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A fast and simple method to prepare the FKBP-rapamycin binding domain of human target of rapamycin for NMR binding assays

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Abstract

Mammalian target of rapamycin (TOR) controls cell growth and metabolism in response to the availability of nutrients, growth factors, and the cellular energy status. Misregulation of TOR can result in a pathogenic increase or decrease in organ size and in cancer. TOR can be inhibited by binding of a complex of rapamycin and FKBP to the FKBP-rapamycin binding (FRB) domain. Rapamycin and derivatives of it have been used as immunosuppressive drugs. Because TOR is further an interesting drug target in cancer research, we established an expression, purification, and refolding protocol for the FRB domain of human TOR (hFRB). hFRB is extracted from inclusion bodies, purified by reversed phase HPLC, and refolded by drop-wise dilution of the denatured protein into a native buffer. The procedure is very simple and can easily be scaled up to prepare large amounts of functional protein for high-throughput cancer drug screening assays by NMR and other techniques.

Keywords: Target of rapamycin; TOR; FKBP; Refolding; Inclusion body; HPLC; NMR

The target of rapamycin (TOR)[1] is a highly conserved eukaryotic ser/thr kinase that controls cell growth in response to nutrient availability and growth factor signals [1,2]. TOR intercepts several different signaling pathways [3,4] and misregulation of these pathways is found in many types of human disorders and cancer [5–9]. Human TOR is 2549 residues long and consists of several functional domains [2]. The N-terminal region contains several HEAT repeats [10] followed by the FAT domain [11] which was suggested to be composed of an α-helical repeat motif. Both regions are assumed to mediate protein–protein interactions [10,11]. The following FKBP-rapamycin domain (FRB) is about 100 amino acids long and is next to the catalytic ser/thr kinase domain, which encompasses about 250 residues. The C-terminal FATC domain always occurs in tandem with the FAT domain and has been shown to influence TOR-stability through its redox state [12]. TOR has been shown to participate in two different complexes, called TORC1 and TORC2 [13]. TORC1 is sensitive to the inhibitor rapamycin and controls cell growth by regulating processes such as ribosome biogenesis, autophagy, transcription and translation [13]. TORC2 is insensitive to rapamycin and regulates the reorganization of the cytoskeleton [14].

Because mTOR has emerged as a central target for anticancer drugs, there is an increasing interest in finding new small molecules that act as mTOR inhibitors. The FRB domain is the target for the immunosuppressant inhibitor complex FKBP-rapamycin and the structurally best characterized functional unit of TOR. Crystal structures of the FRB domain in complex with FKBP-rapamycin [15,16] and of the free form [17] have been described. Recently, the structure of the free FRB domain and its interaction with new potential inhibitors [18,19] and phosphatidic acid [19] have been characterized by NMR. Because the published

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[1] *Abbreviations used:* FKBP, FK506 binding protein, here equivalent to *Sacharomyces cerevisiae* FPR1; GdmCl, guanidinium hydrochloride; GST, glutathione transferase; hFRB, FKBP-rapamycin binding domain of human TOR; min, minute(s); RP HPLC, reversed phase high-performance liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; (m)TOR, (mammalian) target or rapamycin.

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purification protocols are lengthy and have protein at the same time in the soluble and the inclusion body fractions and employ proteolytic digestion [18,20] or leave a non-native N-terminal 26 residue stretch including a 6× His tag [21], we developed a new purification protocol that includes expression of the full human FRB domain (2014–2114 = hFRB) without a fusion-partner, extraction of the protein from inclusion bodies, purification by RP HPLC, and a simple refolding procedure based on drop-wise dilution of the denatured protein into a native buffer. The final refolding buffer is simple (20 mM Tris, 1 mM TCEP, 300 mM NaCl, pH 8) and should not interfere with binding of the FRB domain to inhibitors or cellular binding partners. The procedure can be easily scaled up, which makes the FRB domain amenable for high-throughput drug screening by NMR and other techniques.

Materials and methods

Plasmid cloning

The region encoding for human the TOR FRB domain (hFRB, residues 2014–2114) was amplified by PCR using as a template, a plasmid encoding full-length human TOR (gift from Prof. Hall, Biozentrum, Univ. Basel) and the forward primer 5′CGGAATTC**CATATG**GAGGAGCTGAT CCGAGTG3′ including and NdeI site (bold) and the reverse primer 5′GCGC**GGATCC**CTACTGCTTTGAGATTCGT CGG3′ including a BamHI site (bold). The used Taq Polymerase was obtained from Sigma and the 10 mM dNTP mix from Roche. The respective PCR product was inserted into the TOPO TA cloning® vector pCR®2.1-TOPO® (Invitrogen) which allowed further amplification. The respective amplified plasmid encoding hFRB was digested with NdeI and BamHI and ligated into accordingly digested pET-11a expression plasmid (Novagen) using T4 Ligase (Promega). Isolation of plasmid DNA was carried out using the QIAprep® Spin Miniprep Kit (Qiagen). For all cloning steps TOP10 cells (Invitrogen) were used. The authenticity of the obtained DNA construct was confirmed by DNA sequencing. The expression plasmid for the FKBP-homolog FPR1 from *Sacharomyces cerevisiae* (in pGEX-4T) was a gift from Prof. Hall (Biozentrum, University of Basel).

Physicochemical parameters of hFRB (UniProt Accession No. P42345)

$M_w = 12212.9$ Da, p$I = 6.10$, $\varepsilon_{280nm} = 29160\ M^{-1}\ cm^{-1}$.

Expression of hFRB

hFRB was expressed from *Escherichia coli* Rosetta (DE3) cells (Novagen). One hundred milliliters of LB medium supplemented with 100 μg/ml ampicillin and 40 μg/ml chloramphenicol were inoculated with a single colony of transformed cells and grown overnight at 30 °C. The starter culture was diluted 1:40 in 2 L LB medium or 1:20 in 2 L M9 minimal medium, both supplemented with 100 μg/ml ampicillin and 40 μg/ml chloramphenicol. For growth in minimal medium the cells were centrifuged gently for 5 min at 2000 rpm. The supernatant was discarded and the cells were resuspended in minimal medium. The prepared culture was grown at 37 °C to an OD_{600} of ≈0.8 and induced with 1 mM IPTG. The cells continued growing overnight at 37 °C. Uniformly ^{15}N-labeled hFRB was prepared in minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The used M9 minimal medium contained M9 salts w/o NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1× BME vitamin solution (Sigma), 4 g/L glucose, and 1 g/L $^{15}NH_4Cl$.

Protein extraction and purification of hFRB

Cells expressing hFRB were harvested by centrifugation at 4 °C (20 min, 5000 rpm, Sorvall SLA-3000 rotor). The cell pellet was resuspended in 50 ml lysis buffer (50 mM Tris, 2 mM EDTA, 2 mM benzamidine, 2 mM DTT, pH 8). The cell suspension was supplemented with 1 ml of lysozyme stock solution (10 mg/ml) and incubated for 10 min at room temperature. Cells were disrupted by sonication on ice using a Branson Digital Sonifier and a power level of 40%. During sonication for 6 min, intervals of 3 s pulsing alternated with 7 s breaks. To verify that hFRB is in the inclusion body fraction 100 μl of the sonicated cell suspension were centrifuged and the supernatant and the pellet checked by SDS–PAGE. After that the whole sonicated cell suspension was centrifuged for 30 min at 12,000 rpm (Sorvall SS34 rotor). The supernatant was discarded and the pellet was washed twice with 20 mM Tris, 1 M urea, 10 mM TCEP, pH 8. The washed inclusion body pellet was extracted with 20 ml, 50 mM Tris, 6 M GdmCl, 10 mM TCEP, pH 8 by disrupting the pellet manually and incubating it for 1 h at 4 °C. After extraction the mixture was centrifuged for 30 min at 32,000 rpm and 4 °C using an ultracentrifuge. Protein samples containing 6 M GdmCl were precipitated in the following way to check them by SDS–PAGE: Mix 30 μl protein solution with 30 μl H_2O and 60 μl 10% TCA and leave on ice for 20 min, centrifuge for 15 min in a microfuge (13,000 rpm), discard the supernatant and wash the pellet with 60 μl ice cold ethanol, dry the pellet from ethanol and resuspend it in 60 μl 2× sample buffer.

To further purify hFRB, the inclusion body extract was first diluted 1:3 with inclusion body extraction buffer and then 2:1 with 30% HPLC buffer B (90% acetonitrile/0.1%TFA) and applied to a preparative C4 HPLC column that had been equilibrated with 30% HPLC buffer B. HPLC buffer A was 0.1% TFA in water. To elute the protein a gradient was started that increased the concentration of buffer B by 1.4%/min; hFRB eluted around 52–60% B. Two hundred microliters of each fraction were dried in a SpeedVac and dissolved in 20 μl 2× sample buffer to analyze them by SDS–PAGE. Those containing nearly pure hFRB were frozen in liquid nitrogen, lyophilized and stored at −20 °C. The protein concentration in each fraction was determined based on the absorption at 280 nm. Note that if the concentration