The corticotropin-releasing factor receptor type 2a (CRF$_{2(a)}$ receptor) belongs to the family of G protein-coupled receptors. The receptor possesses a putative N-terminal signal peptide that is believed to be cleaved-off after mediating the endoplasmic reticulum targeting/insertion process, like the corresponding sequence of the homologous CRF$_1$ receptor. Here, we have assessed the functional significance of the putative signal peptide of the CRF$_{2(a)}$ receptor and show that it is surprisingly completely incapable of mediating endoplasmic reticulum targeting, despite meeting all sequence criteria for a functional signal by prediction algorithms. Moreover, it is uncleaved and forms part of the mature receptor protein. Replacement of residue Asn$^{13}$ by hydrophobic or positively charged residues converts the sequence into a fully functional and cleaved signal peptide demonstrating that conventional signal peptide functions are inhibited by a single amino acid residue. Deletion of the domain leads to an increase in the amount of immature, intracellularly retained receptors demonstrating that the sequence has adopted a new function in receptor trafficking through the early secretory pathway. Taken together, our results identify a novel hydroporphic receptor domain in the family of the heptahelical G protein-coupled receptors and the first pseudo signal peptide of a eukaryotic membrane protein. Our data also show that the extreme N termini of the individual CRF receptor subtypes differ substantially.

The corticotropin-releasing factor (CRF)$^3$ receptor family is involved in the regulation of the hypothalamic-pituitary-adrenal stress axis in mammals (1–3). A large body of evidence points to a major role of the receptors in mediating CRF effects in anxiety and depressive disorders and in stress-associated pathologies. Two types of CRF receptors are known, the CRF$_1$ and the CRF$_2$ receptors. The CRF$_1$ receptor is expressed mainly in the pituitary and central nervous system and binds CRF with high affinity. It mediates adrenocorticotropic hormone release from the anterior pituitary and is involved in the endocrine, autonomic, and cognitive responses to stress stimuli. The CRF$_2$ receptors are expressed in the central nervous system but also in the periphery including skeletal muscle cells, cardiac myocytes, and cells of the gastrointestinal tract. Three splice variants of CRF$_2$ receptors have been described: CRF$_{2a}$, CRF$_{2b}$, and CRF$_{2c}$ receptors. They bind CRF with low and the urocortins 1–3 with high affinity. The CRF$_2$ receptors are involved in the regulation of feeding behavior (4) and in recovery from a stress response (5). It is likely that they are also involved in modulating anxiety-related behavior.

The CRF receptors belong to the small subgroup of GPCRs (5–10%) possessing putative N-terminal signal peptides. These peptides are believed to be cleaved-off after mediating the ER targeting/insertion process (6, 7). The majority (90–95%) of the GPCRs do not possess cleavable signal peptides. Here, one of the transmembrane helices (TM) of the mature receptors (usually TM1) mediates ER targeting/insertion as an uncleaved signal anchor sequence (6). The reason as to why some membrane proteins including GPCRs require additional signal peptides, whereas others do not, is not completely understood.

An initial function of a signal sequence (cleaved signal peptide or uncleaved signal anchor sequence) is to stop the cytoplasmic translation of a membrane protein (“elongation arrest”) by its binding to the signal recognition particle (SRP) (8, 9). The resulting complex is targeted to the translocon at the ER membrane and the signal sequence promotes translocon gating. Protein synthesis starts again and the nascent chains are integrated into the bilayer. Without a signal peptide, the N tail of a membrane protein is synthesized in the cytoplasm, because translation continues until the signal anchor sequence (TM1) appears. The N tail must thus be translocated post-translationally. In contrast, in the presence of an additional signal peptide, the N tail of the GPCR is not translated in the cytosol, because SRP binding to the preceding signal peptide stops elongation. Here, the N tail can be translocated co-translationally through the translocon. Taking these considerations into account, it was proposed that signal peptides facilitate N tail translocation of those membrane proteins for which a post-translational translocation of the N tail is impaired (10, 11), for example, because...
rapidly folding domains are present (12). Usually, these proteins contain long N-tails (6).

In the case of GPCRs, it was shown that the signal peptide of the endothelin B (ETB) receptor is indeed essential for N tail translocation across the ER membrane (10). Addition of a cleavable signal peptide also promotes N tail translocation of GPCRs normally containing only signal anchor sequences (11, 13). Signal peptides may also facilitate predominantly ER targeting. Such a function was recently described for the signal peptide of the homologous CRF1 receptor (14). Here, we have assessed the functional significance of the putative signal peptide of the CRF2(a) receptor. We show that this receptor does not possess a conventional cleaved signal peptide like the homologous CRF1 receptor. Instead, it contains a pseudo signal peptide that forms part of the mature receptor protein. This sequence represents a novel hydrophobic receptor domain in the GPCR family.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNA encoding the rat CRF1 and CRF2(a) receptors were a gift from U. B. Kaupp (IBI Forschungszentrum Jülich, Germany). The PrP(A120L) reporter was described (15). [125I-Tyr6]Sauvagine and [3H]cAMP were purchased from PerkinElmer Life Sciences. All peptidic ligands of CRF receptors (sauvagine, urocortin I, and urocortin II) were synthesized in our laboratory (16). Lipofectamine™ 2000 and the vector pSecTag2A were purchased from Invitrogen. The transfection reagent FuGENETM 6 was from Roche Diagnostics. DNA-modifying enzymes, PNgaseF and EndoH, were from New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were purchased from Biotez (Berlin, Germany). The TALON metal affinity resin, vector plasmid pEGFP-N1 (encoding the red-shifted variant of green fluorescent protein (GFP)), and plasmid pECFP-ER were obtained from Clontech Laboratories. The Roti-Load sample buffer was from Carl Roth (Karlsruhe, Germany). All other reagents were from Sigma. Data of the ligand binding assays were analyzed using the program RadLig software 6.0 (Cambridge, UK) and GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA). The materials for the prion protein targeting assay (rabbit reticuloocyte lysate, canine pancreatic rough microsomal membranes, proteinase K, Triton X-100, and N-glycosylation acceptor site peptide inhibitor (NYP, NH2-Asp-Tyr-Thr-COOH)) were described previously (15, 17).

**Cell Culture and Transfection**—Cells were cultured at 37 °C and 5% CO2. HEK 293, COS.M6, and AtT 20 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal calf, penicillin (100 units/ml), and streptomycin (100 µg/ml). In the case of PC12 cells, this cell culture medium was additionally supplemented with 5% (v/v) heat-inactivated horse serum. SH-SY5Y cells were cultivated in Dulbecco’s modified Eagle’s medium/Ham’s (1:1) F-12 medium containing 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 1% (w/v) nonessential amino acids. Transfection of the cells with Lipofectamine™ 2000 or FuGENETM 6 was carried out according to the supplier’s recommendations.

**Confocal Laser Scanning Microscopy and Localization of GFP Fluorescence Signals**—Transiently transfected HEK 293 cells (1×106) grown on glass coverslips (pretreated with 25 µg/ml poly-L-lysine) in 35-mm diameter dishes were transiently trans-
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fected with plasmid DNA (0.8 μg) and FuGENE 6 according to the supplier’s recommendations. Cells were incubated overnight, washed once with PBS, and transferred immediately into a self-made chamber (details on request). Cells were covered with 1 ml of PBS and GFP fluorescence was visualized at room temperature on a Zeiss LSM510-META invert confocal laser-scanning microscope (objective lens: ×100/1.3 oil; optical section, <0.8 μm; multitrack mode; λ$_{excl}$ 488 nm, Argon laser, BP filter: 500–530 nm). Images were imported into Photoshop 5.5 software (Adobe Systems Inc.) and contrast was adjusted to approximate the original image.

Confocal Laser Scanning Microscopy: Colocalization of the Constructs with ER Markers—Stably transected HEK 293 cells expressing the receptor constructs were prepared on glass coverslips in 35-mm diameter dishes. Cells were transiently transfected with 0.8 μg of pECPF-ER plasmid DNA from Clontech Laboratories (encoding a fusion of enhanced cyan fluorescent protein, ECFP, with the ER targeting sequence of calreticulin and the ER retrieval sequence KDEL). The FuGENE 6 transfection reagent was used according to the supplier’s recommendation. The receptor GFP signals and the ER signals of ECFP-ER were analyzed by confocal LSM (objective lens: ×100/1.3 oil; optical section, <0.9 μm; multitrack mode; GFP, λ$_{excl}$ 488 nm, Argon laser, BP filter: 516–601 nm; ECFP-ER, λ$_{excl}$ 458 nm, Argon laser, BP filter: 462–494 nm) and processed as described above.

For rhodamine 6G staining of the ER, the stably transected HEK 293 cells expressing the receptor constructs were washed with PBS, and incubated for 40 min with 50 nM rhodamine 6G in PBS (rhodamine 6G is suitable for ER staining of living cells, see Ref. 19). The receptor GFP signals and the ER signals of ECFP-ER were analyzed by confocal LSM (objective lens: ×100/1.3 oil; optical section, <0.9 μm; multitrack mode; GFP, λ$_{excl}$ 488 nm, Argon laser, BP filter: 494–516 nm; rhodamine 6G, λ$_{excl}$ 543 nm, HeNe laser, LP filter: 560 nm) and processed as described above.

Quantitative Detection of Secreted GFP Fusion Proteins—Secreted GFP fusion proteins from transiently transected HEK 293 cells were analyzed by immunoblotting and fluorimetric measurements as described previously (14).

Purin Protein Targeting Assay—In vitro transcription with SP6 RNA polymerase was performed for 1 h at 40 °C. Translation with rabbit reticulocyte lysate in the presence of [35S]methionine, and translocation into canine rough microsomal membranes were carried out at 40 °C for 1 h as described previously (15, 17). Proteinase K digestion (0.5 mg/ml) was performed at 0 °C; reactions were terminated with 5 mM phenylmethylsulfonyl fluoride. Triton X-100 (1%) was used to permeabilize membranes, and the peptide NYT (NH$_2$-Asp-Tyr-Thr-COOH) (100 μM) to inhibit N-glycosylation of the constructs. All samples were transferred into 10 preheated volumes of 1% SDS and 0.1 M Tris-HCl (pH 8.0) and analyzed by SDS-PAGE on 12% Tris/Tricine gels. Proteins were visualized by autoradiography.

[125I-Tyr]$^6$Sauvagine Displacement Binding Assay—Crude membranes of stably transected HEK 293 cells were incubated in a final volume of 200 μl of Tris/BAME buffer (50 mM Tris-HCl, 2 mM EGTA, 10 mM CaCl$_2$, 15 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 3.2 μg/ml trypsin inhibitor, 210 mg/ml bacitracin, pH 7.2) containing 0.1 nM [125I-Tyr]$^6$]sauvagine alone and increasing concentrations of unlabeled ligand ($1 \times 10^{-11}$-1 \times 10^{-6}$ M). Binding was determined and data were analyzed as described previously (16). Saturation analysis yielded $K_D$ values of 0.33 and 0.28 nM for constructs CRF$_2$α and ASP-CRF$_2$α, respectively. These values were used for calculations of the $K_I$ values of the unlabeled ligands (placement experiments).

[125I-Tyr]$^6$Sauvagine Binding Assay to Intact Cells—HEK 293 cells (1 \times 10^6) grown on 24-well plates were transiently transfected with plasmid DNA (200 ng) and FuGENE 6 according to the supplier’s recommendations. Cells were grown for 24 h and washed once with DPBS (PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$). Then DPBS supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1.4 μg/ml aprotinin, 3.2 μg/ml trypsin inhibitor, 210 mg/ml bacitracin, 0.05% (w/v) bovine serum albumin, and [125I-Tyr]$^6$]sauvagine (15 nM = saturating conditions) was added (final volume 300 μl). For determination of the nonspecific binding, 1 μM sauvagine was added. The samples were incubated at 25 °C for 2 h. Cells were washed 2 times with ice-cold DPBS and lysed with 500 μl of 100 mM NaOH solution. Bound [125I-Tyr]$^6$]sauvagine was quantified using a PerkinElmer 1470 Wizard$^\text{TM}$ liquid scintillation counter.

Immunoprecipitation of GFP-tagged Full-length CRF$_1$ and CRF$_2$α Receptor Constructs and Analysis of Their Glycosylation State—Cells (HEK 293 = 4 \times 10^6; PC12 and SH-SY5Y = 5 \times 10^6; AtT 20 = 3 \times 10^6; COS-M6 = 2 \times 10^6) grown on 100-mm diameter dishes were transiently transfected with plasmid DNA (HEK 293 = 6 μg; AtT 20, COS-M6, PC12, and SH-SY5Y cells = 24 μg) and FuGENE 6 (HEK 293 cells) or Lipofectamine 2000 (AtT 20, COS-M6, PC12, and SH-SY5Y cells) according to the supplier’s recommendations. Cells were cultivated for 24 h, washed twice with PBS (pH 7.4), and lysed for 1 h with 1 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, pH 8.0, supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1.4 μg/ml aprotinin, 3.2 μg/ml trypsin inhibitor). Insoluble debris was removed by centrifugation (15 min, 26,000 × g). The supernatant was supplemented with polyclonal rabbit anti-GFP antiseraum 01 coupled to protein A-Sepharose CL-4B beads and the sample was incubated for 3 h (beads were prepared by equilibrating 10 mg of the beads with lysis buffer and subsequent overnight incubation with 4 μl of polyclonal rabbit anti-GFP antiseraum 01). GFP-tagged receptors were precipitated (2 min, 700 × g), and the beads were washed 2 times with 2 ml of washing buffer 1 (50 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 0.5% (v/v) Triton X-100, pH 8.0) and once with 2 ml of washing buffer 2 (50 mM Tris-HCl, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, pH 7.4). Precipitated receptors were divided into three aliquots. One sample was supplemented with Roti-Load sample buffer, incubated for 5 min at 95 °C, and directly used for SDS-PAGE/immunoblot analysis. The proteins of the second and third sample were treated with PNGaseF or EndoH prior to immunoblot analysis according to the supplier’s recommendations. The GFP-tagged receptors were detected by high resolution SDS-PAGE (8% SDS, 48-cm$^2$ gels) and immunoblotting using a monoclonal mouse anti-GFP antibody and alkaline phos-
RESULTS

The CRF2(a) Receptor Meets All Sequence Criteria for the Presence of a Functional and Cleaved Signal Peptide—Cleavable signal peptides of eukaryotic membrane proteins share characteristic features with signal peptides of eukaryotic secretory proteins (21): a polar and often charged N-terminal (n) region, a central hydrophobic (h) region, and a polar C-terminal (c) region. 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. Fig. 1B shows that both the rat CRF1 receptor and the rat CRF2(a) receptor contain putative signal peptides when monitored with the novel program “SignalP 3.0” (22, 23). Signal peptide probability is 0.99 in the case of the CRF1 receptor and 0.98 in the case of the CRF2(a) receptor. The maximal cleavage site probability for the CRF1 receptor is 0.59 between residues Ser24 and Lys25 and 0.76 for the CRF2(a) receptor between residues Ala18 and Glu19, i.e. cleavage is even better predictable for the CRF2(a) receptor. In the case of the CRF1 receptor, the presence of a cleaved signal peptide has been demonstrated experimentally (14, 24, 25). This signal peptide facilitates one of the early steps of receptor biogenesis such as ER targeting/translocon gating (14).

The Putative Signal Peptide of the CRF2(a) Receptor Is Incapable of Mediating ER Targeting of Heterologous Proteins—To assess whether the putative signal peptide of the CRF2(a) receptor is functional, the sequence for the N tail of the receptor with and without signal peptide was fused with a His-tagged GFP as a soluble marker protein (Fig. 1A; constructs CRF2(a).NT and ΔSP-CRF2(a).NT, respectively). Constructs containing the N tail of the CRF1 receptor and its signal peptide mutant were used as a control (Fig. 1A; constructs CRF1.NT and ΔSP-CRF1.NT, respectively; see also Alken et al. (14)). If a cleavable signal peptide is present in the N tail of the CRF2(a) receptor, it should direct the soluble GFP marker to the ER and subsequently via the secretory pathway to the cell culture medium. If the sequence does not contain a functional signal peptide, the marker protein will remain in the cytosol. HEK 293 cells were transiently transfected with the constructs and the GFP fluorescence signals were localized by confocal LSM (Fig. 2A, upper panel). In the case of ΔSP-CRF1.NT, the GFP signals were detected diffusely throughout the cell, indic-
Pseudo Signal Peptide of the CRF2(a) Receptor

From the same batch of transiently transfected HEK 293 cells, the secreted GFP fusions were purified from the cell culture medium using their His tag and detected fluorimetrically (Fig. 2A, central panel) or by immunoblotting using anti-GFP antibodies (Fig. 2A, lower panel). GFP fluorescence or secreted proteins indicating a functional signal peptide were detectable only in the case of the CRF2(a) receptor, consistent with the data obtained by confocal LSM.

The data that the putative signal peptide of the CRF2(a) receptor is incapable of mediating ER targeting raise the question as to how this receptor is targeted to the ER membrane. It was shown previously (27) that almost every TM of a GPCR can function as a signal anchor sequence. It was thus very likely that TM1 of the CRF2(a) receptor takes over the functions in ER targeting/insertion. If the N tail of the GFP-tagged construct ΔSP-CRF2(a)-NT was elongated by TM of the mature receptor (construct ΔSP-CRF2(a)-NT.TM1; see also Fig. 1A), reticular GFP fluorescence signals were detected by confocal LSM in transiently transfected HEK 293 cells (Fig. 2B). Moreover, the protein was detected in an N-glycosylated form on immunoblots following immunoprecipitation from these cells by a polyclonal anti-GFP antiserum (Fig. 2C) confirming that TM1 of the CRF2(a) receptor is capable of taking over the ER targeting/insertion functions as a signal anchor sequence.

Signal peptide function of the CRF2(a) receptor was also assessed in detail by in vitro transcription/translation experiments using the PrP(A120L) hamster prion protein (15, 17) as a marker. The PrP(A120L) reporter is a modified version of the PrP protein lacking its N-terminal signal peptide. The remaining single transmembrane domain of this construct is unable to mediate ER targeting, making efficient translocation of the protein dependent on the introduction of a signal peptide (17). In this system, [35S]methionine-labeled fusion proteins are synthesized, using an in vitro rabbit reticulocyte lysate translation system in the presence of canine pancreatic rough microsomal membranes. Nascent chains containing signal peptides that fail to target and/or fail to promote binding to the translocon result in an exclusive cytosolic and completely proteinase K-sensitive translation product (Fig. 3A, step 1). In contrast, signal peptides targeting the nascent chain to the translocon lead to the integration of the fusion protein into the ER membrane (Fig. 3A, step 2). Two different orientations of the fusions are possibly dependent on the gating properties of the signal peptide: if the signal peptide opens the translocon efficiently, the Ntm form (Fig. 3A, step 3.1, = N_{exo}-C_{cyst} form) of the construct is synthesized and the signal peptide is cleaved-off after translocation. Complete translocation of the construct into the ER lumen may also take place in this case (Fig. 3A, step 3.1, Lu form). If, however, the signal peptide opens the translocon inefficiently, the TM of the PrP(A120L) reporter takes over the gating function, and the Ctm form (Fig. 3A, step 3.2, = N_{cyst}-C_{exo} form) with an uncleaved signal peptide is synthesized. Proteinase K treatment of the samples lead to smaller Ntm and larger Ctm protease-resistant fragments that are detectable by SDS-PAGE and autoradiography.

To assess the function of the putative signal peptide of the CRF2(a) receptor in this system, the complete N tail of the CRF2(a) receptor was fused to PrP(A120L) (Figs. 1A and 3B, construct CRF2(a)-NT.PrP). An equivalent construct containing the N tail with the functional signal peptide of the ETb receptor (10) was used as a control (Figs. 1A and 3B;
In the case of the ETb.NT.PrP control construct, an abundant product was obtained after in vitro transcription/translation alone (Fig. 3C, lane 1, "a"). Addition of rough microsomal membranes (lane 2) leads to two bands, the glycosylated Ntm form ("b," one glycosylation) and the glycosylated luminal form ("c," three glycosylations). Proteinase K treatment of the sample (lane 3) leads to the digestion of the Ntm form to a protease-resistant fragment ("d"), the luminal form remains unaffected ("e"). Addition of the glycosylation inhibitor NYT (lane 4) leads to a single prominent band ("f"), representing the Ntm and the luminal forms sharing the same apparent molecular mass after removal of their glycosylations. The bands representing these products run faster than the nonglycosylated original translation product (lane 1, "a"), because the signal peptide is cleaved off in both cases. Finally, permeabilization of the microsomes by Triton X-100 treatment (lane 5) causes the complete digestions of all proteins. These results show that the ETb receptor control contains a signal peptide mediating ER targeting/translocon gating. In the case of the putative signal peptide of the CRF2(a) receptor, in vitro transcription/translation alone also leads to a single, prominent band (lane 6, "g"). Addition of rough microsomal membranes, however, did not change the size of the protein (lane 7). Moreover, the product was completely sensitive to proteinase K (lane 8) and remained nonglycosylated (lane 9) demonstrating that the signal peptide of the CRF2(a) receptor fails to mediate ER targeting of the PrP(A120L) reporter. These data are consistent with the in vivo results described above.

The Putative Signal Peptide of the CRF2(a) Receptor Is Uncleaved—The failure of the putative signal peptide to mediate ER targeting raises the question whether this sequence is cleaved-off by the signal peptidases of the ER. To address this point, we used the full-length wild-type CRF2(a) receptor and its signal peptide mutant C-terminal fused with GFP (Fig. 1A, constructs CRF2(a) and ΔSP-CRF2(a), respectively). The GFP tag allows both the immunoprecipitation of the receptors and their subcellular localization by confocal LSM. The GFP tag does not significantly alter the pharmacological and the trafficking properties of the CRF2(a) receptor (data not shown).

To assess for signal peptide cleavage, constructs CRF2(a) and ΔSP-CRF2(a) were immunoprecipitated from transiently transfected HEK 293 cells using a polyclonal anti-GFP antisera, digested with PNGaseF to remove N-glycans, and detected by...
Pseudo Signal Peptide of the CRF$_{2(a)}$ Receptor

FIGURE 4. Analysis of signal peptide cleavage of constructs CRF$_{2(a)}$ and ΔSP-CRF$_{2(a)}$ A, high resolution SDS-PAGE immunoblot analysis of the constructs. Precipitated receptors were digested with PNGaseF and detected by immunoblotting using a monoclonal anti-GFP antibody. Each lane shows the receptors from 2.5 × 10^6 cells. The constructs CRF$_1$ and ΔSP-CRF$_1$, were used as a control for signal peptide cleavage. In the case of the CRF$_1$, derivatives, each lane shows the receptors from 1.25 × 10^6 cells. (−), mock-transfected cells. The immunoblot is representative of three independent experiments. B, high resolution SDS-PAGE immunoblot analysis of constructs CRF$_{2(a)}$ and ΔSP-CRF$_{2(a)}$, obtained from different transiently transfected cell lines. The receptors were precipitated, PNGaseF treated and detected as in A. Each lane shows the receptors from 2.5 × 10^6 cells. (−), mock-transfected cells. The immunoblot is representative of three independent experiments.

High-resolution SDS-PAGE/immunoblotting (Fig. 4). If the putative signal peptide is cleaved, the deglycosylated CRF$_{2(a)}$ and ΔSP-CRF$_{2(a)}$ constructs should comigrate. If the putative signal peptide is uncleaved, the apparent molecular mass of the CRF$_{2(a)}$ construct should be 2 kDa larger than that of ΔSP-CRF$_{2(a)}$. The wild-type GFP-tagged CRF$_1$ receptor and its signal peptide mutant (Fig. 1A; constructs CRF$_1$ and ΔSP-CRF$_1$) were used as a control for signal peptide cleavage in these experiments. In the case of the control constructs CRF$_1$ and ΔSP-CRF$_1$, protein bands with identical apparent molecular masses (67 kDa) were detected, indicative of signal peptide cleavage (Fig. 4A, right panel). In contrast, construct CRF$_{2(a)}$ was 2 kDa larger than ΔSP-CRF$_{2(a)}$ (72 versus 70 kDa, respectively; Fig. 4A, left panel) demonstrating that the signal peptide of the CRF$_{2(a)}$ receptor is indeed uncleaved and thus represents a pseudo signal peptide.

Signal peptide cleavage of the CRF$_{2(a)}$ receptor was also assessed in other transiently transfected cell lines, namely mouse pituitary cells (AtT 20), green monkey kidney cells (COS.M6), rat chromaffine cells (PC12), and rat neuroblastoma cells (SH-SY5Y) (Fig. 4B). In all these cell lines, constructs CRF$_{2(a)}$ and ΔSP-CRF$_{2(a)}$, migrated with 2 kDa different molecular masses. Thus, the absence of signal peptide cleavage was not specific for HEK 293 cells.

The Pseudo Signal Peptide Does Not Influence the Ligand Binding Profile of the CRF$_{2(a)}$ Receptor—The CRF$_1$ receptor possesses a conventional signal peptide, whereas the CRF$_{2(a)}$ receptor contains an uncleaved pseudo signal peptide. The pseudo signal peptide represents a novel receptor domain within the large GPCR family and this raises the question as to the function of this sequence. The CRF$_{2(a)}$ receptor binds urocrin I with a higher affinity than the CRF$_1$ receptor. In addition, the CRF$_{2(a)}$ receptor is selective for urocortin II. The uncleaved pseudo signal peptide may thus have an influence or even determine the ligand binding profile of the CRF$_{2(a)}$ receptor. To address this question, crude membranes of stably transfected HEK 293 cells under saturating conditions (15 nM [125I-TyrO]sauvagine). Specific binding is shown. Data points represent mean values of three independent experiments each performed in triplicate. Unspecific binding contributed up to 20% of total binding. B, [125I-TyrO]sauvagine binding to intact, transiently transfected HEK 293 cells under saturating conditions (15 nM [125I-TyrO]sauvagine). Specific binding is shown. Data points represent mean values of three independent experiments each performed in triplicate (± S.E.). Unspecific binding contributed up to 20% of total binding.

FIGURE 5. Ligand binding profiles of constructs CRF$_{2(a)}$ and ΔSP-CRF$_{2(a)}$. A, displacement binding assay. Membranes from stably transfected HEK 293 cells were incubated for 3 h with [125I-TyrO]sauvagine (0.1 nM). Increasing concentrations of the agonists urocortin I (upper panel) or urocortin II (lower panel) were added to the radioligand. Specific binding of [125I-TyrO]sauvagine is shown. Data represent mean values (± S.E.) of three independent experiments each performed in triplicate. Unspecific binding contributed up to 20% of total binding. B, [125I-TyrO]sauvagine binding to intact, transiently transfected HEK 293 cells under saturating conditions (15 nM [125I-TyrO]sauvagine). Specific binding is shown. Data points represent mean values of three independent experiments each performed in triplicate (± S.E.). Unspecific binding contributed up to 20% of total binding.
the signal peptide is removed (K_i values: CRF_{2(a)} = 0.30 ± 0.05 nm for urocortin I and 0.21 ± 0.02 nm for urocortin II; ΔSP-CRF_{2(a)} = 0.28 ± 0.15 nm for urocortin I and 0.11 ± 0.04 nm for urocortin II). Maximal binding of ΔSP-CRF_{2(a)} was, however, substantially reduced in comparison to the wild-type CRF_{2(a)} construct (urocortin I experiment = 34% of the wild-type level; urocortin II experiment = 33% of the wild-type level). Similar results were obtained when [^{125}I-TyrO]sauvagine binding to intact, transiently transected HEK 293 cells was studied under saturating conditions (using 15 nm [^{125}I-TyrO]sauvagine; Fig. 5B). Because the K_i values do not differ significantly between constructs CRF_{2(a)} and ΔSP-CRF_{2(a)}, the differences in maximal binding indicate differences in the number of functional receptors at the cell surface. Thus, the amount of functional receptors at the plasma membrane seems to be reduced ~4-fold in the case of ΔSP-CRF_{2(a)}. However, those ΔSP-CRF_{2(a)} receptors reaching the cell surface display a wild-type ligand binding profile.

The Pseudo Signal Peptide of the CRF_{2(a)} Receptor Facilitates Trafficking through the Early Secretory Pathway and Consequently Increases Receptor Cell Surface Expression—The data of the binding assays indicate that the pseudo signal peptide influences receptor trafficking. To study receptor trafficking and receptor distribution in more detail, we first determined the glycosylation state of constructs CRF_{2(a)} and ΔSP-CRF_{2(a)}.

Receptors were immunoprecipitated from transiently transfected HEK 293 cells and treated with EndoH to remove high mannose glycans or PNGaseF to remove both complex and high mannose glycans. Receptors were detected by immunoblotting as described above. In the untreated sample from cells expressing CRF_{2(a)}, three immunoreactive protein bands were detected: a broad band with an apparent molecular mass of 100 kDa (Fig. 6A, left panel, “a”), a 78-kDa band (“b”), and a 72-kDa band (“c”). The 100-kDa band was resistant to EndoH treatment, and its size was reduced to 72 kDa upon PNGaseF treatment. The 100-kDa band thus represents the mature, complex-glycosylated receptor, and the 72-kDa band the nonglycosylated receptor. The 78-kDa band was sensitive to both EndoH and PNGaseF and was shifted to 72 kDa. The 78-kDa band thus represents the high mannose form of the receptor. Nonglycosylated receptors and high mannose forms are frequently detectable for GPCRs in transfected cells (e.g. Ref. 28). They represent either transport intermediates en route to the cell surface and/or receptors that are retained as a consequence of overexpression. In the case of ΔSP-CRF_{2(a)} (Fig. 6A, right panel) the pattern of the protein bands was similar. However, the protein band representing the complex-glycosylated receptors was weaker than the corresponding band of CRF_{2(a)} whereas the bands representing the high mannose and in particular the nonglycosylated receptors were stronger. All three protein bands were 2 kDa smaller, consistent with the presence of an uncleaved signal peptide in the case of construct CRF_{2(a)}.

These data show that deletion of the pseudo signal peptide in construct ΔSP-CRF_{2(a)} increases the amount of immature and misfolded receptors. It is conceivable that these receptors are recognized by the quality control system of the early secretory pathway (29, 30). The fact that the majority of these immature receptors are nonglycosylated rather than high mannose-glycosylated suggests that the pseudo signal peptide of the CRF_{2(a)} receptor facilitates N tail translocation of the receptor across the ER membrane.

To confirm that construct ΔSP-CRF_{2(a)} is retained in the early secretory pathway, the intracellular transport of constructs CRF_{2(a)} and ΔSP-CRF_{2(a)} was monitored by confocal LSM (Fig. 6B). The GFP signals of the receptors were recorded (left panels, green) and colocalized with different ER markers (center panels; see the right panels for the overlays). The ER was visualized with either the cotransfected ECFP-ER protein (upper two panels) or a rhodamine 6G stain (lower two panels). In comparison to the wild-type construct, the cell surface GFP

**FIGURE 6. Subcellular localization of constructs CRF_{2(a)} and ΔSP-CRF_{2(a)}**

A, analysis of the glycosylation state in transiently transfected HEK 293 cells. Precipitated receptors were treated with PNGaseF to remove complex and high mannose glycans or EndoH to remove only the high mannose glycans or left untreated (control). Proteins were detected by immunoblotting using a monoclonal mouse anti-GFP antibody. (−), mock-transfected cells. Each lane shows the receptors from 3 × 10^6 cells. The immunoblot is representative of three independent experiments. B, subcellular localization of the constructs by confocal LSM in stably transfected HEK 293 cells. Upper panel, colocalization with the cotransfected ER marker ECFP-ER. The GFP signals of the receptors (left panel, green) and the ER signals of ECFP-ER (central panel, red) were computer-overlaid (right panel). The horizontal (xy) scans show representative cells. Scale bar, 10 μm. Similar data were obtained in three independent experiments. Lower panel, colocalization with rhodamine 6G. The ER of the cells was stained for 40 min with rhodamine 6G (50 nm). The GFP signals of the receptors (left panel, green) and the ER signals of rhodamine 6G (central panel, red) were computer-overlaid (right panel). The horizontal (xy) scans show representative cells. Scale bar, 10 μm. Similar data were obtained in three independent experiments.

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signals of ΔSP-CRF2(a) were weaker and intracellular signals colocalizing with both ER markers were stronger confirming that a substantial amount of ΔSP-CRF2(a) is retained in the early secretory pathway. Whereas the pseudo signal peptide of the CRF2(a) receptor facilitates receptor trafficking through the early secretory pathway, it seems to have no significant influence upon overall receptor biosynthesis (compare e.g. the protein bands of the PNGaseF-treated receptors in Fig. 6A where all glycosylations are removed).

An N13A Mutation of the CRF2(a) Receptor Converts the Pseudo Signal Peptide into a Fully Functional Cleaved Signal Peptide—The pseudo signal peptide contains an N-glycosylation consensus site (Asn\(^{13}\)-Cys\(^{14}\)-Ser\(^{15}\)). Modification of Asn\(^{13}\) may help to keep the hydrophobic domain soluble in the aqueous environment of the ER lumen and the extracellular space and we thus assessed whether the pseudo signal peptide becomes glycosylated. To this end, all the other putative N-glycosylated residues (Asn\(^{41}\), Asn\(^{74}\), Asn\(^{86}\), and Asn\(^{94}\)) in the N tail of the GFP-tagged CRF2(a) receptor were replaced by alanine residues (resulting mutant N4A-CRF2(a); Asn\(^{13}\) remaining as the sole glycosylation site). Receptors were immunoprecipitated from transiently transfected HEK 293 cells, treated with EndoH or PNGaseF, and detected by immunoblotting (Fig. 7A). A PNGaseF and EndoH-sensitive high mannose form of N4A-CRF2(a) was detectable demonstrating that Asn\(^{13}\) in the pseudo signal peptide is indeed glycosylated (the absence of complex-glycosylated forms indicate that the quadruple mutant is retained intracellularly and obviously fails to pass the quality control system).

To assess the significance of the glycosylation of the pseudo signal peptide, single mutants were constructed for each of the five putative N-glycosylation sites in the N tail of the CRF2(a) receptor (mutants N13A-CRF2(a), N41A-CRF2(a), N74A-CRF2(a), N86A-CRF2(a), and N94A-CRF2(a)). Comparison of the apparent molecular masses of the immunoprecipitated and PNGaseF-treated mutant receptors surprisingly revealed that the single mutant N13A-CRF2(a) comigrates with the signal peptide mutant ΔSP-CRF2(a), in contrast to all the other glyco-mutants and in contrast to the wild type CRF2(a) construct (Fig. 7B; see also Fig. 4 above). These results indicate that the pseudo signal peptide of the CRF2(a) receptor is converted into a conventional, cleaved signal peptide if Asn\(^{13}\) is replaced. Fig. 7B also shows that expression of the receptor increases following this conversion.

Glycosylation at Asn\(^{13}\) may prevent signal peptide cleavage. Residue Asn\(^{13}\) may also inhibit the ER targeting/insertion functions of the pseudo signal peptide at the cytosolic side in its nonglycosylated form. To address the latter question, the N13A mutation was introduced into the fusion protein CRF2(a).NT containing the N tail of the receptor fused to GFP (resulting mutant = N13A-CRF2(a).NT; Fig. 1A). This construct allows to study the ER targeting functions of the mutant pseudo signal peptide (see also Fig. 2, above). The wild-type construct CRF\(_{2(a)}\).NT and the corresponding construct of the CRF\(_{1}\) receptor (CRF\(_{1}\).NT) were used as respective negative and positive controls for ER targeting functions. The GFP fluorescence signals of all constructs were localized in transiently transfected HEK 293 cells by confocal LSM (Fig. 7C, upper panel). In the case of CRF\(_{2(a)}\).NT, the GFP fluorescence was again detected diffusely throughout the whole cell indicating that this fusion did not enter the ER membrane (see also Fig. 2A, above). In the case of mutant N13A-CRF\(_{2(a)}\).NT, however, the GFP signals were membrane-bound, similar to those of the control construct CRF\(_{1}\).NT. These results demonstrate that the signal carrying the N13A mutation is not only cleaved-off, but has also regained its ER targeting functions. From the same transiently transfected HEK 293 cells, secreted GFP fusions were purified from the cell culture medium using their His tag and detected fluorimetrically (Fig. 7C, central panel) or by immunoblotting using anti-GFP antibodies (Fig. 7C, lower panel). The construct

![Fluorescence intensity graph](image-url)

**FIGURE 7. Analysis of N-glycosylation of residue Asn\(^{13}\) and of signal peptide functions of the glycosylation mutant N13A-CRF2(a) in transiently transfected HEK 293 cells.** A, Asn\(^{13}\) glycosylation analysis. The precipitated construct N4A-CRF2(a) was treated with PNGaseF or EndoH or left untreated (Control) and detected by SDS-PAGE immunoblotting as described in the legend to Fig. 4A. The signal of the secreted, purified construct N4A-CRF2(a) was used as a control for the targeting functions of a signal peptide. The horizontal (x) scans are representative of four independent experiments. Scale bar, 10 μm. B, analysis of signal peptide cleavage by high resolution SDS-PAGE immunoblot analysis. Constructs CRF\(_{2(a)}\) and CRF\(_{2(a)}\), and the individual mutants of the putative N-glycosylation sites (N13A-CRF2(a), N41A-CRF2(a), N74A-CRF2(a), N86A-CRF2(a), and N94A-CRF2(a)) were precipitated, treated with PNGaseF to remove N-glycosylations, and detected by SDS-PAGE immunoblotting as described in the legend to Fig. 4A. Each lane shows the receptor derived from 2 × 10\(^{6}\) cells. The immunoblot is representative of three independent experiments. C, upper panel, analysis of the ER targeting functions of the signal peptide mutant N13A-CRF2(a). Constructs CRF\(_{2(a)}\).NT and N13A-CRF2(a).NT were localized by confocal LSM. The construct CRF\(_{2(a)}\).NT was used as a control for the targeting functions of a signal peptide. The horizontal (x) scans are representative of four independent experiments. Scale bar, 10 μm. N, nucleus; (-), mock-transfected cells. Center panel, analysis of secretion of the constructs shown in the upper panel by GFP fluorescence measurements. Columns represent the fluorescence of the secreted, purified constructs from 12 × 10\(^{6}\) cells and show mean values of three independent experiments each performed in triplicate ± S.D. Lower panel, detection of secreted, purified constructs by immunoblotting using a monoclonal anti-GFP antibody. In each lane, the isolated protein of 2 × 10\(^{6}\) cells was loaded. The immunoblot is representative of three independent experiments.
N13A-CRF2(a).NT was readily detectable in the cell culture medium confirming these results.

Replacement of Asn13 of the full-length CRF2(a) construct by other hydrophobic residues (mutants N13I-CRF2(a) or N13F-CRF2(a)) or by a positively charged residue (mutant N13R-CRF2(a)) also lead to a cleaved signal peptide (Fig. 8, upper and center panels, respectively; successful ER targeting was confirmed by localizing the corresponding CRF2(a).NT marker constructs; data not shown). In contrast, replacement of Asn13 by negatively charged residues (mutants N13D-CRF2(a) and N13E-CRF2(a)) or a glutamine residue (mutant N13Q-CRF2(a)) preserved the pseudo signal peptide (Fig. 8, lower panel). Taken together, these results demonstrate that Asn13 blocks the conventional signal peptide functions of the CRF2(a) receptor by impairing the ER targeting/insertion process.

The fact that hydrophobic or positively charged residues are Asn13-compatible indicates that glycosylation of Asn13 is not relevant for preserving the pseudo signal peptide. Trafficking of Asn13 mutants maintaining the pseudo signal peptide (N13D-CRF2(a), N13E-CRF2(a), and N13Q-CRF2(a); data not shown) was comparable with the wild-type construct demonstrating that glycosylation of Asn13 is also not involved in facilitating transfer of the receptor through the early secretory pathway, the key function of the pseudo signal peptide.

**DISCUSSION**

We have shown that the CRF2(a) receptor contains an uncleaved pseudo signal peptide that is unable to mediate ER targeting. Conventional signal peptide functions are inhibited by residue Asn13. In contrast, the homologous CRF1 receptor possesses a conventional and cleaved signal peptide (see Fig. 9 for a summary of our results).

It was previously reported for the human cytomegalovirus US11 protein that cleavage of an N-terminal signal peptide may be delayed (31). In the case of the prion protein PrP, the normal protein is synthesized in three topological forms in the ER, one possessing an uncleaved N-terminal signal peptide (32–34). These results show that the signal peptides of some membrane proteins may function inefficiently. However, in all these cases, the signal peptides have been functional and mediated normal
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protein synthesis. In contrast, we describe here the first pseudo signal peptide of a eukaryotic membrane protein.

Deletion of the pseudo signal peptide leads to an increase in the amount of immature, intracellularly retained receptors. The fact that the majority of these immature receptors are nonglycosylated rather than high mannose glycosylated (see Fig. 6A) strongly suggests that the pseudo signal peptide of the CRF$_{2(a)}$ receptor facilitates N tail translocation of the receptor across the ER membrane. The underlying mechanism, however, is unclear. Using fusions of the CRF$_{2(a)}$ receptor N tail with marker proteins (GFP or PrP), we have demonstrated that this domain is unable to target nascent chains to the ER membrane, the first key function of conventional signal peptides (see Figs. 2 and 3). In the case of the CRF$_{2(a)}$ receptor, TM1 thus takes over this function as an unsealed signal anchor sequence (see Fig. 2). However, despite the loss of its targeting functions, the pseudo signal peptide may still be involved in translocon gating, and thereby improve N tail translocation. However, such a residual conventional function should be accompanied by the lateral escape of the signal into the ER membrane and its subsequent cleavage. Taking into account that the signal-like domain at the cytosolic side (see Figs. 7 and 8), it is more likely that this domain is rapidly translocated and facilitates N tail translocation at the luminal side. It may, for example, provide an additional platform for chaperone proteins such as immunoglobulin heavy chain-binding protein helping to pull the N tail through the translocon.

Our data show that Asn$_{13}$ is a key residue of the pseudo signal peptide because it inhibits the ER targeting functions of this domain in the cytosolic side (see Figs. 7 and 8). It is not clear, however, how exactly Asn$_{13}$ impairs this process. One plausible hypothesis is that the presence of Asn$_{13}$ prevents SRP binding. Replacement of Asn$_{13}$ by hydrophobic residues restores ER targeting, whereas negatively charged residues and a polar glutamine residue preserve the pseudo signal peptide (see Fig. 8). Because SRP binding is driven mainly by signal hydrophobicity, these data are consistent with this hypothesis. However, introduction of a positively charged arginine residue also restores ER targeting (see Fig. 8). Whereas positively charged residues may be present in the N region of a signal peptide, they should impair rather than promote direct SRP binding when they are located at the Asn$_{13}$ position. Thus, it may also be speculated that Asn$_{13}$ blocks SRP binding indirectly by mediating association of a protein that in turn prevents SRP/pseudo signal peptide interactions. If positive charges or hydrophobic residues are introduced, binding of this hypothetical protein may be blocked and SRP binding restored.

The result that the CRF$_{2(a)}$ receptor possesses a pseudo signal peptide shows that the extreme N tails of the individual CRF receptor subtypes differ substantially. Whereas our data indicate that the pseudo signal peptide influences CRF$_{2(a)}$ receptor trafficking rather than its ligand binding profile, it is not excluded that this domain plays an additional role even for plasma membrane receptors. The pseudo signal peptide may, for example, participate in mediating protein/protein interactions or in binding of yet unknown ligands. Further experiments are needed to clarify this point.

Acknowledgments—We thank Ramanujan Hegde (National Institutes of Health) for the PrP(A120L) reporter plasmid and for help in preparing the prion protein targeting assay. We are grateful to Doreen Wietfeld for help in cloning of the CRF$_{2(a)}$, receptor cDNA. We thank Hartmut Berger, Michael Bienert, Alexander Oksche, and Ricardo Hermosilla for useful discussions. We thank Gesela Papsdorf of the cell culture facilities of the Leibniz-Institut für Molekulare Pharmacologie and Erhard Klauschen and Barbara Mohs from the DNA sequencing service group for their contributions. We also thank Dagmar Michl, Brunhilde Oczko, and Gabriela Vogelreiter for excellent technical assistance.

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