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The protease-activated receptor 1 possesses a functional and cleavable signal peptide which is necessary for receptor expression

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ABSTRACT

The protease-activated receptor 1 (PAR1) is activated by thrombin cleavage releasing the physiologically-relevant parstatin peptide (residues 1–41). However, the actual length of parstatin was unclear since the receptor may also possess a cleavable signal peptide (residues 1–21) according to prediction programs. Here, we show that this putative signal peptide is indeed functional and removed from the PAR1 resolving the question of parstatin length. Moreover, we show that the sequence encoding the signal peptide may surprisingly play a role in stabilization of the PAR1 mRNA, a function which would be novel for a G protein-coupled receptor.

1. Introduction

Protease-activated receptors (PARs) form a small family of G protein-coupled receptors (GPCRs) mediating responses of cells to extracellular proteases [1]. PAR1, the prototypical receptor of this family, is a predominant mediator of thrombin signaling in many cell types, including human platelets, endothelial cells, fibroblasts and smooth muscle cells [1]. The activation of PAR1 by thrombin occurs through the irreversible cleavage between Arg^{41} and Ser^{42} in the extracellular N terminus of the receptor. The truncated N terminus forms a new domain, binds intra-molecularly and acts as a tethered ligand triggering transmembrane signaling [2].

Proteolytic activation of PAR1 by thrombin also results in the release of a peptide with a potential length of 41 amino acids, namely parstatin. Strikingly, it was recently shown that parstatin mediates significant biological effects. The peptide efficiently blocked angiogenesis in several in vivo, ex vivo and in vitro studies [3]. In established animal models used for evaluation of neovascular ocular diseases, parstatin was found to prevent corneal, choroidal and retinal neovascularization without obvious side effects [4]. Parstatin also exhibited vasodilatory properties and was effective in cardioprotection during ischemia and reperfusion injury. Parstatin treatment before, during or after ischemia significantly decreased infarct size in an in vivo model of myocardial ischemia–reperfusion injury [5]. Although parstatin has attractive properties which may be transformed into therapeutic application in the future, several key questions regarding its sequence and structure are unclear. Most importantly, the precise length of the peptide is still unknown despite some attempts to detect and identify it in cell supernatants [6,7]. While it is clear that the peptide is released by cleavage between Arg^{41} and Ser^{42}, its actual length depends on whether the PAR1 possesses an N-terminal cleavable signal peptide or not.

Cleavable signal peptides of GPCRs and other integral membrane proteins mediate integration of the proteins into the membrane of the endoplasmic reticulum (ER), the initial step of the intracellular transport. Most GPCRs do not possess cleavable signal peptides but instead a non-cleaved signal anchor sequence which takes over signaling functions (usually the first transmembrane domain of the mature receptor). Both types of signal sequences bind to the signal recognition particle (SRP) and mediate targeting of the nascent chains to the translocon complex at the ER membrane. The signal sequences also facilitate opening of the Sec61 protein-conducting channel of the translocon complex in order to integrate the nascent chain into the bilayer. Whereas signal peptides are cleaved off following ER insertion by the signal peptidases of the ER, signal anchor

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sequences form part of the mature protein. However, it was recently shown that even signal peptides may remain as an uncleaved pseudo signal peptide at the N tail of a GPCR [8–10].

Here, we have addressed the question of whether the PAR1 possesses an N-terminal cleavable signal peptide or not. If a cleavable signal peptide is present, the thrombin-released parstatin peptide would only be 20 amino acids long. If not, the actual parstatin length would be 41 amino acid residues. We show that the PAR1 possesses a functional and cleaved signal peptide resolving the question of actual parstatin length. Moreover, we show that the sequence encoding the signal peptide surprisingly plays a role in stabilization of the PAR1 mRNA secondary structure.

2. Materials and methods

2.1. Materials

The PAR1 cDNA was purchased from the Missouri University of Science and Technology (Rolla, MO, USA). The vector plasmid pEGFP-N1 (encoding the enhanced green fluorescent protein), the ER marker plasmid pECFP-ER (encoding the enhanced cyan fluorescent protein fused with a KDEL ER retrieval sequence), TALON metal affinity beads and the monoclonal mouse antibody against the green fluorescent protein (GFP) were from BD Biosciences Clontech (Mountain View, CA, USA). The transfection reagent Lipofectamine 2000, Trizol reagent, SuperScript III First-Strand Synthesis SuperMix kit were from Invitrogen (Karlsruhe, Germany). DNA-modifying enzymes and peptide-N-glycosidase F (PNGaseF) were from New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were purchased from Biotez (Berlin, Germany). Trypan blue was purchased from Seromed (Berlin, Germany). RotiLoad sample buffer was from Carl Roth (Karlsruhe, Germany). TMB (3, 3′, 5, 5′-tetramethylbenzidine) was from Calbiochem (Darmstadt, Germany). Polyclonal rabbit anti-GFP antiserum 02 (raised against a GST-GFP fusion protein) has been described [10]. HRP-conjugated goat anti-mouse IgG was purchased from from Dianova (Hamburg, Germany). Monoclonal mouse anti-FLAG M2 antibody and monoclonal mouse peroxidase-conjugated anti-FLAG M2 antibody Actinomycin D and all other reagents were from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Plasmid constructs

The constructs used in this study are schematically shown in Fig. 1A. Marker protein fusions: plasmids $pCRF_1$.NT and $pCRF_{2(a)}$.NT encode C-terminal his tagged GFP fusions to N tail sequences of the rat corticotropin-releasing factor receptor type 1 (CRF_1R) and type 2(a) ($CRF_{2(a)}R$) in the vector pSecTag2A plasmid, respectively (CRF_1R: fusion at position Ala^{119} predicted N tail length including the signal peptide = 119 residues; $CRF_{2(a)}R$: fusion at position Ala^{121}; predicted N tail length including the pseudo signal peptide = 116 residues). The additional C-terminal His6-sequence at the GFP moiety allowed the purification of all GFP fusion proteins. Plasmid pPAR1.NT was constructed by fusing C-terminally His-Tagged GFP to an N tail sequence of the human PAR1 accordingly (fusion at position Leu^{101}; predicted N tail length including the putative signal peptide = 101 residues). Full length receptor constructs: plasmids $pCRF_1$ encodes the full length CRF_1R C-terminally fused with GFP (position Thr^{413}) in the vector plasmid pEGFP-N1; plasmid $p\Delta SP.CRF_1$ encodes the corresponding signal peptide mutant (deletion of residues 1–24) [8]. Plasmid pPAR1 was constructed by fusing the full length PAR1 C-terminally with GFP (position Thr^{425}); plasmid ΔSP.PAR1 encodes the signal peptide mutant of the latter construct (deletion of residues 1–21). Plasmids pFLAG.CRF_1 and pFLAG.PAR1 encode C-terminally GFP-tagged full length receptors, possessing an additional N-terminal FLAG tag (sequence DYKDDDDK). Plasmid pFLAG.V_2 encodes a C-terminally GFP and N-terminally FLAG-tagged construct of the human vasopressin V_2 receptor (V_2R).

2.3. Cell culture and transfection

HEK 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Transfection of the cells using Lipofectamine 2000 was carried out according to the supplier's recommendations. Equal amounts of plasmid were transfected in each experiment.

2.4. Confocal laser scanning microscopy (LSM) and colocalization of GFP and trypan blue or ECFP-ER fluorescence signals in live cells

For colocalization of the GFP fluorescence signals of the constructs with trypan blue, HEK 293 cells (2×10^5) grown on poly-L-lysine-treated (25 μg/ml) glass coverslips in 35-mm diameter dishes were transiently transfected with the plasmid DNA of the constructs (1.2 μg). Cells were grown overnight, washed and stained with trypan blue at final concentration of 0.05% for 1 min. GFP and trypan blue fluorescence signals were visualized on a Zeiss LSM510-META invert confocal laser-scanning microscope (objective lens: 100×/1.3 oil; optical section:<0.8 μm; multitrack mode; GFP, λ_{exc}: 488 nm, Argon laser, BP filter: 500–530 nm; trypan blue, λ_{exc}: 543 nm, HeNe laser, LP filter: 560 nm). For colocalization of the GFP signals of the constructs with those of the ER marker protein ECFP-ER, a cotransfection was performed accordingly. GFP and ECFP-ER signals were visualized on a Zeiss LSM780-META invert confocal laser-scanning microscope (objective lens: 100×/1.3 oil; optical section:<1 μm; multitrack mode; GFP, λ_{exc}: 488 nm, Argon laser, BP filter: 496–534 nm; ECFP-ER, λ_{exc}: 458 nm, Argon laser, BP filter: 461–500 nm).

2.5. Quantitative detection of secreted GFP fusion proteins

Secreted fusion proteins of transiently transfected HEK 293 cells were purified and detected by SDS/PAGE immunoblotting as described [8] using a monoclonal mouse anti-GFP antibody (dilution 1:3000) and HRP-conjugated anti-mouse IgG (dilution 1:5000). GFP fluorescence intensity of the secreted marker fusions was quantified fluorometrically as described [8] (λ_{exc} = 488 nm, λ_{em} = 510 nm).

2.6. Immunoprecipitation of GFP-tagged full-length receptor constructs

Full length receptor constructs of HEK 293 cells were precipitated using the polyclonal anti-GFP antiserum 02 as described [8]. Precipitated receptors were treated with PNGaseF prior to immunoblot analysis according to the supplier's recommendations. The GFP-tagged receptors were detected by SDS PAGE (10% SDS, 48 cm^2 gels) and immunoblotting using a monoclonal mouse anti-GFP antibody (dilution 1:3000) and an HRP-conjugated anti-mouse IgG (dilution 1:5000).

2.7. Cell surface ELISA assay

HEK 293 cells (20×10^3) grown in a 96 well plate pretreated with 25 μg/ml poly-L-lysine were transiently transfected with plasmid DNA (200 ng/well) and cultivated for 24 h at 37 °C. Cells were washed two times with PBS (containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and fixed for 15 min at room temperature (RT) using 4% paraformaldehyde. Cells were washed three times with PBS and

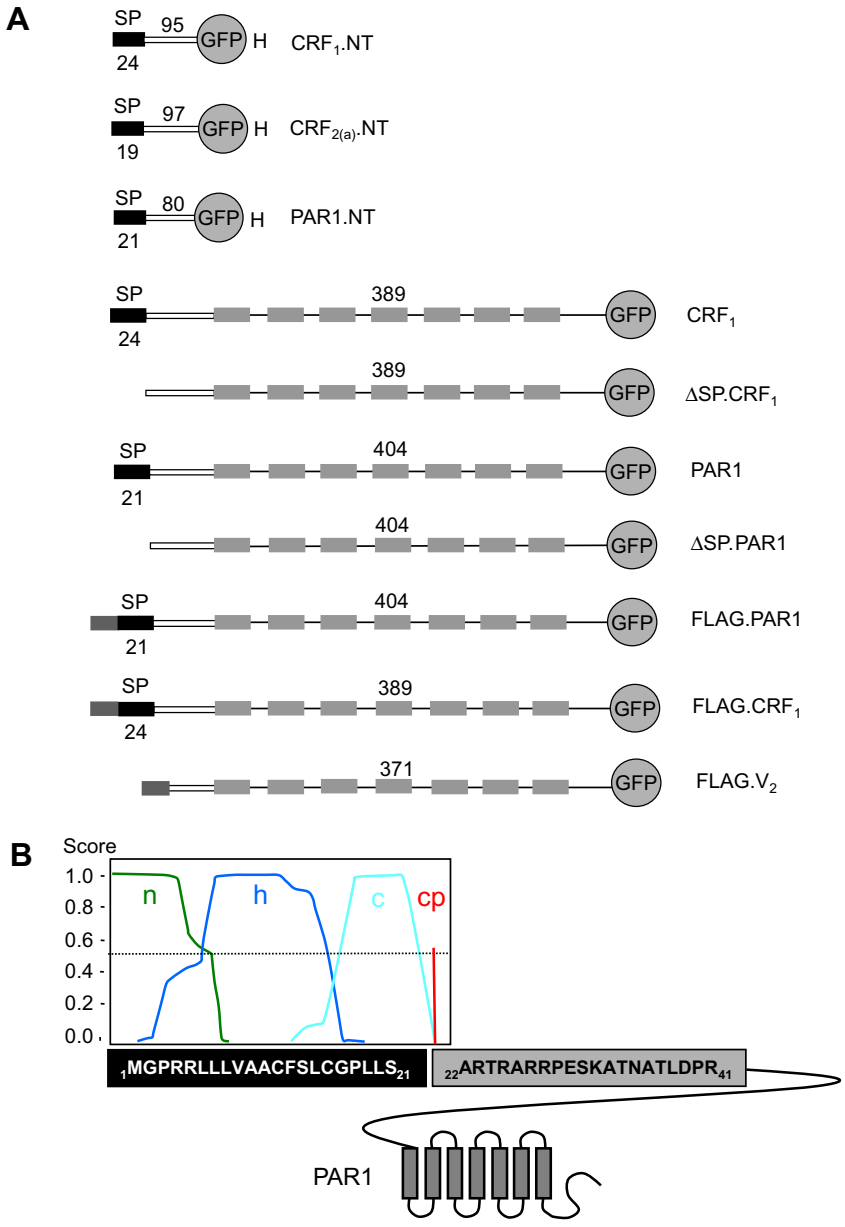

Fig. 1. (A) Schematic representation of the constructs used in this study. The signal peptides (SP) and the transmembrane domains are shown as black and gray boxes respectively. N tail sequences are depicted as open boxes, FLAG tags are indicated by dark gray boxes. The numbers above each construct indicate the predicted number of amino acid residues of the N tails (without signal peptide); the numerals below the constructs indicate predicted signal peptide length. Upper panel: marker protein fusions. Fused GFP and His tags (H) are indicated. Lower panel: full-length receptor constructs. (B) Depiction of the N terminal sequence of the PAR1 including the putative signal peptide (Met^1–Ser^{21}) and the sequence up to the thrombin cleavage site (Ala^{22}–Arg^{41}). The sequence Met^1 to Arg^{41} was originally described as parstatin. For the signal peptide, the probabilities of the presence of n (green), h (blue), and c (light blue) regions and the cleavage probabilities (cp, red) are indicated in a score ranging from 0 to 1.

treated with gelatine blocking reagent for 1h at RT. After washing three times with ELISA buffer (PBS-buffer containing 0.05% Tween 20 and 0.5% BSA) monoclonal, peroxidase-conjugated anti-FLAG M2 antibodies were added (1:2000 in ELISA buffer) and samples were incubated for 1 h at 37 °C. Cells were washed three times with ELISA buffer and incubated with TMB for 30 min at RT in the dark. The reaction was stopped by adding H_2SO_4 at a final concentration of 0.5 N. Optical density (OD) at 450 nm was measured (with correction at 630 nm to eliminate signals resulting from surface impurities on the plates) using a Tecan Safire multi-detection monochrometer microplate reader (Männedorf, Switzerland). Assay reliability was verified by blocking antibody binding using a soluble FLAG epitope peptide.

2.8. Immunofluorescence microscopy

HEK 293 cells (1.5×10^5) grown in 24 well plates with cover slips pretreated with 25 μg/ml poly-L-lysine were transiently transfected with plasmid DNA (1.2 μg). Cells were cultured for 24 h, washed twice in serum free DMEM and incubated with a monoclonal anti-FLAG M2 antibody (dilution 1:1000) for 1 h at 4 °C. Cells were washed three times with PBS containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ and then fixed for 5 min at 4 °C with 4% (w/v) PFA. Cells were washed with PBS containing 1% non fat dry milk and 150 mM sodium acetate (pH 7.0) and incubated for 15 min at RT in the same buffer without sodium acetate. Cells were incubated with Cy3-conjugated goat anti-mouse IgG (dilution

1:500) for 1 h at RT, washed and transferred to LSM analysis (λ_{exc} = 488 nm, λ_{em} = 507 nm).

2.9. Total RNA extraction, cDNA synthesis, qRT PCR and mRNA degradation assay

HEK 293 cells (4×10^6) were grown on 100 mm diameter dishes and transiently transfected with plasmid DNA as described above. RNA extraction was performed using the Trizol reagent. After drying the RNA pellet, the RNAeasy kit (Qiagen, Hilden, Germany) was used for additional purification according to the supplier's recommendations. The RNA pellet was finally dissolved in 35 µl of RNase free water. Synthesis of the cDNA was performed using the SuperScript III First-Strand Synthesis Super Mix kit (Invitrogen, Karlsruhe, Germany) according to the manufacture's protocol. For the qRT PCR reaction, the TaqMan gene expression assay technology and primers were used (Applied Biosystems, Darmstadt, Germany) according to the supplier's protocols. Results were normalized to the endogenous glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) control and compared to a reference sample (untreated HEK 293 cells). Statistical analysis of the data was performed using the DataAssist v2.0 Software provided (Applied Biosystems). For the mRNA degradation assay, HEK 293 cells (4×10^6) grown on 60-mm diameter dishes were transiently transfected with plasmid DNA as described above. Twenty-four hours after transfection, cells were treated with 10 µg/ml Actinomycin D for various time points. Cells were collected and total RNA extraction, cDNA synthesis and qRT PCR were performed as described above.

2.10. In vitro transcription

The EasyXpress Insect kit II (Qiagen, Hilden, Germany) was used for the *in vitro* transcription according to the manufacture's protocol. To determine transcription efficiency, the mRNA was measured at 260 nm using the UV Spectrometer NanoDrop™ 2000 (Peqlab, Erlangen, Germany).

2.11. Inositol phosphate accumulation assay

The experiment was carried out with intact transiently transfected HEK-293 cells as described previously [11]. For PAR1 stimulation, cells were treated with 1 U/ml thrombin.

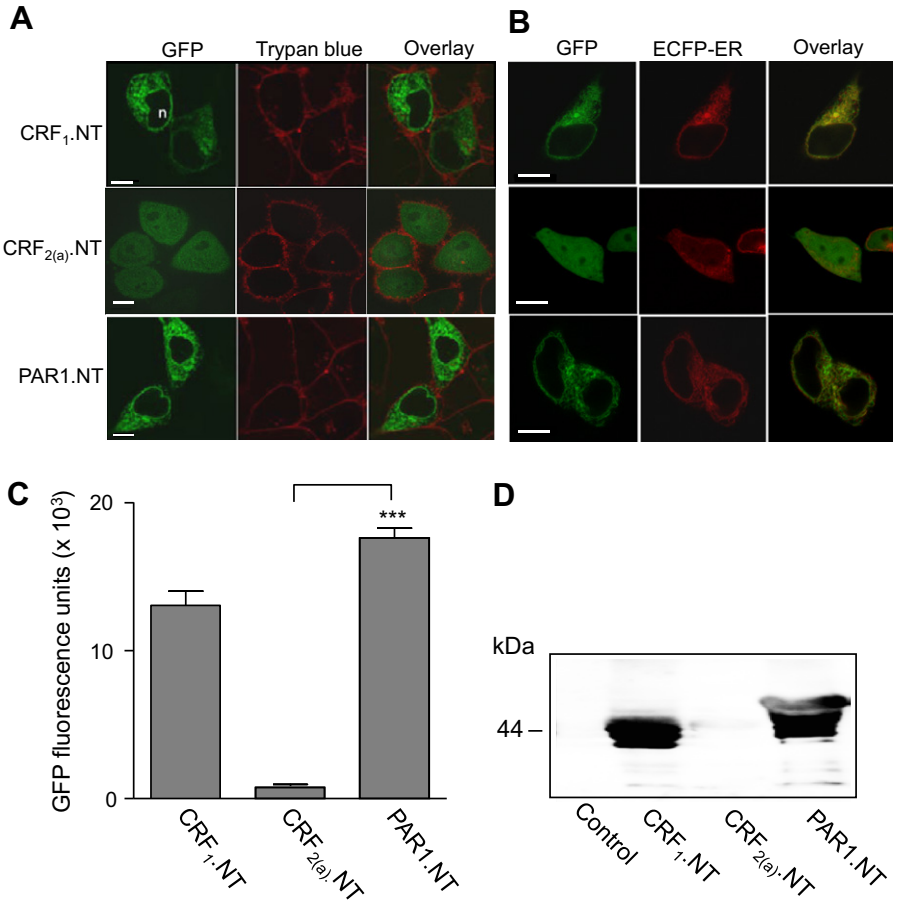

Fig. 2. Analysis of the PAR1 signal peptide cleavage using marker protein fusions. (A) Subcellular localization of the constructs CRF_1.NT, $CRF_{2(a)}$.NT and PAR1.NT in transiently transfected HEK 293 cells using confocal LSM. The GFP signals of the constructs (green, left panels) and the plasma membrane signals following staining with trypan blue (red, central panels) are shown. GFP and trypan blue fluorescence signals were computer-overlayed (right panels). GFP fluorescence is detectable only for transfected cells, whereas all cells show cell surface trypan blue fluorescence. Horizontal xy-scans of representative cells are shown. Scale bar = 10 µm, (n) = nucleus. (B) Co-localization of the constructs CRF_1.NT, $CRF_{2(a)}$.NT and PAR1.NT with the ER marker protein ECFP-ER in transiently transfected HEK 293 cells. The GFP signals of the constructs (green, left panels) and the ECFP-ER signals (red, central panels) were computer-overlayed (right panels). Horizontal xy-scans of representative cells are shown. Scale bar = 10 µm, (n) = nucleus. (C) Fluorometric detection of the constructs in the cell culture supernatant. Columns represent the fluorescence of the secreted, purified constructs from 4×10^6 cells, and show mean values of three independent experiments each performed in triplicates (±SD) ($^{***}P < 0.001$, student's *T*-test). (D) Detection of secreted, purified constructs by immunoblotting using a monoclonal anti-GFP antibody and HRP-conjugated anti mouse IgG. In each lane, the isolated PNGaseF–treated protein of 4×10^6 cells was loaded. The immunoblot is representative of three independent experiments.

3. Results

3.1. The PAR1 meets all the necessary criteria for the presence of a cleavable signal peptide

Cleavable signal peptides of eukaryotic membrane and secretory proteins share characteristic features [12]: a polar and often charged N-terminal (n) region, a central hydrophobic (h) region and a polar C-terminal (c) region containing helix-breaking proline and glycine residues and small uncharged residues at positions-1 and -3 of the cleavage site. Analysis of the N-terminal sequence of the PAR1 with the "SignalP3.0" bioinformatics software [13,14] revealed that the N-terminal sequence Met^{1}–Arg^{23} meets all criteria for the presence of a functional signal peptide which is cleaved by the signal peptidases of the ER (Fig. 1B). Signal peptide probability reached a maximal 1.0 value and a cleavage site seems to be present between residues Ser^{21} and Ala^{22}.

3.2. The PAR1 possesses a functional and cleaved N-terminal signal peptide

Length of the parstatin peptide is dependent on whether the PAR1 possesses a cleavable signal peptide or not. To assess whether the predicted signal peptide is indeed functional, we fused the entire PAR1 N tail (Met^{1}–Leu^{101}) with His-tagged GFP (construct PAR1.NT; see Fig. 1A). If the putative signal peptide is functional, the cytosolic GFP protein should be converted to a secreted protein. As a secretory protein, GFP should appear initially in the ER and, following transport through the secretory pathway, in the cell culture medium. However, if the putative signal peptide is not functional and uncleaved like e.g. the recently described pseudo signal peptide of the $CRF_{2(a)}R$ [8–10], the construct should remain in the cytosol. As controls for the secretion experiments, we used the previously described [8] fusions of the N tails of the CRF_1R (cleaved signal peptide, construct CRF_1.NT, Fig. 1A) and the $CRF_{2(a)}R$ (pseudo signal peptide, construct $CRF_{2(a)}$.NT, Fig. 1A). HEK 293 cells were transiently transfected with the constructs and the GFP fluorescence signals were localized by LSM and by fluorimetric measurements and immunoblotting. In the case of $CRF_{2(a)}$.NT, the signals were detected diffusely throughout the cell including the nucleus demonstrating that these fusions were not targeted to the ER membrane (Fig. 2A). In contrast, in the case of CRF_1.NT and PAR1.NT, reticular signals were detected demonstrating that these fusions were able to enter the ER (Fig. 2A; the validity of this LSM assay has been confirmed previously [8,9]). The reticular signals also colocalized almost completely with the cotransfected ER marker protein ECFP-ER (consisting of ECFP fused to a KDEL ER retrieval sequence) (Fig. 2B). Consistent with these results, the constructs CRF_1.NT and PAR1.NT but not $CRF_{2(a)}$.NT could be purified via their His-tag from cell culture supernatants and detected by fluorometric measurements or by immunoblotting (Figs. 2C and D).

Taken together, these results indicate that the PAR1 possesses a conventional and cleaved signal peptide which is able to direct the GFP marker protein to the ER and via the secretory pathway finally to the cell culture medium.

To confirm these results for the full length PAR1, a FLAG tag was fused N-terminally to the signal peptide of the PAR1 (Fig. 1A, construct FLAG.PAR1). In addition, the receptor was tagged C-terminally with GFP. If the signal peptide is cleaved, the FLAG tag should be removed together with the signal peptide in the early secretory pathway and should no more be detectable at cell surface receptors. As controls for these experiments, we used the N-terminally FLAG-tagged CRF_1R possessing a cleaved signal peptide (Fig. 1A; construct FLAG.CRF_1) and an N-terminally FLAG-tagged vasopressin V_2 receptor (Fig. 1A; construct FLAG.V_2) possessing

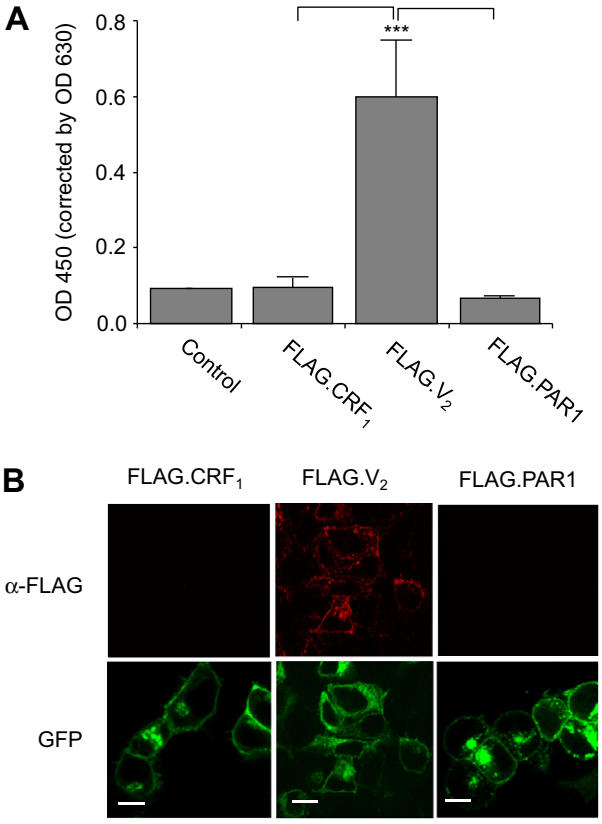

Fig. 3. Analysis of the signal peptide cleavage of the full length PAR1 using N-terminal FLAG tags preceding the signal peptide. HEK 293 cells were transiently transfected with the constructs FLAG.PAR1, FLAG.CRF_1 and FLAG.V_2. (A) ELISA Assay. Cell surface expression on intact cells was quantified using a monoclonal peroxidase-conjugated anti-Flag M2 antibody. Columns represent mean values of three independent experiments (±SD) (***$P < 0.001$, student's T-test). (B) Immunofluorescence microscopy using confocal LSM. Intact cells were assessed for the presence of an uncleaved FLAG epitope using mouse anti-FLAG M2 antibodies and Cy3-conjugated goat anti-mouse IgG (upper panel). Receptor expression was verified by recording the GFP fluorescence signals. (lower panel). Horizontal xy-scans of representative cells are shown. Scale bar = 10 μm.

only a signal anchor sequence. HEK 293 cells were transiently transfected and receptors were detected on the surface of intact cells using anti-FLAG antibodies and an ELISA assay (Fig. 3A) or immunofluorescence microscopy (Fig. 3B). FLAG signals at the cell surface were detectable in both experiments in the case of the control construct FLAG.V_2 but not for constructs FLAG.CRF_1 and FLAG.PAR1, while all three constructs were readily visible when monitoring their GFP signals (Fig. 3B, lower panel). These results demonstrate that the PAR1 possesses a conventional and cleaved signal peptide and that the signal peptide sequence is consequently not included in the parstatin peptide.

3.3. Deletion of the sequence encoding the signal peptide strongly decreases the amount of PAR1 mRNA

It was previously shown that signal peptides of GPCRs may serve different functions in addition to their role in the ER targeting/insertion mechanism. Among these are e.g. regulatory effects on total receptor expression and N tail translocation across the ER membrane [15]. To analyze the functional significance of the signal peptide of the PAR1, a signal peptide mutant was constructed by deleting residues Met^{1}–Ser^{21} of the C-terminally GFP-tagged receptor (Fig. 1A; constructs PAR1 and ΔSP.PAR1, respectively). Deletion of a signal peptide of a GPCR does normally

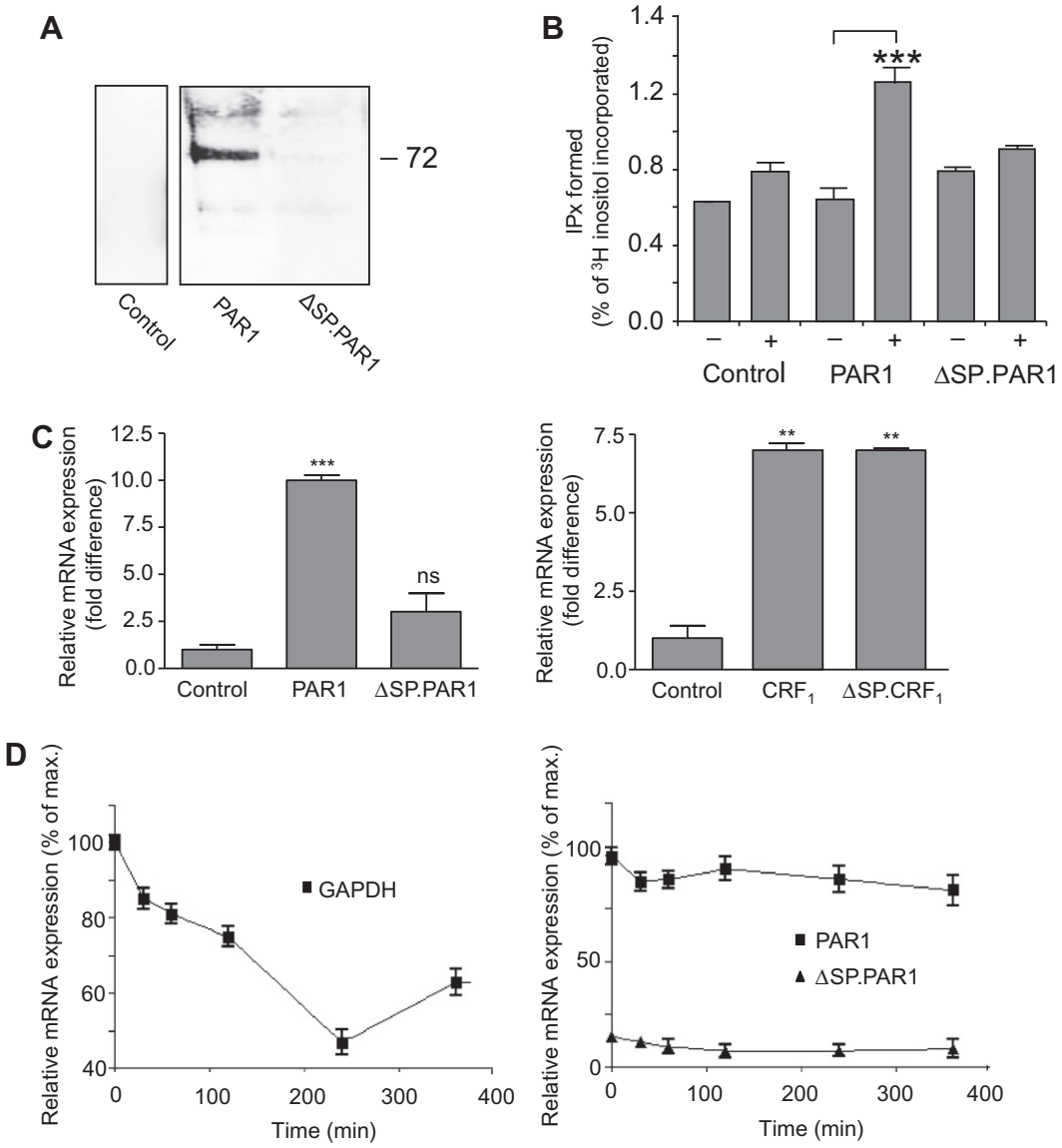

Fig. 4. Analysis of the expression of the full length PAR 1 and its signal peptide mutant ΔSP.PAR1. (A) SDS–PAGE/immunoblot analysis. Receptors were immunoprecipitated from transiently transfected HEK 293 cells, digested with PNGaseF to remove all N glycans and detected using a monoclonal anti-GFP antibody and HRP-conjugated anti-mouse IgG. The immunoreactive protein band with an apparent molecular mass of 70 kDa represents the PAR1. Control = mock-transfected cells. The immunoblot is representative of three independent experiments. (B) Thrombin-mediated inositol phosphate accumulation in intact HEK 293 cells transiently transfected with the constructs PAR1 and ΔSP.PAR1. Cells were treated with thrombin (+) or with vehicle (−). Columns represent mean values of three independent experiments each performed in triplicates (± SD) (***$P < 0.005$, two-way analysis of variance). (C) qRT PCR quantification of the mRNA of constructs PAR1/ΔSP.PAR1 (left panel) and CRF_1/ΔSP.CRF_1 (right panel) in transiently transfected HEK 293 cells. Columns represent fold difference of mRNA expression after normalization to the GAPDH endogenous control compared to the reference sample (untreated cells) and show mean values of three independent experiments each performed in triplicates (±SD) (***P < 0.001; **P < 0.01, student's *T*-test). (D) Degradation of the mRNA of PAR1 and ΔSP.PAR1 in transiently transfected HEK 293 cells treated for different times with the transcription inhibitor Actinomycin D. Data points represent the remaining mRNA of GAPDH (left panel) and that of PAR1 and ΔSP.PAR1 (right panel; normalized to the amount of GAPDH) of three experiments performed in triplicates (±SD).

not abolish receptor expression since one of the transmembrane domains usually takes over ER targeting/insertion functions as a signal anchor sequence. In the case of opsin, for example, it was shown that five of the six transmembrane segments studied could function as a signal anchor sequence [16].

However, when analyzing constructs PAR1 and ΔSP.PAR1 by SDS–PAGE/immunoblotting following immunoprecipitation from transiently transfected HEK 293 cells, we failed to detect significant amounts of ΔSP.PAR1 (Fig. 4A). Moreover, we failed to detect significant GFP fluorescence signals of the signal peptide mutant in the transiently transfected HEK 293 cells (data not shown) and ΔSP.PAR1-mediated inositol phosphate accumulation (Fig. 4B). These results indicate that the signal peptide of the PAR1 surprisingly seems to be necessary for PAR1 expression.

A lack of receptor expression in the case of ΔSP.PAR1 mutant could be caused by a failure of the transmembrane domains to function as signal anchor sequences. On the other hand, the sequence encoding the signal peptide might be necessary for efficient mRNA expression. We thought that it is unlikely that each of the transmembrane domains is unable to function as a signal anchor and thus analyzed whether deletion of the sequence encoding the signal peptide affects mRNA levels. To this end, we first performed an in vitro transcription assay using constructs PAR1 and ΔSP.PAR1 under the control of the T7 promoter. Photometric

measurement of the produced transcripts revealed similar amounts of mRNA for both constructs (PAR1: 1002 ± 145 ng/μl; ΔSP.PAR1: 836 ± 130 ng/μl) indicating that transcription itself is not significantly affected when the sequence encoding the signal peptide is deleted. Next, we performed qRT-PCR using RNAs isolated from HEK-293 cells transiently transfected with PAR1 and ΔSP.PAR1. As a control, we used the C-terminally GFP-tagged full length CRF_1R and its signal peptide mutant (constructs CRF_1 and $\Delta SP.CRF_1$; Fig. 1A). All data were normalized to the endogenous control of the glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH). Substantial amounts of mRNA were detected in the case of PAR1 but not for ΔSP.PAR1. In the case of the control constructs CRF_1 and $\Delta SP.CRF_1$, similar amounts of mRNA were detectable (Fig. 4C), demonstrating that the results were specific for the PAR1.

When mRNA expression is decreased but transcription itself not influenced, the sequence encoding the signal peptide of the PAR1 may increase mRNA stability. To address this question, we performed mRNA decay assays, using Actinomycin D, as an inhibitor of transcription. Quantification of mRNA levels at various time points using qRT-PCR shows that PAR1 mRNA levels remained stable for at least 4 h after actinomycin D treatment (Fig. 4D). In contrast, the very low mRNA levels of construct ΔSP.PAR1 are consistent with a very rapid mRNA degradation.

To address this question also theoretically, we performed bioinformatic analyses of the mRNA secondary structure of PAR1 and ΔSP.PAR1 using the program mFold [17] (Fig. 5). The prediction indicates that the sequence encoding the signal peptide of PAR1 forms a GC-rich long stem loop which is absent in the case of ΔSP.PAR1. Formation of such stem loops has indeed been shown to stabilize mRNA structures consistent with the results above [18,19].

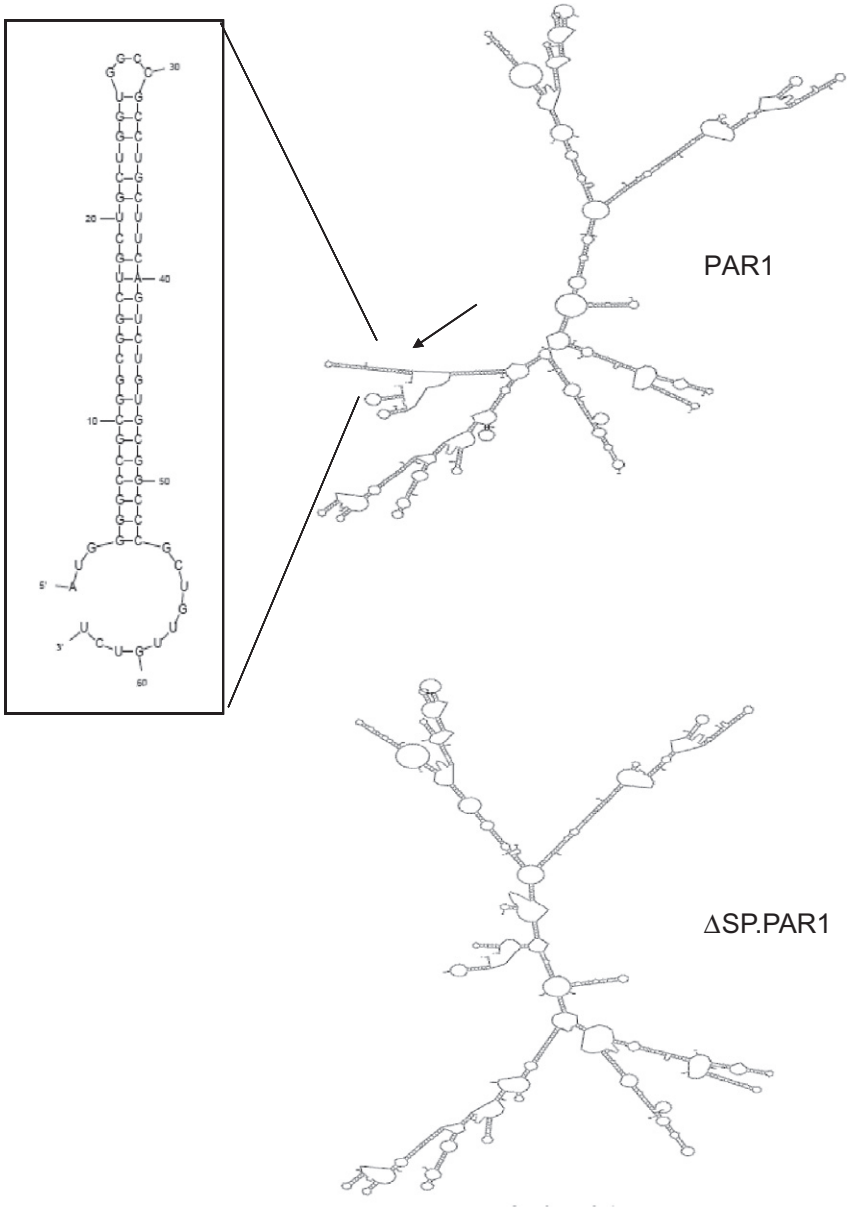

Fig. 5. Bioinformatic analysis of the putative mRNA secondary structure of PAR1 (upper panel) and ΔSP.PAR1 (lower panel). In the case of PAR1, the sequence encoding the signal peptide forms a stem loop structure (arrow and cutaway view) which is missing in the case of ΔSP.PAR1. The analysis was performed using the program mFold.

4. Discussion

We show here that the sequence at the N tail of the PAR1 represents a functional signal peptide which is removed from the receptor following insertion into the ER membrane. Thus, the peptide which is proteolytically cleaved from the mature receptor at the plasma membrane following thrombin activation does not contain the residues forming the signal peptide. Most importantly, and without knowing that the signal peptide of the PAR1 is cleaved off, it was recently shown that the parstatin fragment represented by the sequence Met^{1}–Ala^{26} contains the functional domain of the peptide [20]. In a rat ischemia–reperfusion injury model, it was demonstrated the synthetic parstatin (1–26) to be more effective and potent in the protection of myocardium, compared to full-sized parstatin (1–41). In the same model the synthetic parstatin fragment (24–41) is not functional (unpublished data), indicating that parstatin functions are mediated by the signal peptide alone. Taking into account that the signal peptide is actually cleaved off in the early secretory pathway, parstatin effects must originate from a peptide which is initially embedded in the ER membrane rather than being released extracellularly. In this case, how can the cleaved signal peptide then fulfill its functions? It is known for a long time that in some cases, cleaved signal peptides are not degraded but processed by intramembrane signal peptide peptidases [21,22]. The trimmed peptides may be retranslocated into the cytosol by an as yet incomplete understood mechanism and it was proposed that the peptides have physiological functions following their release [21,22]. In the case of the mouse mammary tumor virus Rem protein, for example, it was shown recently that the released peptide is transported into the nucleus and accumulates in nucleoli [23]. Therefore and based on these observations, it is reasonable to speculate that the cleaved signal peptide of the PAR1 is also released from the ER membrane. It may translocate to the cytosol or may enter the nucleus in a similar way as described for the Rem protein and fulfill its functions. Alternatively, the released signal peptide may have an indirect effect on other proteins in the cytosol or nucleus. Specific analytical experiments are needed to verify the release of the peptide and to further investigate its fate and biological and physiological role.

In the original experiments leading to the description of the parstatin effects, the peptide was added to the extracellular side of the cells [3,5,20]. These results do not conflict with the fact that the relevant peptide obviously does not leave the cells. It is conceivable that the hydrophobic signal peptide may easily enter the cells when it is added into cell culture medium, either by diffusion and/or by endocytosis [3].

For the PAR1, we could also show that the sequence encoding the signal peptide dramatically increases the amount of mRNA, most likely by facilitating the formation of a stem loop and by preventing rapid RNA degradation. It is already known that such stem loops may stabilize the secondary structures of mRNAs [24–26]. In the case of a bacterial protein, a sequence encoding a signal peptide had a similar mRNA-stabilizing effect [27].

In the GPCR protein family, an influence of a sequence encoding a signal peptide on mRNA levels is so far unique. In the case of the ET_BR [28], CRF_1R [29] and $CRF_{2(a)}R$ [8] deletion of the signal peptide sequence did not abolish receptor expression. Future studies must show, whether this function of the signal peptide sequence represents a more general principle in the GPCR protein family.

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