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Improving coiled coil stability while maintaining specificity by a bacterial hitchhiker selection system

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ABSTRACT

The design and selection of peptides targeting cellular proteins is challenging and often yields candidates with undesired properties. Therefore we deployed a new selection system based on the twin-arginine translocase (TAT) pathway of *Escherichia coli*, named hitchhiker translocation (HiT) selection. A pool of α-helix encoding sequences was designed and selected for interference with the coiled coil domain (CC) of a melanoma-associated basic-helix-loop-helix-leucine-zipper (bHLHLZ) protein, the microphthalmia associated transcription factor (MITF). One predominant sequence (iM10) was enriched during selection and showed remarkable protease resistance, high solubility and thermal stability while maintaining its specificity. Furthermore, it exhibited nanomolar range affinity towards the target peptide. A mutation screen indicated that target-binding helices of increased homodimer stability and improved expression rates were preferred in the selection process. The crystal structure of the iM10/MITF-CC heterodimer (2.1 Å) provided important structural insights and validated our design predictions. Importantly, iM10 did not only bind to the MITF coiled coil, but also to the markedly more stable HLHLZ domain of MITF. Characterizing the selected variants of the semi-rational library demonstrated the potential of the innovative bacterial selection approach.

1. Introduction

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development and maintenance (McGill et al., 2002; Palmieri et al., 2009; Pogenberg et al., 2012). It is a member of the Mi/TFE sub-family network and shares a high degree of homology with three other transcription factors: transcription factor E3 (TFE3), transcription factor EB (TFEB) and transcription factor EC (TFEC) (Sardiello et al., 2009; Steingrímsson et al., 2004). All four proteins exhibit sequence similarities in their DNA binding domains and additionally display fairly conserved coiled coil motifs (Verastegui et al., 2000), resulting in specific heterodimerization potential. Generally, TFEB and TFE3 are involved in the biogenesis of lysosomal cell compartments, and malfunction of these factors is known to result in lysosomal disorders (Sardiello et al., 2009). Similarly, MITF is involved in melanogenesis and cell cycle control (McGill et al., 2002). It has been connected to melanocyte differentiation as it is involved in lineage commitment and melanoblast survival (Levy et al., 2010; Opdecamp et al., 1997; Palmieri et al., 2009; Widlund and Fisher, 2003). Mutations in the MITF gene are linked to various diseases, such as Waardenburg syndrome, and MITF is found to be overexpressed in several types of melanoma (Hughes et al., 1994; Tassabehji et al., 1994).

Abbreviations: bHLHLZ, basic helix-loop-helix-leucine-zipper; CC, coiled coil; CD, circular dichroism; HLHLZ, helix-loop-helix-leucine-zipper; HiT, hitchhiker translocation; iM6, interacting peptide of MITF-Clone #6; iM10, interacting peptide of MITF-Clone #10; ITC, isothermal titration calorimetry; LZ, leucine zipper; MITF, microphthalmia-associated transcription factor; ssTorA, signal sequence of trimethylamine oxide reductase; TAT, twin-arginine translocase.

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Although some studies have revealed that MITF is deregulated in several melanoma cell lines (Kido et al., 2009), it is still under debate whether MITF is a driving proto-oncogene (Palmieri et al., 2009; Wellbrock and Marais, 2005). Nevertheless, it is widely accepted that MITF plays a crucial role in melanoma survival (McGill et al., 2002).

Structurally, the transcription factor belongs to the group of basic helix-loop-helix leucine zipper (bHLHLZ) proteins (Pogenberg et al., 2012). This domain of approximately 13 kDa triggers homo and heterodimerization with other proteins of the same structural class and facilitates specific DNA binding (Steingrímsson et al., 1996; Xu et al., 2010). Approximately 15 residues are defined as the *basic region*, harboring several Arg and Lys residues that are important for DNA recognition and also function as a nuclear localization sequence (Krylov et al., 1997). The helix-loop-helix (HLH) sequence combines all necessary components to form a four-helix bundle with the HLH domain of the interaction partner and is thus involved in homo and heterodimer stabilization (Pogenberg et al., 2012). The leucine zipper (LZ) is mainly responsible for dimer recognition and specificity, whereas its precise contribution to the overall complex stability has not been sufficiently described. Nevertheless, several studies have discussed the role of leucine zippers in transcriptional control of bHLHLZ proteins (Morii et al., 2001).

Due to their importance in cellular signaling, leucine zipper-containing proteins have attracted attention and been exploited as biotechnological tools and therapeutics (Chen et al., 2011; Hagemann et al., 2008; Jouaux et al., 2008; Krylov et al., 1997; Mason et al., 2006). During the last decades, their modes of interaction have been well described experimentally, while computational design and prediction software provided further insight (Grigoryan and Keating, 2008; Mason and Arndt, 2004). Although the packing of a coiled coil appears relatively simple with its repetitive heptad repeat $(a–b–c–d–e–f–g)_n$ (Harbury et al., 1993; Mason and Arndt, 2004) and "knobs-into-holes" packing of the hydrophobic interface (Walshaw and Woolfson, 2003), it still remains difficult to rationally control oligomerization, affinity and specificity. Importantly, design of these peptides too often results in candidates with undesired properties, such as insolubility or promiscuous binding behavior (Schreiber and Keating, 2011).

To circumvent these problems, rational design has been combined with selection strategies to reach a new era of synthetic peptide development (Mason et al., 2006). However, depending on the desired properties of the peptides, the selection approaches as well as the library design have to be chosen carefully.

In contrast to approaches such as classical phage display that select for strong binders towards purified and immobilized targets (Bradbury and Marks, 2004; Speck et al., 2012), *in vivo* two-hybrid systems have the advantage of posing selection pressure within the crowded environment of the cell (Joung et al., 2000; Mason et al., 2006; Pellis et al., 2012). One example of a novel two-hybrid system is the hitchhiker translocation (HiT) system (Speck et al., 2012), allowing peptide selection towards affinity and specificity in the cytosol of *Escherichia coli*. This strategy was described before (Waraho and DeLisa, 2009) and further developed in our groups (Speck et al., 2012). Briefly, it constitutes an ampicillin resistance assay based on the fusion of the library helices to a reporter enzyme (β-lactamase) that conveys ampicillin resistance in the periplasm. However, the construct lacks the original signal peptide and requires a second component to be expressed in the cells for complementation. This is the designated target helix, fused to the signal sequence of trimethylamine oxide reductase (ssTorA) that mediates Tat-dependent translocation of folded peptides to the periplasm. Hence, a β-lactamase devoid of its original signal sequence can only facilitate resistance to applied antibiotics if the library peptide and the target helix interact and fold into a native complex. We could show that stronger peptide interactions correlate well with cell survival at higher ampicillin concentrations (Speck et al., 2012).

By using semi-rational libraries in combination with the HiT bacterial two-hybrid selection system, we aimed to engineer a peptide with high affinity and specificity towards the coiled coil domain of the melanoma relevant transcription factor MITF. The peptide library was based on MITF, as it forms homotypic interactions, and modified at key positions of the interaction interface. We previously presented an improved peptide (Interacting Peptide of MITF-Clone #6 [iM6]) gained from this selection (Speck et al., 2012). The winner peptide was further randomized at mainly *e* and *g* positions to generate a second library aiming for a new generation of improved binders. After several selection rounds, one peptide (iM10) was enriched and formed hetero-complexes with the target peptide with nanomolar K_D, whereas undesired binding towards other coiled coils was not observed. Importantly, iM10 formed tightly packed heterodimers with MITF and could thus protect the otherwise highly protease-labile coiled coil of MITF from proteinase K digestion. In summary, these data emphasize that HiT is a very convenient method as it is easy to use, fast, and has the additional feature of a TAT dependent folding control allowing for simultaneous selection of soluble, stable and specific target-binding peptides. Furthermore, we could confirm our semi-rational design strategy which combines prediction and selection to target coiled coil containing bHLHLZ proteins.

2. Materials and methods

2.1. Library cloning and selection

For the first library, two primers were ordered (Microsynth AG, Switzerland), randomized at defined positions with an overlapping region (Forward: GCATTAGCTAGCGCGAKCGTGGATTATATTCGCAA AVTCCAGCGCGAASAGCAGCGCVTCAAAGAACTGGAAAACCGC; reverse: CGATATGGCGCGCCGTGCGCGCGAABCTGCTGTTCCAGTTCCTGARYG CGCTGCAGCAGATGGCGGTTTGCATGTTCCAGTTTTTTCHGGCGGTTT TCCAGTTCTTT.) The second library was based on the selected peptide of the first selection (iM6) (Speck et al., 2012) and was also generated from overlapping oligonucleotides Microsynth AG, Switzerland) (Forward: GCATTAGCTAGCGCGATCGTGGATTAT VAACGCAAARTTCAGCGCRHAVAACAGCGCGTTAAAGAACTGGAAAAC; reverse: CGATATGGCGCGCCGTGCGCGCGAAYCTGCTGTTCCAGTTCC TGAGCGCGCTGTTBCAGATGGCGGTTCKYATGTTCCAGTTTTTTCAGTB TGTTTTCCAGTTCTTTAAC).

The double-stranded library fragments were generated by hybridizing the two overlapping oligonucleotides with a subsequent Klenow fill-in reaction using a thermal cycler (Hybaid Px2, Thermo Scientific): 25 pmol of each oligonucleotide, 2 µl Klenow Buffer (10×), 1 µl dNTPs of 10 mM stock solution were mixed in a final volume of 19.5 µl. After incubation at 94 °C for 3 min, the temperature was decreased to 37 °C at a rate of 0.5 °C s^{-1}. After reaching 37 °C, 0.5 µl Klenow fragment was added and the reaction mixture was incubated at 37 °C for 1 h. The products were gel-purified, digested with *Nhe*I and *Asc*I and cloned into the respective plasmids further described in (Speck et al., 2012). The library was cloned into the vector containing β-lactamase, whereas the wild-type coiled coil sequence was fused to the TorA sequence (for details see Table S1 in the supplement and (Speck et al., 2012)). Subsequently, competent XL-1 blue *E. coli* cells were co-transformed with both vectors and plated on LB agar supplemented with Kan_{50} (50 µg ml^{-1} Kan), Cm_{25} (25 µg ml^{-1} Cm) and 1% glucose to suppress expression leakage. Sufficient amounts of cells were collected to assure a 7 to 10-fold oversampling of the library. The pool was again plated on 0.5 mM IPTG, Kan_{50} and Cm_{25} and increasing ampicillin

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