The signal peptide of the rat corticotropin-releasing factor receptor 1 promotes receptor expression but is not essential for establishing a functional receptor

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Approximately 5–10 % of the GPCRs (G-protein-coupled receptors) contain N-terminal signal peptides that are cleaved off during receptor insertion into the ER (endoplasmic reticulum) membrane by the signal peptidases of the ER. The reason as to why only a subset of GPCRs requires these additional signal peptides is not known. We have recently shown that the signal peptide of the human ET_B-R (endothelin B receptor) does not influence receptor expression but is necessary for the translocation of the receptor's N-tail across the ER membrane and thus for the establishment of a functional receptor [Köchl, Alken, Rutz, Krause, Oksche, Rosenthal and Schüle (2002) *J. Biol. Chem.* **277**, 16131–16138]. In the present study, we show that the signal peptide of the rat CRF-R1 (corticotropin-releasing factor receptor 1) has a different function: a mutant of the CRF-R1 lacking the signal peptide was functional and displayed wild-type properties with respect

to ligand binding and activation of adenylate cyclase. However, immunoblot analysis and confocal laser scanning microscopy revealed that the mutant receptor was expressed at 10-fold lower levels than the wild-type receptor. Northern-blot and *in vitro* transcription translation analyses precluded the possibility that the reduced receptor expression is due to decreased transcription or translation levels. Thus the signal peptide of the CRF-R1 promotes an early step of receptor biogenesis, such as targeting of the nascent chain to the ER membrane and/or the gating of the protein-conducting translocon of the ER membrane.

Key words: corticotropin-releasing factor receptor 1 (CRF-R1), endoplasmic reticulum, functional receptor, G-protein-coupled receptor (GPCR), signal peptide, translocon.

INTRODUCTION

Heptahelical GPCRs (G-protein-coupled receptors) represent the largest protein family in vertebrates and are the targets of many drugs. The first step in the intracellular transport of GPCRs is targeting of the nascent polypeptide chain to the ER (endoplasmic reticulum) membrane and integration into this compartment. ER targeting/insertion of GPCRs follows one of two different pathways [1]. The majority (90–95 %) of the receptors use the first TM domain (transmembrane domain) of the mature receptor as an uncleaved signal anchor sequence for this process. A smaller subgroup (5–10 %), however, possesses additional cleavable signal peptides. Cleavable signal peptides of membrane proteins share characteristic features with signal peptides of secretory proteins [2]: a central hydrophobic region N-terminally and C-terminally flanked by rather polar amino acid residues. The C-terminal end often contains helix-breaking proline and glycine residues and small uncharged residues at positions –1 and –3 of the cleavage site. In the case of secretory proteins, cleavable signal peptides are obligatory for their translocation across the ER membrane.

The reason why some membrane proteins including GPCRs require additional signal peptides, whereas others do not, is not completely understood. We have recently proposed a function of cleavable signal peptides of GPCRs [3]. It is based on current

knowledge of the ER targeting/insertion mechanism of secretory proteins that also contain cleavable signal peptides (for reviews see [4–6]). The intracellular transport of GPCRs with and without signal peptides begins in the cytosol with the synthesis of the receptor's N-tails. Cytosolic translation continues until the first hydrophobic segment, namely a cleavable signal peptide or an uncleavable signal anchor sequence, appears. This leads to the binding of the SRP (signal recognition particle) and to a translation stop in the cytosol ('elongation arrest'). The resulting complexes are then targeted to the ER membrane. The nascent chains are transferred to the translocon (mainly consisting of the protein-conducting channel protein Sec61), translation restarts and the proteins are integrated into the membrane. This mechanism implies differences in the translocation of the N-tails of GPCRs with and without signal peptides. Without a signal peptide, the N-tail of a GPCR is completely synthesized in the cytoplasm, since translation continues until the signal anchor sequence (TM1) appears. The N-tail must thus be translocated post-translationally through the translocon. In contrast, in the case of an additional signal peptide, the N-tail is not translated in the cytosol, since SRP binding to the preceding signal peptide stops elongation. Here, the N-tail is translocated co-translationally through the translocon, i.e. during its synthesis. Taking these considerations into account, we have suggested that signal peptides are necessary for N-tail translocation of those GPCRs for which a post-translational

Abbreviations used: AP, alkaline phosphatase; CRF-R1, corticotropin-releasing factor receptor 1; DPBS, Dulbecco's PBS; ER, endoplasmic reticulum; ET-1, endothelin-1; ET_B-R, endothelin B receptor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HEK-293 cell, human embryonic kidney 293 cell; IP, inositol phosphate; PNGase F, peptide N-glycosidase F; PrP, prion protein; SRP, signal recognition particle; TM domain, transmembrane domain.

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translocation of the N-tail is impaired. Indeed, we have recently shown that the signal peptide of ET_B-R (endothelin B receptor) is essential for an N-tail translocation across the ER membrane and consequently for the establishment of a functional receptor [3]. The signal peptide, however, had no influence on receptor expression. A similar function of the signal peptide can also be assumed for the rat thyrotropin receptor, where deletion of a sequence containing the putative signal peptide led to non-functional receptors [7,8].

A cleavable signal peptide may also promote targeting of the nascent chain-SRP complex to the ER membrane or gating of the translocon and thereby influence receptor expression. In the present study, we show that the signal peptide of CRF-R1 (corticotropin-releasing factor receptor 1) strongly increases receptor expression while not being necessary for establishing a functional receptor. Thus the cleavable signal peptides of GPCRs serve different functions.

EXPERIMENTAL

Materials

The cDNA of CRF-R1 was a gift from U.B. Kaupp (IBI Forschungszentrum Jülich, Jülich, Germany) and the PrP (prion protein) A120L reporter cassette was a gift from R. Hegde (National Institutes of Health, Bethesda, MD, U.S.A.). [¹²⁵I]Tyr⁰-sauvagine and [³H]cAMP were purchased from PerkinElmer LifeSciences (Karlsruhe, Germany). Unlabelled sauvagine was synthesized as described previously [9]. [α -³²P]ATP, [α -³²P]dCTP, *myo*-[2-³H]inositol, the MegaprimeTM DNA labelling system and Microspin G-50 columns were obtained from Amersham Biosciences. [¹²⁵I]ET-1 (where ET-1 stands for endothelin-1) was obtained from NEN Life Science Products (Boston, MA, U.S.A.). LipofectamineTM, TRIzol[®] reagent and vector plasmids pCDNA3 and pSecTag2A were purchased from Invitrogen Life Technologies (Karlsruhe, Germany), and TransFastTM was from Promega (Mannheim, Germany). DNA-modifying enzymes and PNGase F (peptide N-glycosidase F) were purchased from New England Biolabs (Frankfurt am Main, Germany). Trypan Blue was from Seromed (Berlin, Germany). EZ-LinkTM Sulpho-NHS-Biotin (where NHS stands for *N*-hydroxysuccinimide) and immobilized NeutrAvidin were obtained from Pierce (Rockford, IL, U.S.A.). Oligonucleotides were purchased from Biotex (Berlin, Germany). Vector plasmid pEGFP-N1, encoding the red-shifted variant of GFP (green fluorescent protein), the Great EscapeTM SEAP kit, TALON metal-affinity resin and the human β -actin cDNA control probe were purchased from Clontech Laboratories (Heidelberg, Germany). The ULTRAhybTM ultra-sensitive hybridization buffer was purchased from Ambion (Cambridgeshire, U.K.). All other reagents were obtained from Sigma (Taufkirchen, Germany). Data of the ligand-binding, adenylate cyclase activity and IP (inositol phosphate) accumulation assays were analysed using the program GraphPad Prism version 3.02 (GraphPAD Software, San Diego, CA, U.S.A.).

Antibodies

The polyclonal rabbit anti-GFP antiserum 08 raised against a synthetic peptide has been described in [10]. The polyclonal rabbit anti-GFP antiserum 01 was raised against a fusion protein consisting of glutathione S-transferase and GFP. The fusion protein was expressed in *Escherichia coli* and purified with glutathione-Sepharose beads. The specificity of the resulting antiserum was determined by immunodetection of a GFP-tagged marker protein (vasopressin V2 receptor) [11,12] in crude mem-

Figure 1 Amino acid sequence of the N-tail of CRF-R1 and schematic representation of the CRF-R1 constructs used in the present study

(A) Sequence of the N-tail of the CRF-R1 and its cleavable signal peptide (black). (B) Full-length receptor constructs. CRF-R1.GFP and Δ SP.CRF-R1.GFP represent the wild-type GFP-tagged CRF-R1 and its signal peptide mutant respectively. The signal peptide and TMs (numbered) are shown as black boxes. The N-tail is depicted as an open box. (C) Marker protein fusions. NT.GFP and Δ SP.NT.GFP are fusion proteins consisting of GFP and the N-tail with and without signal peptide respectively. For purification, an additional C-terminal His₆-sequence (His) is added. Plasmids NT.AP and Δ SP.NT.AP represent the corresponding AP fusions. Plasmid CRF-R1.PrP encodes a fusion of the N-tail of CRF-R1 to the hamster prion marker protein. Plasmid Δ SP.CRF-R1.PrP encodes the corresponding signal peptide mutant.

branes of transiently transfected HEK-293 cells (human embryonic kidney 293 cells) (results not shown). The monoclonal mouse anti-GFP antibody was purchased from Clontech Laboratories, the monoclonal mouse anti-His antibody was from Roche (Mannheim, Germany). AP (alkaline phosphatase)-conjugated anti-mouse or anti-rabbit IgG and peroxidase-conjugated anti-mouse IgG were purchased from Dianova (Hamburg, Germany). The Lumi-Light Western-blot substrate was obtained from Roche.

Plasmid constructs

The CRF-R1 constructs used in the present study are schematically represented in Figure 1 (details of the cloning procedures are available on request). Plasmid pCRF-R1.GFP encodes the full-length CRF-R1 in the vector plasmid pEGFP-N1. The protein is C-terminally tagged with a GFP moiety at position Val⁴¹⁵ (thereby deleting the stop codon of the CRF-R1). Plasmid p Δ SP.CRF-R1.GFP encodes the signal peptide mutant lacking the N-terminal 24 amino acid residues. Plasmids pNT.GFP and p Δ SP.NT.GFP encode GFP fusions to the N-tail of CRF-R1 (position Ala¹¹⁹) with and without signal peptide respectively in the vector pSecTag2A. In the case of the signal peptide mutants, the N-terminal 25 amino acid residues were deleted. An additional C-terminal His₆-sequence allowed the purification of all GFP fusion proteins. Plasmids pNT.AP and p Δ SP.NT.AP encode the corresponding

AP fusion proteins in the vector pSecTag2A. Plasmid CRF-R1.PrP encodes a fusion of the N-terminal 121 amino acids of CRF-R1 to the hamster PrP marker (PrP A120L reporter cassette) [13]. Plasmid p Δ SP.CRF-R1.PrP encodes the corresponding signal peptide mutant lacking the N-terminal 24 amino acids.

The ET_B-R constructs used in the present study have been described previously [3]. Plasmid pET_B.GFP encodes the C-terminally GFP-tagged full-length ET_B-R, and plasmid pET_B Δ 26.GFP encodes the corresponding signal peptide mutant. Plasmid ET_B134.GFP encodes a truncated receptor fragment comprising signal peptide, N-tail, the first TM and the first intracellular loop of the ET_B-R C-terminally fused to GFP (GFP fusion to Asn¹³⁴). Plasmid ET_B134/ Δ 26.GFP encodes the corresponding signal peptide mutant.

Cell culture and transfection

HEK-293 cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Transfection of the cells with LipofectamineTM or TransFastTM was carried out according to the manufacturer's instructions.

Quantitative detection of secreted GFP fusion proteins

Secreted GFP fusion proteins were analysed by immunoblotting and fluorimetric measurements. HEK-293 cells (4×10^6) grown on 100 mm diameter dishes were transiently transfected with 7 µg of plasmid DNA and 50 µl of LipofectamineTM. Cells were grown for 24 h. The cell-culture medium (8 ml) was collected and cell debris was removed by centrifugation (5 min, 200 g). TALON metal-affinity resin beads (500 µl bed volume) were washed twice with washing buffer (37 mM Na₂HPO₄, 11 mM NaH₂PO₄ and 300 mM NaCl, pH 7.0) and added to the cell-culture medium. The sample was incubated for 30 min to allow coupling of the His-tagged GFP fusion proteins with the beads. Resin beads were collected (2 min, 700 g), washed three times with washing buffer and resuspended in 200 µl of elution buffer (37 mM Na₂HPO₄, 11 mM NaH₂PO₄, 300 mM NaCl and 150 mM imidazole, pH 7.0). The GFP fusion proteins were solubilized for 15 min and the resin beads were separated by centrifugation as described above.

For Western-blot detection of the secreted fusion proteins, 30 µl of the resulting supernatant was treated with PNGase F. To this end, 3 µl of denaturing buffer (5%, w/v, SDS and 10%, v/v, 2-mercaptoethanol) was added and the sample was incubated for 10 min at 95°C. Reaction buffer (4.5 µl; 0.5 M sodium phosphate, pH 7.5), PNGase F (3 µl; 500 units/µl) and Nonidet P40 (4 µl; 10%, v/v) were added, and the proteins were digested for 1.5 h at 37°C. The sample was supplemented with Laemmli buffer (60 mM Tris/HCl, 2%, w/v, SDS, 10%, v/v, glycerol, 5%, v/v, 2-mercaptoethanol and 0.1%, w/v, Bromophenol Blue, pH 6.8), incubated for 3 min at 95°C and proteins were analysed by SDS/PAGE/immunoblotting. Secreted proteins were detected with the polyclonal rabbit anti-GFP antiserum 08 and AP-conjugated anti-rabbit IgG.

For measuring GFP fluorescence, the remaining 170 µl of the elution buffer supernatant (see above) was centrifuged again (2 min, 13 000 g) and directly used for fluorimetric analysis ($\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} \geq 507$ nm).

Quantitative detection of secreted AP fusion proteins

Secreted AP fusion proteins were assessed by immunoblotting and by AP activity assays. HEK-293 cells (7.5×10^5) grown on 60 mm

diameter dishes were transiently transfected with 2.5 µg of plasmid DNA and 19 µl of LipofectamineTM. Cells were grown for 24 h. For immunoblot detection, the cell-culture medium (5 ml) was collected and cell debris was removed by centrifugation (5 min, 200 g). The His-tagged AP fusion proteins of the cell-culture medium were coupled with TALON metal-affinity resin beads (200 µl bed volume), dissolved in 60 µl of the elution buffer and separated from the beads as described above for the GFP fusion proteins. An aliquot of the resulting supernatant (30 µl) was digested with PNGase F and subjected to SDS/PAGE/immunoblotting as described above. The AP fusion proteins were detected with a monoclonal mouse anti-His antibody and AP-conjugated anti-mouse IgG.

AP activity assays were performed using the Great EscAPETM SEAP kit. The cell-culture medium (110 µl) was centrifuged (10 min, 12 000 g) to remove cells and cell debris. To the supernatant (7.5 µl aliquot), 5× dilution buffer was added to achieve a final volume of 26 µl. The samples were incubated for 30 min at 65°C and chilled on ice for 3 min. Assay buffer (25 µl) was added followed by another incubation for 5 min at room temperature (20°C). After addition of the chemiluminescence substrate (25 µl of a 1:20 dilution), the samples were incubated for 10 min at room temperature. A 1:10 dilution of the reaction samples was used for the luminometric measurements.

Adenylate cyclase activity assay

HEK-293 cells (3.6×10^6) grown on 100 mm diameter dishes were transiently transfected with 8 µg of plasmid DNA and 24 µl of TransFastTM. After transfection, cells were grown for 24 h. Cells of three 100 mm dishes were collected and washed once with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.47 mM KH₂PO₄, pH 7.4). Cells were collected (5 min, 100 g) and resuspended in 1.5 ml of a buffer (27%, w/v, sucrose, 2 mM Hepes and 1 mM EDTA, pH 7.8). Cells were homogenized with a Potter S homogenizer (10 strokes, 750 rev./min). The sample was centrifuged (10 min, 20 000 g), and the pellet (crude membranes) was resuspended in 600 µl of a buffer (2 mM Hepes and 1 mM EDTA, pH 7.8). For measurements of adenylate cyclase activity, crude membranes (20 µg of protein) were incubated in a final volume of 100 µl of a buffer containing 27 750 Bq [α -³²P]ATP, 4 mM MgCl₂, 100 µM ATP, 10 µM GTP, 1 mM cAMP, 2 mM EDTA, 1 mM dithiothreitol, 1 mM isobutylmethylxanthine, 1.32 mg/ml creatine kinase, 5.1 mg/ml phosphocreatine, 6 mg/ml BSA and 50 mM Tris/HCl (pH 7.8). The ligand sauvagine was diluted with a buffer (2 mM Hepes, 1 mM EDTA and 2%, w/v, BSA, pH 7.8; final volume 10 µl) and added to the reaction sample at the final concentrations indicated in the Results section. Samples were incubated for 20 min at 32°C, and [³²P]cAMP formation was stopped by the addition of 500 µl of stop solution (4 mM ATP, 1.4 mM cAMP, 2%, w/v, SDS and 500 Bq/500 µl [³H]cAMP). [³²P]cAMP was isolated using the two-column method [14], and radioactivity was quantified with a liquid-scintillation counter (1409; PerkinElmer Wallac, Freemont, CA, U.S.A.).

[¹²⁵I]Tyr⁰-sauvagine binding assay

HEK-293 cells (2.5×10^4) grown on 24-well plates were transiently transfected with 250 ng of plasmid DNA and 0.75 µl of TransFastTM and grown for 62 h. Cells were washed once with DPBS (Dulbecco's PBS; PBS containing 9 mM CaCl₂ and 5 mM MgCl₂). Then, DPBS supplemented with 0.5 mM PMSF, 0.5 mM benzamidine, 1.4 µg/ml aprotinin, 3.2 µg/ml trypsin inhibitor, 213 µg/ml bacitracin, 0.05% (w/v) BSA and [¹²⁵I]Tyr⁰-sauvagine (see the Results section for the individual concentrations) was

added (final volume 450 μ l). For determination of the non-specific binding, 1 μ M sauvagine was added. The samples were incubated at 25°C for 2 h. Cells were washed three times with ice-cold DPBS and lysed with 500 μ l of 100 mM NaOH solution. Bound [125 I]Tyr⁰-sauvagine was quantified using a PerkinElmer 1470 WizardTM liquid-scintillation counter.

[125 I]ET-1 binding assay

The experiment was carried out with crude membranes of transiently transfected HEK-293 cells as described previously for stably transfected Chinese-hamster ovary cells [15].

IP accumulation assay

The experiment was carried out with intact transiently transfected HEK-293 cells as described previously for stably transfected HEK-293 cells [16].

Visualization of the GFP-tagged CRF-R1 in transiently transfected HEK-293 cells

Cells (1×10^4) grown on glass coverslips in 35 mm diameter dishes were transiently transfected with 1 μ g of plasmid DNA and 7.5 μ l of LipofectamineTM and incubated for 24 h. The receptor GFP signals and the plasma-membrane Trypan Blue signals were analysed by confocal laser scanning microscopy as described previously [3] using a Zeiss LSM 510 Meta microscope (Carl Zeiss, Jena, Germany). The quantification of the fluorescence signals was analysed using the software version 3.2 SP2 of this microscope.

Biotinylation of cell-surface proteins

HEK-293 cells (3.6×10^6) grown on 100 mm diameter dishes were transiently transfected with 2.5 μ g of plasmid DNA and 50 μ l of LipofectamineTM and maintained for another 24 h. Cells were washed twice with PBS (pH 7.4) and cell-surface proteins were biotinylated on ice for 30 min with 4 ml of a solution containing 0.5 mg/ml EZ-LinkTM Sulpho-NHS-Biotin in PBS (pH 7.4). The labelling reaction was stopped by replacing the biotin solution with 4 ml of stop solution (50 mM NH₄Cl in PBS). Cells were incubated for 10 min, washed three times with PBS and supplemented with 3 ml of lysis buffer (0.5 mM PMSF, 0.5 mM benzamidin, 1.4 μ g/ml aprotinin, 3.2 μ g/ml trypsin inhibitor, 150 mM NaCl, 1 mM EDTA, 1%, v/v, Triton X-100, 0.1% SDS and 50 mM Tris/HCl, pH 8.0). Samples were incubated for 30 min, and insoluble debris was removed by centrifugation (20 min, 20 000 g). Biotinylated proteins were recovered from the supernatant with 50 μ l of NeutrAvidinTM agarose beads (1.5 h incubation). Beads were sedimented (5 s, 17 000 g), washed three times with 1 ml of washing buffer 1 (500 mM NaCl, 1 mM EDTA, 0.5%, v/v, Triton X-100, 0.1%, w/v, SDS and 50 mM Tris/HCl, pH 8.0) and once with 1 ml of washing buffer 2 (1 mM EDTA, 0.5%, v/v, Triton X-100, 0.1%, w/v, SDS and 50 mM Tris/HCl, pH 7.4). The proteins were separated from the beads using 80 μ l of Laemmli buffer, denatured for 5 min at 95°C and subjected to SDS/PAGE/immunoblotting. The cell-surface-bound GFP-tagged receptors were detected with the polyclonal rabbit anti-GFP antiserum 01 and peroxidase-conjugated anti-rabbit IgG.

Immunoprecipitation of GFP-tagged full-length CRF-R1 and ET_B-R fragments

HEK-293 cells (3.6×10^6) grown on 100 mm diameter dishes were transiently transfected with 2.5 μ g of plasmid DNA and

50 μ l of LipofectamineTM and maintained for 24 h. Cells were collected (5 min, 100 g), washed once with PBS (pH 7.4) and lysed for 1 h with 2 ml of lysis buffer. Insoluble debris was removed by centrifugation (20 min, 20 000 g). The supernatant was divided into two aliquots, and each sample was supplemented with 20 μ l of polyclonal rabbit anti-GFP antiserum 01 and incubated for 2 h. After the addition of 10 mg of Protein A-Sepharose CI-4B beads (equilibrated with lysis buffer), GFP-tagged receptors were pulled down (5 s, 17 000 g), and the beads were washed three times with 1 ml of washing buffer 1 and once with 1 ml of washing buffer 2. One sample was supplemented with Laemmli buffer, incubated for 3 min at 95°C and directly used for SDS/PAGE/immunoblotting. The proteins of the second sample were treated with PNGase F before immunoblot analysis as described above (except that denaturation was carried out for 15 min at 40°C). The precipitated GFP-tagged receptors were detected by immunoblotting using a monoclonal mouse anti-GFP antibody and peroxidase-conjugated anti-mouse IgG.

Northern-blot analysis and *in vitro* transcription/translation assay

HEK-293 cells (4×10^6) grown on 100 mm diameter dishes were transiently transfected with 7 μ g of plasmid DNA and 50 μ l of LipofectamineTM and maintained for another 24 h after transfection. Total RNA was isolated using the TRIzol[®] reagent according to the manufacturer's instructions. RNA denaturation, agarose gel electrophoresis and Northern blotting were carried out using standard procedures. DNA probes against the cDNA of CRF-R1 were generated using [α - 32 P]dCTP and the MegaprimeTM DNA labelling system. The labelled probes were purified using Microspin G-50 columns. Prehybridization and hybridization were carried out using the ULTRAhybTM hybridization buffer according to the manufacturer's instructions. The Northern blots were analysed using a STORM 830 phosphor imager from Molecular Dynamics (Piscataway, NJ, U.S.A.).

In vitro transcription and translation of the PrP fusion proteins with SP6 RNA polymerase and rabbit reticulocyte lysate in the presence of [35 S]Met were performed as described in [17]. Proteins were separated by SDS/PAGE and detected by autoradiography.

Miscellaneous

Standard DNA preparations and manipulations were carried out. The nucleotide sequences of DNA fragments were verified by sequencing, using the FS Dye Terminator kit from PerkinElmer (Weiterstadt, Germany).

RESULTS

Experimental proof of the signal peptide cleavage of CRF-R1

Signal peptides of membrane proteins are predictable by computer programs such as SPScan (Genetics Computer Group, Madison, WI, U.S.A.). Computer predictions as well as previous experimental results indicate that CRF-R1 contains a cleavable signal peptide [18,19]. We first verified the presence of the signal peptide of CRF-R1 by a simple method. The N-terminal 119 amino acid residues of the receptor containing the putative signal peptide were fused to the GFP or *E. coli* AP as marker proteins (constructs NT.GFP and NT.AP respectively, see Figure 1 and the Experimental section). The corresponding signal peptide mutants lacking the N-terminal 25 amino acid residues (constructs Δ SP.NT.GFP and Δ SP.NT.AP, see Figure 1) were used as controls. If a cleavable signal peptide is indeed present in the N-tail of CRF-R1, it should direct the marker proteins via the secretory pathway to the cell-culture medium (Figure 2A). Here,

Figure 2 CRF-R1 possesses a cleavable signal peptide

(A) Schematic representation of a method to detect cleavable signal peptides of membrane proteins. Putative signal peptides are fused to marker proteins (AP or GFP). In the presence of a signal peptide, the marker proteins are directed via the secretory pathway to the extracellular space. Secreted marker proteins are detectable by their autofluorescence (GFP) or by an enzyme activity assay (AP). Ri, ribosome. (B) Upper panel: GFP fluorescence assay. HEK-293 cells were transiently transfected with fusion proteins consisting of GFP and the N-tail of CRF-R1 with signal peptide (NT.GFP) and without signal peptide (ΔSP.NT.GFP). Untransfected HEK-293 cells were used as a control. The fusion proteins were purified from the supernatant and the GFP fluorescence signals were monitored. Results shown are the means $\pm$ S.D. for three independent experiments each performed in triplicates. Lower panel: detection of the same purified fusion proteins by immunoblotting using a polyclonal rabbit anti-GFP antibody and AP-conjugated anti-rabbit IgG. (C) Upper panel: AP activity assay. HEK-293 cells were transiently transfected with fusion proteins consisting of AP and the N-tail of CRF-R1 with signal peptide (NT.AP) and without signal peptide (ΔSP.NT.AP). Untransfected HEK-293 cells were used as a control. The AP activity of each supernatant was quantified by an enzymatic assay. Results shown are the means $\pm$ S.D. for three independent experiments each performed in triplicates. Lower panel: detection of the same fusion proteins by immunoblotting using a monoclonal mouse anti-His antibody and AP-conjugated anti-mouse IgG. The fusion proteins were purified before SDS/PAGE/immunoblotting.

the secreted protein should be easily detectable by fluorimetric measurements (GFP) or by enzyme activity assays (AP). If the sequence, however, contains an uncleaved signal peptide, the marker proteins will become integral membrane proteins. If the sequence contains no signal peptide at all, the marker proteins will become cytoplasmic proteins.

HEK-293 cells were transiently transfected with the constructs and the secreted GFP-tagged proteins were purified from the cell-culture medium using their His tag and detected fluorimetrically or by immunoblotting (Figure 2B). The secreted AP-tagged proteins were detected directly by an AP activity assay or by immunoblotting after purification (Figure 2C). GFP fluorescence or AP activity in the cell-culture medium was detectable only if the putative signal peptide of the CRF-R1 was present (constructs

NT.GFP and NT.AP respectively). Likewise, immunoreactive proteins were found only in the medium of the constructs containing signal peptides. The apparent molecular masses of NT.GFP (41 kDa) and NT.AP (70 kDa) were in good agreement with the calculated sizes of the constructs (41 385 and 68 807 Da respectively). The presence of the marker proteins in the cell-culture medium shows that the N-tail of CRF-R1 indeed contains a cleavable signal peptide. This simple methodology may also be used in future studies to detect cleavable signal peptides of other GPCRs and unrelated membrane proteins.

The signal peptide of CRF-R1 is not necessary for establishing a functional receptor

A cleavable signal peptide may be necessary for translocation of a receptor's N-tail across the ER membrane. It may also promote ER targeting/insertion of the nascent chain or stabilize it against early proteolytic degradation and thereby increase receptor biogenesis. In the case of CRF-R1, a ligand-binding study using a mutant lacking a signal peptide and a wild-type receptor is a useful initial experiment to distinguish these two possibilities. When the signal peptide is removed, TM1 of the mature receptor should assure the signalling properties for ER targeting/insertion as a signal anchor sequence (it was shown by Audigier et al. [20] that almost every TM of a GPCR can function as a signal anchor sequence if it is placed in the TM1 position). If the signal peptide is necessary for N-tail translocation, the signal peptide mutant should be binding-defective. If the signal peptide does not influence N-tail translocation but receptor expression, a binding-competent mutant should result, displaying an altered maximal binding ($B_{\max}$) value. The K_D value should not be influenced since signal peptide mutant and wild-type receptor sequences are identical once the signal peptide of the wild-type is cleaved off in the ER (except for the first amino acid residue, which is methionine for the signal peptide mutant).

To assess the functional significance of the signal peptide of CRF-R1, we deleted the N-terminal 24 residues of the receptor (construct ΔSP.CRF-R1.GFP, see Figure 1). The wild-type receptor was used as a control (construct CRF-R1.GFP). Both receptors were C-terminally tagged with GFP to allow their intracellular localization (see below). The wild-type receptor and the signal peptide mutant were transiently expressed in HEK-293 cells, and [¹²⁵I]Tyr⁰-sauvagine-binding assays were performed (Figure 3A, left panel). We also performed adenylate cyclase activity assays to assess receptor function (Figure 3A, right panel). Both the wild-type receptor and the signal peptide mutant were able to bind the radioligand and to stimulate the adenylate cyclase system. These results demonstrate that the signal peptide of CRF-R1 is not essential for establishing a functional receptor, in contrast with the signal peptide of ET_B-R [3]. As expected in the case of a functional signal peptide mutant, its K_D and EC_{50} values were similar to those obtained for the wild-type receptor (K_D = 0.84 nM versus 0.97 nM; EC_{50} = 2.03 nM versus 0.72 nM). The $B_{\max}$ value of the signal peptide mutant was substantially lower than that of the wild-type (0.24 pmol/mg versus 0.55 pmol/mg), indicating that the receptor number at the cell membrane was reduced. The decreased number of receptors at the cell membrane is nevertheless sufficient to achieve maximal adenylate cyclase stimulation (receptor reserve). We have also performed binding experiments with the corresponding untagged CRF-R1 and its signal peptide mutant and a similar reduction in the number of ligand-binding sites of the signal peptide mutant was observed (results not shown).

The result that deletion of the signal peptide of CRF-R1 led to functional receptors is different from what we have observed for

Figure 3 Pharmacological properties of the GFP-tagged CRF-R1 and ET_B-R and their respective signal peptide mutants

(A) Left panel: [¹²⁵I]Tyr⁰-sauvagine-binding profiles of intact transiently transfected HEK-293 cells expressing CRF-R1.GFP (wild-type CRF-R1) and the signal peptide mutant ΔSP .CRF-R1.GFP. Specific binding is shown. Data points represent the means $\pm$ S.E.M. for three independent experiments each performed in triplicates. Unspecific binding contributed up to 10 % of the total binding. The calculated K_D and B_{max} values are indicated. Right panel: adenylate cyclase activity assay with crude membranes of transiently transfected HEK-293 cells expressing the same constructs as in the left-hand panel. Data points represent the means $\pm$ S.E.M. for three independent experiments each performed in triplicates. The calculated EC_{50} values are indicated. (B) Left panel: [¹²⁵I]ET-1-binding profiles of intact transiently transfected HEK-293 cells expressing ET_B.GFP (wild-type ET_B-R) and its signal peptide mutant ET_B $\Delta 26$.GFP. Specific binding is shown. Data points represent the means $\pm$ S.E.M. for three independent experiments each performed in triplicates. Unspecific binding contributed up to 15 % of the total binding. The calculated K_D and B_{max} values are indicated. Right panel: ET-1-mediated IP accumulation in intact transiently transfected HEK-293 cells expressing the same constructs as in the left-hand panel. Data points represent the means $\pm$ S.E.M. derived from a single experiment performed in triplicate. The dose-response curve is representative of two independent experiments. The calculated EC_{50} values are indicated.

ET_B-R where the signal peptide was necessary for N-tail translocation across the ER membrane and consequently for a functional receptor [3]. To stress this difference in receptor functionality, we have taken transiently transfected HEK-293 cells expressing the previously described GFP-tagged ET_B-R (construct ET_B.GFP) and its signal peptide mutant (construct ET_B $\Delta 26$.GFP) [3], and show again by [¹²⁵I]ET-1 binding (Figure 3B, left-hand panel) and ET-1-mediated IP accumulation assays (Figure 3B, right-hand panel) that the signal peptide of ET_B-R is essential for the formation of a functional receptor.

To confirm that the reduced B_{max} value of the signal peptide mutant of CRF-R1 results from a decreased number of cell-surface receptors, biotinylation assays with intact, transiently transfected HEK-293 cells expressing the GFP-tagged receptors were performed. Plasma-membrane proteins were labelled with biotin, isolated with NeutrAvidin and analysed by SDS/PAGE/immunoblotting (Figure 4). For both the wild-type receptor and the signal peptide mutant, an immunoreactive protein band with an apparent molecular mass of 100 kDa was detected, corresponding to the N-glycosylated form of the receptors (see Figure 6 for PNGase F digestions). The band representing the signal peptide mutant was much weaker, indicating that the number of receptors at the plasma membrane is substantially reduced (to ~ 12 % of the wild-type level according to densitometric analysis; results not shown).

The signal peptide of CRF-R1 promotes receptor expression

Two interpretations are possible to explain the decreased amount of the signal peptide mutant at the cell surface. First, the expression of the mutant is normal but receptor folding and consequently transport to the cell surface are impaired. The signal

Figure 4 Cell-surface biotinylation assay with transiently transfected HEK-293 cells expressing CRF-R1.GFP and its signal peptide mutant ΔSP .CRF-R1.GFP

Plasma-membrane proteins of intact cells were labelled with biotin. Biotinylated proteins were isolated with NeutrAvidin, and labelled receptors were detected by immunoblotting, using a polyclonal rabbit anti-GFP antiserum and peroxidase-conjugated anti-rabbit IgG. Untransfected HEK-293 cells were used as a control. The immunoblot is representative of three independent experiments.

peptide may, for example, favour an early folding event before its cleavage and signal peptide deletion may thus lead to an increased amount of misfolded receptors that are recognized by the quality control system of the early secretory pathway. In this case, a substantial amount of the protein should accumulate intracellularly and be degraded by the ER-associated degradation pathway. An influence of the signal peptide on the later transport steps is unlikely since the sequences of the receptors are identical once the signal peptide is removed. The second possibility is that transport of the mutant is normal, but receptor expression is reduced. In this case, the mutant should not accumulate intracellularly.

Figure 5 Confocal laser scanning microscopy of transiently transfected HEK-293 cells expressing CRF-R1.GFP and its signal peptide mutant Δ SP.CRF-R1.GFP

(A) Localization of the GFP fluorescence signals. Shown are horizontal (xy) scans. GFP signals are shown in green (left panels) and Trypan Blue signals of the cell surface of the same cells in red (central panels). GFP and Trypan Blue fluorescence signals were computer-overlaid (right panels; overlap is indicated by yellow). The scans show representative cells. Scale bar, 10 μ m. Similar data were obtained in three independent experiments. (B) Quantification of the plasma-membrane GFP fluorescence signals of the cells. The GFP signals were analysed by horizontal scans (left panels). The GFP signal intensities were measured using an 8 bit grey scale (ranging from 0 to 250) and transformed into a colour scale (central panel; see the Figure for colour scale). The coloured signal intensities are also shown using a three-dimensional plot of the cells (right panels). (C) Statistical analysis. Results shown are the means $\pm$ S.D. for the plasma-membrane GFP fluorescence intensities of cells ($n = 30$). Fluorescence intensities of the individual cells were recorded as shown in (B).

To distinguish between these possibilities, GFP fluorescence signals of the wild-type receptor and the signal peptide mutant were localized in living, transiently transfected HEK-293 cells by laser scanning microscopy (Figure 5A, left panel, in green).

The cell surface of the same cells was visualized by the use of Trypan Blue (Figure 5A, central panel, in red). Co-localization is indicated by yellow (Figure 5A, right panel). In the case of the wild-type receptor, strong GFP signals were detected at the

Figure 6 Immunoprecipitation of GFP-tagged CRF-R1 and ET_B-R constructs and their respective signal peptide mutants

(A) CRF-R1 constructs. Crude membranes of transiently transfected HEK-293 cells expressing CRF-R1.GFP and ΔSP.CRF-R1.GFP were isolated and receptors were immunoprecipitated using a polyclonal rabbit anti-GFP antiserum. Precipitated receptors were detected by immunoblotting using a monoclonal mouse anti-GFP antibody and peroxidase-conjugated anti-mouse IgG. Membranes were either treated with PNGase F to remove N-glycosylations (+) or left untreated (–). The immunoblot is representative of three independent experiments. #, complex-glycosylated receptors; >, high-mannose forms; *, non-glycosylated receptors. (B) ET_B-R constructs. Crude membranes of transiently transfected HEK-293 cells expressing ET_B 134.GFP and ET_B 134/Δ26.GFP were isolated. Receptor fragments were immunoprecipitated and detected as described above. Membranes were either treated with PNGase F to remove N-glycosylations (+) or left untreated (–). The immunoblot is representative of two independent experiments.

cell surface. GFP signals at the plasma membrane were also detected in the case of the signal peptide mutant. However, they were significantly weaker than the wild-type signals, consistent with the cell-surface biotinylation assay (12% of the wild-type level according to plasma-membrane fluorescence intensity measurements; Figures 5B and 5C). An intracellular accumulation of the signal peptide mutant was not observed, indicating that the transport of the signal peptide mutant through the early secretory pathway is normal but that expression of the mutant is reduced. Consistent with these results, we failed to detect an increased turnover rate of the signal peptide mutant in pulse-chase experiments, which would indicate an activation of the ER-associated degradation pathway (results not shown).

To corroborate these results, the total amount of receptor protein synthesized was determined. Crude membranes of transiently transfected HEK-293 cells were prepared, and receptors were immunoprecipitated and analysed by SDS/PAGE/immunoblotting (Figure 6A). PNGase F digestions were used to test for the presence of N-glycosylated receptors. For both the wild-type receptor and the signal peptide mutant, two immunoreactive protein bands were detectable in the untreated samples: a broad band with an apparent molecular mass of 100 kDa ('#' in Figure 6A) and a smaller 72 kDa band ('>' in Figure 6A). Both bands were shifted to 62 kDa by PNGase F treatment ('*' in Figure 6A). The 100 kDa protein bands were also detectable at the plasma membrane (see Figure 4) and thus represent the mature, complex-glycosylated receptors. The 72 kDa forms were not detectable at the plasma membrane (see Figure 4). It is conceivable that they represent the high-mannose forms of the receptors, present only in the early secretory pathway. The 62 kDa protein band represents non-glycosylated GFP-tagged receptors. This protein band migrated faster than calculated (calculated molecular mass 72 325 Da). The discrepancy between the apparent and calculated molecular masses is probably caused by incomplete unfolding of the receptor portion in the presence of SDS. In the case of the signal peptide mutant, both the 100 kDa and the 72 kDa band were substantially weaker in the untreated sample than the corresponding wild-type bands, demonstrating again that receptor expression is strongly decreased.

Upon PNGase F treatment, a substantial amount of immunoreactive protein of the signal peptide mutant and the wild-type

Figure 7 Northern-blot analysis and translation assay

(A) Northern-blot analysis of CRF-R1.GFP and the signal peptide mutant ΔSP.CRF-R1.GFP. Total RNA was isolated from transiently transfected HEK-293 cells and Northern-blot analysis (20 μg of RNA/lane) was performed with ³²P-labelled CRF-R1 cDNA. Blots were stripped and reprobed with a ³²P-labelled cDNA fragment specific for actin. Untransfected cells were used as a control. The Northern blot is representative of three independent experiments. (B) *In vitro* transcription/translation assay of the N-tail of CRF1-R1 with and without signal peptide fused to the hamster PrP as a marker (constructs CRF-R1.PrP and ΔSP.CRF-R1.PrP respectively). [³⁵S]Met was incorporated into the proteins using SP6 RNA polymerase and rabbit reticulocyte lysate. Labelled proteins were detected by autoradiography. The experiment is representative of two independent experiments.

receptor is lost. This may be due to the formation of SDS-insoluble aggregates upon glycan removal, since there appears to be more material on top of the gel under these conditions.

We have performed equivalent precipitation experiments using the previously described ET_B-R constructs ET_B 134.GFP and ET_B 134/Δ26.GFP [3] (Figure 6B). ET_B 134.GFP represents a truncated receptor fragment comprising signal peptide, N-tail, the first TM and the first intracellular loop of ET_B-R C-terminally fused to GFP (truncated receptor fragments must be used in the case of ET_B-R to allow the detection of N-glycosylation that increases the apparent molecular mass of this receptor by only 4 kDa [3]). ET_B 134/Δ26.GFP represents the corresponding signal peptide mutant. The wild-type ET_B-R construct (43 kDa, glycosylated) and its signal peptide mutant (39 kDa, non-glycosylated) were expressed in similar amounts, demonstrating that the signal peptide of the ET_B-R has no influence on receptor expression, in contrast with that of CRF-R1. The failure of glycosylation of the signal peptide mutant ET_B 134/Δ26.GFP demonstrates again that the signal peptide of ET_B-R is necessary for translocation of the receptor's N-tail across the ER membrane, as described previously [3].

A reduced receptor expression of the CRF-R1 may result if ER targeting/insertion of the receptor is impaired in the absence of the signal peptide. Alternatively, it may also result from decreased transcription or translation. To exclude the latter possibilities, we performed Northern-blot analyses with total RNA derived from transiently transfected HEK-293 cells (Figure 7A). For the wild-type receptor and the signal peptide mutant, RNA bands of 1.97 and 1.90 kb were detected respectively (the smaller size in the case of the signal peptide mutant is due to the deletion of the sequence encoding the signal peptide). Both transcripts were present in similar amounts, demonstrating that differences in transcription does not account for the reduced expression of the signal peptide mutant. To assess the influence of the signal peptide on the translation process, we fused the N-terminal 121 amino acids of the CRF1-R1 with and without signal peptide to the hamster PrP as a marker (constructs CRF-R1.PrP and ΔSP.CRF-R1.PrP respectively) and performed *in vitro* transcription translation

assays (Figure 7B). Proteins of 32 kDa (wild-type N-tail) and 30 kDa (N-tail of the signal peptide mutant) could be detected in similar amounts in the samples, indicating that the signal peptide does not influence translation. Taken together, our results indicate that the signal peptide of CRF-R1 improves one of the early steps of receptor biogenesis such as ER targeting and/or insertion.

DISCUSSION

In the present study, we show that the signal peptide of CRF-R1 is not essential for establishing a functional receptor. However, it strongly increases receptor expression. Cell-surface biotinylation (Figure 4) and laser scanning microscopy (Figure 5) consistently revealed that expression of the signal peptide mutant is decreased by approx. 90%. $B_{\max}$ values obtained in ligand-binding experiments, however, suggest that the number of ligand-binding sites is reduced by only 50% (Figure 3A). The discrepancy can be explained by a larger amount of uncoupled receptors present in the case of the wild-type CRF-R1. If the highly expressed wild-type receptor depletes the amount of available G-proteins, the proportion of uncoupled, low-affinity receptors at the cell surface increases and these receptors will escape detection in our ligand-binding experiment. Indeed, it was recently described that in HEK-293 cells transfected with the wild-type CRF-R1, the proportion of low-affinity ($K_D = 14.7$ nM) uncoupled receptors was larger than 85% [21].

We have excluded the possibility that the sequence encoding the signal peptide has an effect on mRNA level or translation efficiency (see Figure 7). Thus the CRF-R1 signal peptide influences one of the processes of the early secretory pathway. It may facilitate targeting of the nascent chain–SRP–ribosome complex to the ER membrane or one of the steps occurring shortly after targeting ('translocon gating'): (i) the binding of the ribosome to the translocon, (ii) the formation of the seal between the translocon and the ribosomal nascent chain exit site or (iii) the opening of the translocon towards the lumen of the ER. Statistical analyses revealed that mammalian signal sequences are remarkably diverse in length, net charge and hydrophobicity [2,22,23]. Recent data demonstrated that different signal peptides display only minimal differences in their targeting functions but a substantial degree of variation in translocon gating [13]. Thus the signal peptide of CRF-R1 may belong to a group of signal peptides that strongly promote gating. Unfortunately, there is no obvious correlation between the sequence of a signal peptide and its gating properties [13].

An alternative explanation of the results is that the signal peptide prevents an early degradation process. Although we have excluded the possibility that the signal peptide decreases proteolytic degradation once the receptor is integrated into the ER membrane, a role in decreasing the co-translational degradation processes [24] cannot be excluded.

Two functions of signal peptides of GPCRs are now known. The first function is to mediate N-tail translocation across the ER membrane if the N-tail of a receptor cannot be translocated post-translationally, as observed for ET_B-R [3]. Removal of such a signal peptide leads to non-functional receptors. For ET_B-R, the signal peptide had no influence on receptor expression. The second function, as described here for CRF-R1, is to modulate receptor expression by an as yet incompletely understood mechanism. For CRF-R1, the signal peptide precedes an N-tail that can be translocated post-translationally. It is not required for the formation of a functional receptor. It remains, however, possible that signal peptides that serve both functions may be described.

The observation that the signal peptide of CRF-R1 strongly influences receptor expression may also have biotechnological

implications, e.g. for the overexpression of GPCRs and other membrane proteins, which is a prerequisite for e.g. structural studies. It is long known that the expression of GPCRs that normally do not contain signal peptides may be increased by up to 3-fold by the fusion of a cleavable signal peptide from a secretory protein, although the molecular basis for this effect was not understood [25–28]. We have shown in the present study that the signal peptide of CRF-R1 increases expression of this receptor by 10-fold. The CRF-R1 signal peptide may thus become a tool for high-level expression of recombinant membrane proteins. Previous studies for proteins other than GPCRs indicate that signal peptides may also have functions after their cleavage. The signal peptides of preprolactin and the HIV 1 envelope protein p-gp 160 are further processed after cleavage. The resulting fragments are transported back to the cytosol and interact efficiently with calmodulin [29,30]. This interaction provides a molecular basis for modulating calcium signalling. Future studies should also address the question of whether the signal peptides of GPCRs modulate the signal transduction cascades mediated by them.

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