of proteins by transcribing and translating their DNA, thus mitochondria have their own functions and machinery and contribute greatly to the life a cell.1) Therefore, a mutation in mtDNA or the down-regulation of protein expression can result in the loss of mitochondrial function and the development of mitochondrial diseases, neurodegenerative diseases and type 2 diabetes. An investigation of a drug delivery system (DDS) for addressing and overcoming these types of mitochondrial diseases is, therefore, an important issue.3–6) Mitochondrial targeting ligands are important in terms of achieving active targeting to mitochondria to control the intracellular trafficking of drugs and some ligands have been reported in the past.7) Triphenylphosphonium (TPP) is a hydrophobic cationic molecule.8) It can bind to mitochondrial membrane via electrostatic interactions, penetrate mitochondrial membrane with membrane potential and reach to matrix in mitochondria. A conjugate of coenzyme Q10 and TPP (MitoQ) is used in the treatment of Parkinson diseases.9) A TPP–lipid conjugate was synthesized and used as a ligand for liposomes and has been used as a drug delivery system, by virtue of the fact that it can bind well to mitochondria.10) Mitochondrial targeting signals (MTS) are peptides which are used in living cells to send proteins encoded by a nuclear genome to mitochondria. MTS is usually located on the N-terminal of peptides and sends these proteins to mitochondria via translocase of the outer membrane (TOM)–translocase of the inner membrane (TIM) complex.11) MTS is also used as a ligand for targeting to mitochondria by conjugating it with exogenous proteins.12) MTS has also been used effectively for liposomal DDS.13) The D-arm is a part of a tyrosine encoded transfer RNA and can be transferred to mitochondria. This tRNA binds to the tubulin antisense-binding protein (TAB) and the resulting complex can enter mitochondria and is also used as a ligand for DDS.14,15) There are, however, problems associated with the use of such ligands. TPP acts via electrostatic interactions and ideally cannot be applied for use in damaged mitochondria. Because MTS and D-arm are naturally occurring peptides and RNA, respectively, they are susceptible to digestion under physiological conditions. Aptamers are short DNA, RNA or peptide molecules that can bind to small molecules, proteins, viruses and cells via an induced fit mechanism. Aptamers can be easily chemically modified to make them stable toward nucleases and have a low immunogeneity compared to antibodies. Because of these reasons, aptamers are frequently considered to be new classes of ligand. Actually, aptamers have been used as ligands in nanoparticles that are used for cancer therapy.16) The systematic evolution of ligands by exponential enrichment (SELEX) method is used to identify aptamers. This method was first reported by Ellington’s group and Tuerk’s group independently in 1990 and many types of modified SELEX have since been reported.17–19) Among these methods, cell-SELEX represents one of the most promising approaches.20) In the cell-SELEX method, cells themselves are used as the target. This method has some advantages, in that it is a better method to direct the aptamer toward membrane proteins and sometimes the target protein, since this kind of ligand is a membrane protein. When the aptamer is used to target mitochondria, the identity of the target protein is unclear, but, using the mitochondria-based SELEX method, it is possible to isolate the desired aptamer, regardless of the target protein. In 2010, Kolesnikova et al. tried SELEX to mitochondria to improve the efficacy of importable yeast and human tRNA by changing a part of sequence in natural tRNA.21) They showed excellent results and their aptamers could be bound and imported to mitochondria. By the way, the target protein of their aptamer might be same as the protein which the yeast tRNA would be bound. Compared to their research, we focused on whole proteins on mitochondria and try to get aptamers only using the mechanism of SELEX procedure and we could get new class of sequences by applying the SELEX approach to mitochondria. Therefore we...
applied the cell-SELEX method to mitochondria and refer to this method as mitochondria based SELEX (Fig. 1).

MATERIALS AND METHODS

The Isolation of Mitochondria from Rat Liver  
Mitochondria were isolated from livers obtained from adult male Wistar rats (6–8 weeks of age). Rats were sacrificed and the livers were taken after bleeding had largely subsided. Rat liver was placed in approximately 20 mL of ice-cold mitochondrial isolation buffer plus ethylenediaminetetraacetic acid (EDTA) Mitochondria Import Buffer (MIB) (+); 250 mM sucrose, 2 mM Tris–HCl, 1 mM EDTA, pH 7.4) per 10 g of liver. All subsequent steps were carried out on ice. The liver was chopped into small pieces and the suspension was homogenized in a glass homogenizer (50 mL capacity) with a pestle. Three complete up and down cycles with the pestle were done. The pestle was motor-driven and operated at approximately 550 rpm. The homogenate was diluted approximately 1 : 3 with MIB (+) and centrifuged at 800 × g for 5 min. The supernatant was transferred into ice-cold tubes and centrifuged at 7500 × g for 10 min. The pellets were washed once with 5 mL of MIB (+), and then once with 5 mL of MIB (−) which is mitochondria isolation buffer didn’t contained EDTA. Concentrations of mitochondrial proteins were determined using a bicinchoninic acid (BCA) protein assay kit. Respiratory control index was measured according to the procedure.[22] All animal protocols were approved by the institutional animal care and research advisory committee at the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Preparation of RNA Library  
A Durascribe T7 in vitro transcription kit (Epicentre Biotechnologies) was used to transcribe the RNA pool from the double stranded DNA template. Two microliter of water, 4 µL of 100 bp DNA template (1.0 µg), 2 µL of 10X Reaction Buffer, 2 µL each of 50 mM ATP, GTP, 2′-deoxy-2′-fluorouridine 5′-triphosphate (2′-F-CTP), 2′-deoxy-2′-fluorouridine 5′-triphosphate (2′-F-UTP), 2 µL 100 mm dithiothreitol (DTT), 2 µL of Durascribe T7 enzyme mix was incubated for 6 h at 37°C. The sequence of non-template DNA was 5′-AGCTCACATTCTATACGACTCATAGGGAGGACGATCAGTGCTGTAA-3′. One microliter (1 unit) of DNase I was added the mixture and was further incubated for 30 min. An equal volume of phenol–CHCl₃–isoamyl alcohol (25 : 24 : 1) (100 µL) was added to in vitro transcription product and mixed well by vortexing, spun at room temperature for 2 min. The aqueous layer was collected and an equal volume of chloroform was mixed well by vortexing and spun at room temperature for 2 min. The aqueous layer was collected and added 0.1 volume of 3 M NaOAc, 4.0 µL glycogen (5 mg/mL) and 2.5 volume of ethanol was added. This mixture was incubated at −20°C overnight to precipitate RNA. The resulting solution was centrifuged at 15000 × g for 30 min under 4°C. The resulting RNA pellet was washed with 600 µL of 70% ethanol and spun again under same conditions. The pellet was allowed to dry at room temperature and was then dissolved with diethylpyrocarbonate (DEPC) treated water. RNA was purified using a NAP-5 column (GE Healthcare) and concentrated using with Amicon-filter (MWCO 3000).

Mitochondria-Based SELEX  
Eighty nmoles ssRNAs (500 pmol) contained random sequences were dissolved in 1× selection buffer (20 mM Tris–HCl, 2 mM KCl, 40 mM NaCl, 1 mM MgCl₂, 250 mM sucrose, pH=7.5) and heated to 80°C for 10 min and cooled down slowly to 4°C. The RNAs were mixed with isolated mitochondria (0.5 mg), bovine serum albumin (BSA) (500 µg) and yeast tRNA (500 µg). This mixture was incubated at 4°C for 45 min, centrifuged (8000 × g, 4°C, 10 min) and the supernatant was removed. Collected mitochondria were washed with 1× selection buffer (500 µL) and lysed with 0.1% triton/1× selection buffer (200 µL). This mixture was added 1 Molecular Biology Unit (MBU) of DNase I and RNase A, respectively and incubated 37°C
for 15 min. This mixture was applied for phenol–chloroform extraction and recovered RNAs were collected with ethanol precipitation. The RNAs were reverse-transcribed with one-step reverse transcription-polymerase chain reaction (RT-PCR) kit (Qiagen) following with their procedure with forward primer (5'-TCA TAG GGA GAA CAA TCA GA-3') and reverse primer (5'-TGT ACT CAG CGA CGA CTG AA-3') to prepare 80 bp double-stranded DNA (dsDNA). This dsDNA was again amplified with TakaraTaq polymerase also following their procedure with same reverse primer and 40 nt forward primer (5'-AGC TCA ATT CTA AGA CTC ATA GGG AGG ACA ATC AGA-3') to prepare 99 bp dsDNA.

Cloning and Sequencing After 7 rounds of the selection, recovered dsDNA was applied for PCR with 30 min final extension time. This A-overhanging dsDNA was applied as the substrate to TOPO-TA cloning kit (Invitrogen). Plasmid DNA (pDNA) was transformed to *Escherichia coli* (DH5α) and detected desired colonies using colony PCR. pDNAs with insert were collected by Miniprep (Qiagen) following their procedure and applied for sequencing.

Binding Assay of Mitomers to Mitochondria FAM-tagged Mitomer1, Mitomer2, short Mitomer2, D-arm (GGGACUGUAGCUCAUUGUGAGCAU)14) and random sequence were purchased from Sigma Genosys. These oligonucleotides were dissolved in MIB(−) to 2 μM concentrations, heated to 80°C for 10 min and cooled down to 4°C. To this solution, BSA (200 μg), yeast tRNA (200 μg) and isolated mitochondria (200 μg) were added and mixed for 30 min. These mixtures were centrifuged (8000×g, 4°C, 10 min) and unbound RNAs were removed. Mitochondria were lysed with 0.1% triton/MIB(−) and bound RNAs were recovered with phenol–chloroform extraction and ethanol precipitation. Collected RNAs were dissolved in MIB(−) and measured their fluorescent intensities (Ex. 494 nm, Em. 519 nm).

**RESULTS AND DISCUSSION**

In investigations of ligands for mitochondria, it is sometimes difficult to choose the target receptor when the SELEX is applied to some recombinant mitochondrial proteins, because it is necessary to have information on a suitable protein that expressed well on the surface of mitochondria and to prepare a recombinant protein, which has the same tertiary structure on the surface of the mitochondrial membrane. On the other hand, when isolated mitochondria themselves are used as the target, it is not necessary to have complete and detailed knowledge concerning the target molecule and also the higher-order structure of surface proteins do not need to be reproduced. Additionally, it is possible to identify several kinds of aptamers that can target different receptors. Based on these considerations, we attempted to initiate studies related to the use of mitochondria-based SELEX, *i.e.*, to use isolated mitochondia as the target.

For mitochondria-based SELEX, the isolation of mitochondria is an important step in this process, because if mitochondria are damaged, the structure of the proteins on the mitochondrial surface is not guaranteed and the findings would be invalid. In our experiments, we carefully isolated mitochondria from rat livers using literature procedures22,23 and estimated the intactness of mitochondria using the value for the respiratory control index (RCI). RCI represents the ratio of the rate of oxygen consumption of mitochondria with or without ADP in the presence of suitable substrates and a higher value is indicative of a higher intactness. In our experiments, only mitochondria in which the RCI was higher than 4 were used for selection. Next, an RNA library was prepared for the selection. When aptamers are used as ligands, the stability of the aptamers is important because there are several nucleases and phosphodiesterases in the cytosol. Non-modified RNAs are usually very susceptible to digestion by nucleases, therefore RNA in which the pyrimidine nucleotide 2'-hydroxyl groups were modified by fluorine was employed for our mitochondria-based SELEX to increase nuclease resistance.24 Firstly, the initial RNA library was *in vitro* transcribed with Durascript T7 RNA polymerase which can incorporate 2'-F pyrimidine nucleotide to RNAs. The diversity of initial RNA library was around 1014 and this is enough diversity for SELEX. Mitochondria-based SELEX (Fig. 1) was started by mixing RNA libraries and isolated mitochondria at 4°C, 45 min using a buffer that approximated osmotic stress using sucrose (250 mm). BSA and yeast RNA were added to reduce non-specific binding. After the incubation, unbound RNAs were removed by centrifugation and the bound RNAs were recovered by heating to denature all of the RNAs and proteins. Contaminating DNAs and RNAs from mitochondria were digested by DNase I and RNase A. APTamer candidate RNAs didn’t cleave under such conditions because 2'-F modified RNAs are completely intact toward these enzymes.25,26 The desired RNAs were purified by phenol–chloroform extraction and ethanol precipitation. The resulting aptamer candidate RNAs were reverse-transcribed to dsDNA and *in vitro* transcribed to prepare ssRNA for the next round using the DurascriptT7 RNA kit which incorporates 2'-F CTP and UTP to ssRNA. Seven cycles of selection were applied and one counter selection was applied using isolated nuclei.27 On this counter selection, RNAs that did not bind to nuclei, but did bind to mitochondria were collected. ssRNA was recovered and amplified using the same method as previously described. After the completion of selection, dsDNA was incorporated to pDNA via TA cloning and the sequences were determined.

Two sequences were picked up from the results of sequencing and are referred to as Mitomer1 (5'-CACAG AU CAC GGU UU CCCU CGC AGG UAA GGU GA-3') and Mitomer2 (5'-UCCCGA AU ACGUA AUCU UAG CCC AU AGCU GGCGC-3'). The binding affinities of these aptamers were investigated by checking the FAM-labeled mitomers and isolated mitochondria. Mitomers were mixed with isolated mitochondria at a temperature below 4°C for 30 min. Unbound mitomers were removed by centrifugation and the mitochondria were washed, lysed and used in the fluorescent intensity measurements. The D-arm was used as a positive control RNA which is known as the sequence taken from *Leishmania* tRNA. A random sequence that can bind non-specifically to mitochondria was used as the control. Mitomer2 showed a high affinity for mitochondria compared to Mitomer1. The secondary structure of Mitomer2 contained one stem-loop and a non-arranged structure (Fig. 2), as calculated by mfold.28 From this structure, the sequence responsible for binding was found to be only 5'-CUUAGCCAAUGCUUGGCUCG-3' therefore Mitomer2 was truncated to a short Mitomer2 (5'-CUUAGCCAAUGCUUGGCUGC-3') and binding affin-
ity was evaluated. The short Mitomer2 was checked and its binding affinity was found to be significantly better than that for the full length of Mitomer2 and the binding activity was close to that for D-arm which is a known ligand for targeting mitochondria (Fig. 3). It was therefore concluded that mitochondria-based SELEX was established and that the isolated aptamer called short Mitomer2 represents a good candidate for mitochondria targeting because of its binding affinity and resistance to degradation by nucleases.

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REFERENCES

1) Gray MW. Mitochondrial evolution. Cold Spring Harb. Perspect. Biol., 4, a011403 (2012).
2) Holt IJ, Reyes A. Human mitochondrial DNA replication. Cold Spring Harb. Perspect. Biol., 4, a012971 (2012).
3) Yamada Y, Harashima H. Mitochondrial drug delivery systems for macromolecule and their therapeutic application to mitochondrial diseases. Adv. Drug Deliv. Rev., 60, 1459–1462 (2008).
4) Selfridge JE, E L, Lu J, Swerdlow RH. Role of mitochondrial homeostasis and dynamics in Alzheimer’s disease. Neurobiol. Dis., 51, 3–12 (2013).
5) Naviaux RK. Mitochondrial DNA disorders. Eur. J. Pediatr., 159 (Suppl. 3), S219–S226 (2000).
6) Johannsen DL, Ravussin E. The role of mitochondria in health and disease. Curr. Opin. Pharmacol., 9, 780–786 (2009).
7) Hoye AT, Davoren JE, Wipf P, Fink MP, Kagan VE. Targeting mitochondria. Acc. Chem. Res., 41, 87–97 (2008).
8) Ross MF, Kelso GF, Blakie FH, James AM, Cochemé HM, Filipovska A, Da Ros T, Hurd TR, Smith RAJ, Murphy MP. Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology. Biochemistry (Mosc.), 70, 222–230 (2005).
9) Snow BJ, Rolfe FL, Lockhart MM, Frampton CM, O’Sullivan JD, Fung V, Smith RA, Murphy MP, Taylor KM, Protect Study Group. A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson’s disease. Mov. Disord., 25, 1670–1674 (2010).
10) Biswas S, Dodwadkar NS, Deshpande PP, Torchilin VP. Liposomes loaded with paclitaxel and modified with novel triphenylphosphonium–PEG–PE conjugate possess low toxicity, target mitochondria and demonstrate enhanced antitumor effects in vitro and in vivo. J. Control. Release, 159, 393–402 (2012).
11) Schatz G. The protein import system of mitochondria. J. Biol. Chem., 271, 31763–31766 (1996).
12) Shkolenko IN, Alexeyev MF, LeDoux SP, Wilson GL. TAT-mediated protein transduction and targeted delivery of fusion proteins.

Fig. 2. Secondary Structures of Mitomers
Secondary structures of the mitomers were calculated using the mfold software. The URL for the mfold website is http://mfold.rna.albany.edu.

Fig. 3. Binding Affinities of Truncated Mitomer2 (Short Mitomer2)
Binding affinities of FAM-labeled mitomers toward isolated mitochondria were investigated. Comparisons between multiple treatments were made by one-way ANOVA, followed by the Student–Newman–Keuls test. Values are the mean±S.D. of at least three different experiments (n=3, **p<0.01).
13) Kawamura E, Yamada Y, Yasuzaki Y, Hyodo M, Harashima H. Intracellular observation of nanocarriers modified with a mitochondrial targeting signal peptide. J. Biosci. Bioeng., 116, 634–637 (2013).

14) Mahapatra S, Ghosh S, Bera SK, Ghosh T, Das A, Adhya S. The D arm of tRNA\(^{137}\) is necessary and sufficient for import into Leishmania mitochondria \textit{in vitro}. Nucleic Acids Res., 26, 2037–2041 (1998).

15) Mukherjee S, Mahata B, Mahato B, Adhya S. Targeted mRNA degradation by complex-mediated delivery of antisense RNAs to intracellular human mitochondria. Hum. Mol. Genet., 17, 1292–1298 (2008).

16) Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R. Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy \textit{in vivo}. Proc. Natl. Acad. Sci. U.S.A., 103, 6315–6320 (2006).

17) Ellington AD, Szostak JW. \textit{In vitro} selection of RNA molecules that bind specific ligands. Nature, 346, 818–822 (1990).

18) Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science, 249, 505–510 (1990).

19) Brody EN, Gold L. Aptamers as therapeutic and diagnostic agents. J. Biotechnol., 74, 5–13 (2000).

20) Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW, Mallikaratchy P, Sefah K, Yang CJ, Tan W. Aptamers evolved from live cells as effective molecular probes for cancer study. Proc. Natl. Acad. Sci. U.S.A., 103, 11838–11843 (2006).

21) Kolesnikova O, Kazakova H, Comte C, Steinberg S, Kamenski P, Martin RP, Tarassov I, Entelis N. Selection of RNA aptamers imported into yeast and human mitochondria. RNA, 16, 926–941 (2010).

22) Shinohara Y, Almofri MR, Yamamoto T, Ishida T, Kita F, Kanzaki H, Ohnishi M, Yamashita K, Shimizu S, Terada H. Permeability transition-independent release of mitochondrial cytochrome \(c\) induced by valinomycin. Eur. J. Biochem., 269, 5224–5230 (2002).

23) Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H. MITO-Porter: A liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim. Biophys. Acta, 1778, 423–432 (2008).

24) Ruckman J, Green LS, Beeson J, Waugh S, Gillette WL, Henninger DD, Claesson-Welsh L, Janiči N. 2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. J. Biol. Chem., 273, 20556–20567 (1998).

25) Meis JE, Chen F. \textit{In vitro} synthesis of 2'-fluoro-modified RNA transcripts that are completely resistant to RNase A digestion using the DuraScribe\textsuperscript{™} T7 transcription kit. Epicentre Forum 9, 10–11 (2002).

26) Capodici J, Karikó K, Weissman D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol., 169, 5196–5201 (2002).

27) Ikeda M, Omukai Y, Hosokawa K, Senoo T. Difference in extractability of estradiol- and tamoxifen-receptor complex in the nuclei from MCF-7 cells with Nonidet P-40. Steroids, 43, 481–489 (1984).

28) Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res., 31, 3406–3415 (2003).