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Destabilization domain approach adapted for regulated protein expression in the protozoan parasite *Entamoeba histolytica*

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ABSTRACT

A plethora of information has been gained by sequencing the genome of the human parasite *Entamoeba histolytica*, however a lack of robust genetic tools hampers experimental elucidation of gene functions. We adapted the destabilization domain approach for modulation of protein levels in *E. histolytica* using the destabilization domains of FK506 binding protein (ddFKBP) and dihydrofolate reductase (ddDHFR), respectively. In our studies, the ddFKBP appears to be more tightly regulated than ddDHFR, with minimal detectable protein in trophozoites in the absence of the stabilizing compound. The on- and off-rate kinetics for ddFKBP were rapid, with stabilization and degradation within 3 h of addition or removal of stabilizing compound, respectively. The kinetics for ddDHFR was different, with rapid stabilization (within 3 h of stabilizing compound being added) but much slower degradation (protein not destabilized until 24 h after compound removal). Furthermore, we demonstrated that for the ddFKBP, the standard stabilizing compound Shield-1 could be effectively replaced by two cheaper alternatives (rapamycin and FK506), indicating that the more cost-effective alternatives are viable options for use with *E. histolytica*. Thus, the destabilization domain approach represents a powerful method to study protein functions in *E. histolytica* and adds to the catalog of genetic tools that could be used to study this important human pathogen.

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1. Introduction

Entamoeba histolytica is a protozoan parasite and the causative agent of amebiasis, a major health problem affecting 50 million people and causing an estimated 100,000 deaths annually (WHO, 1997). Despite its global importance, insufficient data are available on the molecular basis of amebic pathogenesis. The plethora of available information from *E. histolytica* genome sequencing still requires further experimental validation of annotated gene functions (Loftus et al., 2005). While genetic tools for expression of amoebic or exogenous proteins have been developed for *Entamoeba* spp., due to variable number of nuclei, polyploidy and lack of homologous recombination in *E. histolytica* (Lopez-Revilla and Gomez, 1978; Marquez-Monter et al., 1990; Willhoeft and Tannich, 1999), knock-out technology is not currently feasible. Multiple gene knockdown approaches have been developed including regulated antisense gene expression (Sahoo et al., 2003), a double-stranded (ds)RNA-based silencing method (Kaur and Lohia, 2004), and a number of RNA interference (RNAi)-based methods (Vayssie et al., 2004; Abed and Ankri, 2008; Solis and Guillen, 2008; Linford et al., 2009; Morf et al., 2013). However, specific tools for modulation of protein abundance that could assist in elucidation of protein functions have not yet been developed.

Destabilization domain (DD)_technology enables regulation of gene product at the protein level. In the DD approach, the gene of interest is coupled to a DD, which leads to degradation of the fused protein by the proteasome (Banaszynski et al., 2006; Sellmyer et al., 2009; Egeler et al., 2011). In the presence of a stabilizing compound, the DD changes its structure, leading to a stable fusion protein (Banaszynski et al., 2006; Egeler et al., 2011). The DD is based on the mutated protein versions of FK506 binding protein (ddFKBP; Banaszynski et al., 2006) and dihydrofolate reductase (ddDHFR; Iwamoto et al., 2010; Muralidharan et al., 2011). The stabilizing compound can be Shield-1, rapamycin or FK506 for the ddFKBP system, and Trimethoprim (TMP) for the ddDHFR system. Recently, a new DD system was established based on the oestrogen receptor with one of two synthetic ligands, CMP8 or 4-hydroxytamoxifen, as a stabilizing compound (Miyazaki et al., 2012).

DD approaches have been tested as fusions to diverse proteins such as kinases, cell cycle regulation proteins and small GTPases,

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suggesting broad applicability (Banaszynski et al., 2006). Additionally, this approach has been applied to a wide variety of different organisms including the apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii*, and the kinetoplastids *Leishmania* spp. and *Trypanosoma cruzi* (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Herm-Gotz et al., 2007; Madeira da Silva et al., 2009; Muralidharan et al., 2011; Ma et al., 2012).

Here, we explore the DD approach as a method for genetic manipulation of regulated protein expression in *E. histolytica*. We show that regulated protein levels are achievable and we characterise the kinetics and efficiency of DD-induced protein degradation. Importantly, we determine that in *E. histolytica* trophozoites the ddFKBP approach is more tightly regulated than ddDHFR, with almost no stable protein detectable in trophozoites with the ddFKBP in the absence of a stabilizing compound. We also confirmed that stabilizing compound Shield-1 can be effectively replaced by the cheaper alternatives rapamycin and FK506. Furthermore, we determined that the two DD approaches showed different off-rate kinetics in *E. histolytica*. Overall we expect that this approach will expand the arsenal of tools for genetic manipulation in *E. histolytica*.

2. Materials and methods

2.1. Plasmid generation

Constructs were generated to express fusion proteins of yellow fluorescent protein (YFP) with N- and C-terminal DDs based on mutated ddFKBP and ddDHFR and a haemagglutinin (HA) tag (Fig. 1). The YFP, DD and HA inserts were directly amplified and cloned from mammalian over-expression plasmids provided by the Wandless group (Rakhit et al., 2011) into the *Nhe*I site of the *Entamoeba* over-expression plasmid pKT-3M (Saito-Nakano et al., 2004) and named pE8 to pE11. In order to generate a ddDHFR-YFP-HA expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE8 using the forward primer OLE12 and the reverse primer OLE13, and cloned into pKT-3M using the *Nhe*I site (Saito-Nakano et al., 2004), resulting in plasmid pE19 (Fig. 1). To generate an HA-YFP-ddDHFR expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE9 using the forward primer OLE14 and reverse primer OLE15, and cloned into pKT-3M using the *Nhe*I site (Saito-Nakano et al., 2004), resulting in plasmid pE20 (Fig. 1). To generate a ddFKBP-YFP-HA expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE10 using the forward primer OLE16 and the reverse primer OLE17, and cloned into pKT-3M using the *Nhe*I site (Saito-Nakano et al., 2004), resulting in plasmid pE21 (Fig. 1). To generate an HA-YFP-ddFKBP expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE11 using the forward primer OLE18 and reverse primer OLE19, and cloned into pKT-3M using the *Nhe*I site (Saito-Nakano et al., 2004), resulting in plasmid pE22 (Fig. 1). Correct orientation of all inserts was confirmed by sequencing. Sequences of all primers are listed in Table 1.

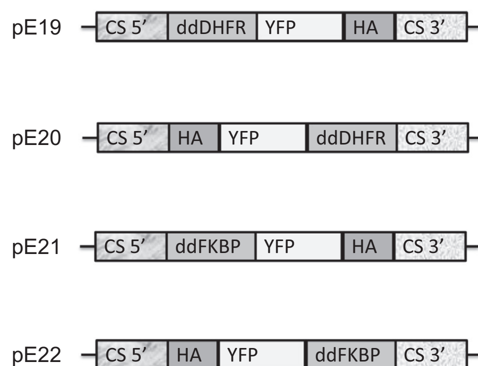

Fig. 1. Establishment of a destabilization domain approach in *Entamoeba histolytica.* (A) Generation of plasmids for establishment of destabilization domain in *E. histolytica* with yellow fluorescent protein (YFP) fused to a destabilization domain and tagged with N or C-terminal haemagglutinin (HA) tags. Plasmids for mammalian expression were obtained from Banaszynski et al. (2006) and Iwamoto et al. (2010), and modified to generate plasmids pE19-pE22 which were used for testing the destabilization domain approach in *E. histolytica*. Cysteine synthase regulatory regions were used to drive protein expression. ddDHFR, destabilization domain based on mutated dihydrofolatereductase; ddFKBP, destabilization domain based on mutated FK506 binding protein.

2.2. Parasite culture and transfection

For generation of transgenic parasites, *E. histolytica* strain HM-1:IMSS trophozoites were transfected using a previously published protocol (Baxt et al., 2010). Briefly, trophozoites were seeded in 35 mm Petri dishes and transfected with 10–20 μg of plasmid DNA using SuperFect (Qiagen, USA) reagent. The transfected parasites were allowed to grow for 24 h, and drug selection started at 1 μg/ml of G418 drug selection and then increased in a stepwise manner to 6 μg/ml of G418 until a stable cell line was achieved. All experiments were done with trophozoites grown at 12–48 μg/ml of G418.

2.3. Western blot analysis

Western blot analysis was performed as described previously (Pearson et al., 2013). Briefly, parasites were harvested, washed once and lysed in lysis buffer containing different protease inhibitors. Lysates (40–80 μg of total protein) were subjected to 12% SDS–PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked, incubated in primary anti-HA antibody (Cell Signaling, USA) (1:1,000 dilution) overnight at 4 °C and secondary HRP-conjugated anti-mouse antibody (Jackson Immuno Research, USA) (1:10,000 dilution) for at least 1 h at room temperature before detection with ECL+ (GE Healthcare, USA) on the Kodak Image Station 4000R (Kodak, USA) or with film (GE Healthcare). After mild stripping, the same membrane was used to detect actin using a pan-specific mouse anti-actin antibody (MP Biomedicals, USA) (1:1,000 dilution) for at least 1 h at room temperature and the secondary HRP-conjugated anti-mouse antibody (Jackson Immuno Research) (1:10,000 dilution) for at least 1 h at room temperature.

Table 1
Oligonucleotides for cloning of yellow fluorescent protein (YFP) fused to destabilization domain (DD) (YFP-DD) to generate plasmids pE19-pE22 in this study.

Primer name	Sequence	Resulting construct
OLE12	ATG<u>GCTAGC</u>GCCACCATGATCAGTCTGATT	ddDHFR-YFP-HA
OLE13	CGG<u>GCTAGC</u>TCATGCGTAGTCTGGTACG	ddDHFR-YFP-HA
OLE14	GCC<u>GCTAGC</u>GCCACCATGTATCCGTACG	HA-YFP-ddDHFR
OLE15	GTA<u>GCTAGC</u>GGTCATCGCCGCTCCAG	HA-YFP-ddDHFR
OLE16	GCC<u>GCTAGC</u>GCCACCATGGGAGTGCAG	ddFKBP-YFP-HA
OLE17	GCC<u>GCTAGC</u>TTAGTCGAGTGCGTAGTCTGG	ddFKBP-YFP-HA
OLE18	GCC<u>GCTAGC</u>GCCACCATGTATCCGTAC	HA-YFP-ddFKBP
OLE19	GCC<u>GCTAGC</u>TCATTCCAGTTCTAGAAGCTC	HA-YFP-ddFKBP

The *Nhe*I restriction site is underlined.
ddDHFR, destabilization domain based on mutated dihydrofolate reductase; ddFKBP, destabilization domain based on mutated FK506 binding protein.

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