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Selection of RNA aptamers against the *M. tuberculosis* EsxG protein using surface plasmon resonance-based SELEX

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ABSTRACT

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, remains one of the most prevalent infectious diseases worldwide which causes high morbidity and mortality. However, there is still limited understanding of the physiological processes that allow *M. tuberculosis* to survive in its host environment. One of the challenges is the limited availability of molecular probes that can be used to study some of the complex systems in mycobacteria. One such system is the ESX-3 secretion system, a specialized type VII secretion (T7S) system. This system is essential for optimal growth of pathogenic mycobacteria in low iron environments similar to that encountered by mycobacteria in macrophages during infection. EsxG, a protein of unknown function, is both encoded within the *ESX-3* locus and secreted by the ESX-3 system.

There are currently no molecular probes with high affinity and specificity to the EsxG protein that can be used to study it. Here we demonstrate the use of surface plasmon resonance-based systematic evolution of ligands by exponential enrichment (SELEX) to identify two aptamers, G43 and G78 that bind EsxG with high affinities, K_D of 8.04 ± 1.90 nM and 78.85 ± 9.40 nM, respectively. Moreover, these aptamers preferentially bind EsxG over its homologue EsxA. Availability of such probes enables biological investigation of the role of this protein in mycobacteria and its potential as a biomarker for TB diagnosis.

1. Introduction

Tuberculosis, which is caused by *Mycobacterium tuberculosis*, remains one of the major infectious diseases that lead to morbidity and death worldwide. Despite the widespread availability of effective antibiotics against drug sensitive *M. tuberculosis*, the World Health Organisation (WHO) estimates that in 2011 there were between 8.3 and 9.0 million incidences of TB globally [1]. A better understanding of the biological processes of mycobacteria will improve the control of the spread of tuberculosis by enabling the development of better therapeutic interventions and diagnostic tests. One such process is the iron acquisition pathway of mycobacteria.

Iron is an essential nutrient for most microorganisms. At least 40 enzymes encoded by the *M. tuberculosis* genome require iron as a cofactor [2]. One of the ways that mycobacteria has evolved to acquire iron is by the use of small-molecule compounds, called siderophores, for high affinity chelating of ferric iron [3]. Siderophores are generally secreted by the organism to compete for the available iron in their environment [4]. The genus of mycobacteria has two kinds of siderophores, mycobactin and exochelin [5,6]. The synthesis of the siderophores is highly regulated and occurs exclusively under low iron conditions [7]. In order to maintain iron homeostasis, *M. tuberculosis* uses a transcription factor, the iron-dependent regulator (IdeR) protein. In the presence of iron, IdeR binds to the promoters and represses the transcription of the *mbt* genes which are essential for siderophore synthesis [8]. As a result, the uptake of iron from the surrounding environment is inhibited.

The IdeR protein also acts on another target, the ESX-3 secretion system. This secretion system is one of the five specialised type VII secretion (T7S) system of mycobacteria [9]. The IdeR protein has been shown to repress the transcription of the *ESX-3* gene cluster in both *M. tuberculosis* [7] and *M. smegmatis* [10], while low iron conditions induce the transcription of the *ESX-3* cluster in *M. tuberculosis* [7]. This is indicative of the participation of this system in the acquisition of iron by mycobacteria under low iron conditions. In addition, a study by Siegrist and colleagues demonstrated that a

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M. smegmatis mutant which lacks ESX-3 (ΔESX-3) is unable to be rescued by the exogenous addition of purified siderophore, mycobactin, indicating that the mutant is unable to use iron-bound mycobactin [11]. Similar findings were also reported by Serafini and colleagues using a *M. tuberculosis* ESX-3 conditional mutant. Serafini and colleagues further hypothesised that ESX-3 is responsible for the secretion of some unknown factor(s) which is required for the optimal uptake of iron and zinc. These factors have not been identified yet [12].

One of the ESX-3 secreted substrates is the EsxG protein, which is homologous to the well characterised EsxA protein secreted by the paralogous ESX-1 system. While there is evidence to show that EsxA is required for pathogenicity in *M. tuberculosis* [13–16], such evidence does not exist for EsxG. Also, while genetic studies have been able to demonstrate the involvement of ESX-3 in iron acquisition, they have been limited in investigating the role of individual ESX-3 secreted substrates in this pathway. For this reason we sought to develop specific aptamers against one of the substrates of ESX-3, namely EsxG.

Aptamers are nucleic acid probes, which are known to bind and in some cases inhibit function of proteins [17–20]. The availability of such reagents will enable the investigation of the role of this protein in mycobacteria growth and/or iron acquisition.

2. Materials and methods

2.1. Expression of EsxG protein

EsxG from *M. tuberculosis* H37Rv was cloned into the bacterial vector pET41A (Eric Rubin, unpublished data). The vector was used to transform *Escherichia coli* BL21 (DE3) cells and recombinant clones were selected on Kanamycin (50 μg/ml). Expression was induced by addition of IPTG to a final concentration of 0.5 mM to the Luria broth (LB), followed by incubation at 30 °C for 4 h. The cells were harvested by centrifugation using the Sorvall RC-5B super speed centrifuge at 10,000 RPM for 20 min at 4 °C.

2.2. Purification of EsxG protein

The cells were lysed using B-PER (in phosphate buffer, 50 mM [pH 7.5]) bacterial protein extraction reagent (Thermo Fisher Scientific, Rockford, Illinois). The recombinant protein was purified using immobilised metal affinity chromatography (IMAC) packed with Protino® Ni-TED resin (Machery-Nagel, Düren, Germany) as previously described [21]. The purity of protein was assessed in a 12% SDS/PAGE gel stained with Coomassie blue and the identity of EsxG was confirmed using mass spectrometry and the *Thorough* search algorithm in Protein Pilot software (ABSCIEX, CA, USA). An identification confidence of 95% was selected during searches.

2.3. Western blotting

Proteins separated by SDS/PAGE were transferred to Hybond-P PVDF transfer membrane (GE Healthcare Lifesciences, Buckinghamshire, United Kingdom) with a Bio-Rad Transblot semi-dry transfer cell at 12 V for 2 h. To confirm the presence of the histidine-tagged EsxG protein the protein bands were detected using a 1:10,000 dilution of the penta·His-HRP conjugated antibody cat no. 34460 (Qiagen, Hilden, Germany). A pre-stained ladder was used for the Western blot and the detection was accomplished with a colorimetric HRP substrate, TMB (3,3′,5,5′-tetramethylbenzidine) (Thermo Fisher Scientific, Rockford, Illinois).

2.4. In vitro transcription and selection of RNA aptamers against EsxG protein using the NTA system on the Biacore 3000

In vitro transcription of the RNA aptamers was performed as previously described by Khati and colleagues [22]. The SELEX process was performed using surface plasmon resonance on the Biacore 3000 instrument. The HBS-P (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 0.005% Surfactant P20) continuous flow buffer (GE Healthcare Lifesciences, Ohio, USA) was used in all the Biacore experiments on the nitrilotriacetic acid (NTA) chips. Briefly, the surface of the NTA chip was activated as per suppliers' recommendation (GE Healthcare Life Sciences, Ohio, USA). Following surface activation, 30 μl of 200 nM of EsxG (His_6) was injected at 2 μl/min. The transcribed RNA pool (representing approximately 10^{14} unique molecules) was denatured at 95 °C for 4 min and refolded at room temperature in HMCKN buffer (10 mM Hepes [pH 7.4], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2.7 mM KCl, 150 mM NaCl) for an additional 4 min. The RNA pool was injected for 2 min into the flow cell at a flow rate of 20 μl/min. After each selection cycle the bound RNA was eluted from the NTA using the elution buffer (7 M urea, 5 mM EDTA). The recovered RNA was then converted to cDNA by reverse transcription using the RevertAid M-MuLV Reverse Transcriptase (Fermentas, USA) according to suppliers' protocol. The cDNA was amplified by PCR and later transcribed to RNA. After five rounds of selection, the enriched RNA pool was reverse transcribed; the resulting cDNA amplified by PCR and cloned into the TA cloning vector using the pGEM-T Easy cloning kit (Promega, Wisconsin, USA) as per manufactures instruction. The individual clones were screened for the binding to EsxG using the Biacore.

2.5. Binding assay using the amine coupling on the Biacore

A standard amine coupling protocol using the CM5 chip was followed according to manufacturer's instructions (Biacore, GE Healthcare Lifesciences, Ohio, USA). The Biacore 3000 biosensor instrument (GE Healthcare Lifesciences, Ohio, USA) flow rate was set at 10 μl/min and the carboxymethyl groups on the chip were activated by injecting 50 μl of 0.5 M *N*-hydroxysuccinimide (NHS), 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The protein (either EsxG or EsxA) was diluted into 10 mM sodium acetate, pH 4.5 to a final concentration of 2 μM and injected onto the activated surface, resulting in amine coupling of the protein to the activated surface. The remaining activated carboxymethyl groups were blocked by injecting 70 μl of 1 M ethanolamine–HCl, pH 8.0, which also helped elute non-covalently bound ligand.

2.6. Binding kinetics analysis using the Biacore

Affinity measurements were performed at room temperature (25 °C), which was the same temperature used during selection. EsxG was covalently immobilised on the chip (1000–1200 RU) using amine coupling chemistry. Each concentration in the dilution series (5–500 nM) of the aptamer (20 μl, starting with the lowest) was injected at 10 μl/min and allowed to dissociate over 600 s. To ensure that there was no residual RNA left on the surface before the next concentration injection, the ligand was regenerated by injecting 10 μl of freshly prepared 100 mM NaOH. Each experiment was performed in triplicate using three neighbouring flow cells, and the fourth flow cell served as a negative control. The negative control flow cell was subtracted as a baseline and the data analysed using the BIAevaluation 3.0 (Biacore, GE Healthcare UK Ltd, Buckinghamshire, England) and GraphPad Prism 5.0 (GraphPad software Inc., USA) software. The affinity constant was calculated based on a *Langmuir* one-on-one curve fit using the BIAevaluation 3.0.

3. Results

3.1. Assessment of EsxG expression

His-tagged EsxG was expressed in soluble form in *E. coli* cells and subsequently purified using IMAC. The concentration of the purified protein was approximately 300 μg/ml and the total yield was 0.6 mg/L of culture as quantified by the bicinchoninic acid (BCA) assay (Thermo Scientific, MA, USA). The apparent molecular mass of the purified EsxG was estimated at 11 kDa when compared to the protein standards (Biorad, CA, USA) on 12% SDS–PAGE gel (Fig. 1A), which is very close to the expected weight of 10.5 kDa. The presence of EsxG protein was validated by Western blot using the horseradish peroxidase-conjugated penta-histidine antibody (Fig. 1B). In addition, mass spectrometry-based peptide sequencing was used to confirm protein identity. The results from the gel digestion of the protein bands and an in-solution digest of the purified fraction identified EsxG with 100% and 89.3% sequence coverage, respectively. Taken together, these results indicate that EsxG was successfully purified using nickel chromatography.

3.2. Screening of monoclonal aptamers binding to the EsxG

To identify the successful isolation of aptamers against EsxG, the resulting PCR product from the fifth round of the SPR-based SELEX was cloned into pGEM-T Easy vector using TA cloning and used to transform DH5α *E. coli* cells. Fifty-seven individual clones were successfully sequenced. The aptamer clones were arbitrarily numbered, with the prefix G. Of the 57 successful sequences, five clones had identical sequences (G31 and G34; G36 and G71; G43 and G94; G70 and G76; G78 and G98), and the rest had no detectable similarity to each other. The individual aptamer sequences that were represented more than once during sequencing were screened for binding to the recombinant EsxG protein using amine coupling chemistry on the CM5 chip. Four of the five aptamers (Table 1) that were present more than once during sequencing were able to bind EsxG when coupled to the CM5 chip (Fig. 2). The two aptamers that showed the highest binding signal to EsxG during screening, G43 and G78, were further characterised for binding kinetics and specificity.

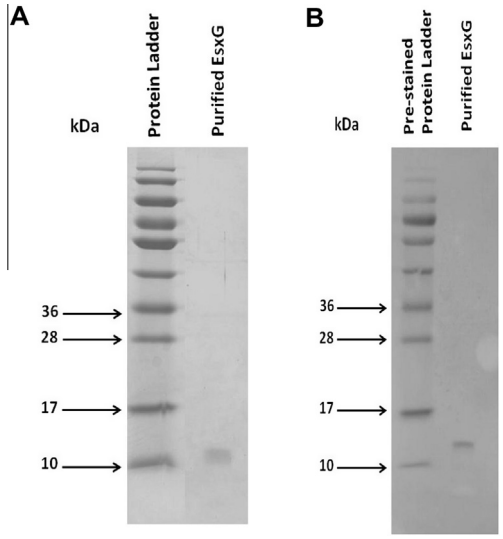

Fig. 1. Assessment of EsxG expression. (A) Coomassie stained 12% SDS–PAGE gel of purified EsxG after purification. (B) Western-blot of EsxG using the penta·His-HRP conjugated antibody. A pre-stained ladder was used for the Western blot and the detection was accomplished with a colorimetric substrate, TMB.

3.3. Assessment of aptamers G43 and G78 binding kinetics to EsxG

Binding kinetics to EsxG of the two selected aptamers was analysed using a Biacore instrument. Each concentration of the analyte (5–500 nM) was injected at 10 μl/min and allowed to dissociate over 600 s. The affinity constant was calculated based on a *Langmuir* one-on-one curve fit using the BIAevaluation 3.0 software. Representative curves of the kinetic analysis obtained from the Biacore are shown in Fig. 3A and B. The chi-square values for all the kinetic analyses were between 4.2 and 9.8. Typically, a chi-square value of less than 10 is indicative of good fitting to the model used, which means that the *Langmuir* model used for our aptamer kinetics study adequately describes our data [23,24]. The dissociation constants of the G43 and G78 aptamers were 8.04 ± 1.90 and 78.85 ± 9.40 nM, respectively. Thus, the affinity of aptamer G43 to EsxG was about ten times higher than the affinity of aptamer G78 (Fig. 3A and B).

3.4. Assessment of aptamersG43 and G78 binding to EsxA

To determine if the aptamers G43 and G78 are able to discriminate EsxG from its closely related protein, EsxA, their binding to both proteins was assessed using the Biacore. The ligand, EsxG or EsxA, was immobilised using amine coupling chemistry on a CM5 chip and 500 nM of each aptamer was injected over the immobilised proteins. The binding experiments were done in triplicate. A two-tailed, unpaired *t*-test was used to analyse significance in the difference between binding of the aptamers to EsxG and EsxA. Response units measurements taken 100 s after aptamer injection had ended, showed that both aptamers had significantly higher binding (G43: $p < 0.05$; G78: $p < 0.01$) to immobilised EsxG as compared to their binding to EsxA (Fig. 3C). While aptamer G43 showed no significant binding to EsxA, aptamer G78 showed some binding to EsxA, though a significantly lower binding signal was observed ($p < 0.01$) when compared to that of EsxG.

4. Discussion

While antibodies are the traditional molecular probes, aptamers have now been explored as alternative recognition molecules for basic research and diagnostic applications [25–27]. These artificial nucleic acid probes offer many advantages which include their ease of synthesis that does not require animals or cell lines, making them cheaper to produce when compared to antibodies [28]. Also, they can be potent inhibitors of protein function [29], which makes them applicable as ideal reagents to be used in drug development as well as pathway elucidation in scientific research. For instance, the RNA aptamer Macugen, an inhibitor of vascular endothelial growth factor (VEGF), is approved by the FDA for the treatment of age-related macular degeneration (AMD) [30].

In this study, we performed a surface plasmon resonance-based SELEX, to select RNA aptamers that bind to the ESX-3 secreted protein, EsxG. Our data shows that after five rounds of selection, we selected at least four RNA sequences that bind to EsxG. In this study, we employed the same protocol and library that was successfully used previously to identify RNA aptamers against the HIV g120 glycoprotein. [22]. Two of the aptamers selected in this study, G43 and G78, were analysed for equilibrium kinetics and had K_D in the nanomolar range. Aptamer G43 had a K_D of 8.04 ± 1.90 nM while G78 had a K_D of 78.85 ± 9.40 nM.

M. tuberculosis has five homologous T7S system [31,32]. ESX-1 is the most studied of these systems and is implicated in virulence of *M. tuberculosis* [15,16]. The ESX-1 secretion system secretes the EsxA protein which is homologous to the EsxG protein secreted by the ESX-3 system. To investigate whether the selected aptamers can dis-

Table 1
Sequences of selected aptamers.

Aptamer name	Sequence format	Nucleotide (nt) sequence
G43	T7 SELEX primer-(**50 random nucleotide sequence**)-T3 SELEX primer	TAATACGACTCACTATAGGGAGACAAGACTAGACGCTCAA (AATAGGAACTTAGAACAACCCTCTCCCTCATGTAGCGAACCCGAACCAGG) TTCGACATGAGACTCACAACAGTTCCCTTTAGTGAGGGTTAATT
G78		TAATACGACTCACTATAGGGAGACAAGACTAGACGCTCAA GCGTTTAAACGTTGCACCGTCGTTGACGGGACCGCTTGAGACATTCGCTC TTCGACATGAGACTCACAACAGTTCCCTTTAGTGAGGGTTAATT
G31		TAATACGACTCACTATAGGGAGACAAGACTAGACGCTCAA TAACGACTCACCAACACATCGACTAATTTCCCTAAATGACTTAACTGAAT TTCGACATGAGACTCACAACAGTTCCCTTTAGTGAGGGTTAATT
G70		TAATACGACTCACTATAGGGAGACAAGACTAGACGCTCAA ACTCGTGACACTTACGAACGACCTATGGCGGGAGAATCCTAGCCGCTACG TTCGACATGAGACTCACAACAGTTCCCTTTAGTGAGGGTTAATT

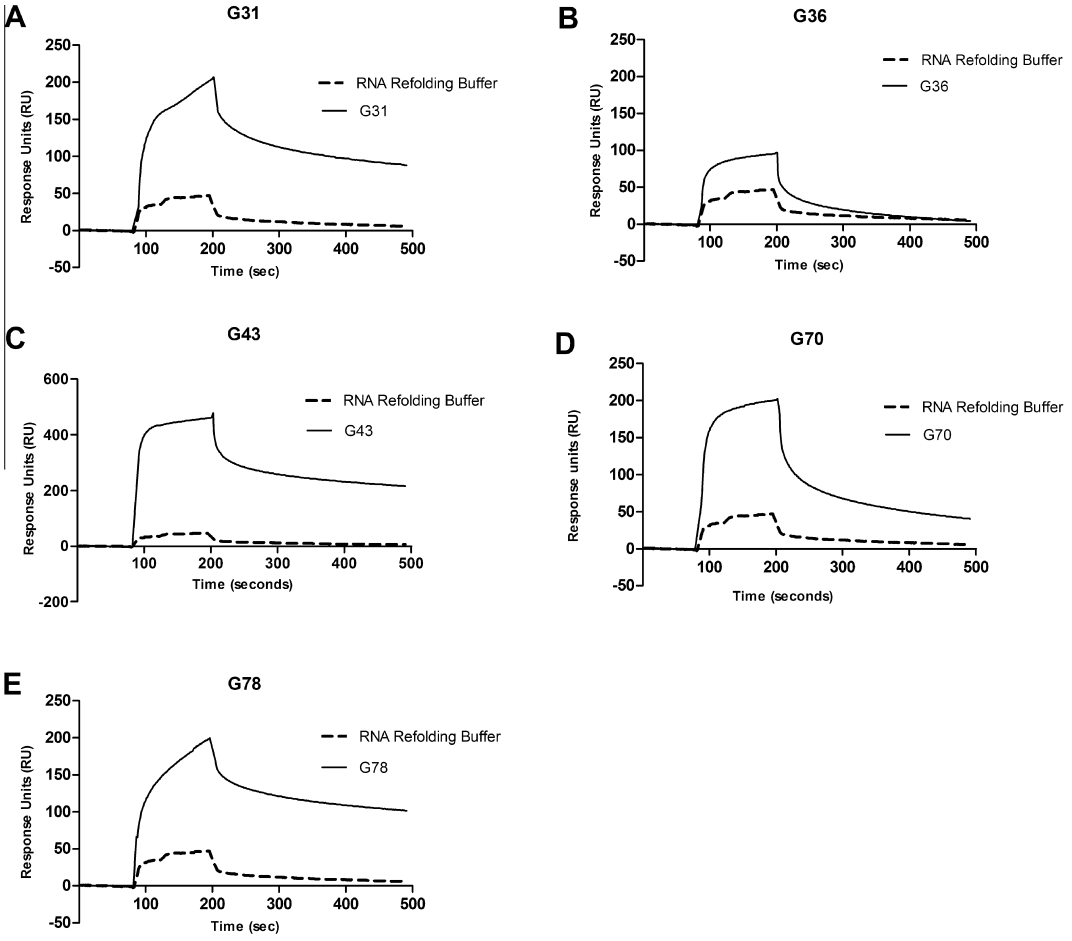

Fig. 2. Screening for binding of selected aptamers on the CM5 chip. Surface plasmon resonance sensorgram for EsxG interactions with representative aptamers of the pairs that were duplicated during sequencing (A–E). The thick black lines represent the aptamer binding to EsxG, and the dotted lines the buffer control.

criminate amongst closely related proteins, we evaluated the binding of these aptamers to EsxA. Our data showed that the selected aptamers have varying specificity for the homologous protein EsxA. That is, aptamer G43 did not bind significantly to EsxA, while aptamer G78 showed some binding to EsxA, even though this binding signal was significantly lower ($p < 0.01$) when compared to that of EsxG. Taken together, this data demonstrate that in addition to aptamers G43 and G78 discriminate recognition of their target, these aptamers also bind their target with high affinity, in the nanomolar range. Thus making these aptamers, suitable recognition probes to investigate the biological role of EsxG in *M. tuberculosis*.

Also, the availability of the aptamers selected in this study enables the investigation of EsxG as a potential biomarker for TB. Recent studies have demonstrated that the EsxA protein is a potential candidate in the diagnosis of TB. Hong and colleagues demonstrated the presence of EsxA in urine samples from TB patients [33]. Moreover, Rotherham and colleagues showed that DNA aptamers raised against the EsxA protein are able to detect infection in sputum samples with a sensitivity of 100% and a specificity of 68.75% [21]. Taken together, this indicates that a protein homologous to EsxG can be detected in clinical samples from infected patients. However, due to the unavailability of high affinity and

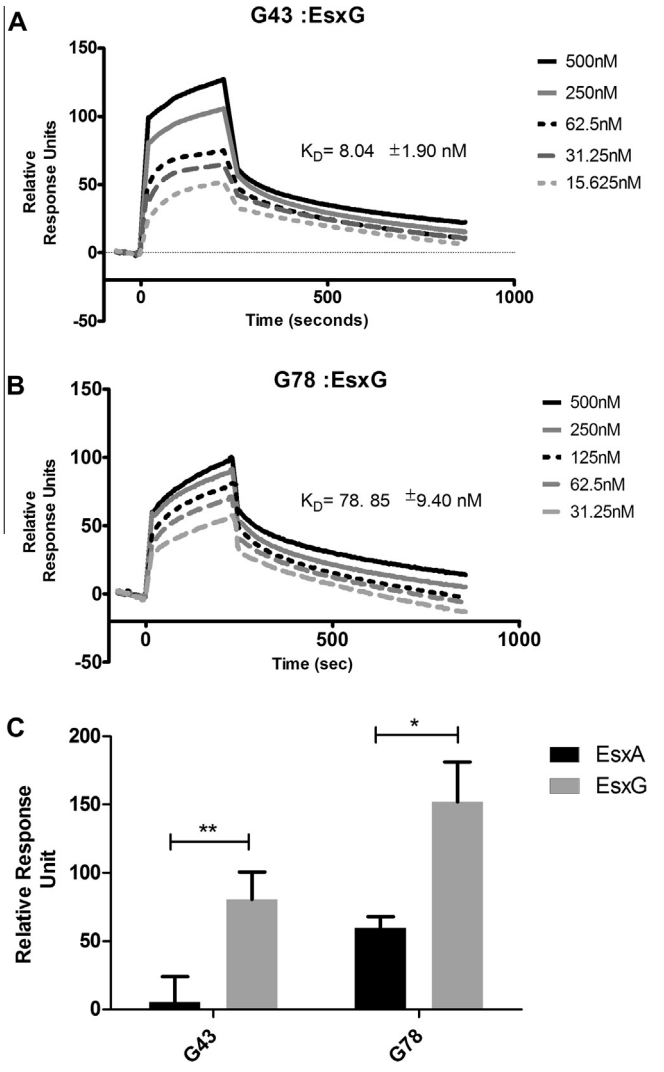

Fig. 3. Binding kinetics and specificity of EsxG aptamers G43 and G78. Equilibrium kinetics of RNA aptamers (A) G43 and (B) G78 binding to immobilised EsxG using the Biacore. Binding kinetic parameters were obtained using various concentrations of aptamer between 0 and 500 nM. The graphs are a representative and standard deviations were calculated from three experiments. (C) RNA aptamers discriminate amongst closely related proteins, EsxG and EsxA by Biacore. Relative response units were taken 100 s post injection of 500 nM G43 and G78 binding to immobilised EsxG and EsxA. A two-tailed, unpaired *t*-test was used to analyse significance ($^{**}p < 0.01$, $^{*}p < 0.05$).

specific detection probes for EsxG, this protein has not been investigated as a potential biomarker for TB diagnosis.

In conclusion, this is the first study to demonstrate the selection of RNA aptamers against a low abundance mycobacteria protein, EsxG. The selected aptamers have high affinity and specificity to their target EsxG. As a result, they can now be exploited to investigate the role of EsxG in the iron acquisition pathway of mycobacteria and potentially in TB diagnosis.

Author contributions

Conceived and designed the experiments: N.A.C.N., L.G., E.J.R., A.P., M.K. Performed the experiments: N.A.C.N., L.G. Analysed the data: N.A.C.N., L.G., E.J.R., A.P., M.K. Wrote the manuscript: N.A.C.N. Reviewed the manuscript: L.G., E.J.R., A.P., M.K.

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References

[1] World Health Organisation, Global Tuberculosis Control, WHO Report, 2012.
[2] S.T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S.V. Gordon, K. Eiglmeier, S. Gas, C.E. Barry 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M.A. Quail, M.A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J.E. Sulston, K. Taylor, S. Whitehead, B.G. Barrell, Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence, Nature 393 (1998) 537–544.
[3] M. Miethke, M.A. Marahiel, Siderophore-based iron acquisition and pathogen control, Microbiol. Mol. Biol. Rev. 71 (2007) 413–451.
[4] J.J. De Voss, K. Rutter, B.G. Schroeder, C.E. Barry 3rd, Iron acquisition and metabolism by mycobacteria, J. Bacteriol. 181 (1999) 4443–4451.
[5] J. Gobin, M.A. Horwitz, Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall, J. Exp. Med. 183 (1996) 1527–1532.
[6] D.K. Wong, J. Gobin, M.A. Horwitz, B.W. Gibson, Characterization of exochelins of *Mycobacterium avium*: evidence for saturated and unsaturated and for acid and ester forms, J. Bacteriol. 178 (1996) 6394–6398.
[7] G.M. Rodriguez, M.I. Voskuil, B. Gold, G.K. Schoolnik, I. Smith, ideR, An essential gene in *mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response, Infect. Immun. 70 (2002) 3371–3381.
[8] B. Gold, G.M. Rodriguez, S.A. Marras, M. Pentecost, I. Smith, The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages, Mol. Microbiol. 42 (2001) 851–865.
[9] A.M. Abdallah, N.C. Gey van Pittius, P.A. Champion, J. Cox, J. Luirink, C.M. Vandenbroucke-Grauls, B.J. Appelmelk, W. Bitter, Type VII secretion – mycobacteria show the way, Nat. Rev. Microbiol. 5 (2007) 883–891.
[10] A. Maciag, A. Piazza, G. Riccardi, A. Milano, Transcriptional analysis of ESAT-6 cluster 3 in *Mycobacterium smegmatis*, BMC Microbiol. 9 (2009) 48.
[11] M.S. Siegrist, M. Unnikrishnan, M.J. McConnell, M. Borowsky, T.Y. Cheng, N. Siddiqi, S.M. Fortune, D.B. Moody, E.J. Rubin, Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 18792–18797.
[12] A. Serafini, F. Boldrin, G. Palu, R. Manganelli, Characterization of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc, J. Bacteriol. 191 (2009) 6340–6344.
[13] K.M. Guinn, M.J. Hickey, S.K. Mathur, K.L. Zakel, J.E. Grotzke, D.M. Lewinsohn, S. Smith, D.R. Sherman, Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*, Mol. Microbiol. 51 (2004) 359–370.
[14] T. Hsu, S.M. Hingley-Wilson, B. Chen, M. Chen, A.Z. Dai, P.M. Morin, C.B. Marks, J. Padiyar, C. Goulding, M. Gingery, D. Eisenberg, R.G. Russell, S.C. Derrick, F.M. Collins, S.L. Morris, C.H. King, W.R. Jacobs Jr., The primary mechanism of attenuation of bacillus Calmette–Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 12420–12425.
[15] K.N. Lewis, R. Liao, K.M. Guinn, M.J. Hickey, S. Smith, M.A. Behr, D.R. Sherman, Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette–Guerin attenuation, J. Infect. Dis. 187 (2003) 117–123.
[16] A.S. Pym, P. Brodin, R. Brosch, M. Huerre, S.T. Cole, Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*, Mol. Microbiol. 46 (2002) 709–717.
[17] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566.
[18] I. Hirao, K. Madin, Y. Endo, S. Yokoyama, A.D. Ellington, RNA aptamers that bind to and inhibit the ribosome-inactivating protein, pepocin, J. Biol. Chem. 275 (2000) 4943–4948.
[19] A. Rhie, L. Kirby, N. Sayer, R. Wellesley, P. Disterer, I. Sylvester, A. Gill, J. Hope, W. James, A. Tahiri-Alaoui, Characterization of 2′-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion, J. Biol. Chem. 278 (2003) 39697–39705.
[20] D.M. Tasset, M.F. Kubik, W. Steiner, Oligonucleotide inhibitors of human thrombin that bind distinct epitopes, J. Mol. Biol. 272 (1997) 688–698.
[21] L.S. Rotherham, C. Maserumule, K. Dheda, J. Theron, M. Khati, Selection and application of ssDNA aptamers to detect active TB from sputum samples, PLoS ONE 7 (2012) e46862.

[22] M. Khati, M. Schuman, J. Ibrahim, Q. Sattentau, S. Gordon, W. James, Neutralization of infectivity of diverse R5 clinical isolates of human immunodeficiency virus type 1 by gp120-binding 2′F-RNA aptamers, J. Virol. 77 (2003) 12692–12698.
[23] F.A. Baglia, K.O. Badellino, D.H. Ho, V.R. Dasari, P.N. Walsh, A binding site for the kringle II Domain of prothrombin in the apple 1 domain of factor XI, J. Biol. Chem. 275 (2000) 31954–31962.
[24] Y. Liu, Y. Pan, Y. Xu, Binding investigation of integrin Î ± vÎ²3 with its inhibitors by SPR technology and molecular docking simulation, J. Biomol. Screen. 15 (2010) 131–137.
[25] S. Centi, S. Tombelli, M. Minunni, M. Mascini, Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads, Anal. Chem. 79 (2007) 1466–1473.
[26] E. Heyduk, T. Heyduk, Nucleic acid-based fluorescence sensors for detecting proteins, Anal. Chem. 77 (2005) 1147–1156.
[27] Y. Xiao, A.A. Lubin, A.J. Heeger, K.W. Plaxco, Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor, Angew. Chem. Int. Ed. Engl. 44 (2005) 5456–5459.
[28] S.E. Osborne, I. Matsumura, A.D. Ellington, Aptamers as therapeutic and diagnostic reagents: problems and prospects, Curr. Opin. Chem. Biol. 1 (1997) 5–9.
[29] P.K. Kumar, K. Machida, P.T. Urvil, N. Kakiuchi, D. Vishnuvardhan, K. Shimotohno, K. Taira, S. Nishikawa, Isolation of RNA aptamers specific to the NS3 protein of hepatitis C virus from a pool of completely random RNA, Virology 237 (1997) 270–282.
[30] J.H. Lee, M.D. Canny, A. De Erkenez, D. Krilleke, Y.S. Ng, D.T. Shima, A. Pardi, F. Jucker, A therapeutic aptamer inhibits angiogenesis by specifically targeting the heparin binding domain of VEGF165, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 18902–18907.
[31] N.C. Gey Van Pittius, J. Gamieldien, W. Hide, G.D. Brown, R.J. Siezen, A.D. Beyers, The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G + C Gram-positive bacteria, Genome Biol. 2 (2001) (RESEARCH0044).
[32] F. Tekaia, S.V. Gordon, T. Garnier, R. Brosch, B.G. Barrell, S.T. Cole, Analysis of the proteome of *Mycobacterium tuberculosis* in silico, Tuber. Lung Dis. 79 (1999) 329–342.
[33] S.C. Hong, J. Lee, H.-C. Shin, C.-M. Kim, J.Y. Park, K. Koh, H.-J. Kim, C.L. Chang, J. Lee, Clinical immunosensing of tuberculosis CFP-10 in patient urine by surface plasmon resonance spectroscopy, Sens. Actuators B Chem. 160 (2011) 1434.