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Supplemental material

Expression studies for constructs of the Thyrotropin receptor hinge region

We performed a systematic approach of expression studies for several different TSHR hinge region constructs in *Escherichia coli* and in *Pichia pastoris*. The designed hinge region constructs are different in length (construct descriptions are given in S-Figure 1 and S-Figure 2).

Expression studies in E. coli

Methods

Construction of E. coli expression plasmids

All *E.coli* strains and expression vectors were purchased from "Novagen". The ligation independent cloning was used to facilitate the construction of fusion protein expression vectors. The template for the PCR was the cDNA of the TSHR-gene. The purified amplicon was then cloned into the expression vectors pET30, pET32 and pET40 by the ligation independent cloning procedure. The entire coding region of each cDNA construct was sequenced.

Over-expression in E. coli and protein preparation

Expression-tests were done in small-scale 5ml culture. For expression following *E. coli* strains were used: BL21(DE3), BL21(DE3) Rosetta2, BL21(DE3)pLysS, RosettagamiB (DE3). Single antibiotic resistant colonies of cells transformed with the accordant expression vectors were used to inoculate 2 ml of Luria broth supplemented with 100 µg/ml kanamycine or 60 µg/ml carbenicilline. These cultures were grown as shaking over night culture at 37°C and 170 rpm. The over night cultures were then diluted 100 fold into 5 ml of fresh medium. When the cells reached the log phase (OD_{600} 0.6) the temperature was set up to 37°C / 30°C/ 25°C / 16°C and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1mM, 0.5 mM and 1 mM. Cells were recovered after 16 hours induction by centrifugation at 4500g for 15 min and stored at -80°C for following protein-preparation or SDS-PAGE.

All preparation steps were carried out at 4°C. E. coli cell paste was suspended in ice-cold Lysis Buffer (50 mM sodiumphosphat pH 8.0/150 mM NaCl /0.1% Brji35) containing Complete EDTA-free protease inhibitor cocktail ("Roche"). Cells were lysed by ultrasonification (3x3 min 20% / 0.5 sec intervals). After cell lysis Benzonase was added and cell-lysate was shaked at 4°C for one hour. The supernatant was recovered by centrifugation at 15 000 g for 30 min at 4°C. Supernatant was loaded onto a spin column containing Ni-NTA matrix equilibrated with buffer. The column was then washed three times with one column volume of washing buffer containing 10 mM imidazole and finally recombinant protein was eluated with elution buffer containing 250 mM imidazole. SDS PAGE was performed to confirm the result of protein isolation.

Recombinant protein solution was dialyzed against Cleavage-Buffer (for enterokinase cleavage buffer contains 0.02 M Tris-HCl, 0.1 M NaCl, 0.002 M $CaCl_2$ pH 8.0; for Thrombin cleavage buffer contains 0.02 M Sodiumphosphat, 0.2 M NaCl pH 7.5), 10 units per mg protein of protease was then added to the solution to cleave off the accordant fusion partner.

Cleavage was carried out for 16 hours at room temperature. SDS PAGE was performed to confirm the result of the protein cleavage.

Results

Aim of the expression studies was to find expression conditions for soluble over-expression of the isolated hinge region of the TSHR for further NMR structural studies. The DNA of the hinge region constructs (S-Table 1, S-Figure 1) were different in length and endings were cloned into the expression vectors pET30, pET32 and pET41 to achieve an over-expression as fusion proteins with the N-terminal fusion partner His-S-tag, thioredoxin-His-S-tag (Trx-tag) and glutathione-S-transferase-His-S-tag (GST-tag). All of the hinge region fragments were cloned successfully into the expression vectors pET30 and pET41. For over-expression as Trx- fusion protein only for constructs TSHR_ht2, 3 positive clones were selected. For expression vectors were transformed into the *E. coli* strains BL21(DE3), BL21(DE3)Rosetta 2, BL21(DE3)pLysS and RosettaGamiB(DE3). Over-expression was carried out under different temperature and induction conditions. Soluble over-expression of the His- and GST-hinge region fusion proteins was achieved in the *E. coli* strain BL21(DE3) (0.2 mg from 5 ml culture). The Trx-fusion proteins were expressed as inclusion-bodies. The over-expression rate of all hinge region fusion proteins in the *E. coli* strain BL21(DE3)Rossetta2 was higher but resulted in complete formation of inclusion bodies. Only the GST-fusion proteins were soluble expressed. In comparison to the over-expression in the E. coli strains BL21(DE3) and BL21(DE3)Rosetta2 the expression rate was strongly reduced and soluble protein expression was achieved only at 16°C (0.05 mg from 5 ml culture). In the E. *coli* strain BL21(DE3)pLysS only for GST-constructs moderate soluble protein over-expression was observed. In the *E. coli* strain RosettaGamiB(DE3) no expression was detected. After Isolation and dialysis of the four soluble expressed constructs (His- taged TSHR_ht2, GST-taged TSHR_ht2,3,5) (S-Table 2) all of them showed stability over several days at 4°C. After cleavage of the accordant tags the hinge region proteins subjected very fast degradation due to incorrect folding of the hinge region.

Expression studies in P. pastoris

Methods

Construction of P. pastoris expression plasmids

The *P. pastoris* strain X33 and all expression vectors were purchased from Invitrogen. The expression vector pPICZb was prepared for ligation by restriction with the enzymes *EcoR*I and *Xba*I. The expression vector pPICZαA containing the N-terminal Yeast a-factor signal peptide as fusion partner was prepared for ligation by restriction with the enzymes *Xho*I and *Xba*I. The template for the PCR was the cDNA of the TSHR-gene. For using α-factor signal peptide as fusion partner, the TSHR construct DNA was amplified without native N-terminal signal peptide. The purified amplicon was restricted with the correspondent restriction enzymes and cloned into the linearized expression vectors pPICZb and pPICZαA. The GFP-fusion constructs were prepared by Splicing overlap extension PCR (SOE-PCR). GFP cDNA was amplified by conventional PCR from the plasmid expression vector pEGFP-N1. The entire coding region of each DNA construct was sequenced. Recombinant expression vectors were propagated using DH5α *E. coli* strain.

Transformation and over-expression in P. pastoris

The 14 different integrative expression vectors were linearized using the restriction enzyme *Pme*I and transformed into X33 *P. pastoris* strain using the "Pichia easy comp Kit" from Invitrogen. Recombinant clones were selected on YPDS agar plates (1% yeast extract, 2% peptone, 2% dextrose, 1M Sorbitol, 2% agar) containing 100 µg/ml Zeocine. In a second step aimed at selecting for multicopy transformants, positive clones were grown on YPD agar plates containing various concentrations of Zeocine.

Representative clones exhibiting resistance to various Zeocine concentrations were checked for the Mut phenotype. Genomic DNA was prepared by phenole-chloroform extraction according to Hoffman and Winston 1987 (suppl. ref. 1). Conventional PCR using the 5'AOX1 and 3'AOX1 primers were used to determine the Mut^S and Mut^+ phenotype. Mut^S clones showed one amplicon, the expression cassette integrated by gene replacement at AOX1. Mut^+ clones showed two amplicons the AOX1 gene and the expression cassette integrated by gene insertion.

For Protein over-expression, cells were precultured in BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base without amino acid, 0.00004% biotin, 1% glycerol, 0.1 M phosphate buffer at pH 6) at 30°C and 250 rpm until an OD600 of 2–6 was reached. As standard condition, induction was achieved in 5 ml BMMY medium (same as BMGY with 0.5% methanol instead of glycerol) at 30°C from an initial OD_{600} of 1. Every 24 hours post induction start, cells were recovered by centrifugation at 3000g for 5 min and stored at -80°C. Protein production was determined by SDS-PAGE and Western Blot analysis. Cells expressing GFP fused hinge region proteins were checked by Light-scattering-microscopy (LSM) before harvesting.

All following work was carried out at 4°C. Cells were washed once with ice-cold breaking buffer (50 mM sodium-phosphate buffer at pH 7.4, 150 mM NaCl, 1 mM PMSF) and resuspended to 30 % wet weight. After 0.5-mm glass beads were added to the cell suspension. Yeast cells were broken by vortexing at 4°C 3 times for 3 min. Breaking efficiency was checked by qualitative Bradford Assay. Cell debris were separated from the membrane suspension by low-speed centrifugation (3000g, 5 min, 4°). Supernatant was checked for expressed protein by SDS PAGE and Western Blot using specific antibodies against the extracellular part of the TSHR.

Results

Expression in P. pastoris

To achieve potentially a stabilization of the hinge region, the hinge region fragment was cloned with the N-terminal Leucine-rich repeat domain and C-terminal with the first transmembrane helix and/or with GFP. To achieve an over- expression in the host *P. pastoris*, different constructs (S-figure 2) were cloned with native signal peptide or with Yeast α–factor signal peptide into the expression vectors pPICZB and pPICZαA and then transformed into X33 *P. pastoris* strain. 24 clones of each transformants were then selected for multicopy transformants, by growing on YPD agar plates containing various concentrations of Zeocine. Best growing 12 clones were then checked for their Mut-phenotype by conventional PCR.

Four Mut^+ and four Mut^S clones of each transformants were checked for over-expressing recombinant hinge region constructs. Cells were grown in small scale (5 ml culture) in complex media for 72 hours. Samples were taken every 24 hours after induction start. No over-expression of any hinge region construct could be detected, neither by SDS-PAGE then by Western Blot using specific antibodies against extracellular part of the TSHR. No GFP fused protein could be detected by LSM (S-Table 2).

S-Table 1

fusion partner	hinge region constructs	cloning	protein-expression	protein-expression soluble fraction	stability after isolation	stability after cleavage
His-tag	TSHR_ht1	+	+	-		-
	TSHR_ht2	+	+	+	+	-
	TSHR_ht3	+	+	-	-	-
	TSHR_ht4	+	-	-	-	-
	TSHR_ht5	+	+	-	-	-
	TSHR_ht6	+	-	-	-	-
GST-tag	TSHR_ht1	+	+	-	-	-
	TSHR_ht2	+	+	+	+	-
	TSHR_ht3	+	+	+	+	-
	TSHR_ht4	+	-	-	-	-
	TSHR_ht5	+	+	+	+	-
	TSHR_ht6	+	-	-	-	-
Trx-tag	TSHR_ht1	-	-	-	-	-
	TSHR_ht2	+	+	-	-	-
	TSHR_ht3	+	+	-	-	-
	TSHR_ht4	-	-	-	-	-
	TSHR_ht5	-	-	-	-	-
	TSHR_ht6	-	-	-	-	-

+ positive result
– a negative result
Results are shown for expression in *E. coli* strain BL21(DE3) for 16 hours at 25°C, no improved results were achieved by expression in *E. coli* strains BL21(DE3) Rosetta2, BL21(DE3)pLysS, RosettaGamiB(DE3).

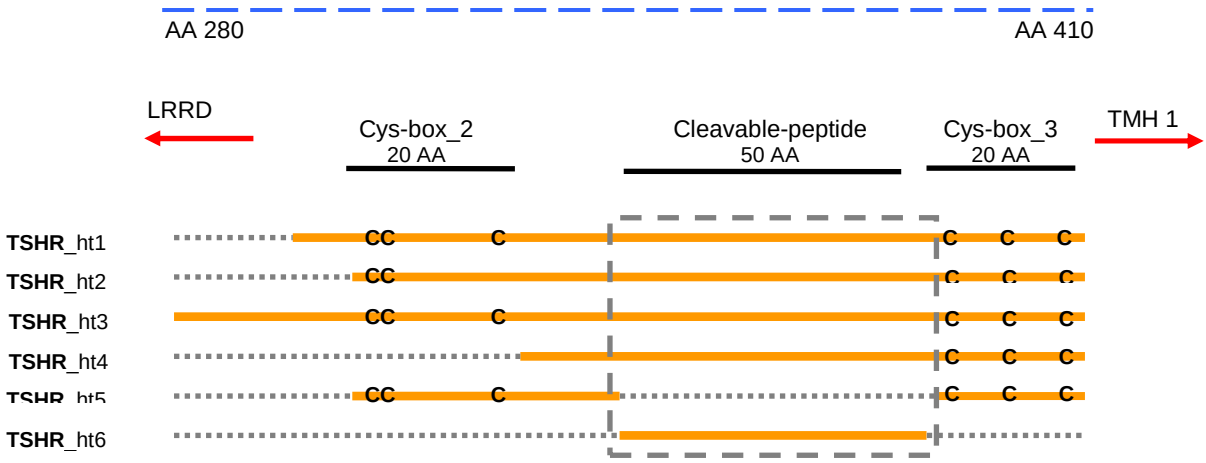
S-Table 2

Hinge Region Constructs	Cloning with native signalpeptide	Cloning with α-factor signalpeptide	Expression in Mut^+ clones	Expression in Mut^S clones
hTSHR	+	+	-	-
hTSHR-GFP	+	+	-	-
LRR-HingeR	+	+	-	-
LRR-HingeR-Xa	+	+	-	-
LRR-HingeR-GFP	+	+	-	-
LRR-HingeR-TMH1	+	+	-	-
LRR-HingeR-TMH1-GFP	+	+	-	-

+ positive result
– expression could not be detected neither by SDS-PAGE and Western Blot nor by Light Scattering Microscopy (LSM)

S-Figure 1

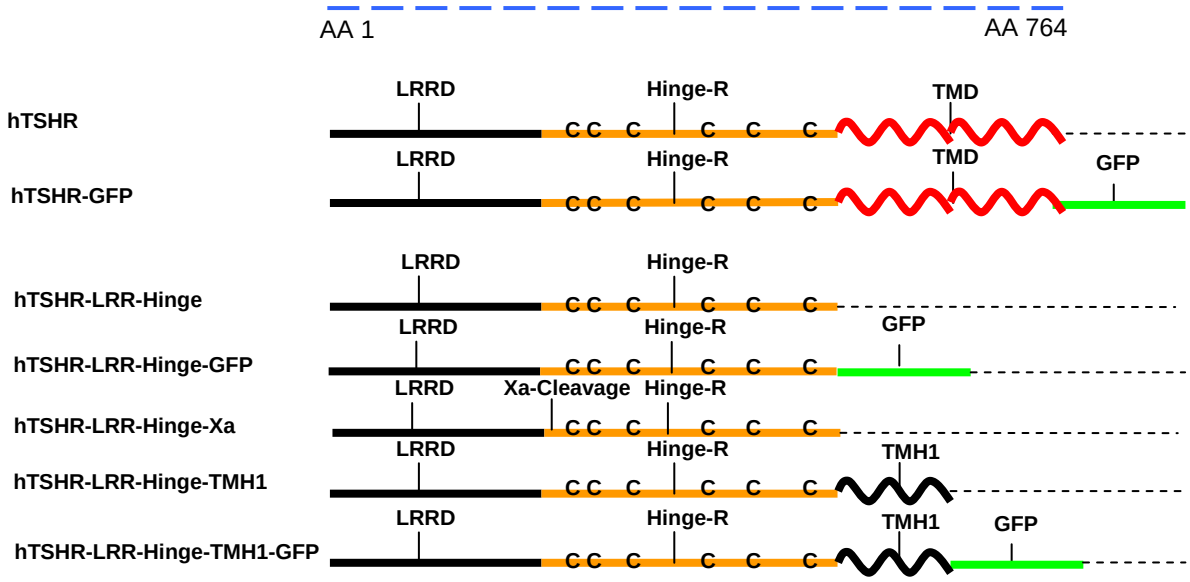
TSHR hinge region constructs for protein-overexpression in E.coli



S-Figure1: Used hinge region constructs for over-expression in E. coli
The hinge region is localized between the Leucine-rich repeat domain (LRRD) and the first transmembrane helix (TMH1). Six cysteines are localized at the N- and C- terminus. In the central part the cleavable peptide of around 50 amino acids (AA) is described.

S-Figure 2

TSHR hinge region constructs for protein-overxpression in P.Pastoris



S- Figure 2: List of used hinge region constructs for over-expression in P. pastoris
The TSHR hinge region is localized between the Leucine-rich repeat domain (LRRD) and the transmembrane domaine (TMD). TMH1 - first transmembrane helix, GFP - green fluorescent protein.

Supplemental Material References

1. Hoffman CS & Winston F., 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57 (2-3), 267-272