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Functional properties of cell-free expressed human endothelin A and endothelin B receptors in artificial membrane environments

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ABSTRACT

The human endothelin receptors are members of the rhodopsin class A of G-protein coupled receptors and key modulators of blood pressure regulation. Their functional *in vitro* characterization has widely been limited by the availability of high quality samples. We have optimized cell-free expression protocols for the human endothelin A and endothelin B receptors by implementing co-translational association approaches of the synthesized proteins with supplied liposomes or nanodiscs. Efficiency of membrane association and ligand binding properties of the receptors have systematically been studied in correlation to different membrane environments and lipid types. Ligand binding was analyzed by a number of complementary assays including radioassays, surface plasmon resonance and fluorescence measurements. High affinity binding of the peptide ligand ET-1 to both endothelin receptors could be obtained with several conditions and the highest Bmax values were measured in association with nanodiscs. We could further obtain the characteristic differential binding pattern of the two endothelin receptors with a panel of selected agonists and antagonists. Two intrinsic properties of the functionally folded endothelin B receptor, the proteolytic processing based on conformational recognition as well as the formation of SDS-resistant complexes with the peptide ligand ET-1, were observed with samples obtained from several cell-free expression conditions. High affinity and specific binding of ligands could furthermore be obtained with non-purified receptor samples in crude cell-free reaction mixtures, thus providing new perspectives for fast *in vitro* screening applications.

1. Introduction

G-protein coupled receptors (GPCRs) are one of the most frequent classes of membrane proteins encoded by the human genome and they represent important drug targets [1,2]. The human endothelin system comprises the two receptors endothelin A (ETA) and endothelin B (ETB) which mediate the effect of the three natural endothelin isopeptides ET-1, ET-2 and ET-3 [3]. Differential tissue specific expression patterns of the two receptors regulate vasoconstriction or vasodilatation [4]. The endothelin system is the main modulator in cardiovascular regulation and dysfunctions can result into pathological conditions like chronic heart failure or pulmonary hypertension [5,6].

The biochemical characterization and development of drugs directed against endothelin receptors are prerequisite for the clinical treatment of several vascular disorders [7,8]. *In vitro* studies have so far been problematic due to notorious difficulties in the preparative scale production of high quality GPCR samples. By using continuous exchange cell-free (CECF) expression systems, a number of intrinsic problems common to the classical cell-based *in vivo* expression systems can be avoided [9,10]. CF expression is therefore one of the most efficient approaches for the overexpression of the human endothelin receptors and probably also for other GPCRs [11–14]. We previously showed the preparative scale CF expression of ETA and ETB receptors either co- or post-translationally solubilized in detergents [15–17]. However, interaction with the peptide ligand ET-1 revealed significant lower binding affinities if compared with data from the receptors *in vivo* [15].

CF expression is a relatively new technology and it allows the modulation of GPCR sample quality by manifold variations of the

Abbreviations: Aso-PC, soybean L-α-phosphatidylcholine; BTE, brain total extract; CECF, continuous exchange cell-free; CF, cell-free; CHS, cholesteryl hemisuccinate; CVs, column volumes; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; EPL, *Escherichia coli* polar lipids; ETA, endothelin A receptor; ETB, endothelin B receptor; FM, feeding mix; GPCR, G-protein coupled receptor; HTL, heart total lipids; IMAC, immobilized metal affinity chromatography; LMPG, 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]; LPPG, 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]; LT-SDS-PAGE, low temperature SDS-PAGE; MSP, membrane scaffold protein; ND, nanodisc; PBS, phosphate buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PR, proteorhodopsin; RM, reaction mix; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoreses; sGFP, red shifted green fluorescent protein; SPR, surface plasmon resonance

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expression environment [10,15]. GPCRs can be synthesized as initial precipitates (P-CF mode) followed by subsequent post-translational solubilization in detergents. Alternatively, proteomicelles could be produced co-translationally by supplementing appropriate detergents directly into the CF reaction (D-CF mode). The solubilized GPCRs can then be reconstituted into membranes by classical *in vitro* techniques. The co-translational reconstitution could furthermore be attempted by addition of lipids either as preformed liposomes or as nanodiscs (NDs) into the CF reaction (L-CF mode). In particular interesting is the combination of CF expression with the new ND technology employing discoidal membrane structures of 10–20 nm in diameter formed by a lipid bilayer core and surrounded by the membrane scaffold protein (MSP) [17]. Considering the variety of potentially useful detergents, lipids and other hydrophobic compounds, CF expression represents currently the most variable production technique for membrane proteins.

Lipids are known as frequent modulators of membrane protein function. We have studied the ligand binding of the two CF expressed endothelin receptors in different lipid environments with complementary techniques and by implementing post- as well as co-translational reconstitution. The binding properties of a panel of agonists and antagonists to ETA and ETB have been studied. In addition, the characteristic SDS-stable complex formation of ETB with the ET-1 ligand as well as its conformation-specific proteolysis was analyzed.

2. Materials and methods

2.1. Materials

Wild type ET-1, its biotinylated derivative Lys^9-biotin-ET-1 (bET-1), 4-alanine(1,3,11,15)mutant ET-1 (4-Ala-ET-1) and biotin9-ET-3 (b-ET-3) were obtained from the Leibniz Institute for Molecular Pharmacology (FMP, Berlin, Germany); Radioactively labeled [^{125}I]Tyr^{13}-ET-1 (2200 Ci/mmol) was purchased from PerkinElmer (Rodgau-Jügesheim, Germany). The ligands BQ-123 and IRL-1620 were purchased from Tocris Bioscience (Bristol, United Kingdom).

The lipids L-α-phosphatidylcholine from soybean, Type IV-S (Aso-PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), heart total lipid extract, *Escherichia coli* polar lipids, cholesteryl hemisuccinate and brain total extract were purchased from Avanti Polar Lipids (Alabaster, USA).

Detergents used for resolubilization were 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LPPG) or 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) (Avanti Polar Lipids, Alabaster, AL). In the D-CF mode, the detergents Brij78, Brij35, digitonin (Sigma Aldrich, Taufkirchen, Germany) and Chaps (Roth, Karlsruhe, Germany) were used.

2.1.1. DNA techniques

DNA templates were constructed by standard PCR using the corresponding oligonucleotide primers and Vent-DNA Polymerase (New England Biolabs, Frankfurt, Germany) (Table 1). Purified DNA fragments were cloned with *BamHI* and *XhoI* (New England Biolabs, Frankfurt, Germany) into pET21a(+). For the construction of sGFP fusions, PCR fragments of the sGFP coding region were cloned into the *XhoI* restriction site of pET21a(+) vectors containing the coding sequence for a poly(His)$_{10}$ tag as well as for either ETA, ETB or proteorhodopsin (PR).

2.2. Cell-free expression

Basic CF expression protocols were previously described [18]. Bacterial S30 extracts were prepared from *E. coli* strain A19 and analytical scale reactions were performed in mini-CECF reactors [19]. Dialysis membranes with a cut-off of 12–14 kDa (Roth, Karlsruhe, Germany) were used to separate the reaction mixture (RM) from the feeding mixture (FM). The RM volume was 55 μl and the ratio RM/FM was 1:15. The reactions were incubated for 16–20 h on a suitable shaker at 30 °C. CF expressed proteins were collected either as a precipitate (P-CF, L-CF + liposomes) or as supernatant (D-CF, L-CF + NDs) by centrifugation at 18,000 ×*g* for 10 min at 4 °C.

P-CF precipitates were washed two times in buffer (20 mM Tris–HCl, pH 7.5 and 150 mM NaCl) and resuspended in buffer supplemented with detergents in a volume equal to the corresponding RM. Protein solubilization was achieved by incubation with gentle shaking for 1 h at 30 °C. Residual precipitate was removed by centrifugation at 18,000 ×*g* for 10 min. Detergents used for resolubilization were LPPG or LMPG. In the D-CF mode, the detergent Brij78 at a final concentration of 1% was directly added into the RM.

Lipids for L-CF expression were Aso-PC, DMPC, DOPC, heart total lipid extract and *E. coli* polar lipid mixture. The lipids were solubilized in chloroform, evaporated under vacuum to a thin film and completely dried overnight. The film was resuspended in liposome buffer (20 mM potassium phosphate, pH 7.0, 150 mM NaCl) to a final concentration of 40 mg/ml, briefly sonicated in a sonication bath and extruded to a size of 0.2 μm. The formed liposomes were then directly added into the RM to a final concentration of 4 mg/ml. For the L-CF mode in the presence of liposome/detergent mixtures, the detergents Brij35 (0.04%), Brij78 (0.04%), digitonin (0.2%) and Chaps (0.5%) were pre-mixed with pre-formed Aso-PC liposomes and added directly into the RM. For L-CF expression in the presence of NDs, pre-formed NDs were directly added into the RM. MSP1D1 and MSP1E3D1 were expressed in *E. coli* BL21 (DE3) cells and purified by Ni^{2+}chelate affinity chromatography as described [17,20]. NDs were assembled with DMPC, POPC, DMPG, Aso-PC, Aso-PC + 5% cholesterol hemisuccinate and brain total extract. The lipids were solubilized in chloroform, evaporated under vacuum and dried overnight. The lipid film was

Table 1
Constructs and oligonucleotides.

Construct (kDa)	Sequence	Modifications	Primer
ETB	E_{27}–S_{443}	N-T7	F: CGG GGA TCC GAG GAG AGA GGC TTC CCG CCT GAC
(47)		C-His$_{10}$	R: CGG CTC GAG AGA TGA GCT GTA TTT ATT ACT GGA
ΔETB	S_{65}–S_{443}	N-T7	F: CGG GGA TCG TTG GCA CCT GCG GAG GTG CCT AAA
(43)		C-His$_{10}$	R: CGG CTC GAG AGA TGA GCT GTA TTT ATT ACT GGA ACG
ETA	D_{20}–N_{427}	N-T7	F: CGG GGA TCC ATG GAA ACC CTT TGC CTC AGG GCA
(48.7)		C-His$_{10}$	R: CGG CTC GAG GTT CAT GCT GTC CTT ATG GCT GCT
ETB-sGFP (74)	E_{27}–S_{443}	N-T7	F: CGG CTC GAG CTA AAG GTG GAA GAA TTA TTC ACT TGG
		C-His$_{10}$	R: CGG CTC GAG TTA TTT GTA CAA TTC ATC CAT ACC ATG
ETA-sGFP	D_{20}–N_{427}	N-T7	F: CGG CTC GAG CTA AAG GTG GAA GAA TTA TTC ACT TGG
(75.7)		C-His$_{10}$	R: CGG CTC GAG TTA TTT GTA CAA TTC ATC CAT ACC ATG

resuspended in buffer (20 mM Tris–HCl, 100 mM NaCl, 100 mM Na-cholate) to a final concentration of 50 mM. The lipids were mixed with the purified MSPs in the presence of 0.1% DPC and incubated for 15 min at room temperature. Removal of detergents and formation of NDs were performed by dialysis for 24 h in buffer (20 mM Tris–HCl, 100 mM NaCl).

2.3. Protein analysis

Protein samples supplemented with SDS loading buffer (7.5% SDS (w/v), 50% (v/v) glycerol, 25% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromophenol blue in 0.3 M Tris–HCl pH 6.8) were analyzed by SDS-PAGE. Samples were separated by 12% or 16% (w/v) SDS gels and stained with Coomassie blue or analyzed by immunoblotting. Gels were blotted on activated 0.45 μm immobilon-P membrane (PVDF, Millipore, Eschborn, Germany) in wet Western blot apparatus (MiniProtean IV, Bio-Rad, Munich, Germany). The membrane was then blocked for 1 h in phosphate buffered saline supplemented with 4% skim milk powder and 0.05% Tween-20 followed by incubation with the first antibody (anti C-terminal His-tag (dilution 1:2000); anti N-terminal T7-tag (dilution 1:2000) or anti-biotin peroxidase conjugate (1:1000)) at 4 °C overnight with gentle shaking in antibody incubation buffer (phosphate buffered saline supplemented with 1% skim milk powder and 0.05% Tween-20). For the anti His-tag and anti T7-tag blots, after three washing steps in wash buffer (phosphate buffered saline and 0.005% Tween-20), the secondary antibody horse radish peroxidase conjugate was added in antibody incubation buffer at a final concentration of 1:5000 for 1 h at room temperature. After extensive washing in washing buffer, the membranes were analyzed by chemiluminescence in a Lumi-Imager F1 (Roche Applied Science, Mannheim, Germany). Protein concentrations were routinely estimated by 280 nm absorption using Nanodrop technology (Peqlab, Erlangen, Germany) and Lambert–Beer equations. For sGFP tagged proteins, samples were analyzed for fluorescence using a Tecan Genios fluorescence reader and concentrations of protein samples were determined using a calibration curve.

Low temperature-SDS-PAGE: SDS resistant receptor/ligand complexes were monitored by low temperature SDS-PAGE. ETB and ΔETB receptors were D-CF (Brij78 1%) expressed and affinity purified by metal chelate chromatography (Brij35 0.1%). Protein aliquots (0.5 μg to 2 μg) were incubated for 1 h at 4 °C with 10 ng of b-ET-1 in phosphate buffered saline in a total volume of 30 μl. Samples were then mixed with SDS-PAGE loading buffer and separated on 16% SDS-PAGE at 10 °C. The gels were blotted on PVDF membranes and analyzed with anti-biotin peroxidase conjugate antibodies (Millipore, Eschborn, Germany).

2.4. Protein purification

After D-CF expression, the first detergent Brij78 could be exchanged against a second detergent upon immobilized metal affinity chromatography. Samples were centrifuged at 18,000 ×*g* for 10 min in order to remove precipitate. The supernatant was diluted 1:5 in equilibration buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole and detergent at 5–10× critical micellar concentration (CMC)) and mixed with pre-equilibrated NTA-agarose beads (Qiagen, Hilden, Germany) loaded with Ni^{2+} ions. The mixture was incubated overnight at 4 °C with gentle shaking and applied on an empty gravity flow column. The beads were washed with 10 column volumes (CVs) of washing buffer 1 (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 30 mM imidazole and detergent at 5–10× CMC), 10 CVs of washing buffer 2 (washing buffer 1 with 80 mM imidazole) and 10 CVs of washing buffer 3 (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and detergent at 5–10× CMC). Proteins were finally eluted with elution buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 400 mM imidazole and detergent at 5–10× CMC) in 4–5 fractions of 1 CV each. Immobilized metal affinity chromatography was further applied to purify co-translationally formed GPCR/ND complexes. In this case the same buffers described previously were used but in the absence of any detergent.

2.5. Reconstitution of liposomes

Reconstitution of proteoliposomes was done with P-CF produced GPCR samples directly after resolubilization with 1% LMPG in liposome buffer at a concentration of approx. 1.5 mg/ml. For D-CF expressed samples, the detergent used for expression (Brij78 1%) was exchanged against second detergents upon immobilized metal affinity chromatography. *E. coli* polar lipids or Aso-PC lipids were solubilized in 100% chloroform, evaporated under vacuum to a thin film and completely dried under vacuum overnight. After resuspension of the lipidic film in liposome buffer to a final concentration of 20 mg/ml, the liposomes were extruded to a size of 0.2 μm. Protein samples and liposomes were then mixed at different molar ratios (from 1:250 to 1:4000). The mixture was incubated 1 h at room temperature and the detergent removed by addition of 500 mg Biobeads SM-2 (Bio-Rad, München, Germany) pre-equilibrated with liposome buffer. After 1 h incubation, a new aliquot of Biobeads was added and the sample incubated for up to 48 h at 18 °C. Proteoliposomes were separated from empty liposomes in a discontinuous sucrose gradient of 10–20–30–40% sucrose in liposome buffer by ultracentrifugation at 83,000 ×*g* (SW28 swing bucket rotor, Beckman Coulter, Krefeld, Germany) for 16 h at 4 °C. The pelleted proteoliposomes were resuspended in liposome buffer, shock frozen in liquid nitrogen and stored at −80 °C.

2.6. Ligand affinity chromatography

Avidin agarose beads (Pierce, Bonn, Germany) were prepared following the manufacturer's recommendations and subsequently incubated with 100 μg of b-ET-1 for each 100 μl of matrix. Peptide loading was continued for 1 h at 4 °C in matrix buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Brij35). Unbound ligand was removed by washing with 3 CVs of matrix buffer. Purified ΔETB (3 μM) in 0.1% Brij35 was diluted 1:3 in matrix buffer and loaded on the matrix. The matrix was then incubated for 2 h at room temperature with gentle shaking. The flow through was collected and the matrix was washed 10 times with 1 CV of matrix buffer in order to remove unbound protein. Elution of the ligand bound receptor was then performed using 2 mM D-desthiobiotin in matrix buffer. One step of 0.5 CV was followed by 8 elution steps of 1 CV. Aliquots of 32 μl (for the flow through and the first elution fraction) and of 55 μl (for the washing and elution fractions) were analyzed by 12% SDS-PAGE followed by immunoblotting.

2.7. Ligand binding assays

For radioassays of proteoliposomes, 500 nM protein/well was incubated with 500 pM [^{125}I]Tyr^{13}ET-1 (specific activity 2200 Ci/mmol) (PerkinElmer, Rodgau-Jügesheim, Germany) in a final volume of 50 μl binding buffer (20 mM potassium phosphate, pH 7.0, 150 mM NaCl, 0.1% (w/v) bovine serum albumin) for 1 h at room temperature in 96-well microplates. In order to determine unspecific binding, the samples were pre-incubated for 1 h at 4 °C with 4 μM final concentration of unlabeled ET-1 before addition of labeled ligand. Reactions were stopped by addition of cold binding buffer and samples were transferred to GF/B glass fiber filters (Millipore, Eschborn, Germany) pre-treated for 10 min with 0.3% polyethyleneimine and washed with 500 μl binding buffer. Unbound ligand was removed by washing filters three times with 150 μl of binding buffer. Dry filters were finally collected and the retained radioactivity was determined in a gamma counter (Cobra II Auto gamma, PerkinElmer, Rodgau-Jügesheim,

Germany). Ligand binding controls were performed using Chem1 mammalian cell membrane preparations containing recombinant human ETA or ETB receptor (Chemiscreen GPCR membrane preparation, Millipore, Eschborn, Germany). Control membrane aliquots of 5 μg were incubated with 500 pM of [^{125}I]Tyr^{13}ET-1 for 1 h at room temperature. Unspecific binding was determined in the presence of 4 μM unlabeled ET-1.

For saturation radioassay experiments with NDs, 0.25 μl (7 ng) of purified ETB-sGFP/NDs (DMPC) (30 μg/ml stock according to sGFP fluorescence), resulting into 5 nM estimated final protein concentration per well was incubated with increasing concentrations of [^{125}I]Tyr^{13}ET-1 in a final volume of 20 μl in binding buffer (50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA) for 1 h at room temperature. For competition experiments, same amounts of NDs or proteoliposome samples were pre-incubated for 1 h at room temperature with increasing concentrations of unlabeled ET-1 before adjusting to 500 pM [^{125}I]Tyr^{13}ET-1. After 1 h incubation, the samples were transferred to GF/B glass fiber filters pre-treated for 20 min with 0.3% polyethyleneimine and washed with 300 μl washing buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 0.1% BSA). Unbound ligand was removed by washing the filters five times with 150 μl washing buffer. Radioactivity was finally measured with the dried filters. Unspecific binding was determined in the presence of 2 μM unlabeled ET-1.

For the determination of Cy3-ET-1 binding, 2 nM of immobilized metal affinity chromatography purified ETB-sGFP/ND (DMPC) or PR-sGFP/ND (DMPC) (measured according the sGFP fluorescence) were incubated for 1 h with 10 nM Cy-3-ET-1 in a final volume of 25 μl in binding buffer (phosphate buffer saline, pH 7.5). The mixture was then applied on a Sephadex G50 spin column and centrifuged at 770 ×*g* for 2 min. The GPCR/ligand complex was collected in the flow-through while unbound ligand remained in the column. Fluorescence of Cy-3 was then measured using a Tecan Genios fluorescence plate reader (Excitation 535 nm, emission 612 nm) in order to determine the percentage of active receptor in a 1:1 binding model (RFU measured in the flow-through/511 RFU X 100, where 511 RFU are the RFU measured for 2 nM Cy3-ET-1).

2.8. Confocal microscopy

Images were acquired using a Zeiss LS 510 confocal microscope with an oil immersion Plan-Apochromat 63×/1.4. Excitation was provided by an argon lamp filtered with standard bandpass excitation/emission filter cubes.

2.9. Surface plasmon resonance (SPR)

Measurements were conducted with a Biacore X100 (Biacore, Uppsala, Sweden) using SA chips. 50 resonance units (RUs) of b-ET-1 or b-ET-3 were immobilized on chips. ETA, ETB and ETB derivatives co-translationally associated with MSP1E3D1-NDs were either metal affinity purified or directly injected in the CF RMs without any previous purification. In single cycle kinetic experiments, concentration series of the analyte were injected without chip regeneration in between the injections [21,22]. Kinetic parameters were analyzed using a 1:1 interaction model. In multi cycle kinetic experiments, the chips were regenerated with regeneration buffer (50 mM NaOH, 1 M NaCl) in between sample injections. Competition experiments were performed using multi cycle kinetic experiments. Constant concentrations of GPCR/ND samples (5 nM final protein concentration according to sGFP fluorescence) were incubated before injection with increasing ligand concentrations from 0.01 nM to 6 μM for 1 h at 4 °C. In all sensograms a report point at 10 s after the end of the injection was placed and the RU measured at the report point were plotted against the logarithm of the ligand concentration. Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and processed by nonlinear regression curve fitting.

3. Results

3.1. N-terminal processing and SDS resistant complex formation of CF synthesized ETB with ET-1

The ETB receptor was either expressed by co-translational solubilization in D-CF reactions supplemented with 1% Brij78, in P-CF reactions with subsequent solubilization in 1% LMPG or in L-CF reactions in the presence of MSP1E3D1-NDs (DMPC). ETA was D-CF synthesized in the presence of 0.5% Brij35. In P-CF and D-CF modes, the primary detergent of the samples was exchanged to 0.1% Brij35 upon metal affinity chromatography. While a single ETB band corresponding to a MW of approximately 40 kDa was detectable by SDS-PAGE analysis from P-CF samples, two bands corresponding to approximately 40 kDa and 35 kDa were observed with the D-CF and L-CF samples (Fig. 1A). In contrast, only one band of approximately 41 kDa was detected after D-CF as well as after P-CF expression of ETA (Fig. 1A). The formation of the ETB double band was found to be independent from the selection of supplied detergents as well as from the presence of 100 nM agonist ET-1. The samples were analyzed after Western blotting by immunodetection with an anti-His antibody and the detection of both bands of the D-CF samples indicated the presence of intact C-terminal ends in the two ETB derivatives (Fig. 1B). ETB further contains an N-terminal T7-tag and anti-T7-tag antibodies reacted after Western blotting only with the larger 40 kDa band, indicating an N-terminal deletion of the 35 kDa ETB derivative (Fig. 1B). A specific cleavage of the first 64 amino acids of ETB produced in HEK293 cells by putative metalloproteases has been reported [23]. In order to analyze whether the origin of the second band is based on a similar N-terminal cleavage, the mutant ΔETB carrying a deletion of the first 64 amino acids was prepared. The D-CF expression of ΔETB resulted in a single band after SDS-PAGE analysis apparently co-migrating with the lower 35 kDa band of full-length ETB D-CF samples (Fig. 1A). Edman sequencing of the 35 kDa band indicated the residues alanine, proline and glutamic acid in positions 3, 4 and 6, supporting the hypothesis of the specific cleavage of ETB at amino acid position 64 (data not shown).

D-CF samples of ETB and ΔETB as well as D-CF produced samples of ETA in Brij35 (0.1%) were incubated with the biotin labeled peptide agonist b-ET-1 for 1 h. After separation by low temperature SDS-PAGE, the samples were immunoblotted with anti-biotin antibodies. A clear signal of b-ET-1 was detectable with both bands of full-length ETB. The signal with the truncation ΔETB was weaker but still detectable, while no bound ligand was observed with ETA (Fig. 1B and C). The results gave evidence of SDS-resistant ETB/b-ET-1 complexes, while putatively formed ETA/b-ET-1 complexes were SDS sensitive. Aliquots of 13 nmol of the agonist b-ET-1 were immobilized on monomeric avidin agarose columns and 0.67 nmol of purified D-CF (1% Brij78) synthesized and into 0.1% Brij35 transferred ΔETB was loaded (Fig. 1D). After washing, the bound ΔETB/b-ET-1 complexes were eluted with D-desthiobiotin and quantified by immunoblotting with anti-His antibodies. A binding competence of approximately 25% was measured for ΔETB which is comparable to the previously determined efficiency of full-length ETB at similar conditions [15].

3.2. ET-1 binding after post-translational reconstitution of ETB into liposomes

Purified ETB samples after P-CF expression and solubilization in 1% LMPG were *in vitro* reconstituted into liposomes composed out of *E. coli* polar lipids or Aso-PC mixtures and analyzed for their binding of radiolabeled [^{125}I]Tyr^{13}ET-1 in filter binding assays (Table 2).

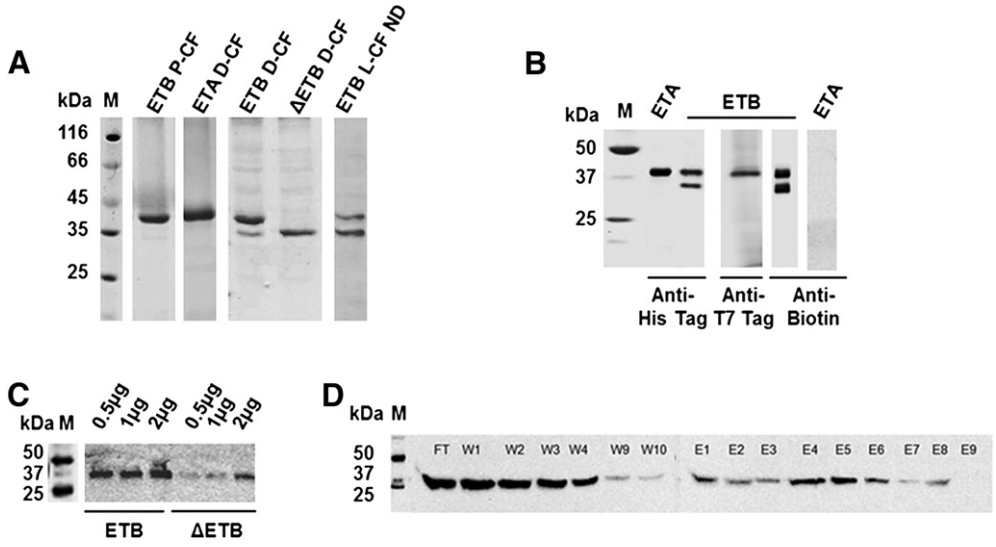

Fig. 1. Analysis of CF expressed ETA and ETB. A, metal affinity purified ETA and ETB derivatives after CF expression in P-CF, D-CF (1% Brij78) and L-CF (MSP1E3D1-ND DMPC) modes. Separation by 12% SDS-PAGE and Coomassie-blue staining. B, immunoblot of D-CF (1% Brij78) produced ETB and ETA. Sample volumes of 1 µl were separated by 12% SDS-PAGE and immunoblotted with anti-His or anti-T7 antibodies. For immunodetection with anti-biotin antibodies, 1 µl samples were first incubated for 1 h at 4 °C with 100 nM of b-ET-1 in phosphate buffered saline, separated by 12% SDS-PAGE at 10 °C and then blotted. C, formation of SDS-resistant ET-1 complexes with D-CF produced ETB and ΔETB. Different concentrations of purified protein samples were incubated with 100 nM b-ET-1 in phosphate buffered saline for 1 h at 4 °C. Samples were then separated by 16% SDS-PAGE at 10 °C and immunoblotted with anti-biotin antibodies. D, ligand affinity chromatography of D-CF produced ΔETB. 1 µM purified receptor in 0.1% Brij35 was incubated with 100 µg of b-ET-1 immobilized on monomeric avidin agarose matrix, washed and eluted with 2 mM D-desthiobiotin. Samples of all fractions were separated by 16% SDS-PAGE and immunoblotted with anti-His-tag antibodies. M, marker; FT, flow-through fraction; W, wash fractions; E, elution fractions.

Non-specific binding of [^{125}I]Tyr^{13}ET-1 to the liposome samples was determined after blocking of specific binding sites by preincubation with an excess of 4 µM non-labeled ET-1. The results indicate that the functional reconstitution of P-CF synthesized ETB strongly depends on the ratio of protein to lipid. The highest specific [^{125}I]Tyr^{13}ET-1 binding of 49% with a Bmax of 2.1 pmol/mg was obtained with EPL in a protein to lipid ratio of 1 to 1000 and in homologous competition experiments with the same sample a K_i of 2.1 µM was measured (Table 2).

D-CF (1% Brij78) synthesized ETB samples were transferred into a variety of different secondary detergents and reconstituted into *E. coli* polar lipids at a protein to lipid ratio of 1 to 1000 or 1 to 2000. In all

Table 2
^{125}I-ET-1 binding to ETB.

Mode	1st detergent[a]	2nd detergent[b]	Lipids[c]	P/L-ratio	Specific binding[d] (%)	Specific activity (pmol/mg)	K_D (nM)
P-CF	LMPG, 1%	–	EPL	1:250	32 ± 2	2 ± 0.2	–
			EPL	1:500	26 ± 1	1.2 ± 0.1	–
			EPL	1:1000	49 ± 3.5	2.1 ± 0.3	2100 (K_i)
			EPL	1:2000	26 ± 1	0.8 ± 0.03	–
			EPL	1:4000	15 ± 3.5	0.3 ± 0.1	–
			Aso-PC	1:1000	36 ± 2	1.7 ± 0.1	–
D-CF	Brij78, 1%	LPPG (0.05%)	EPL	1:1000	7 ± 3	0.1 ± 0.01	–
		LMPG (0.05%)	EPL	1:1000	30 ± 5	0.4 ± 0.02	–
		DPC (0.1%)	EPL	1:1000	34 ± 6	0.46 ± 0.2	–
		DPC (0.1%)	EPL	1:2000	26 ± 7	0.37 ± 0.1	–
		DDM (0.05%)	EPL	1:1000	7 ± 2	0.08 ± 0.02	–
		Chaps (1%)	EPL	1:2000	14 ± 2.5	0.16 ± 0.03	–
		Brij78 (0.1%)	EPL	1:1000	6 ± 3	0.05 ± 0.02	–
L-CF	Brij35, 0.04%	–	Aso-PC	4 mg/ml[e]	38 ± 1	3.3 ± 0.1	–
	Brij78, 0.04%				39 ± 2	1.5 ± 0.15	–
	Digitonin, 0.2%				25 ± 1	1.3 ± 0.1	–
	Chaps, 0.5%				25 ± 8	1.6 ± 0.4	–
	–	–	EPL		31 ± 3	2.6 ± 0.3	–
			HTL		27 ± 4	1.1 ± 0.2	–
			DOPC		21 ± 3	1.2 ± 0.08	–
			DMPC		32 ± 1	1.6 ± 0.2	–
			Aso-PC		36 ± 2	3.5 ± 0.3	1300 (K_i)
	–	–	ND	40 µM[5]	67.5 ± 1.5	31.6 ± 0.5	0.45
Positive CTR (Chemiscreen ETA membrane preparation)					87.5 ± 3	1.4 ± 0.2	0.09
Positive CTR (Chemiscreen ETB membrane preparation)					85 ± 2	0.5 ± 0.05	0.06

[a] Detergent used for resolubilization of P-CF generated pellets or as supplement for D-CF or L-CF expression.
[b] Detergent used for elution upon metal affinity purification.
[c] EPL: *E. coli* polar lipids; HTL: heart total lipid extract; ND: MSP1E3D1-NDs (DMPC).
[d] Percentage of the specific binding on the total binding.
[e] Final concentration in the RM.

analyzed cases, a background binding of [^{125}I]Tyr^{13}ET-1 significantly higher than 50% was detected and best values of approximately 34% specific binding were obtained with ETB samples in 0.1% DPC as secondary detergent, but at a relatively low Bmax of 0.46 pmol/mg (Table 2). In contrast, commercial positive control membrane fractions containing overexpressed human ETA or ETB receptor showed a high specific [^{125}I]Tyr^{13}ET-1 binding of approximately 87.5% and 85%, respectively, while the Bmax values were also in the range of approximately 1 pmol/mg (Table 2).

3.3. ET-1 binding after co-translational association of ETB with liposomes

Contact with detergent could impair the functional folding of CF expressed ETB and as alternative strategy, the detergent-free co-translational reconstitution of ETB into supplied preformed liposomes was analyzed in the L-CF expression mode. For faster optimizing and monitoring, an ETB-sGFP fusion construct was used. P-CF expressed ETB-sGFP completely precipitates into a non-fluorescent pellet. The detection of folded and fluorescent sGFP could therefore serve as preliminary indicator for the association of synthesized ETB-sGFP with the provided liposomes. Preformed liposomes composed out of different lipids were supplied into the CF reaction at a final concentration of 4 mg/ml and the sGFP fluorescence was measured after the expression reaction (Fig. 2). Fluorescence of sGFP was only detected in the precipitate, indicating the co-precipitation of the supplied lipids with associated ETB-sGFP. Analysis of a representative L-CF (Aso-PC) precipitate sample by fluorescence microscopy revealed large heterogeneous liposomes in size ranges in between 5 and 20 μm (Fig. 4A). Fluorescent ETB-sGFP particles were only detected in association with the liposomes, indicating a positive effect of the lipids on the folding of at least the sGFP fusion partner.

Comparable fluorescence intensities corresponding to a calculated total of approximately 40 μg of ETB-sGFP per ml RM were determined after addition of liposomes composed out of Aso-PC, *E. coli* polar lipids or DOPC. The addition of liposomes composed out of heart total lipid extract or DMPC yielded only approximately 10 μg/ml of fluorescent ETB-sGFP. The initial mixing of supplied lipids with 0.5% Chaps followed by subsequent dilution of the detergent into the FM during the expression reaction reduced the formation of fluorescent ETB-sGFP.

The binding of [^{125}I]Tyr^{13}ET-1 to selected L-CF produced samples was analyzed by filter binding assays. The non-specific background binding of [^{125}I]Tyr^{13}ET-1 was again very high and the best specific binding within the range of 31–36% was obtained with liposomes composed out of Aso-PC or *E. coli* polar lipids at Bmax values of 3.5 pmol/mg and 2.6 pmol/mg, respectively (Table 2). In homologous competition experiments using proteoliposomes composed out of Asp-PC lipids a K_i of 1.3 μM was measured (Table 2). L-CF generated complexes of the control protein PR-sGFP with supplied *E. coli* polar lipid liposomes showed approximately 27% of [^{125}I]Tyr^{13}ET-1 signal over the background, but at a low Bmax of only 0.02 pmol/mg (Fig. 3).

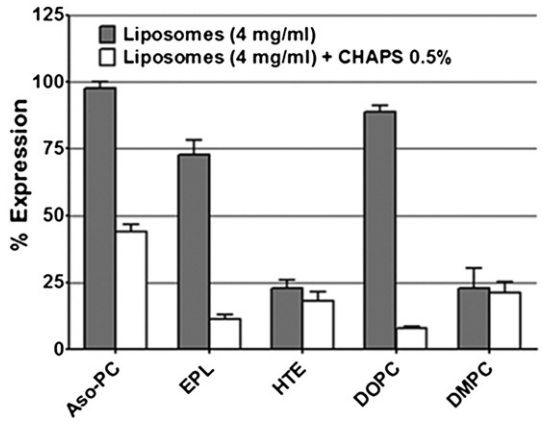

Fig. 2. L-CF expression screening of ETB-sGFP. Co-translational association of ETB-sGFP with different liposomes. Fluorescence values were normalized according to the maximum expression (40 μg/ml of ETB-sGFP with Aso-PC liposomes). Values for L-CF and L/D-CF with initial presence of 0.5% CHAPS in the RM are given.

3.4. Co-translational formation of ETB/ND and ETA/ND complexes

Prepared NDs (DMPC) of two size ranges by using either MSP1D1 or MSP1E3D1 were supplied into the CF reaction. For the initial optimization of the integration/association efficiency, the ETB-sGFP fusion was used and fluorescence in supernatant and pellet was quantified after the expression reactions (Fig. 5A). The amount of soluble sGFP fluorescence increased with increasing concentrations of MSP1E3D1-NDs in the RM and reached a plateau at concentrations in between 60 and 180 μM (Fig. 5A). According to the sGFP fluorescence, approximately 150 μg/ml of ETB-sGFP protein was synthesized having at least a functionally folded sGFP moiety. A MSP1E3D1-ND concentration of approximately 40 μM was already sufficient to obtain all detectable fluorescence in the supernatant. With the smaller MSP1D1-NDs, approximately 25% of the fluorescent ETB-sGFP still remained in the pellet at ND concentrations of 40 μM. Fluorescence microscopy revealed larger clusters of NDs in the pellets together with associated ETB-sGFP protein (Fig. 4B). The MSP1E3D1-NDs in the supernatant had a higher apparent homogeneity while the presence of smaller cluster can still not be excluded (Fig. 4B).

The membrane composition of MSP1E3D1-NDs was modified and all prepared NDs were supplied into CF reactions at a final concentration of 40 μM. The obtained ETB-sGFP fluorescence was quantified in the supernatants as well as in the pellets. NDs with membranes composed out of the anionic lipid DMPG increased the yield of fluorescent ETB-sGFP to a level twice as high if compared with NDs (DMPC) (Fig. 5B). NDs containing membranes composed out of all other analyzed lipids such as POPC, lipids isolated from brain total extracts or Aso-PC mixtures reduced the yield of fluorescent ETB-sGFP if compared with NDs (DMPC). A large fraction of the complexes of

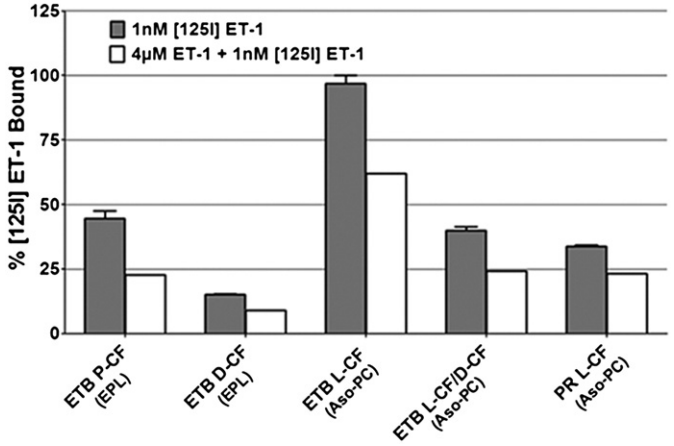

Fig. 3. Radioligand binding of CF produced ETB samples post- (P-CF, D-CF) or co-translationally (L-CF) associated with liposomes. Samples were incubated with 500 pM [^{125}I]-ET-1 for 1 h and then assayed. In competition experiments, the samples were pre-incubated with an excess of 4 μM unlabeled ET-1. Values were normalized according to the maximum count (ETB L-CF ± 35,000 CPM). ETB P-CF: resolubilized in 1% LMPG and reconstituted 1 to 1000 in EPL; ETB D-CF: expression in the presence of 1% Brij78, detergent exchange to 0.1% DPC and reconstitution 1 to 1000 in EPL; ETB L-CF: expression in the presence of 4 mg/ml Aso-PC; ETB L/D-CF: expression in the presence of 0.04% Brij78 and 4 mg/ml Aso-PC; PR L-CF: expression in presence of 4 mg/ml Aso-PC.

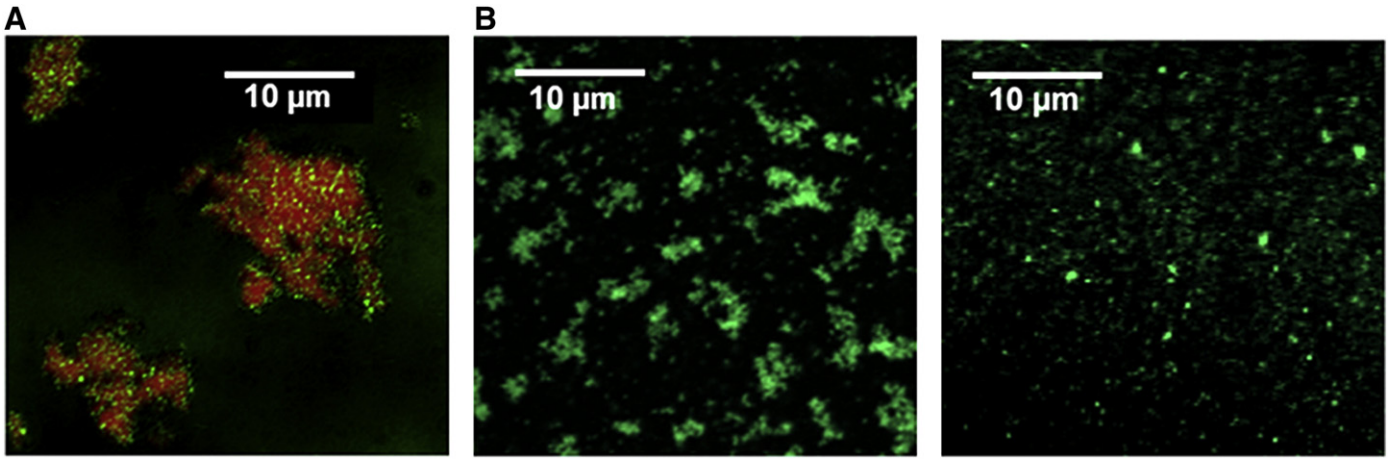

Fig. 4. Fluorescence microscopy of L-CF expressed ETB-sGFP samples. A, precipitate of L-CF expressed ETB-sGFP with Aso-PC liposomes. Green, ETB-sGFP; red, Aso-PC labeled with rhodamine. B, L-CF expression of ETB-sGFP with MSP1E3D1-NDs (DMPC). Left: pellet; right: supernatant.

fluorescent ETB-sGFP and NDs (brain total extract) precipitated in the CF reactions, while complexes with NDs containing any of the other analyzed lipids quantitatively stayed in the supernatant.

A corresponding screening was performed for the expression optimization of ETA-sGFP (Fig. 5B). At 40 µM MSP1D1-NDs (DMPC) most of the fluorescent protein fraction was recovered in the pellet. With the larger MSP1E3D1-NDs, approximately 25 µg/ml of fluorescent ETA-sGFP could be obtained in the supernatant. Similar to ETB-sGFP, with NDs (DMPG) the fluorescence yield of ETA-sGFP was twice if compared to expression experiments with NDs (DMPC) (Fig. 5B).

3.5. Binding of Cy3-ET-1 to ETB-sGFP/ND complexes

In order to evaluate the ligand binding ability of the produced ETB-sGFP/MSP1E3D1-NDs (DMPC), a binding experiment using the fluorescent ET-1 analog Cy3-ET-1 was performed. ND samples were purified by metal affinity chromatography and 2 nM of protein (according to sGFP fluorescence) was incubated with 10 nM of Cy3-ET-1 for 1 h at room temperature. Free ligand was then removed with Sephadex G50 spin columns and the amount of ligand binding active receptor in the ETB-sGFP/ND (DMPC) sample corresponded to 24 ± 3% according to the measured Cy3-ET-1 fluorescence. Approximately 75% of the Cy3-ET-1 binding could be blocked by preincubation of the receptor sample with 2 µM unlabeled ET-1 for 1 h at room temperature. The total Cy3-ET-1 binding of PR-sGFP/MSP1E3D1-NDs (DMPC) or empty MSP1E3D1-ND (DMPC) controls was less than 1.5% (Fig. 6).

3.6. Differential binding of ETA/ND and ETB/ND complexes to the natural agonists ET-1 and ET-3

The ligand binding of ETB-sGFP/MSP1E3D1-ND (DMPC) complexes was analyzed in saturation assays using [^{125}I]Tyr^{13}ET-1. Experiments were performed with metal affinity purified complexes at a final concentration of 5 nM as estimated by sGFP fluorescence. A K_D of 0.45 nM and a Bmax of 58.6 pmol/mg were determined (Fig. 7A). Competition experiments with 5 nM of ETB-sGFP/ND complexes preincubated for 1 h at RT with increasing concentrations of unlabeled ET-1 and subsequent addition of 500 pM of [^{125}I]Tyr^{13}ET-1 revealed a K_i of 6.1 nM (Fig. 7B).

ETB-sGFP/MSP1E3D1-ND (DMPC) complexes are highly soluble and therefore SPR measurements could be performed as complementary

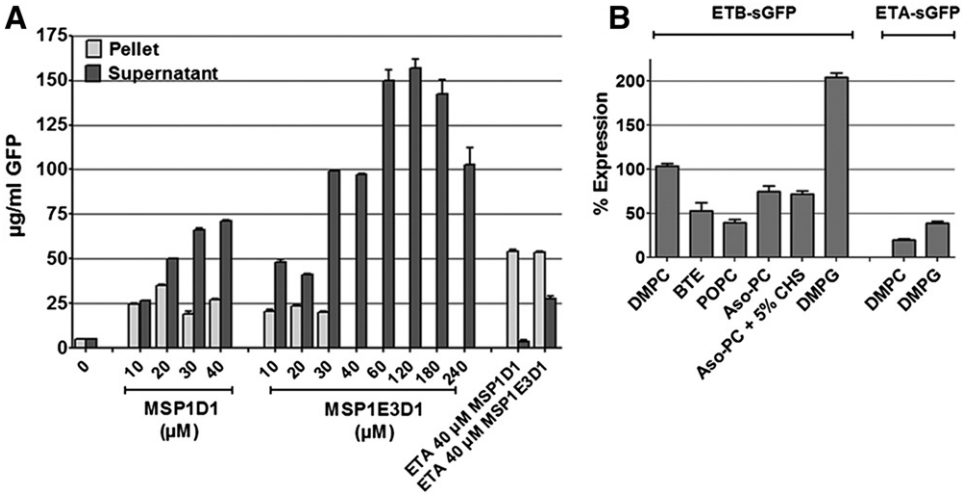

Fig. 5. L-CF (ND) expression optimization of ETB-sGFP and ETA-sGFP. A, L-CF expression in the presence of MSP1D1-NDs (DMPC) or MSP1E3D1-NDs (DMPC). Aliquots of 3 µl of supernatant or pellet were analyzed for sGFP fluorescence and protein was quantified according to a calibration curve. B, ETB-sGFP and ETA-sGFP complex formation with NDs assembled with membranes of different lipid compositions. Complex formation was analyzed at a final ND concentration of 40 µM. Values were normalized according to the ETB-sGFP complex formation with MSP1E3D1-NDs (DMPC).

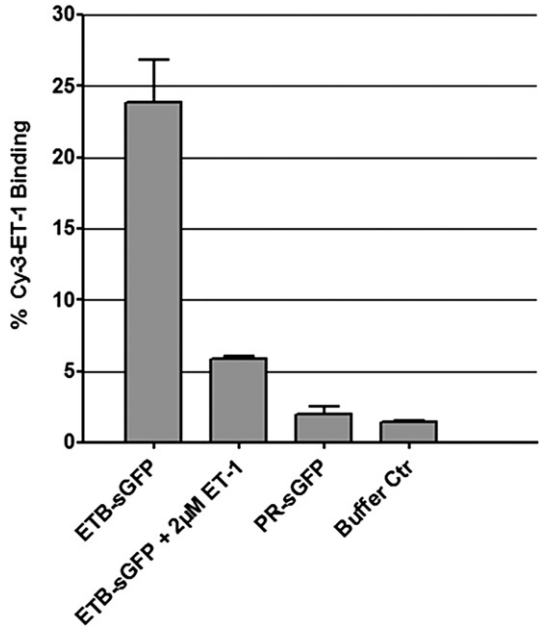

Fig. 6. Cy-3-ET-1 binding to ETB-sGFP/MSP1E3D1-ND (DMPC) complexes. 2 nM of metal affinity purified ETB-sGFP/MSP1E3D1-NDs (DMPC) or PR-sGFP/MSP1E3D1-ND (DMPC) complexes (quantified by sGFP fluorescence) was incubated with 10 nM Cy-3-ET-1. Unbound ligand was removed by Sephadex G50 spin columns and the amount of fluorescent ligand eluted in the flow-through together with the protein/ND complexes was measured.

approach in order to study the ligand binding kinetics of the GPCR samples. Amounts of 50 RUs of the ETA/ETB specific ligand b-ET-1 or of the ETB specific ligand b-ET-3 were immobilized on SA biosensor chips and the binding characteristics of ETA, ETB and the corresponding GPCR-sGFP fusions co-translationally associated with MSP1E3D1-NDs were determined. Supernatants from L-CF reactions containing ETB-sGFP or ETA-sGFP complexes with MSP1E3D1-NDs (DMPC) were directly analyzed without any previous purification. For non-purified ETB-sGFP, an apparent K_D value for the interaction with b-ET-1 of 0.5 nM was obtained (Table 3). The apparent K_D value of IMAC purified ETB-sGFP/MSP1E3D1-ND (DMPC) complexes was similar and determined at 0.4 nM. Accordingly, the non-fused ETB and the truncated derivative ΔETB showed similar K_D values of 0.5 nM and 0.6 nM, respectively. For reaction supernatants containing non-purified ETA-sGFP/MSP1E3D1-ND (DMPC) complexes, an apparent K_D value for the interaction with ET-1 of approximately 1.7 nM was determined. However, the K_D values of corresponding GPCR samples were clearly different for their interaction with the peptide b-ET-3. ETB-sGFP still showed high affinity with an apparent K_D of 0.2 nM, whereas the

Table 3
Apparent K_D values determined with GPCR derivatives in MSP1E3D1-NDs of different lipid compositions and analyzed by SPR measurements or by radioassays.

Lipid	Ligand	ETB-sGFP	ETB-sGFP (pure)	ETB	ΔETB	ETA-sGFP
DMPC	b-ET-3	0.2	–	–	–	3400
DMPC	b-ET-1	0.5	0.4	0.5	0.6	1.7
DMPC	[^{125}I]Tyr^{13}ET-1	0.45	–	–	–	–
PC	b-ET-1	0.69	–	–	–	–
PC + 5% CHS	b-ET-1	0.47	–	–	–	–
POPC	b-ET-1	0.73	–	–	–	–
BTE	b-ET-1	0.53	–	–	–	–

apparent K_D value for ETA-sGFP was with 3.4 μM significantly higher, indicating low affinity (Table 3).

The effect of the membrane composition on the ligand binding activity of ETB-sGFP complexed with MSP1E3D1-NDs was further analyzed (Table 3). ETB-sGFP was expressed in the presence of NDs of different lipid compositions and the resulting ETB-sGFP/ND complexes were metal affinity purified. In ND complexes with membranes composed out of either Aso-PC or POPC a similar strong affinity of ETB-sGFP to b-ET-1 with apparent K_D values of approximately 0.7 nM was obtained. The apparent K_D values became slightly decreased to approximately 0.5 nM if cholesterol hemisuccinate was added into Aso-PC membranes or when brain total extract lipid mixtures were used. Unfortunately, despite the relatively high fluorescence of ETB-sGFP in DMPG membranes, the ligand binding could not further be analyzed due to strong interactions of b-ET-1 with the anionic lipid.

3.7. Differential binding of ETA-sGFP/ND and ETB-sGFP/ND complexes to artificial agonists and antagonists

A panel of ligands comprising modifications of the natural ET-1 agonist as well as artificial non-peptide antagonists that show distinct differential binding patterns to ETA and ETB is known. While the peptide agonist ET-1 is non-selective for both receptors, the two peptide derivatives ALA-4-ET-1 and IRL-1620 are highly specific agonists for ETB with more than 1000 fold reduced binding to ETA. *Vice versa*, the antagonist BQ-123 is highly selective for ETA.

The interaction of these ligands with ETA-sGFP and ETB-sGFP in complexes with MSP1E3D1-NDs (DMPC) was analyzed in b-ET-1 competition binding assays using SPR (Fig. 8A and B). Fixed concentrations (5 nM) of ETA-sGFP/NDs or ETB-sGFP/NDs were preincubated with increasing concentration of ligands in the range from 0.01 nM to 6 μM for 1 h and injected on SA chips containing immobilized b-ET-1 ligand. For ETB-sGFP/ND complexes, the determined IC_{50} values were 9.6 nM for ET-1, 4.1 nM for ALA-4-ET-1, 0.005 nM for

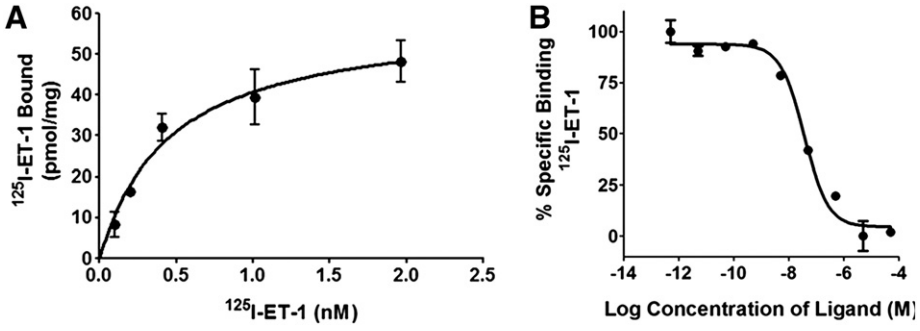

Fig. 7. Radioligand binding assay of ETB-sGFP/MSP1E3D1-ND (DMPC) complexes. A, saturation binding assay. 5 nM of purified ETB-sGFP/MSP1E3D1-NDs (DMPC) was incubated with increasing concentrations of [^{125}I]Tyr^{13}ET-1. Unspecific binding was determined in the presence of 2 μM unlabeled ET-1 and subtracted. B, competition binding assay. 5 nM of purified ETB-sGFP/MSP1E3D1-NDs (DMPC) was incubated with increasing concentrations of unlabeled ET-1 in the presence of 500 pM [^{125}I]Tyr^{13}ET-1. Data are triple measurements of two independent experiments.

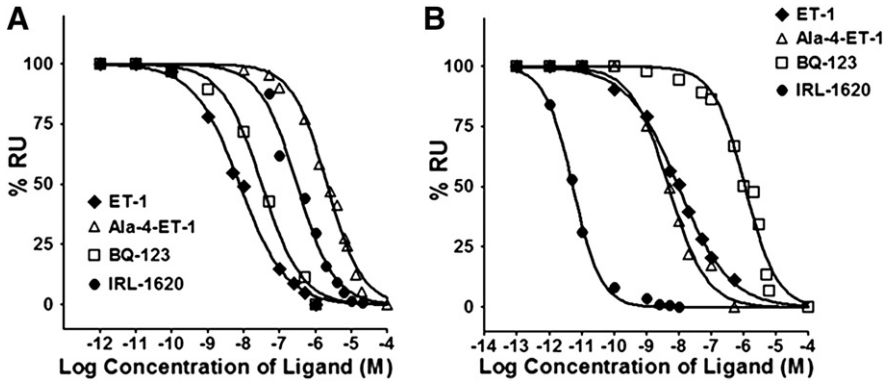

Fig. 8. Differential ligand binding of ETA-sGFP and ETB-sGFP MSP1E3D1-ND (DMPC) complexes. 50 RUs of b-ET-1 were immobilized on SA chips. A, ETA-sGFP; B, ETB-sGFP. Apparent IC_{50} values were determined in competition assays with 5 nM of complex pre-incubated with increasing concentrations (1 pM to 20 μM) of ET-1, 4-Ala-ET-1, BQ-123 or IRL-1620 1 h before injection. Report points at 10 s after injection were plotted against the logarithms of the corresponding ligand concentrations. Data were analyzed with non-linear regression curve fitting using GraphPad Prism 5. The measured apparent values are summarized below the figure.

IRL-1620 and 1.4 μM for the ETA specific antagonist BQ-123 (Fig. 8B). For ETA-sGFP/ND complexes, the IC_{50} value for the cognate ligand ET-1 was 8 nM and for the specific antagonist BQ-123 38 nM. The IC_{50} values for the ETB specific agonists ALA-4-ET-1 and IRL-1620 were 2.6 μM and 300 nM, respectively (Fig. 8A).

4. Discussion

Functionally folded ETB receptor shows a number of intrinsic characteristics. The N-terminal domain is *in vivo* subject to proteolytic cleavage in between the amino acid residues R64 and S65 [23,24]. The cleavage is catalyzed by a yet unidentified metalloprotease and recognition is based on receptor conformation rather than on the primary sequence of the cleavage site [24]. Efficiency of the *in vivo* cleavage and its physiological relevance is still not fully analyzed. The affinities of ETB and ΔETB to the ET-1 peptide are similar *in vivo* which is in accordance with our results with the CF expressed proteins. However, ΔETB is not able to induce EGF receptor transactivation, indicating differences in the functionality of the two forms [23]. The N-terminal cleavage of the ETB domain was only detectable upon soluble expression in the D-CF and L-CF (MSP1E3D1-ND (DMPC)) modes. The precipitation of ETB in the P-CF mode therefore either restricts access to the proteolytic site or it alters the receptor conformation thus preventing recognition by the protease. Conformational selection mechanisms by recognition of three-dimensional structural motifs are known from other metalloproteases like the collagenase superfamily or from the amyloid precursor protein cleaved by α-secretase [25]. The processing during soluble CF expression gives evidences that ETB adopts a native-like conformation resulting into the conformational recognition by an endogenous protease in the *E. coli* S30 extract with a similar specificity as the yet unidentified human protease.

A further specific property of ETB, again not shared with ETA, is the formation of highly stable and even SDS resistant complexes with ET-1 [26–28]. CF produced samples of ETB and ΔETB showed persistent complexes with b-ET-1 after low-temperature-SDS-PAGE, while in contrast b-ET-1 complexes with ETA were SDS sensitive. The physiological relevance of this feature could be the potential role of ETB to rapidly clear ET-1 from the vascular system after signal induction.

Lipid association efficiencies and ligand binding characteristics of ETB were analyzed after post- as well as after co-translational reconstitution approaches. Similar [^{125}I]Tyr^{13}ET-1 binding was determined with proteoliposomes obtained either after post-translational reconstitution with P-CF synthesized ETB or after co-translational reconstitution in the detergent-free L-CF mode. [^{125}I]Tyr^{13}ET-1 binding efficiencies with proteoliposomes obtained with D-CF expressed ETB samples were generally lower despite significant variations dependent on the selected detergents could be observed. ETB samples obtained after D-CF expression in the presence of Brij78 are more homogenous and have a higher ET-1 binding competence if compared with samples obtained after P-CF expression [12]. The increased functional reconstitution of P-CF samples could therefore give evidence of an improved lipid association of partially unfolded proteins accompanied by subsequent folding. The ETB-sGFP proteoliposomes produced by co-translational reconstitution in the L-CF mode almost quantitatively precipitated during the reaction, presumably due to rearrangements and fusions of the supplied liposomes upon association with the newly synthesized proteins [29–31]. A special reaction modification by forming liposomes upon slow reduction of initial detergent concentrations in the reaction mixture did not improve this effect [32]. The detection of fluorescent ETB-sGFP exclusively in association with liposomes further supports the evidence of functional reconstitution and indicates sGFP as useful fusion partner for the initial screening of functional GPCR expression in CF systems.

Specific activities in [^{125}I]Tyr^{13}ET-1 binding comparable to commercial mammalian membranes containing overexpressed ETB were obtained with ETB proteoliposomes produced by a number of CF expression conditions. However, a clear difference was the generally higher unspecific background binding of [^{125}I]Tyr^{13}ET-1 to CF produced proteoliposomes. Specific [^{125}I]Tyr^{13}ET-1 binding was mostly in between 25% and 40%, while the control membranes containing overexpressed ETA or ETB reached levels of approximately 85% of specific [^{125}I]Tyr^{13}ET-1 binding. We speculate that tight lipid association of only partially folded ETB receptor that still retain some residual ligand binding competence might account for the observed high background binding of [^{125}I]Tyr^{13}ET-1. This effect could also result into the relatively high K_i values of ET-1 within the μM range observed in [^{125}I]Tyr^{13}ET-1 competition assays with proteoliposome samples obtained after post-translational reconstitution of P-CF synthesized ETA or ETB as well as after co-translational L-CF expression of ETB [15].

The post-translational assembly of NDs with purified GPCRs or other membrane proteins is a new and rapidly evolving approach and becomes most promising for the preparation of homogenous protein samples in lipid environments [33–38]. An interesting emerging modification of this process is the co-translational association of membrane proteins with NDs by CF expression as it avoids any contact of the membrane protein with detergents [20,39–42]. Detergent sensitive membrane proteins such as the *E. coli* MraY translocase could be synthesized by this strategy in stable and functionally folded conformation [20]. The co-translational formation of ligand binding

GPCR/ND complexes was successfully applied so far with the Neurokinin 1 Receptor (NK1R), the Dopamine D1 Receptor (DRD1) and the β2-adrenergic receptor [43] as well as with derivative of the β2-adrenergic receptor having the third intracellular loop replaced by T4 lysozyme [41]. We could show for the first time the production of ligand binding active ND complexes with the two endothelin receptors having their native full-length amino acid sequence.

The larger MSP1E3D1-NDs were clearly more efficient for ETA and ETB association and this is in accordance to previous findings with the L-CF (ND) expression of other larger polytopic membrane proteins [20]. Compared to the L-CF expression mode in the presence of liposomes, three to four times more fluorescent ETB-sGFP could be obtained by using NDs. The stable and more homogenous dispersion of the NDs as well as the membrane accessibility from both sides might therefore support the association of folded ETB-sGFP protein. However, membrane association or insertion mechanisms in this artificial system are still largely unclear. ETB-sGFP as well as ETA-sGFP are both expressed at similar levels and without any signal sequence. Nevertheless, the formation of fluorescent ETB-sGFP was approx. twice as high as with ETA-sGFP in the presence of equal concentrations of MSP1E3D1-NDs (DMPC). The two receptors show roughly 63% amino acid homology with conserved transmembrane segments and higher degrees of variation within the extracellular regions [44]. One striking difference is the presence of a mainly hydrophilic region in the N-terminal domain of ETA (His66-Ser77), compared to a hydrophobic region in ETB (Pro87-Glu98). A further hydrophilic stretch is found in the first extracellular loop of ETA (Asp149-Phe153). These structural differences, which are suspected to play a role in the differential ligand binding properties [45,46], may also modulate their association with NDs.

The lipid composition determines thickness, fluidity and surface charge of the membrane and can therefore strongly modulate the association of membrane proteins with NDs as well as their enzymatic activity [20]. The fluorescence yields of ETB-sGFP were twice as high with NDs (DMPG) if compared with NDs (DMPC). The anionic lipid DMPG was also shown to be most effective in the co-translational formation of ND complexes with functionally folded MraY protein [29] as well as for the post-translational insertion of bacteriorhodopsin into NDs [37]. Unfortunately, high interaction of peptide ligands with the DMPG membranes prevented so far more detailed analysis of ETB binding characteristics in this environment and DMPC as one of the most prevalent eukaryotic lipids was selected as environment. The binding of ET-1 to ETB-sGFP/MSP1E3D1-NDs appeared to be relatively independent from the selected lipid environments. Accordingly, also the ligand binding of the neurotensin-1 receptor in ND complexes was found to be unaffected by the lipid composition, whereas Gq protein coupling was correlated to the POPG content [37]. In addition, the interaction of arrestin with rhodopsin/ND complexes was modulated by acidic lipid head groups [47]. Effects of lipid composition on signal transduction or extended differential ligand bind of the endothelin receptors can therefore currently not be excluded and will be subject of further investigation.

Ligand binding assays using the fluorescent analog Cy3-ET-1 and metal affinity purified ETB-sGFP/MSP1E3D1-ND (DMPC) complexes revealed approx. 20% of active receptor. Further estimations are possible from the radioligand binding assays. If Bmax values obtained with the control membranes (approx. 4200 CPM/μg) are compared with those obtained with ETB-sGFP/MSP1E3D1-ND (DMPC) samples (approx. 1200 CPM/μg), approx. 28% active receptor can be calculated. Alternatively, if one binding site per ETB-sGFP (MW = 74,000 Da) is assumed, approx. 650 ng active receptor per total yield of 150 μg/ml can be calculated from the binding kinetics. The specific activity of 58.6 pmol/mg with ETB-sGFP/MSP1E3D1-ND (DMPC) complexes is higher if compared with membrane fractions of ETB overexpressing CHO cells (0.5 pmol/mg), while total amounts are similar to those obtained from COS cells (60 pmol/mg) [26]. SF9 cells appear to be more productive with 100 pmol/mg (15–20 nmol/L) for ETB, whereas other GPCRs such as the β-adrenergic or muscarinic receptors are expressed in SF9 cells at similar concentrations in between 5 and 30 pmol/mg [48].

NDs allow the analysis of associated membrane proteins by techniques established for soluble proteins such as SPR measurements [22,49–51]. Our report is one of the first examples of SPR analysis of GPCR/ND complexes. The sGFP fusions of ETA and ETB were shown to be useful for protocol development and did not affect ligand binding as control experiments with non-fused receptors gave similar affinities to b-ET-1 and comparable to expected values from the literature [52]. The differential ligand binding of the endothelin receptors *in vivo* is well characterized and the two physiological relevant ligands ET-1 and ET-2 have comparable high affinities of 0.1–0.8 nM for both proteins [53,54]. The determined affinity of b-ET-1 to the CF expressed ETA/ND and ETB/ND complexes matches nicely with the expected values. Again in accordance with the literature, the expected K_D value of b-ET-3 is in the same low range for ETB, but >2 μM for ETA. The IC_{50} values analyzed in ET-1 binding competition with cellular membranes containing ETB are 0.3 nM for ALA-4-ET-1, 0.1–50 nM for IRL-1620 and >1.5 μM for BQ-123. The corresponding values with membranes containing ETA are >2 μM for ALA-4-ET-1, >0.5 μM for IRL-1620 and 1–3 nM for BQ-123 [55–57]. In agreement with these data, we could demonstrate high affinity binding of BQ-123 to CF expressed ETA-sGFP/MSP1E3D1-NDs (DMPC) complexes, while the other three ligands showed only low affinity. *Vice versa*, IRL-1620, ALA-4-ET-1 and ET-3 showed high affinity binding to CF expressed ETB-sGFP/MSP1E3D1-ND (DMPC) complexes whereas the apparent K_D value of BQ-123 was >1 μM. The ligand affinity of endothelin receptors is some 10-fold lower in isolated membranes if compared with intact cells [57]. One known exception is the significantly enhanced binding of the peptide agonist IRL-1620 to isolated membranes containing ETB. It is interesting to note that we can observe this effect also with the CF produced ETB-sGFP/MSP1E3D1-ND (DMPC) complexes and an apparent approx. value of some 5 pM could be obtained.

5. Conclusions

Our results document a new process to produce the two endothelin receptors, ETA and ETB, in a ligand-binding competent form and to enable the characterization of GPCR signaling properties. The CF produced GPCR/MSP1E3D1-ND (DMPC) complexes could be directly analyzed by SPR measurements without previous purification steps. CF expression of the endothelin receptors and subsequent ligand binding studies can be completed within two to three days, thus opening new possibilities for GPCR screening approaches in natural lipid environments.

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