

# Proteolytic cleavage of the EMR2 receptor requires both the extracellular stalk and the GPS motif

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Received 26 March 2003; accepted 2 June 2003

First published online 20 June 2003

Edited by Irmgard Sinning

**Abstract** EMR2 is a human myeloid-restricted member of the EGF-TM7 receptor family that contains a highly conserved G protein-coupled receptor proteolysis site (GPS) in the membrane-proximal region. Here the post-translational proteolytic cleavage of EMR2 at GPS was investigated. We show the cleavage occurs at Leu<sup>517</sup>-Ser<sup>518</sup> and is independent of the transmembrane domains. The non-covalent association of the resulting extracellular  $\alpha$ -subunit and transmembrane  $\beta$ -subunit requires a minimum of eight amino acids in the  $\beta$ -subunit. The GPS motif is necessary, but not sufficient for receptor cleavage, which requires the entire extracellular stalk. Thus, an alternatively spliced EMR2 isoform with a truncated stalk fails to undergo proteolytic cleavage. Alternative splicing therefore provides a means to regulate GPS cleavage, producing receptors with two distinct structures.

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**Key words:** Post-translational modification; Proteolytic cleavage

## 1. Introduction

In recent years, a novel group of seven-transmembrane (7TM) receptors, termed long N-terminal family B G protein-coupled receptor (GPCR)-related 7TM (LNB-TM7) receptors, containing a class B GPCR-related 7TM moiety and a large N-terminal extracellular domain (ECD), has attracted much attention due to several unique features [1–3]. In addition to the 7TM domain that may transduce cellular signals, the ECD of these molecules contains multiple repeats of protein modules that are thought to be involved in protein–protein interactions. Furthermore, the expression of LNB-TM7 molecules is restricted to specific cell types or tissues such as leukocytes, smooth muscle cells, epididymal epithelial cells or brain [1]. Thus, these unique receptors are believed to

play important roles in cell type/tissue-specific functions through cellular adhesion via the ECD, followed by signal transduction through the 7TM domain.

Another common characteristic of the LNB-TM7 molecules is a highly conserved Cys-rich domain in the membrane-proximal region [1]. A post-translational proteolytic cleavage event within the Cys-box has been linked to the generation of heterodimeric receptors composed of an extracellular  $\alpha$ -subunit and a 7TM  $\beta$ -subunit [4]. Hence, the Cys-box has also been named the GPCR proteolysis site (GPS) [4,5]. However, a search in the protein databases has yielded several other proteins outside the LNB-TM7 family that also contain the conserved GPS motif [6]. These include PKD-1, the human polycystic kidney disease protein 1 [7,8], suREJ3, a channel-like 11-span transmembrane protein [9] and hPKDREJ, the human homologue of suREJ3 [10], suggesting that the GPS motif and its associated proteolytic cleavage activity are widely used by cell surface receptors. Although the functional significance of the GPS motif-associated proteolysis remains elusive, the presence of the highly conserved GPS motif in such a diverse array of receptors is suggestive of a common role in receptor function or regulation.

The epidermal growth factor-like domain containing TM7 (EGF-TM7) receptors [11,12] belong to a subgroup of the LNB-TM7 family and consist of tandem repeats of N-terminal EGF-like domains and a stalk region at the extracellular region. The EGF-like domains of the EGF-TM7 receptors have been shown to mediate cell–cell interaction by binding to specific cellular proteins [13–17]. The stalk region contains a high percentage of Ser and Thr residues that are potential O-glycosylation sites [11,12]. In addition, multiple potential N-glycosylation sites have also been identified in the stalk. Thus, the stalk region of the EGF-TM7 receptors is thought to be highly glycosylated and act as a mucin-like domain with a rigid structure. EMR2 is a member of the EGF-TM7 family restricted to human myeloid cells including monocytes, macrophages and granulocytes [18,19]. A cellular ligand specific to the EGF-like domains of the largest EMR2 isoform has been identified recently (Stacey et al., Blood, in press). Within the EGF-TM7 family, EMR2 is most related to CD97 in the EGF-like domains (97.5% identity) and to EMR3 in the TM7 domain (85% identity). All EGF-TM7 receptors except EMR1 contain a consensus GPS motif at the most C-terminal end of the stalk region very close to the TM domain. In recent years, proteolytic cleavage of several EGF-TM7 receptors including CD97, ETL and EMR4 has been demonstrated

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**Abbreviations:** ECD, extracellular domain; FACS, fluorescence-activated cell sorting; Fc, fragment crystallisable; GPCR, G protein-coupled receptor; GPS, GPCR proteolysis site; LNB-TM7, long N-terminal family B GPCR-related 7TM receptor; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane

[16,20,21]. Using a newly developed EMR2 stalk-specific monoclonal antibody (mAb), we have recently shown that EMR2 is also proteolytically cleaved [19]. To understand this novel post-translational modification further, we herein report the role of the extracellular stalk and the GPS motif in EMR2 processing.

## 2. Materials and methods

### 2.1. Materials

All chemicals and reagents were obtained from Sigma unless otherwise specified. Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). EMR2-specific 2A1 mAb (mouse IgG1 subtype) was affinity-purified from hybridoma supernatant as previously described [19].

### 2.2. Construction of expression vectors

All expression vectors were constructed on pcDNA3.1(+) (Invitrogen) unless otherwise specified. For the construction of vectors encoding truncated EMR2(1, 2, 5) proteins, EMR2-TM1 and sEMR2, gene-specific primers were used to amplify the desired cDNA fragments and subcloned into pcDNA3.1(+). For the construction of vectors encoding various EMR2-mouse *fragment crystallisable* (mFc) fusion proteins, a previously described expression construct containing a mFc DNA fragment and a biotinylation signal was used [16]. A panel of gene-specific primers was used to generate DNA fragments encoding various truncated EMR2 stalk regions, which were subsequently subcloned to generate the following EMR2-mFc constructs: EMR2(529)mFc, EMR2(526)mFc, EMR2(522)mFc, EMR2(518)mFc, EMR2(310)mFc, EMR2Δ(260–487)mFc, EMR2Δ(260–479)mFc, EMR2Δ(478–487)mFc, EMR2Δ(260–449)mFc, EMR2Δ(260–420)mFc, EMR2Δ(260–380)mFc, EMR2Δ(260–340)mFc and EMR2Δ(260–290)mFc. EMR2(534)mFc represents the fusion protein with a full-length extracellular domain. A mFc expression construct, pSec-mFc, encoding only the mFc protein was generated for use as a positive control by subcloning the mFc fragment into the pSecTag2-A vector (Invitrogen). The site-directed EMR2-mFc mutants, EMR2(1, 2, 5)-S518A-mFc and EMR2(1, 2, 5)-S519A-mFc were made according to the protocols suggested by the manufacturer (GeneEditor Mutagenesis System, Promega).

### 2.3. Cell culture

All culture media were supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. All cells were incubated at 37°C in a 5% CO<sub>2</sub>, 95% humidity incubator. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium and CHO-K1 cells in Ham's F-12 medium. EMR2 expression constructs were transfected into cells cultured in 100 mm dishes using Lipofectamine<sup>®</sup> (Life Technologies) as previously described [18]. 16–18 h post transfection, cells were washed and fed with fresh Opti-MEM I for a further 2–3 days before collecting protein samples.

### 2.4. Purification of soluble EMR2-mFc fusion proteins

HEK293T cells were transfected with 40 µg DNA per 175 cm<sup>2</sup> flask using calcium phosphate precipitation as previously described [22]. The medium was replaced with 25 ml serum-free Opti-MEM I 16–18 h post transfection and incubated for a further 72 h. Conditioned medium was collected, spun and passed through a 0.45 µm filter, followed by protein A-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) column purification as previously described [16].

### 2.5. Protein analysis

Conditioned media from transfected cells were spun at 2000 rpm at 4°C for 20 min followed by 100 000 rpm at 4°C for 20 min. The supernatant was collected and stored at –80°C until use. Total cell lysates were collected in cell lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM aminoethyl benzenesulfonyl fluoride, 5 mM levanisole, 1×Complete<sup>®</sup> (Roche) protease inhibitors). Similar amounts of EMR2 protein samples were subjected to standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis as described previously [16] using 8% gels and 2A1 mAb or

an anti-mouse Fc-specific mAb (Sigma) followed by appropriate secondary antibodies for enhanced chemiluminescence detection (Amersham). For N-terminal amino acid sequencing, 10 µg of the purified EMR2-mFc fusion protein was separated on a 10% Novex Bis-Tris NuPAGE precast gel (Invitrogen) at 200 mA per gel in a Novex XCell II Mini-cell gel apparatus. The gel was electroblotted to a Novex 0.2 µm polyvinylidene difluoride membrane (Invitrogen) and stained with Coomassie brilliant blue. The desired ~37 kDa band was excised, washed extensively with 10% methanol and subjected to sequencing on an Applied Biosystems 494A 'Procise' protein sequencer (Perkin Elmer, Applied Biosystems Division, Warrington, UK) using standard sequencing cycles. The fluorescence-activated cell sorting (FACS) analysis of the cell surface EMR2 expression was done as previously described [18].

## 3. Results and discussion

### 3.1. EMR2 is a heterodimeric cell surface receptor derived from a 7TM-independent proteolytic cleavage event

To examine the proteolytic cleavage of EMR2 further, expression constructs encoding the full-length as well as truncated EMR2 proteins were generated (Fig. 1A). Western blot analysis showed that a broad band of ~45–75 kDa was observed in total cell lysates but not in conditioned media of EMR2-TM7- or EMR2-TM1-transfected cells (Fig. 1B). In cells transfected with sEMR2, a protein band of 60–75 kDa was observed in conditioned media while a ~40–60 kDa band was detected in total cell lysates, believed to be the immature protein precursor of the mature soluble protein. The broad bands are due to protein glycosylation as predicted from the amino acid sequences. This was subsequently confirmed by de-glycosylation experiments (Chang et al., unpublished data). No visible bands were identified in samples from mock-transfected cells (Fig. 1B). Detection of similar-sized EMR2 proteins from cells transfected with EMR2 proteins of different expected lengths (sEMR2, EMR2-TM1 and EMR2-TM7) suggested that EMR2 is indeed proteolytically cleaved and that the TM region is not required for cleavage. Furthermore, the detection of EMR2 in total cell lysates but not in conditioned media indicated that the cleaved extracellular subunit remains associated with the TM subunit on the cell surface.

To confirm these findings, an EMR2-mFc fusion protein composed of the entire EMR2 extracellular region and an mFc fragment was generated. Analysis of supernatants from cells transfected with either control mFc or EMR2-mFc produced protein bands of similar sizes when probed with anti-mFc mAb, while no signal was observed in samples containing sEMR2 proteins (Fig. 1C). On the other hand, when probed with 2A1 mAb, EMR2-mFc was shown to produce a protein species the same size as that of sEMR2 protein (Fig. 1D). These results indicate that the EMR2 fusion protein is cleaved in close proximity to the mFc fragment at the C-terminus of the stalk, similar to the cleavage of the membrane form of EMR2 proteins. Furthermore, as the fusion protein did not possess any TM moiety, the cleavage event is clearly TM-independent. Finally, the association of the cleaved extracellular subunit with the TM subunit on the cell surface was confirmed by cell surface immunostaining of cells transfected with either EMR2-TM7 or EMR2-TM1 (data not shown and Fig. 3). It is therefore evident that the post-translational modification of EMR2 protein involves TM-independent proteolytic cleavage and non-covalent association of two cleaved subunits on cell surface.

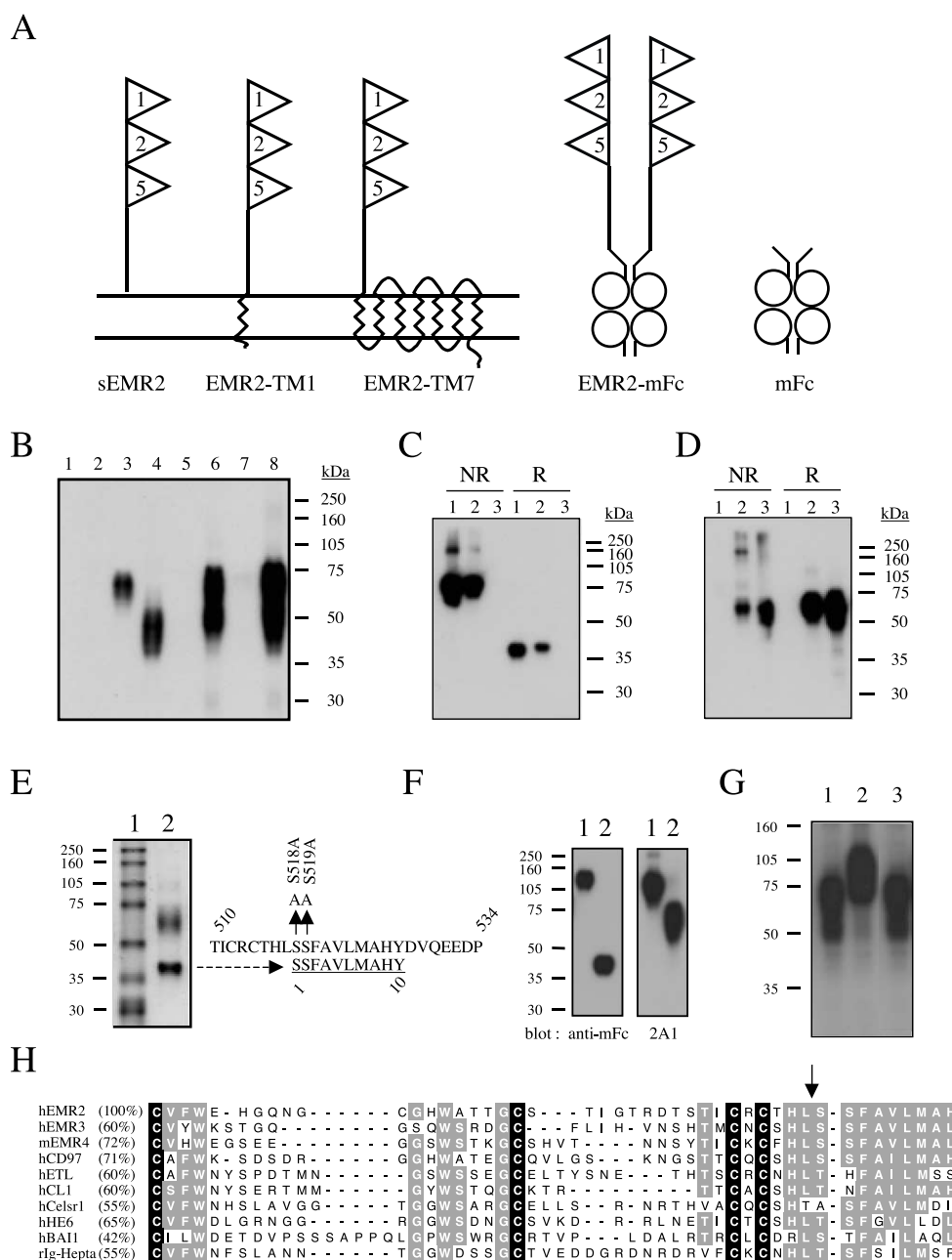


Fig. 1. EMR2 is a heterodimeric cell surface receptor. **A**: Schematic representation of the genetically engineered EMR2 proteins. The EGF-like motif is represented by a triangle, the stalk region by a black line and the TM region by a zig-zag line. The mFc fragment is shown as a dimer of two circles. The number denotes the individual EGF-like domain. **B**: Western blot analysis showing the detection of EMR2 protein by 2A1 mAb in conditioned media (lanes 1, 3, 5, 7) and total cell lysates (lanes 2, 4, 6, 8) of HEK293T cells transfected with sEMR2 (lanes 3, 4), EMR2-TM1 (lanes 5, 6), or EMR2-TM7 construct (lanes 7, 8). Samples from mock-transfected cells (lanes 1, 2) were used as a negative control. **C,D**: Western blot analysis of conditioned media from HEK293T cells transfected with mFc (lane 1), EMR2-mFc (lane 2), or sEMR2 (lane 3) under reducing (R) and non-reducing (NR) conditions using either anti-mFc (C) or 2A1 mAb (D). **E**: Coomassie brilliant blue staining of the purified EMR2-mFc protein (lane 2) reveals two protein fragments of ~37 kDa and ~60–70 kDa. The ~37 kDa fragment was excised for N-terminal sequencing, which produced 10 amino acid residues (underlined) that are identical to residues 518–527 of the EMR2 protein. Lane 1 contains molecular weight standard. **F**: Western blot analysis of conditioned media from HEK293T cells transfected with site-directed mutants, EMR2-S518A-mFc (lane 1) and EMR2-S519A-mFc (lane 2), using anti-mFc or 2A1 mAb. **G**: Western blot analysis of cell lysate from HEK293T cells transfected with the wild-type EMR2-TM7 (lane 1), site-directed mutants, EMR2-S518A-TM7 (lane 2) and EMR2-S519A-TM7 (lane 3), using 2A1 mAb. **H**: Amino acid sequence alignment of the GPS motif of LNB-TM7 proteins. The four conserved Cys residues are boxed with black background whereas the other conserved residues are boxed with grey background. The consensus proteolytic cleavage site is indicated by arrow. The letter before each protein denotes the species from which the protein is derived: h, human; m, mouse; r, rat. The number in parentheses indicates the sequence homology of the aligned GPS domain in comparison to that of EMR2.

### 3.2. Mapping of the EMR2 cleavage site

To locate the exact cleavage site, EMR2(534)mFc fusion protein was purified and resolved on a 10% polyacrylamide gel. Two products representing the extracellular domain

(~60–70 kDa) and the mFc fragment (~37 kDa) were visible (Fig. 1E). N-terminal amino acid sequencing of the mFc-containing fragment produced 10 residues, SSFAVLMMAHY, which are identical to residues 518–527 of EMR2 (Fig. 1E).

The cleavage therefore appeared to take place at the peptide bond between Leu<sup>517</sup> and Ser<sup>518</sup>. This is consistent with the cleavage sites identified previously for CL1 [4], ETL [21], Ig-hepta [23] and mEMR4 [16] (Fig. 1H). To confirm this, site-directed mutant EMR2-mFc fusion proteins containing a Ser518Ala mutation or a Ser519Ala mutation were generated. Western blot analysis showed that the EMR2-S518A-mFc protein, but not the EMR2-S519A-mFc protein, failed to undergo cleavage and was manifested as an intact single chain protein (Fig. 1F). The same result was obtained when the membrane form of the molecules (EMR2-TM7, EMR2-S518A-TM7 and EMR2-S519A-TM7) was examined (Fig. 1G).

### 3.3. The role of the GPS motif and extracellular stalk in the proteolytic cleavage of EMR2

Although the consensus GPS cleavage site is established here and elsewhere [4,16,21], little is known about the involvement of the GPS motif and stalk in the cleavage process. Recently, a functional role for the GPS domain in receptor cleavage has been shown in CL-1, where mutations in the GPS domain render the molecule resistant to intracellular cleavage [24]. Likewise, germline point mutations at the extra-

cellular REJ domain prevent PKD-1 cleavage, leading to polycystic kidney diseases [25]. To evaluate the role of the GPS motif and the stalk in EMR2 cleavage, various EMR2-mFc constructs with truncated stalk regions were employed (Fig. 2A). Western blot analysis showed that the proteolytic cleavage occurred in EMR2(526)mFc, EMR2(529)mFc and the EMR2(534)mFc protein that contains the full-length ECD (Fig. 2B). However, EMR2(518)mFc and EMR2(522)mFc proteins were not proteolytically processed indicating that in the context of an intact stalk, a minimum of eight additional amino acid residues C-terminal to the cleavage site is required for cleavage (Fig. 2B). Interestingly, these eight amino acid residues are highly conserved among all known GPS motifs (Fig. 1H).

Next, the requirement of the GPS motif was demonstrated using EMR2Δ(260–479)mFc, EMR2Δ(260–487)mFc and EMR2Δ(478–487)mFc fusion proteins, containing a complete GPS motif, a partial GPS motif, and a stalk with a minimally deleted GPS motif, respectively (Fig. 2A,C). All three proteins failed to undergo proteolytic cleavage, indicating that the GPS motif is necessary, but not sufficient for the cleavage process. It also suggested that the rest of the stalk might be involved. The requirement of the stalk was confirmed by a series of

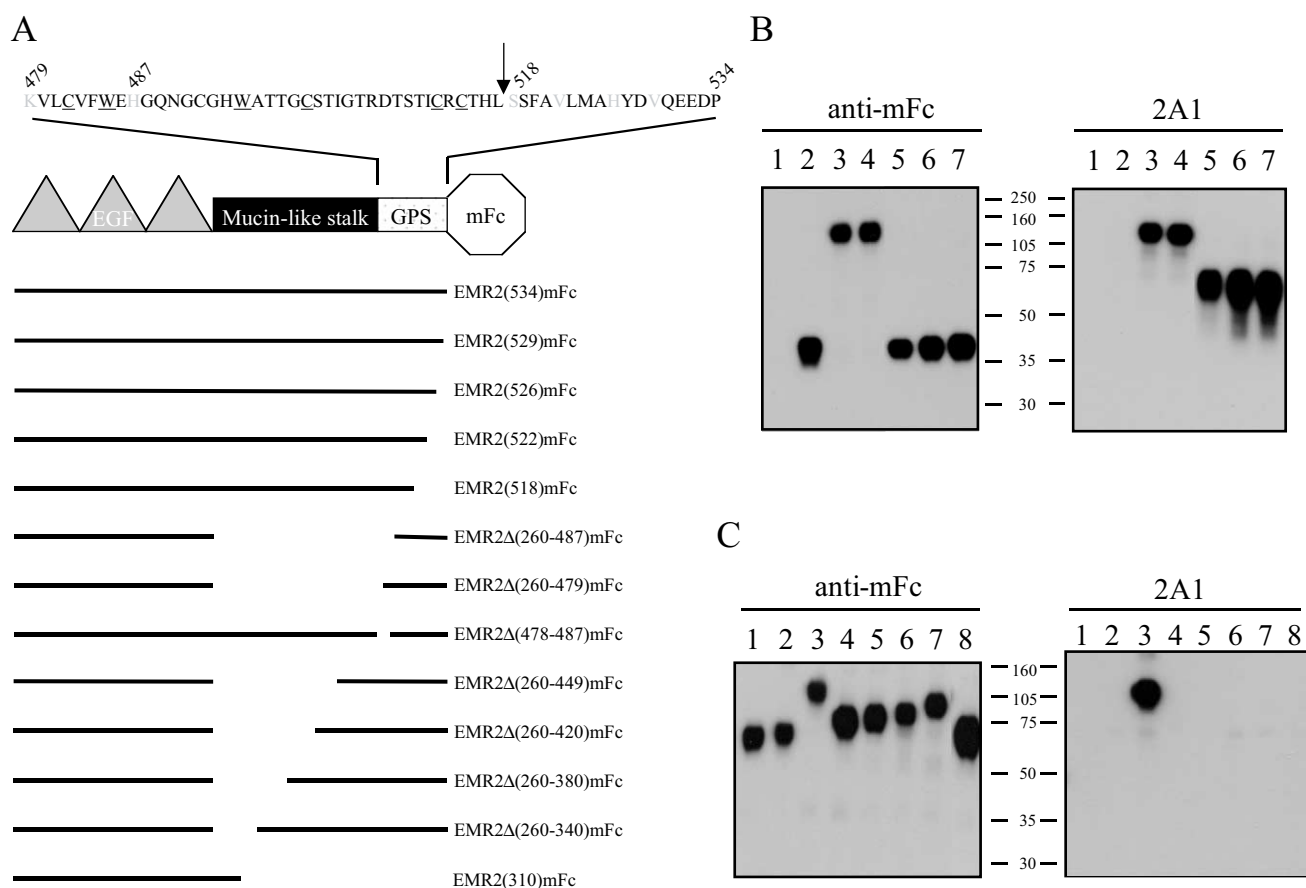


Fig. 2. The GPS motif and the stalk are essential for the proteolytic cleavage of EMR2. A: Schematic representation of the truncated EMR2 proteins used for the GPS cleavage study. The sequence of the GPS motif is shown with residues highlighted in grey corresponding to the positions of the truncation. The conserved residues are underlined. The cleavage site is indicated by arrow. B: Western blot analysis of conditioned media from cells transfected with empty vector (lane 1), mFc (lane 2), EMR2(518)mFc (lane 3), EMR2(522)mFc (lane 4), EMR2(526)mFc (lane 5), EMR2(529)mFc (lane 6) or EMR2(534)mFc (lane 7). Blots were probed with anti-mFc (left panel) or 2A1 mAb (right panel). C: Western blot analysis of conditioned media from cells transfected with EMR2Δ(260–487)mFc (lane 1), EMR2Δ(260–479)mFc (lane 2), EMR2Δ(478–487)mFc (lane 3), EMR2Δ(260–449)mFc (lane 4), EMR2Δ(260–420)mFc (lane 5), EMR2Δ(260–380)mFc (lane 6), EMR2Δ(260–340)mFc (lane 7) or EMR2(310)mFc (lane 8). Blots were probed with anti-mFc (left panel) or 2A1 mAb (right panel).



stalk-truncated mutants, EMR2 $\Delta$ (260–449)mFc, EMR2 $\Delta$ (260–420)mFc, EMR2 $\Delta$ (260–380)mFc, EMR2 $\Delta$ (260–340)mFc and EMR2 $\Delta$ (260–290)mFc, all of which were produced as a unprocessed single polypeptide chain (Fig. 2C). Together these results indicate that in addition to the GPS motif, the entire stalk is also required for the cleavage process. Furthermore, based upon the reactivity of 2A1 to the stalk-truncated mutants, the epitope of the EMR2 stalk-specific 2A1 mAb is likely to be located between residues 310 and 340 (Fig. 2). However, a discontinuous epitope sequence cannot be completely ruled out at present.

To shed more light on the importance of an intact stalk for

receptor proteolysis, the cleavage of a EMR2-mFc fusion protein containing a naturally truncated stalk was evaluated (Fig. 3). Due to the differential usage of splicing acceptors at exon 12, this alternatively spliced stalk lacked 11 amino acid residues (amino acids 396–414) in comparison to the full-length stalk (Fig. 3A) [18]. As shown in Fig. 3B, EMR2-mFc fusion protein containing this alternatively spliced stalk was not cleaved, strengthening the notion that a full-length intact stalk is essential for the proteolytic processing of EMR2. Again, the membrane form of this EMR2 isoform is also not cleaved (data not shown). This result shows that alternative splicing in the stalk region can regulate the receptor cleavage at the

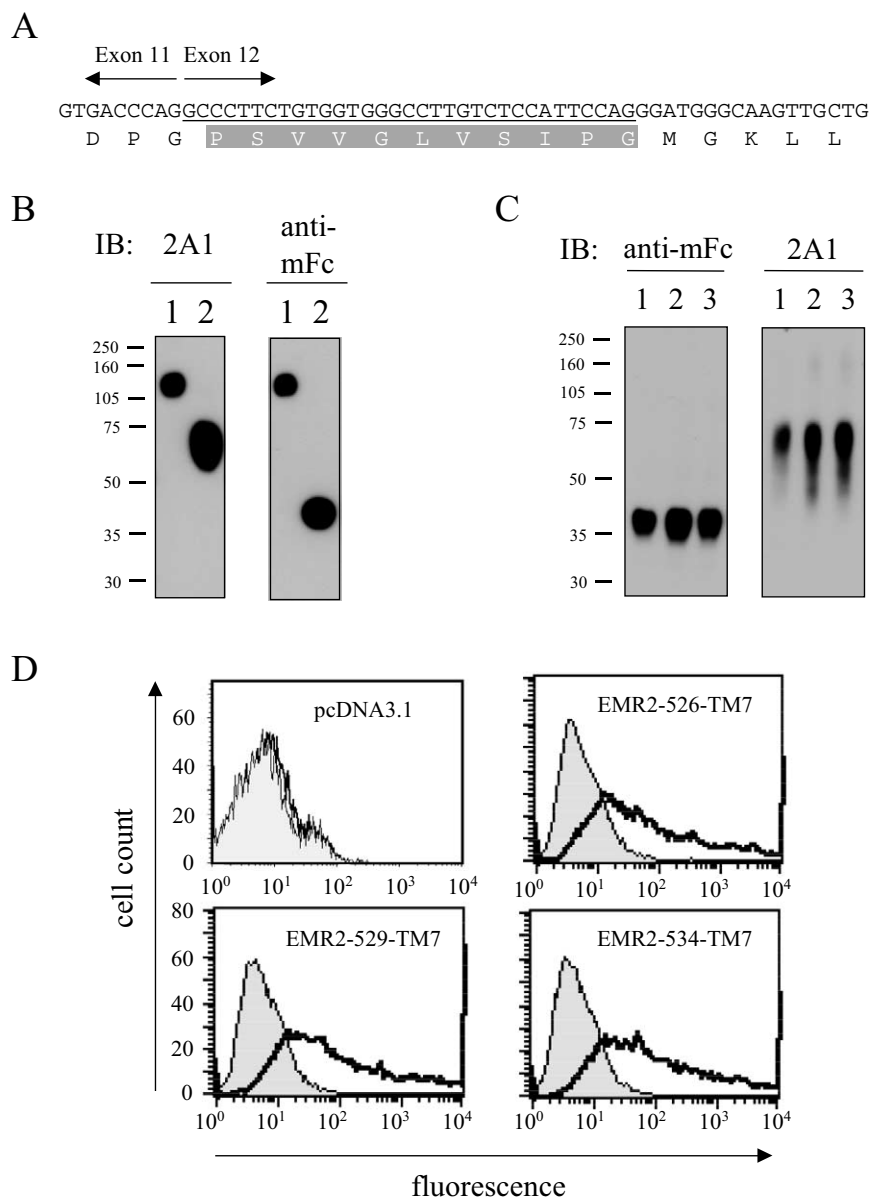


Fig. 3. An alternatively spliced EMR2 isoform containing a truncated stalk was not cleaved. A: Nucleotide and amino acid sequences showing the alternative splicing at exon 12 of EMR2. The 33 nucleotides and the corresponding 11 amino acid residues that are spliced out are underlined and boxed with grey background, respectively. The boundary between exons 11 and 12 is also shown. B: Western blot analysis of conditioned media from cells transfected with EMR2-mFc constructs containing the alternatively spliced stalk (lane 1) or the full-length stalk (lane 2). C,D: Association of EMR2- $\alpha$  and - $\beta$  subunits in vitro (C) and in vivo (D). C: EMR2(526)mFc (lane 1), EMR2(529)mFc (lane 2), and EMR2(534)mFc (lane 3) proteins were immunoprecipitated by protein A Dynabeads, washed, and subjected to SDS-PAGE and Western blotting using 2A1 mAb (right panel) or anti-mFc mAb (left panel). D: FACS analysis of cell surface EMR2 expression on EMR2(526)-TM7-, EMR2(529)-TM7- and EMR2(534)-TM7-transfected CHO-K1 cells using 2A1 mAb (dark solid line). An isotype-match (mouse IgG1) antibody (grey area) was used as a negative control. Cells transfected with pcDNA3.1 were used as a negative control for transfection.

GPS motif and produce either a heterodimeric or a single-chain receptor. The functional difference between the two receptor isoforms remains to be determined.

### 3.4. The sequence requirement for the association of EMR2 heterodimeric subunits

Using the stalk-truncated EMR2-mFc fusion proteins described above, the sequence requirement for subunit association was investigated. The  $\alpha$ -subunits of EMR2(526)mFc, EMR2(529)mFc and EMR2(534)mFc are readily detected from protein A-immunoprecipitated samples, due to the association of the  $\alpha$ -subunit with the mFc fragment (Fig. 3C). Likewise, the  $\alpha$ -subunits of the EMR2(526)-TM7, EMR2(529)-TM7 and EMR2(534)-TM7 proteins were detected on the cell surface (Fig. 3D), but not in the conditioned media (data not shown). This indicates that as few as eight amino acid residues in the  $\beta$ -subunit are sufficient for the cleavage, as well as the non-covalent association of  $\alpha$ - and  $\beta$ -receptor subunits.

The GPS motif was originally named due to its involvement in the proteolytic cleavage of latrophilin/CL-1 [4,5] and has since been identified in a wide variety of cell surface proteins including members of the LNB-TM7 family as well as PKD-1, suREJ3, and hPKDREJ [9,10,26]. The presence of the highly conserved GPS motif in such a diverse array of cell surface proteins suggests that the GPS motif-associated proteolysis is evolutionarily conserved and might be important for the function or regulation of these receptors. Consistent with this notion, Krasnoperov et al. have shown that mutations in the GPS domain make CL-1 resistant to the cleavage and impair its trafficking to the cell surface [24]. Moreover, cleavage of PKD-1 at the conserved GPS site has recently been demonstrated and germline point mutations at the extracellular REJ domain adjacent to the GPS motif prevent PKD-1 cleavage, leading to polycystic kidney disease [25]. Since PKD-1 is an 11-span TM protein, the GPS cleavage process is apparently not restricted to a particular TM conformation. Therefore, the TM conformation-independent cleavage and the requirement of an intact extracellular stalk for GPS cleavage seem to represent common characteristics of this unique post-translational protein modification (Figs. 1 and 2). It is possible that the specific recognition of the intact stalk, probably through correct folding, provides a structural determinant for receptor cleavage. Finally, the requirement of only eight conserved amino acid residues in the  $\beta$ -subunit for receptor cleavage and subunit association is also intriguing (Fig. 3). It is noted that the majority of these residues are small and hydrophobic, a feature that might be relevant to their dual roles in the cleavage of the GPS motif and the association of the cleaved subunits (Figs. 2 and 3). Since the putative protease responsible for GPS cleavage remains unknown, it is hoped that the information obtained from the present study on sequence requirement and substrate specificity will facilitate the identification of the protease involved and further contribute to the understanding of receptor proteolysis at the GPS motif.

**Acknowledgements:** We would like to thank Mr Antony Willis for performing N-terminal amino acid sequencing and Dr D.H. Wreschner for his insightful discussion. Dr J. Davies is thanked for critical reading of the manuscript. This study is supported by grants from British Heart Foundation (PG/02/144 to H.-H.L.) and Wellcome Trust (G.-W.C. and M.S.). H.-H.L. has been supported in part by a research grant from Celltech R&D. S.G.'s research is supported by grants from the Medical Research Council, UK. J.H. is a fellow of the Royal Netherlands Academy of Arts and Sciences.

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