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Expression, fermentation and purification of a predicted intrinsically disordered region of the transcription factor, NFAT5

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ABSTRACT

Hypertonicity stimulates Nuclear Factor of Activated T-cells 5 (NFAT5) nuclear localization and transactivating activity. Many transcription factors are known to contain intrinsically disordered regions (IDRs) which become more structured with local environmental changes such as osmolality, temperature and tonicity. The transactivating domain of NFAT5 is predicted to be intrinsically disordered under normal tonicity, and under high NaCl, the activity of this domain is increased. To study the binding of co-regulatory proteins at IDRs a cDNA construct expressing the NFAT5 TAD was created and transformed into *Escherichia coli* cells. Transformed *E. coli* cells were mass produced by fermentation and extracted by cell lysis to release the NFAT5 TAD. The NFAT5 TAD was subsequently purified using a His-tag column, cation exchange chromatography as well as hydrophobic interaction chromatography and then characterized by mass spectrometry (MS).

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1. Introduction

Nuclear Factor of Activated T-cells 5 (NFAT5) is an osmoregulatory transcription factor that is bidirectionally regulated by change in tonicity, such as variation in NaCl [2,14]. Hypertonicity activates NFAT5 by increasing its RNA and protein abundance [9,10], its nuclear localization [9,10] and its transactivating activity [6]. Hypertonic activation of NFAT5 increases the RNA and protein abundance of its target genes including those proteins that synthesize or transport multiple organic osmolytes, stabilize other proteins, or function in water balance [2]. The result is protection of cells from the deleterious effects of hypertonicity found, in particular, in the normal functioning of the kidney medulla. NFAT5 also has far reaching roles in immunity [3], carcinoma invasion [4], and atherosclerosis [8] that apparently, are independent of its response to tonicity.

High NaCl increases both the activity and phosphorylation of the transactivation domain (TAD) located between amino acids 872-1271 of NFAT5 [6]. As is characteristic of all transcription factors, the TAD of NFAT5 is the region where co-regulatory proteins, co-activators or -repressors, would be expected to bind and as such the TAD is critical to NFAT5 function.

Transcription factors contain intrinsically disordered (ID) regions where binding interactions that control transcription occur. Binding of co-regulatory proteins at ID regions accompanies transition from disorder to order and is critical to initiate and sustain transcriptional activity [5,7,12]. The TAD region of NFAT5 is predicted to be intrinsically disordered (GlobPlot 2 Version 2.3 (http://globplot.embl.de/) (Algorithm: Lusselle/Linding definition) but, currently, there is no published crystal structure. This prompted our purification of the NFAT5 TAD for structural and binding interaction studies.

A cDNA construct expressing the NFAT5 TAD was created and transformed into *Escherichia coli* cells. Transformed *E. coli* cells were mass produced by fermentation and extracted by cell lysis to release the NFAT5 TAD. The NFAT5 TAD was subsequently purified using a His-tag column, cation exchange chromatography as well as hydrophobic interaction chromatography and then characterized by mass spectrometry (MS).

2. Materials and methods

2.1. Plasmids, DNA constructs and oligonucleotides

pET-41b was purchased from EMD Millipore and modified to create pET-41b-MBP-AcTEV-872-1271 F1005W AcTEV (pET-MBP-872-1271 and Fig. 1). Human NFAT5 cDNA clone KIAA0827 was a gift from Dr. Takahiro Nagase (Kazusa DNA Research Institute,

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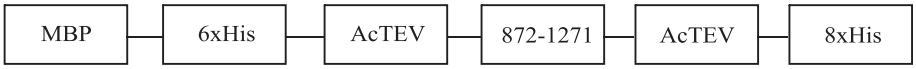

Fig. 1. Sequence map for pET-MBP-872-1271.

ENTLSNQQQQQQQQQQVMESSAAMVMEMQQSICQAAAQIQSELFPSTASANGNLQQSP
VYQQTSHMMSALSTNEDMQMQCELFSSPPAVSGNETSTTTTQQVATPGTTMFQTSSSG
DGEETGTQAKQIQNSVWQTMVQMQHSGDNQPQVNLFSSTKSMMSVQNSGTQQQGNG
LFQQGNEMMSLQSGNFLQQSSHSQAQLFHPQNPIADAQNLSQETQGSLFHSPNPIVHSQT
STTSSEQMQPPMFHSQSTIAVLQGSSVPQDQQSTNIFLSQSPMNNLQTNTVAQEAFFAAP
NSISPLQSTSNSEQQAAFQQQAPISHIQTPMLSQEQAQPPQQGLFQPQVALGSLPPNPMPQ
SQQGTMFQSQHSIVAMQSNSPSQEQQQQQQQQQQQQQQQQQQSILFS

NFAT5 872-1271: 44786.77 Daltons

Fig. 2. Amino acid sequence of NFAT5 872-1271.

Chiba, Japan). All cDNA inserts used in cloning were generated using GeneArt Gene Synthesis (Life Technologies, Carlsbad, CA).

Sequence coding for maltose binding protein (MBP, pMAL-p5E, New England Biolabs) was cloned into pET-41b using the NdeI and SpeI restriction sites. MBP insertion replaced sequence coding for glutathione-S-transferase (GST) but retained that coding for a 6× His tag. Then, sequence coding for AcTEV protease binding site followed by amino acids 872-1271 of NFAT5 protein (KIAA0827) with an F1005W mutation (TTT to TGG), and a second AcTEV protease binding site was inserted between the EcoRV and XhoI restriction sites. A second His tag (8×) was retained as found in pET-41b between the XhoI and AvrII restriction sites. All constructs were generated by using standard cloning procedures and verified by restriction enzyme digestion and DNA sequencing (GeneWiz, Frederick, MD).

2.2. Bacterial expression and fermentation

pET-MBP-872-1271 was transformed into HMS174 *E. coli* competent cells (EMD4Biosciences, USA). 20 ng of plasmid was added to 50 μL competent cells in a 1.5 mL micro-tube and incubated on ice for 10 min. The cell mix was heat-pulsed in a 42 °C water bath for 90 s and plated on LB agar with 50 mg/L kanamycin. A single colony was chosen from the plate after overnight incubation at 37 °C, and inoculated into 100 mL modified LB[1] with 50 mg/L kanamycin in a 500 mL baffled shake flask. Following incubation at 37 °C 220 rpm overnight, the culture was used to inoculate the large fermenter.

A 14 L BioFlo 110 fermenter (New Brunswick Scientific, Edison, NJ) with a working volume of 10 L was used for large-scale fermentation. The fermenter was batched with 10 g/L tryptone, 5 g/L YE, 5 g/L NaCl, 3.5 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 12 g/L K_2HPO_4, 50 mg/L kanamycin, pH 7.0. Temperature was controlled at 37 °C, pH was controlled at 7.0 with 30% NH_4OH, and dissolved oxygen (DO) was controlled at 30%. BioCommand Plus software from NBS was used for data collection. Cells were induced with 0.5 mM IPTG at 37 °C when OD_{600} reached above 3.0. Addition of 2.4 g/L of glucose was added at the time of induction and one hour later. 2–3 h after induction, cells were harvested using a Sharple continuous centrifuge at a flow rate of 250 mL/min. Cell pellets were stored at −80 °C until protein purification.

2.3. Cell lysis of pET-MBP-872-1271

Frozen pellets of *E. coli* cells (10 g) were suspended in 100 mL 25 mM NaPi, pH 7.4, 150 mM NaCl, 1 mM EDTA buffer containing a cocktail of protease inhibitors (Complete, EDTA, Roche, USA) and lysed using a microfluidizer at 17,000 psi (2 cycles) at 4 °C. The cell lysate was centrifuged (19,000 rpm for 35 min) and the supernatant was discarded. The pellets were then re-suspended with 25 mM NaPi, pH 8.0, and 1 mM EDTA buffer (native buffer) and lysed again using a microfluidizer at 17,000 psi. The lysate was centrifuged (19,000 rpm for 35 min) to remove debris and the resulting supernatant was retained for cleavage by protease AcTEV. Each step of lysis was analyzed using SDS–PAGE.

2.4. Proteolytic cleavage of pET-MBP-872-1271

The enzymatic cleavage of pET-MBP-872-1271 was completed with 1 unit of AcTEV per 10 units (29 mg) of pET-MBP-872-1271 in 25 mM NaPi, pH 8.0, 1 mM EDTA buffer, containing a cocktail of protease inhibitors (Complete, EDTA, Roche, USA) and incubated at 4 °C overnight with rocking to give NFAT5 872-1271 (Fig. 2).

2.5. Purification of 872-1271

Digested samples were passed through a 0.22 μm filter and mixed with pre-equilibrated Ni Sepharose Excel resin (GE Health Care Life Sciences, Pittsburgh, PA) to remove the free pET-MBP-6× His-tag and 8× His-tag, and shaken (180 rpm) for 2 h at 6 °C. The resin was first washed with 10 column volumes (CVs) of 25 mM NaPi, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM Imidazole buffer (W1), followed by 10 CVs of 25 mM NaPi, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM Imidazole buffer (W2). The His-tag was eluted with 5 CVs of 25 mM NaPi, pH 7.4, 150 mM NaCl, 1 mM EDTA, 250 mM Imidazole buffer. All fractions were analyzed using SDS–PAGE.

Fractions containing 872-1271 (W1) were dialyzed into 25 mM NaPi, pH 7.4, 1 mM EDTA, filtered through a 0.22 μm filter and loaded onto a pre-equilibrated chromatography column containing DEAE resin (TOSOH, TSK-gel, 13 μm, 21.5 mm × 15 cm) to remove remaining MBP-His-tag as well as remaining impurities with a flow rate of 2.0 mL/min and a 0–600 mM NaCl gradient over 60 min. The flow through was concentrated (30×) using a 10 kDa centrifugal filter unit (Amicon Ultra-15, Merck Millipore, Billerica, MA). 600 mM NaCl was added to the 30× concentrated protein and the soluble protein was loaded onto a chromatography column containing Phenyl-5PW resin (TSK, 10 μm, 7.5 mm × 7.5 cm) to complete purification with a flow rate of 0.5 mL/min and a 0.6–0 mM NaCl over 60 min and 0.5 mL fractions were collected and analyzed with SDS–PAGE. Fractions containing NFAT5 were buffered (25 mM NaPi, pH 7.4, 1 mM EDTA), concentrated (10×) using a 10 kDa centrifugal filter unit (Amicon Ultra-0.5 Merck Millipore, Billerica, MA) and dialyzed into 25 mM NaPi, pH 7.4, 1 mM EDTA

[1] Formula for modified LB: 10 g/L tryptone, 5 g/L YE, 5 g/L NaCl, 3.5 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 12 g/L K_2HPO_4, pH 7.0.