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**Deliverable 3.2-1:**  
**Production of recombinant protein 1 (for structural analysis**  
**and activity assays with Hit compounds)**  
**D - 4**

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**Authors:** Pedro Castanheira (BIOCANT), Pedro J. B. Pereira (IBMC), Carlos Faro (BIOCANT), Sandra de Macedo Ribeiro (IBMC)

**Responsible authors:** Sandra de Macedo Ribeiro (IBMC)

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## 1. Summary

Overweight and obesity are escalating worldwide and, if not prevented or treated, represent a major risk for serious health problems such as diabetes mellitus, cardiovascular disease, hypertension and stroke. The main focus of this project is the identification of new compounds with potential anti-obesity and anti-diabetes properties. Those compounds will be directed towards three major proteins involved in cellular metabolism, energetics and quality control, identified as potential targets associated with obesity and diabetes, two conditions collectively included in the group of metabolic disorders. Diabetes and Obesity Related gene product (DOR) is a protein thought to be associated with the pathology of metabolic disorders.

Development of novel compounds with high specificity and reduced secondary effects often requires the knowledge of the three-dimensional structure of the selected targets. The compounds (ligands) with potential biological effects can be precisely designed to have a shape and chemical properties complementary to their target macromolecule. The most common method used to determine the three-dimensional structure of proteins is X-ray crystallography. In order to solve the structure of a protein by this method the protein must first be purified to homogeneity and then crystallized. Crystallization is often the rate-limiting step for solving a protein structure by X-ray crystallography, and it is crucially dependent upon the quality of the protein used.

To obtain DOR protein with high quality for crystallization and three-dimensional structure determination, we have established a protocol for its expression in a bacterial host and for its purification. The final goal is to obtain crystals of this protein for structure determination and subsequently rational drug design.



## 2. Introduction

Obesity is increasing exponentially worldwide and represents a serious public health problem. The links between diabetes and obesity are firmly established, further strengthening the need for new approaches to their treatment and prevention. This project is based on the early drug discovery phase for new effective therapies and aims to **develop new tools for the rational design of new compounds with anti-obesity or anti-diabetes activities.**

The main goal of this specific aim (Task 2) within Group task 3 is the determination of the three-dimensional structure of target proteins by X-ray crystallography. Those three-dimensional structures will be crucial tools for the rational, structure-aided design of novel compounds to be screened for their potential anti-diabetic and anti-obesity properties. The determination of the 3D structures of proteins by X-ray crystallography comprises several steps, starting with the expression of the target protein in sufficient amounts for crystallization trials, often resorting to the usage of heterologous (bacterial, yeast, insect or mammalian cells) expression systems.

The selected target proteins are Diabetes and obesity related gene protein (DOR), Mitofusin-2 (Mfn2) and Vascular adhesion protein 1 (VAP-1). Research data from partners at IRB and INSERM hints that those proteins may play a central role in obesity and/or diabetes, therefore constituting new potential drug targets (Yraola et al, 2006; Zorzano et al, 2009).

DOR (or Tumor protein p53 inducible nuclear protein 2 - tp53inp2) is a 24kDa nuclear protein required for autophagy in mammalian cells. Recent reports have shown that it binds to the LC3 and LC3-related proteins



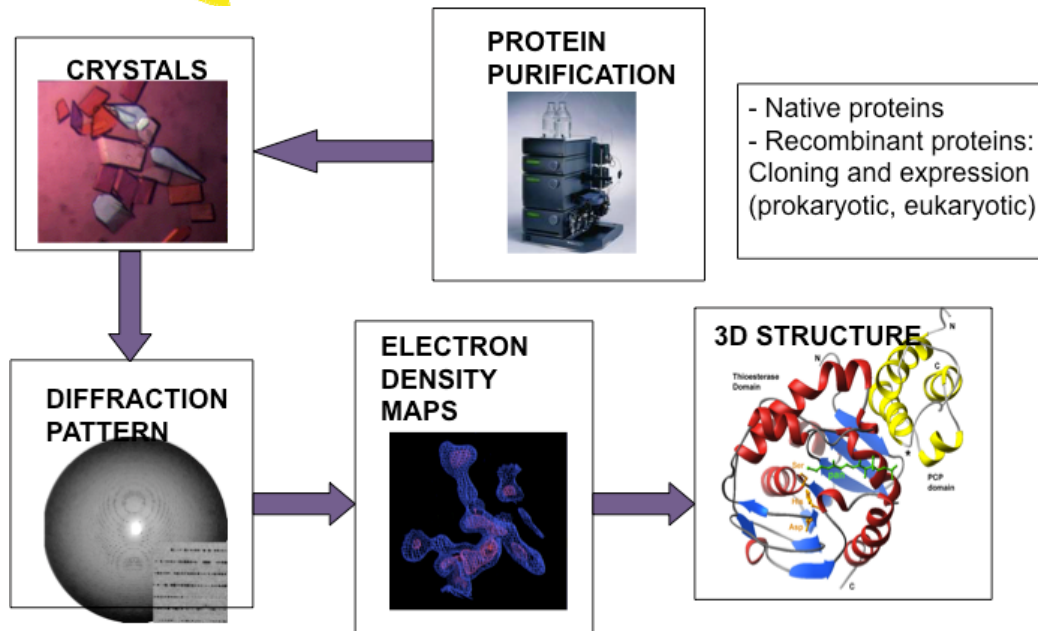
(Mauvezin et al, 2010; Nowak et al, 2009). DOR gene expression is high in skeletal muscle, heart and brain, and specifically repressed in the muscle of Zucker Diabetic rats (ZDF) compared to lean non diabetic animals (Baumgartner et al, 2007). Preliminary unpublished results have shown that its expression is reduced in skeletal muscle of obese individuals and of patients with diabetes, hinting that an increment of its activity might be a possible therapeutic approach for these disorders. It has no homology with other proteins of known 3D structure.

In this deliverable report we show that we were able to successfully express in *E. coli* and to purify to homogeneity recombinant DOR, as well as its interacting protein partners LC3 and GATE16 for protein crystallization trials.

### **3. Objectives and methodology**

#### **3.1. Overview**

The main goal of this group task is to determine the shape or three-dimensional structure of selected proteins within target metabolic and signalling pathways correlated with obesity and type-2 diabetes. Those structures will provide a 3D framework for the structure-based design of specific molecules (ligands) with complementary shape and/or charge. The main method used for determining the three-dimensional structures of proteins is X-ray crystallography whose main steps are represented in Fig. 1.



**Figure 1-** Main steps necessary to obtain the 3D structure of a protein by X-ray crystallography. The main bottleneck in this approach is the growth of protein crystals with the ability to diffract X-rays. The quality of the crystals is highly dependent on the quality (purity and homogeneity) of the protein sample used. Most commonly the protein is expressed in a heterologous system (eukaryotic or prokaryotic) where higher levels of protein production can be achieved

### 3.2. Specific objectives:

To produce recombinant target protein (DOR) in milligram amounts for crystallization screenings.

### 3.3. Methodology

#### 3.3.1. Selection of expression host

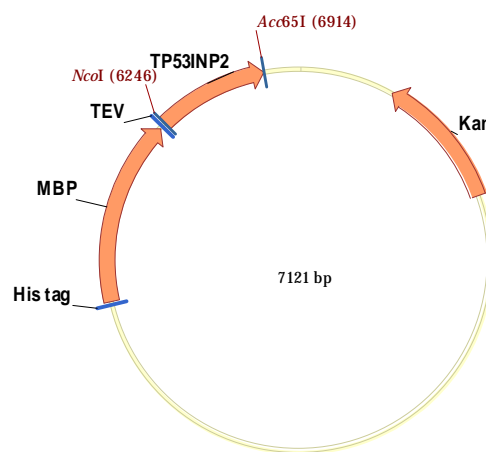
In order to obtain large amounts of pure recombinant protein we have selected *E. coli* as the expression host due to its favourable yield/cost ratio and lack of heterogeneous post-translational modifications. Since the expression of eukaryotic proteins in bacterial systems is often non-trivial, we have ordered a synthetic cDNA coding for DOR optimized for expression in the chosen host. This optimization takes into account several aspects affecting different stages of protein expression, such as codon adaptability,



mRNA structure and various *cis*-elements in transcription and translation which in many cases leads to a more than 10 fold increase in the protein expression level (Burgess-Brown et al, 2008; Makrides, 1996).

### 3.3.2. Selection of expression system

Successful production and purification of soluble and stable recombinant proteins is often dependent on the use of fusion proteins and tags (Makrides, 1996). Fusion proteins can increase expression level, stability, enhance solubility and can be used for affinity protein purification. For this reason, to improve our chances of success, we have chosen a set of expression vectors based on pET28a (Novagen) that have been modified to express N-terminal tags and fusion proteins (Fig. 2). As a common feature they all code for an N-terminal hexahistidine tag, and a Tobacco Etch Virus (TEV) Protease recognition sequence before the cloning site of the protein of interest to allow for fusion tag/protein removal after protein purification. The fusion tags selected were Maltose Binding Protein (MBP), Thioredoxin, Nus A, GB1 and Z2 domain.



**Figure 2-** Schematic representation of DOR cloned onto a modified pET28a coding for an N-terminal His-tag followed by the Maltose Binding Protein (MBP), a TEV protease recognition sequence and the DOR protein. The cloning was done between the Nco I and Acc65 I restriction sites.



### 3.3.3. Screening for protein expression conditions: an incomplete factorial approach

Small-scale tests were performed in order to find the best set of expression conditions yielding soluble protein. The variables tested were: Fusion partner (Maltose Binding Protein (MBP), Thioredoxin, Nus A, GB1 and Z2 domain); *E. coli* strain; expression medium (LB, 2YT, ZMY5052); IPTG concentration and temperature (20 - 37°C). Since the number of experimental points of the full combination of variables increases exponentially, we have decided to use an incomplete factorial approach to decrease the number of combinations to test while retaining the statistical significance of the full factorial (Benoit et al, 2007). Expression levels were analysed by SDS-PAGE and Western-blot using an anti-hexahistidine tag antibody. The construct/expression conditions yielding the higher levels of soluble recombinant protein were selected for experiment scale-up.

### 3.3.4. Protein expression and purification

The MBP-DOR fusion construct was expressed in *E. coli* (BL21star) grown in LB medium (2-4L) at 25°C after induction with 0.1 mM IPTG. Cells were resuspended in lysis buffer (20 mM sodium phosphate pH 7.5, 500 mM sodium chloride, 10 mM imidazole, 1 % (v/v) glycerol, 5 mM 2-mercaptoethanol, EDTA-free Complete protease inhibitor cocktail (Roche)) and disrupted by freeze-thawing. After a centrifugation step for clarification, the MBP-DOR-containing extract was loaded onto a 5 ml HisTrap (GE-Healthcare) column equilibrated with buffer A (20 mM sodium phosphate pH 7.5, 500 mM sodium chloride, 10 mM imidazole, 1 % (v/v) glycerol, 5 mM 2-mercaptoethanol). After a washing step, the protein was eluted stepwise with increasing concentrations of imidazole in buffer A and protein purity was assessed by SDS-PAGE. The protein eluting from the HisTrap column was further purified on a Superdex 200 gel filtration



chromatography column equilibrated in GF buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 5 mM 2-mercaptoethanol).

#### 3.3.5. TEVP cleavage assay

One of the features of the selected expression constructs used is the possibility of removing the fusion tag/partner by tobacco etch virus (TEV) protease cleavage. Purified MBP-DOR was cleaved by recombinant TEV protease in order to remove the MBP fusion partner. As TEV protease was also expressed with a hexahistidine tag, released DOR can be easily purified by immobilized-Ni chromatography of the digestion mixture. The cleavage assay was performed at 4° C (3h) in GF Buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol), using a 1:25 enzyme/substrate ratio.

#### 3.3.6. Expression and purification of LC3 and GATE 16 for co-purification with DOR

Often the stability of proteins is increased and their flexibility is decreased by formation of complexes with their cellular partners. The addition of macromolecular partners to the crystallization setup has allowed the successful crystallization of several intrinsically unstable or very flexible proteins (Volkman et al, 2002; Warke & Momany, 2007).

Recent data from Dr. Zorzano's lab (Mauvezin et al, 2010) showed that LC3 and LC3-like proteins interact with DOR. GATE 16 and LC3 cDNAs (kindly provided by Dr. Zorzano) were subcloned into the Nco I / Acc65 I restriction endonuclease sites of the MBP- and Trx-coding vectors that were previously used to clone DOR. Since DOR was expressed with an MBP fusion partner, GATE 16 and LC3 were expressed as N-terminal thioredoxin fusions, which allowed for the purification of the DOR/GATE 16 and DOR/LC3 complexes by MBP affinity chromatography.



Briefly, LC3 and GATE 16 were expressed with the same procedure used for expression of DOR. After cell resuspension in lysis buffer (20 mM Sodium Phosphate pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 % (v/v) glycerol, 5 mM 2-mercaptoethanol, EDTA-free Complete protease inhibitor (Roche)), cells were disrupted by freeze-thawing and the resulting extract was combined with that from MBP-DOR expression. After a clarification step by centrifugation, protein was loaded onto a 5 ml HisTrap column equilibrated with buffer A (20 mM Sodium Phosphate pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 % (v/v) glycerol, 5 mM 2-mercaptoethanol). The proteins were eluted stepwise with increasing concentrations of imidazole in buffer A and protein purity was assessed by SDS-PAGE. The protein eluting from the HisTrap column was applied onto a 5 ml MBPTrap column (GE-Healthcare) equilibrated with binding buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol). After a washing step, bound protein was eluted with buffer A supplemented with 10 mM maltose. The DOR/GATE 16 and DOR/LC3 complexes herein purified were finally applied to an analytical Superdex 200 10/300 GL gel filtration chromatography column (GE-Healthcare) equilibrated in GF buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 5 mM 2-mercaptoethanol).

#### 3.3.7. Crystallization trials

The purified MPB-DOR fusion protein both alone and in complex with Trx-LC3 or Trx-GATE16, was used for preliminary crystallization trials using the vapour diffusion sitting drop method and a set of commercial crystallization solutions. Briefly, 1 microliter of protein was added to 1 microliter of crystallization solution and equilibrated against 300 microliters of reservoir solution in a closed container. The crystallization plates were incubated at 20°C and visualized every 2-4 days using a stereomicroscope with polarized light.

## 4. Results achieved

### 4.1. Expression constructs

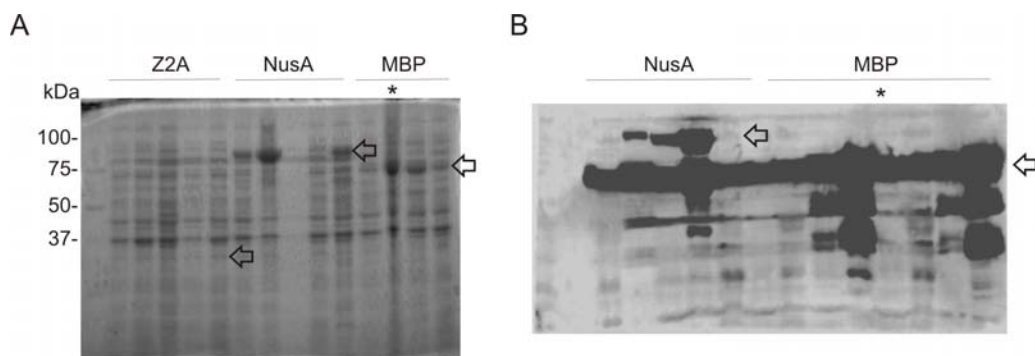
The cDNA encoding for DOR was cloned in five different plasmids for bacterial expression of the following fusion proteins:

MBP-DOR  
Trx-DOR  
-NusA-DOR  
-Ztag2-DOR  
-GB1-DOR

All constructs were verified by restriction analysis and DNA sequencing.

### 4.2. Optimization of expression conditions

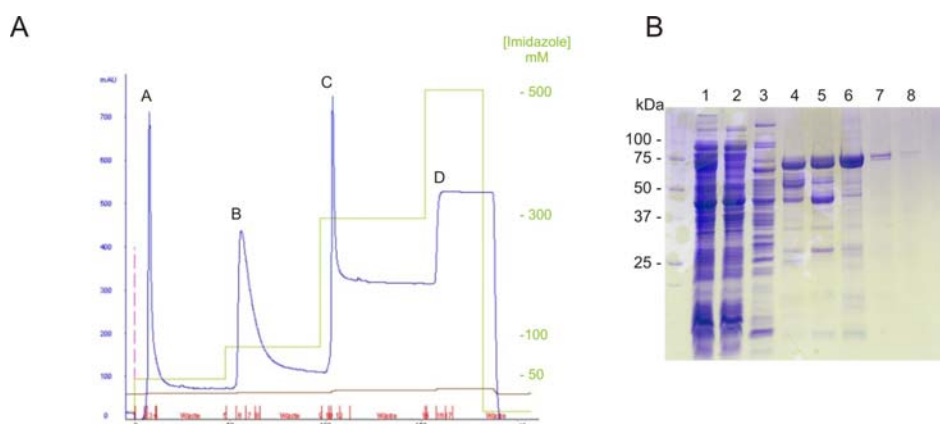
The conditions with the highest expression of soluble DOR were obtained using the MBP-DOR fusion, LB medium, *E. coli* strain BL21star and 0.1 mM IPTG at 25°C (Fig. 3). High levels of soluble NusA-DOR were also obtained, although significant levels of protein degradation were observed for this fusion construct (Fig. 3).



**Figure 3** - SDS-PAGE (A) and Anti-6xHis Western-blot (B) analysis of DOR (TP53INP2) soluble expression conditions. The best conditions found are indicated with an asterisk. Arrows highlight the expected molecular weight of corresponding fusion proteins.

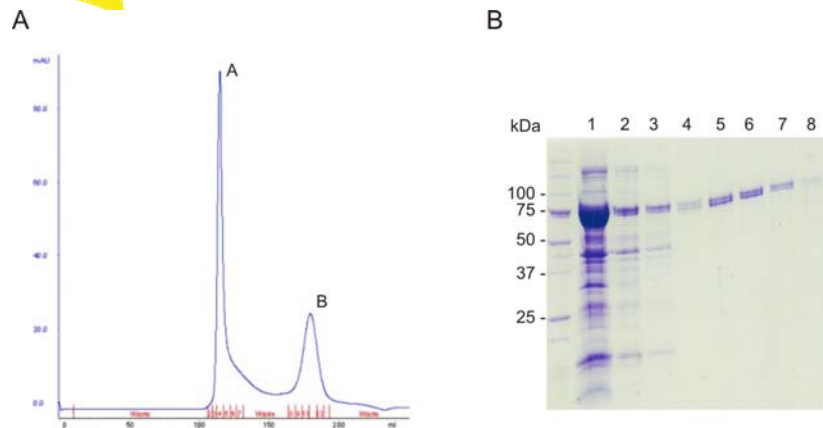
### 4.3. Large scale protein expression and purification

The MBP-DOR fusion protein expression was carried out in 2-4L of cell culture, and the clarified cell extract was applied to a HisTrap column. The purest fusion protein eluted with 300 mM imidazole (Fig. 4B; lane 6).



**Figure 4** - Purification of MBP-DOR. A - HisTrap HP (5 ml) chromatogram; B - SDS-PAGE analysis. 1 - Clarified extract applied to HisTrap; 2 - HisTrap Flow-through; 3 -peak A; 4 and 5 - peak B; 6 and 7 - peak C; 8 - peak D.

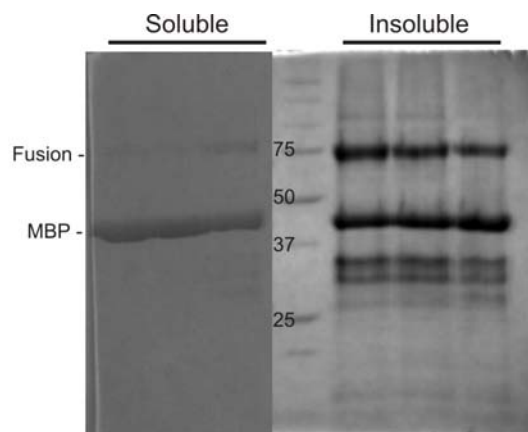
The protein eluting from the HisTrap column was applied to a Superdex 200 GL 10/300 gel filtration chromatography column (Fig. 5 - A). Although some of the protein eluted in the void volume, a significant amount eluted at a volume compatible with monomeric MBP-DOR (peak B in Fig. 5 - A). The fractions composing the second peak were pooled and further concentrated by ultrafiltration on a centrifugal concentration device, down to a concentration of approximately 4 mg/ml and stored at -80°C prior to crystallization trials. The total protein yield is typically ~0.5 mg per 2L of cell culture.



**Figure 5** - MBP-DOR gel filtration chromatography (A) and SDS-PAGE analysis (B). 1 - Protein applied to Superdex 200 column; 2 to 4 - fractions from peak A; 5 to 8 - fractions from peak B.

#### 4.4. Tevp cleavage assays

The results shown in Fig. 6 revealed that DOR precipitates after TEV protease cleavage. Due to this fact, we have decided to proceed with the crystallization studies using the MBP fusion protein, which can also function as aid in maintaining protein stability and in protein crystallization (Smyth et al, 2003).



**Figure 6**- Analysis of MBP-DOR TEVP cleavage. After TEV cleavage the solution was centrifuged and both the supernatant and the pellet were analyzed by SDS-PAGE.

#### 4.5. Purification of DOR / GATE 16 complex

For co-purification of the MBP-DOR /Trx-GATE 16 complex 2L of each fusion protein were expressed separately and after cell lysis the two cell extracts were combined. After removing the insoluble material by centrifugation, the supernatant containing the soluble fraction was applied to a 5 ml HisTrap HP column (Fig. 7 - A). The purified complex was further applied to a MBP-Trap column (Fig. 7 - B)

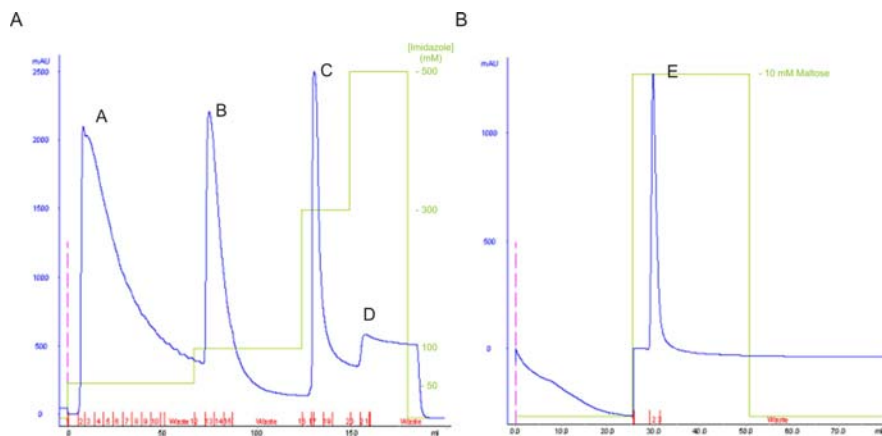


Figure 7- MBP-DOR / Trx-GATE 16 Purification. A - HisTrap purification. B - MBPTrap purification of HisTrap peak C.

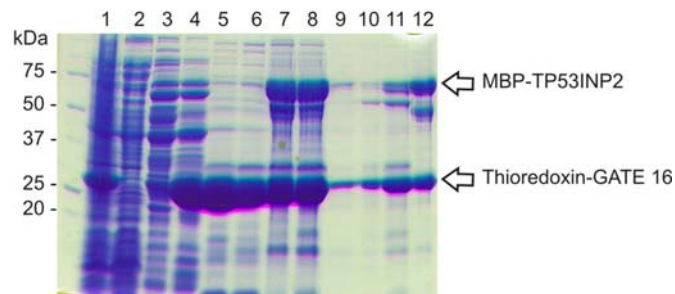


Figure 8- SDS-PAGE analysis of MBP-DOR / Trx-GATE 16 purification. 1 - Clarified cell extract applied to HisTrap; 2 - HisTrap flow-through; 3 and 4 - Peak A from HisTrap 5 and 6 - peak B from HisTrap; 7 and 8 - Peak C from HisTrap; 9 - Peak D from HisTrap; 10 - MBPTrap flow-through; 11 - MBPTrap first washing step; 12 - Peak E from MBPTrap.

The protein eluting from the maltose affinity column was then applied to an analytical gel filtration column (Fig. 9).



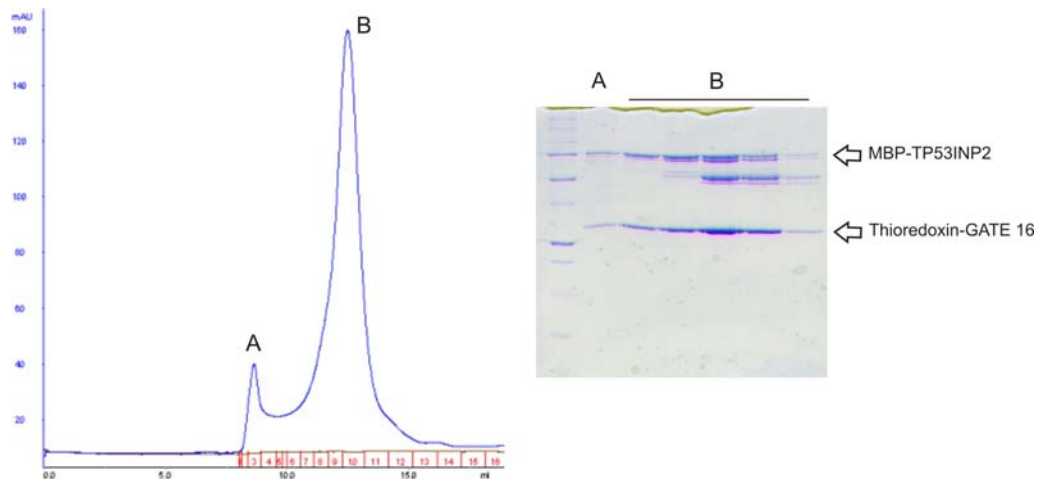
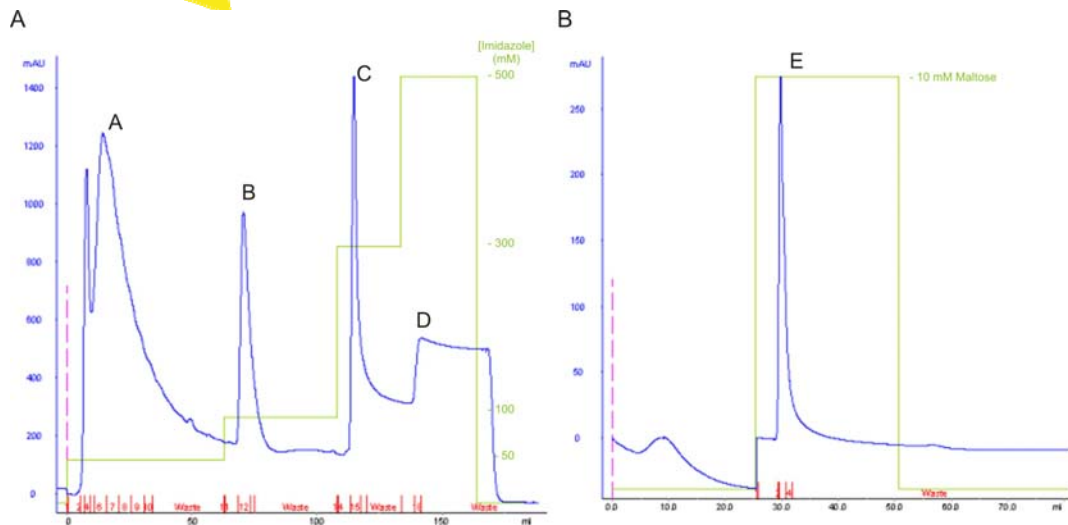


Figure 9- Analytical gel filtration chromatography (left) and SDS-PAGE analysis (right) of purified MBP-DOR / Trx- GATE 16 complex.

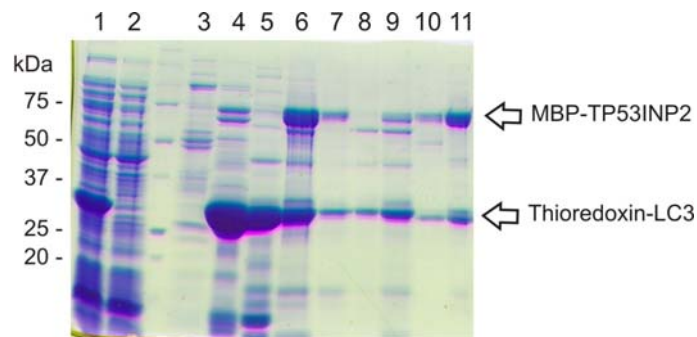
Most of the complex is eluted at an elution volume compatible with a dimeric form (Fig. 9 - B, lanes B). Partial protein degradation is observed for this complex. The overall yield of the complex was 1mg per 2L each of MBP-DOR and Trx-GATE 16 expression.

#### 4.6. Purification OF DOR / LC3 complex

For the purification of the MBP-DOR / Trx-LC3 complex, we followed a similar strategy to the one described for the MBP-DOR /GATE 16 complex co-purification. The fusion proteins were expressed independently and the cells were lysed together before centrifugation and application to the HisTrap column (Fig. 10). The purest fraction, as analysed by SDS-PAGE (Fig. 11) was then applied to the MBP-trap column (Fig. 10).

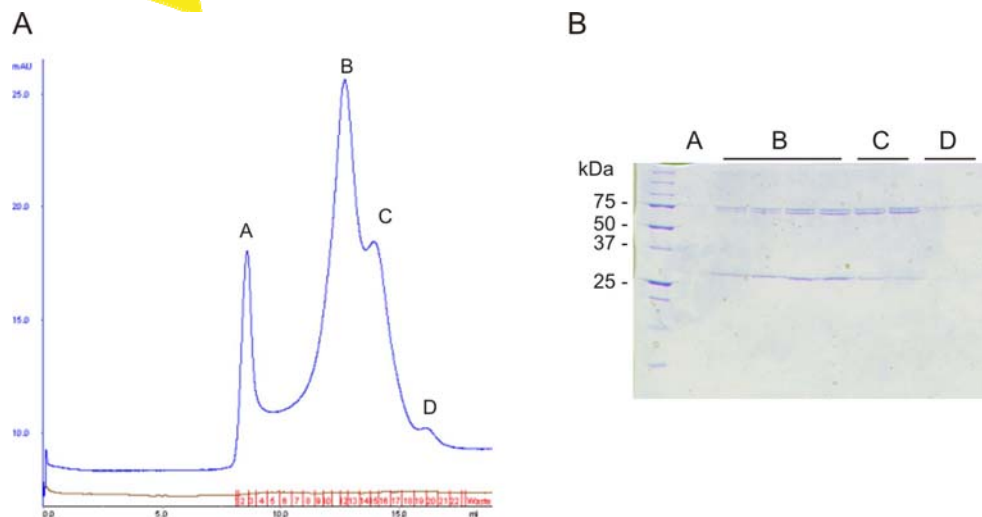


**Figure 10** - MBP-DOR / Trx-LC3 Purification. A - HisTrap purification. B - MBPTrap purification of HisTrap peak C.



**Figure 11** - SDS-PAGE analysis of MBP-DOR / Trx- LC3 purification. 1 - Clarified cell extract applied to HisTrap; 2 - HisTrap flowthrough; 3 and 4 - Peak A from HisTrap 5 - peak B from HisTrap; 6 - Peak C from HisTrap; 7 - Peak D from HisTrap; 8 - MBPTrap flowthrough; 9 - MBPTrap first washing step; 10 and 11 - Peak E from MBPTrap.

The SDS-PAGE results revealed that the MBP-DOR and Trx-LC3 co-eluted from the MBPTrap column, confirming the presence of the expected complex, which was further analysed by analytical gel filtration chromatography (Fig. 12 - A).



**Figure 12-** Analytical gel filtration chromatography (left) and SDS-PAGE analysis (right) of purified MBP-DOR / Trx-LC3 complex.

For the MBP-DOR / Trx-LC3 complex two peaks (B and C from Fig. 12 - A) are observed. Based on the column calibration with known molecular weight markers (not shown) these peaks likely correspond to dimeric (B) and monomeric (C) forms of the complex. The overall yield of the complex was 1mg per 2L each of MBP-DOR and Trx-GATE 16 expression.

#### 4.7. Preliminary crystallization trials

The unliganded MBP-DOR (4 mg/ml) protein was used to setup a 48 crystallization condition screen using the first half (solutions 1 to 48) of the PGA Screen kit (Molecular Dimensions), using the sitting drop method.

#### 4.8. Main conclusions

We have succeeded in establishing a protocol for the production of pure and homogeneous (monomeric) MBP-DOR for utilization in crystallization screenings. The protein purification protocol is being further optimized (e.g. by addition of protease inhibitors along the purification pathway) to



increase the protein yield. Furthermore we have obtained monomeric MBP-DOR/ Trx-GATE-16 and dimeric MBP-DOR/ Trx-LC3 complexes. Those complexes are very promising for utilization in co-crystallization trials.

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