Recognition sequences and structural elements contribute to shedding susceptibility of membrane proteins

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Although regulated ectodomain shedding affects a large panel of structurally and functionally unrelated proteins, little is known about the mechanisms controlling this process. Despite a lack of sequence similarities around cleavage sites, most proteins are shed in response to the stimulation of protein kinase C by phorbol esters. The signal-transducing receptor subunit gp130 is not a substrate of the regulated shedding machinery. We generated several chimaeric proteins of gp130 and the proteins tumour necrosis factor α (TNF- α), transforming growth factor α (TGF- α) and interleukin 6 receptor (IL-6R), which are known to be subject to shedding. By exchanging small peptide sequences of gp130 for cleavage-site peptides of TNF- α , TGF- α and IL-6R we showed that these short sequences conferred susceptibility to spontaneous and phorbol-ester-induced shedding of gp130. Importantly, these chimaeric gp130 proteins were functional, as shown by the phosphorylation of gp130 and the activation of signal transduction and activators of transcription 3 ('STAT3')

on stimulation with cytokine. To investigate minimal requirements for shedding, truncated cleavage-site peptides of IL-6R were inserted into gp130. The resulting chimaeras were susceptible to shedding and showed the same cleavage pattern as observed in the chimaeras containing the complete IL-6R cleavage site. Surprisingly, we could also generate cleavable chimaeras by exchanging the juxtamembrane sequence of gp130 for the corresponding region of leukaemia inhibitory factor ('LIF') receptor, a protein that like gp130 is not subject to regulated or spontaneous shedding. Thus it seems that there is no minimal consensus shedding sequence. We speculate that structural changes allow the access of the protease to a membrane-proximal region, leading to shedding of the protein.

Key words: cleavage site, interleukin 6 receptor, protease, tumour necrosis factor α .

INTRODUCTION

Many cell-surface proteins, including interleukin 6 receptor (IL-6R), are cleaved proteolytically and release their extracellular domains (ectodomains) into the cell medium, a process referred to as ectodomain shedding [1–3]. These proteins are structurally and functionally diverse and include membrane-anchored growth factors [4], membrane-bound precursors of cytokines [5] and cytokine receptors [6,7] as well as ectoenzymes [8,9] and cell adhesion molecules [10]. Ectodomain shedding seems to be involved in various pathophysiological events such as host defence and wound healing [11], rheumatoid arthritis [12] and Alzheimer's disease [13] but little is known about its regulation [14]. The cleavage proteases, often designated as convertases, sheddases or secretases, have several characteristics suggesting a common machinery for the release of cell-surface proteins [3,14,15]. Shedding is enhanced by the phorbol ester activation of protein kinase C [14,16], occurs in close proximity to the cell membrane [9] and is sensitive to hydroxamate-based metalloprotease inhibitors [17]. For instance, protease inhibitors that were initially developed to block the proteolysis of pro-(tumour necrosis factor α) (pro-TNF- α) [18–20] were subsequently shown to inhibit shedding of other proteins such as IL-6R, L-selectin and transforming growth factor α (TGF- α) [7,17].

Shedding of the Alzheimer protein is more complicated because three different enzymes are involved: the α -secretase is a metalloprotease [13], the β -secretase and the γ -secretase are aspartic proteases [21,22].

Although ectodomain shedding seems to be controlled by a common mechanism, there is no apparent sequence similarity at the cleavage site of different proteins; similarly, structural features determining (cell-specific) shedding have not yet been identified [9,23]. Mutagenesis of residues around the cleavage site of the Alzheimer precursor protein (β -APP) [24], the 60-kDa TNF- α receptor [25], IL-6R [23], L-selectin [10] and TNF- α [26] revealed no strict sequence specificity for cleavage. For instance, it was possible to abolish the shedding of pro-TNF- α only by deletion of more than 10 amino acid residues around the cleavage site [26], suggesting that the length of a stalk sequence between the membrane and the first extracellular domain of the membrane protein is important for shedding. For pro-TNF- α , however, we have recently shown that length is not the only determinant for shedding of this molecule [27]. An exchange of the pro-TNF- α cleavage domain for the IL-6R stalk sequence of the same length resulted in a chimaeric protein resistant to shedding [27]. In contrast, an insertion of juxtamembrane sequences of pro-TGF- α and β -APP into the non-cleavable protein β -glycan led to chimaeras that were susceptible to shedding [28].

We therefore asked whether the insertion of various cleavage-site peptides into a protein resistant to the shedding conferred susceptibility to shedding. We chose gp130, the signal-transducing receptor subunit of the IL-6 type cytokines [29], to test this question, because gp130 shedding is several orders of magnitude lower than that of the IL-6R [30,31]. Gp130 is the common signal-transducing receptor subunit of the IL-6 family of cytokines. This family comprises IL-6, IL-11, leukaemia

Abbreviations used: ADAM, a disintegrin and metalloprotease; β -APP, Alzheimer precursor protein; IL, interleukin; LIF, leukaemia inhibitory factor; mAb, monoclonal antibody; R, receptor; STAT, signal transduction and activators of transcription; TGF- α , transforming growth factor α ; TNF- α , tumour necrosis factor α .

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inhibitory factor (LIF), oncostatin M, cardiotrophin 1 and novel neurotrophin 1 [29,32–35]. IL-6 and IL-11 act through a homodimer of gp130, whereas LIF, ciliary neurotrophic factor, cardiotrophin 1 and oncostatin M induce a heterodimer of gp130 and the related protein LIFR on the cell surface. Interestingly, oncostatin M can additionally induce the formation of a heterodimer of gp130 and the related protein oncostatin M receptor [36].

Chimaeric proteins were generated by exchanging a seven-residue part of the juxtamembrane region of gp130 with cleavage-site regions of pro-TNF- α , pro-TGF- α and IL-6R. Introduction of these short peptides rendered gp130 susceptible to cleavage by a regulated shedding system. Phosphorylation analysis revealed that the chimaeras were still functioning in signal transduction after stimulation with IL-6. Our results show that both recognition sequences and structural elements contribute to shedding susceptibility of membrane proteins.

EXPERIMENTAL

Cells and reagents

COS-7 cells were bought from A.T.C.C. (Manassas, VA, U.S.A.). Aprotinin, leupeptin, pepstatin and D-biotinoyl-L-aminohexanoic acid *N*-hydroxysuccinimide ester were purchased from Roche Diagnostics (Mannheim, Germany). PMA and Nonidet P40 were from Sigma (Taufkirchen, Germany). Tran³⁵S-label (44 TBq/mmol) was obtained from ICN (Meckenheim, Germany). A polyclonal monospecific antiserum against human gp130 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), a monoclonal antibody (mAb) against human gp130 (B-S12) [37] was purchased from Diaclone (Besançon, France), an mAb against signal transduction and activators of transcription (STAT3) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.), anti-phosphotyrosine mAb 4G10 was from UBI (Lake Placid, NY, U.S.A.), and the anti-mouse peroxidase-coupled mAb was from Biosource (Calmarillo, CA, U.S.A.). The restriction enzymes used were obtained from AGS (Heidelberg, Germany), and calf intestinal phosphatase was bought from Roche Diagnostics (Mannheim, Germany). T4 DNA ligase was purchased from New England Biolabs (Frankfurt, Germany). Oligonucleotides were bought from Eurogentec (Searing, Belgium).

Construction of gp130 cleavage-site chimaeras

Standard cloning procedures were performed as described by Sambrook et al. [38]. The gp130 cleavage-site chimaeras were generated by using double-stranded oligonucleotide adapters. First, a *NheI* site was introduced into the juxtamembrane region of gp130 by generating the point mutation Q615S. The 12-residue cleavage-site peptides of pro-TNF- α , pro-TGF- α and IL-6R respectively were introduced via the *NheI* and *EcoRI* sites. The chimaeric proteins enclosing the truncated IL-6R cleavage-site peptides and peptides containing the corresponding region of LIFR were generated similarly.

cDNA species and COS-7 cell transfection

COS-7 cells were routinely grown in DMEM (Dulbecco's modified Eagle's medium) (Gibco, Eggenstein, Germany) supplemented with 10% (v/v) foetal calf serum, 50 i.u./ml penicillin and 50 μ g/ml streptomycin in a water-saturated air/CO₂ (19:1) atmosphere. Cells were transiently transfected with the DEAE-dextran method as described previously [39,40]. gp130 and chimaera cDNA species were subcloned into the p409 expression vector (a gift from Immunex, Seattle, WA, U.S.A.) [41]. The

human IL-6R was in the pCDM8 expression vector (Invitrogen), STAT3 in pSVL (Pharmacia, Freiburg, Germany).

Metabolic labelling, biotinylation and immunoprecipitation of cell-surface proteins

For pulse-chase experiments, transfected cells, 48 h after transfection, were incubated in methionine/cysteine-free DMEM at 37 °C for 30 min. The cells were labelled with 50 μ Ci of Tran³⁵S-label in methionine/cysteine-free medium for 2 h. After labelling, cells were chased in the presence or absence of PMA for 2 h. Supernatants were collected and cells were washed with PBS, then lysed in PBS containing 1% (v/v) Nonidet P40 and the proteinase inhibitors aprotinin (2 μ g/ml), leupeptin (0.5 μ g/ml), pepstatin (0.7 μ g/ml) and PMSF (0.7 μ g/ml). For biotinylation of cell-surface proteins, cells were washed with PBS and then incubated for 15 min in PBS containing 50 ng/ml D-biotinoyl-L-aminohexanoic acid *N*-hydroxysuccinimide ester. Biotinylation was blocked with 10 mM NH₄Cl for 5 min. After cells had been washed three times for 5 min with PBS they were lysed in PBS containing 1% (v/v) Nonidet P40 and proteinase inhibitors. Supernatants and cell lysates were pretreated with pansorbin (Calbiochem, Bad Soden, Germany) and subsequently incubated with an antibody against gp130 for 1 h at 4 °C. Immune complexes were precipitated with Protein A-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany), separated by SDS/PAGE [10% or 12.5% (w/v) gel] and analysed by fluorography or Western blotting.

Western blot analysis

Proteins separated by SDS/PAGE were transferred for 1 h at 1 mA/cm² to nitrocellulose or PVDF membranes. The membranes were blocked for 1 h in PBS/0.05% Tween 20 (PBST) with 5% (v/v) skimmed-milk powder and then washed three times in PBST for 5 min each. The filters were then incubated with an appropriate antibody before being labelled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed with the Amersham enhanced chemiluminescence (ECL[®]) kit in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech).

Molecular modelling

With the use of fold recognition algorithms [41a] we found that gp130D6 and LIFRD8 share the fibronectin type-III-like fold. The sequential alignment produced by this method was used to build molecular models for these two domains using the structure of the tenth domain of fibronectin (PDB accession no. 1fna) as a template. On the basis of this alignment, amino acid residues were exchanged in the template. Insertions and deletions in gp130D6 and LIFRD8 respectively were modelled with a database search approach included in the software package WHATIF [42]. The database was searched for a peptide sequence of the appropriate length, which was fitted to the template. All loops were selected from the database to minimize the root-mean-square distance between the ends of the loops and the sheets. Loops with unfavourable backbone angles or van der Waals clashes were excluded. Finally these model structures were energy-minimized, with the use of the steepest-descent algorithm implemented in the GROMOS force field (W.F. van Gunsteren, distributed by BIOMOS Biomolecular Software, Laboratory of Physical Chemistry, University of Groningen, The Netherlands). The final energies for the two molecular models were of the same order of magnitude as the value calculated for the template structure.

Tyrosine phosphorylation analysis

Transfected COS-7 cells were starved overnight in serum-free medium before stimulation with IL-6. After stimulation, cells were lysed in 50 mM Tris/HCl (pH 7.5)/100 mM NaCl/50 mM NaF/3 mM Na₃VO₄ containing 1% (v/v) Nonidet P40. Insoluble material was pelleted and the supernatants were immunoprecipitated overnight with B-S12 anti-gp130 (2 µg/ml). The complexes were isolated with Protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) and the supernatants were subjected to a second immunoprecipitation with anti-phosphotyrosine mAb 4G10 (5 µg/ml). The precipitates were subjected to SDS/PAGE and analysed by Western blotting as described above.

RESULTS

Juxtamembrane sequences as determinants of shedding

Recently it has been shown that the cleavage-site regions of pro-TGF- α and β APP are sufficient to render the otherwise uncleavable protein β -glycan susceptible to shedding [28]. To further investigate the sequence specificity of shedding, we generated a panel of chimaeric proteins of gp130, in which we replaced the juxtamembrane seven residues with 12-residue cleavage-site peptides of pro-TNF- α , pro-TGF- α and IL-6R (Figure 1A). Because pro-TNF- α , in contrast with pro-TGF- α and IL-6R, is a type II transmembrane protein, the cleavage-site peptide was inserted in two directions into gp130 (Figure 1A). gpTN130 contained the pro-TNF- α cleavage-site peptide in the original orientation, whereas gpRT130 contained it in the reverse direction. gpTG130 contained the IL-6R cleavage-site peptide and gpIL130 contained 12 amino acids of the IL-6R cleavage site. To investigate shedding of these chimaeric molecules, we transiently transfected and metabolically labelled COS-7 cells and performed a chase in the presence or absence of PMA (Figure 1B). gp130 was precipitated as a single band of 130 kDa (filled arrowhead), whereas the mutant proteins migrated as double bands at 130 kDa (filled arrowhead) and 120 kDa (open arrowhead). The intensity of the 120 kDa band was similar for all mutant proteins but the intensity of the 130 kDa band varied between the chimaeras. PMA showed no effect on shedding of wild-type gp130 (Figure 1B, lane 2), which could be detected in the cell lysates but not in the medium (lanes 1 and 2). In contrast, all chimaeric proteins were susceptible to shedding. gpTN130 showed a weak cellular expression in the absence of PMA (lane 3), which vanished after treatment with PMA (lane 4). However, in the supernatants soluble gpTN130 could be detected before the application of PMA (lane 3) and was stimulated approx. 2-fold after treatment with PMA (lane 4). Thus shedding of surface gpTN130 seemed to occur spontaneously and to be increased further by stimulation with phorbol ester. In contrast, gpRT130 containing the pro-TNF- α cleavage site in reverse orientation showed a cellular expression comparable to that of wild-type gp130 (lane 5), whereas the level of soluble gpRT130 in the supernatant was slightly lower than that of gpTN130 before activation with PMA (lane 5). Stimulation with PMA led to a complete loss of gpRT130 in the cell lysate and to a marked increase in the protein in the supernatant (lane 6). The chimaeric protein gpTG130 behaved similarly to gpTN130 (lanes 7 and 8): in the absence of PMA, gpTG130 was cleaved spontaneously from the cell surface and was detectable in the supernatant (lane 7). Stimulation with phorbol ester led to complete release of gpTG130 into the supernatant (lane 8). In contrast, gpIL130 was only minimally shed in the absence of PMA (lanes 9 and 10), whereas induction with PMA led to an almost complete cleavage of the chimaeric protein (lane 10). These results demonstrate that the insertion of

12 residues conferred susceptibility to shedding: gpTN130 and gpTG130 showed high spontaneous shedding, whereas this was lower in gpRT130 and almost absent from gpIL130. However, quantitative shedding could be induced by PMA in all mutants.

We cannot formally exclude the possibility that the observed shedding was due to the action of a metalloenzyme during cell preparation. However, we feel that this is unlikely because shedding is PMA-dependent and depends largely on the presence of the ADAM (for 'a disintegrin and metalloprotease') protease TNF- α convertase (TACE). In cells lacking a functional gene for TACE, PMA-induced shedding of the IL-6R was decreased 6-fold [27].

The appearance of a double band in the metabolically labelled cell lysates of cells transfected with the chimaeric proteins might indicate that part of the molecules were processed differentially, possibly owing to incorrect folding. To test for cell-surface expression of the gp130 chimaeric proteins, surface biotinylation of COS-7 cells transiently transfected with either gp130 or the chimaeric proteins was performed (Figure 1C). Immunoprecipitation and Western blot analysis showed that only the higher-molecular-mass band was still detectable, indicating that the lower band represented a precursor that did not reach the cell surface. The lower-molecular-mass band presumably reflected different glycosylation, suggesting that the different mutations introduced into gp130 resulted in proteins with different intracellular trafficking properties.

To exclude the possibility that the results obtained from the shedding experiment originated from a structural disturbance of the chimaeras, we checked the functional integrity of the chimaeras by analysing receptor phosphorylation after the stimulation of transfected cells with IL-6. The cellular response to IL-6 was reconstituted in COS-7 cells by transient transfection of cDNA clones coding for IL-6R, STAT3 and gp130 or for the chimaeric proteins [43]. Tyrosine phosphorylation of gp130 and the chimaeras was detected after stimulation with IL-6 (Figure 1D). Untransfected COS-7 cells showed only weak gp130 phosphorylation after stimulation with IL-6, possibly resulting from a weak interaction of endogenous gp130 with the antibody used (Figure 1D, lane 1). Phosphorylation of gp130 was detected in transfected cells only after stimulation with IL-6 (lane 3). Transfection with the chimaeric molecules also led to phosphorylation after treatment with IL-6 (lanes 5, 7, 9 and 11). The chimaeras were also able to activate downstream signalling cascades as revealed by the phosphorylation of STAT3 after stimulation with IL-6 (Figure 1D).

Cleavage susceptibility of chimaeras is not due to an inserted point mutation

For construction of the chimaeric gp130 proteins, the point mutation Q615S was introduced. To test whether this single point mutation alone might have rendered the chimaeric proteins susceptible to the shedding machinery, a mutant protein gp130Q615S was generated, into which only the point mutation Q615S had been introduced (Figure 2A). Pulse-chase experiments in the presence or absence of PMA revealed that gp130Q615S was not susceptible to shedding (Figure 2B). Lanes 1 and 2 of Figure 2(B) show gp130 before and after treatment with PMA; no soluble gp130 was detected in the medium. In contrast, gpIL130 was found only in the cell lysate of cells not treated with PMA (lane 3), whereas induction with PMA led to a marked increase in soluble gpIL130 in the supernatant (lane 4). gp130Q615S showed the same pattern as gp130. No soluble gp130Q615S was detected in the medium (lanes 5 and 6). Tyrosine phosphorylation of gp130Q615S and subsequent

Figure 1 Shedding of gp130 cleavage-site chimaeras

(A) Schematic representation of the cleavage-site peptides (12-mers) of pro-TNF- α , pro-TGF- α and IL-6R introduced into a membrane-proximal region of gp130. Cleavage sites are marked by arrowheads. Abbreviation: TM, transmembrane domain. (B) The chimaeras shown in (A) were expressed in transiently transfected COS-7 cells; 2×10^6 cells were metabolically labelled with 50 μ Ci of Tran³⁵S-label for 2 h. Cells were incubated in the presence or absence of PMA (10 ng/ml). gp130 proteins were immunoprecipitated from cell lysates and culture medium and analysed by SDS/PAGE and fluorography. (C) The chimaeras shown in (A) were expressed in transiently transfected COS-7 cells; 48 h after transfection, cells were incubated with a non-permeable biotin ester. gp130 proteins were immunoprecipitated from cell lysates and analysed by SDS/PAGE and Western blot analysis with streptavidin coupled to peroxidase for detection. (D) Reconstitution of the IL-6R/gp130/STAT3 signalling system in COS-7 cells. Tyrosine phosphorylation of gp130, chimaeric proteins and STAT3 was measured after transfection with human cDNA species for IL-6R, STAT3 and gp130 or the chimaeric gp130 proteins after stimulation with 50 ng/ml IL-6. Upper panel: tyrosine phosphorylation of gp130. Lower panel: tyrosine phosphorylation of STAT3. Note that weak gp130 phosphorylation is seen in untransfected COS-7 cells on stimulation with IL-6, which is most probably due to the presence of endogenous gp130 and IL-6R proteins in these cells. Abbreviations: α -gp130, anti-gp130; α P-Tyr, anti-phosphotyrosine; α STAT3, anti-STAT3; IP, immunoprecipitation; WB, Western blotting. In (B–D) the positions of molecular mass markers are indicated (in kDa) at the left.

activation of STAT3 showed that the inserted point mutation did not interfere with IL-6 induced signal transduction (Figure 2C).

Truncation of cleavage sites has no effect on susceptibility to shedding

As shown above, the insertion of cleavage-site peptides into a non-cleavable protein led to chimaeras susceptible to shedding,

suggesting that the cleavage site alone bears the information for shedding. We therefore investigated the minimal sequence requirements for transferring cleavability to gp130. We generated four additional chimaeric proteins, into which we inserted truncated IL-6R cleavage-site peptide sequences (Figure 3A). Two of them still contained the original cleavage site of IL-6R, Gln⁶¹²-Asp⁶¹³ [23], whereas the other two did not. gp8IL contained an eight-residue cleavage-site peptide instead of the 12-

Figure 2 Shedding of the gp130 point mutant gp130Q615S

(A) Schematic representation of gp130, gpIL130 and gp130 Q615S. The cleavage site in gpIL130 is marked by an arrowhead. Abbreviation: TM, transmembrane domain. (B) The proteins shown in (A) were expressed in transiently transfected COS-7 cells; 2×10^6 cells were metabolically labelled with 50 μ Ci of Tran³⁵S-label for 2 h. Cells were incubated for 1 h in the presence or absence of PMA (10 ng/ml). gp130 proteins were immunoprecipitated from cell lysates and culture and analysed by SDS/PAGE and fluorography. (C) Reconstitution of the IL-6R/gp130/STAT3 signalling system in COS-7 cells. Tyrosine phosphorylation of gp130, chimaeric proteins and STAT3 was measured after transfection with human cDNA species for IL-6R, STAT3 and gp130 or the chimaeric gp130 proteins after stimulation with 50 ng/ml IL-6. Upper panel: tyrosine phosphorylation of gp130. Lower panel, tyrosine phosphorylation of STAT3. Abbreviations: α -gp130, anti-gp130; α P-Tyr, anti-phosphotyrosine; α STAT3, anti-STAT3; IP, immunoprecipitation; WB, Western blotting. In (B) and (C) the positions of molecular mass markers are indicated (in kDa) at the left.

residue peptide in gpIL130. A further truncation led to gp4IL130, in which the IL-6R cleavage-site peptide was only four residues long. In gp Δ IL130 the four central residues (Val⁶¹¹-Gln⁶¹²-Asp⁶¹³-Ser⁶¹⁴) of the IL-6R cleavage site were removed, whereas an eight-residue peptide representing each of the four N-terminal and C-terminal residues of the site remained. The IL-6R peptide inserted into gp Δ IL130 lacked the eight central residues of the original 12-residue cleavage-site peptide, leaving only four residues flanking the original site (Thr⁶⁰⁷-Ser⁶⁰⁸-Val⁶¹⁷-Pro⁶¹⁸).

To investigate the shedding of these chimaeric proteins, we transiently transfected and metabolically labelled COS-7 cells and performed a chase in the presence or absence of PMA

(Figure 3B). gp130 was not cleaved from the cell surface (Figure 3B, lanes 1 and 2), whereas shedding of gpIL130 was strongly induced by PMA (lanes 3 and 4). The chimaeras containing the truncated IL-6R cleavage-site peptides were all susceptible to shedding (lanes 5–12). In all four chimaeras, induction with PMA led to a marked increase in soluble gp130. Therefore the original, complete IL-6R cleavage site was not necessary for rendering gp130 susceptible to shedding. The structural integrity of the chimaeric proteins was again demonstrated by showing an intact tyrosine phosphorylation of the chimaeric molecules and activation by STAT3 after stimulation of the cells with IL-6 (Figure 3C).

Figure 3 Effect of cleavage-site truncation of gp130 chimaeras

(A) Schematic representation of the truncated cleavage-site peptides (tetramers and octamers) of IL-6R introduced into the same region of gp130 as the cleavage-site peptides shown in Figure 1(A). Cleavage sites are marked by arrow-heads. Abbreviation: TM, transmembrane domain. (B) The chimaeras shown in (A) were expressed in transiently transfected COS-7 cells; 2×10^6 cells were metabolically labelled with $50 \mu\text{Ci}$ of Tran^{35}S -label for 2 h. Cells were incubated for 1 h in the presence or absence of PMA (10 ng/ml). gp130 proteins were immunoprecipitated from cell lysates and culture medium, then analysed by SDS/PAGE and fluorography. (C) Reconstitution of the IL-6R/gp130/STAT3 signalling system in COS-7 cells. Tyrosine phosphorylation of gp130, chimaeric proteins and STAT3 was measured after transfection with human cDNA species for IL-6R, STAT3 and gp130 or the chimaeric gp130 proteins after stimulation with 50 ng/ml IL-6. Upper panel: tyrosine phosphorylation of gp130. Lower panel: tyrosine phosphorylation of STAT3. Abbreviations: α -gp130, anti-gp130; α -P-Tyr, anti-phosphotyrosine; α -STAT3, anti-STAT3; IP, immunoprecipitation; WB, Western blotting. In (B) and (C) the positions of molecular mass markers are indicated (in kDa) at the left.

Insertion of LIFR peptides leads to shedding of gp130

The previous experiments showed that gp130 could be rendered susceptible to shedding by inserting full-length or truncated cleavage-site peptides from which the original cleavage site had been deleted. One possible explanation could be that the insertion of any peptide sequence might confer cleavability to a membrane

protein, possibly owing to alterations in the three-dimensional structure of the juxtamembrane domain. We therefore inserted equivalent peptides from the related protein LIFR. LIFR is a transmembrane protein that is not shed from the cell membrane after stimulation with PMA (P. Vollmer and K.-J. Kallen, unpublished work). The fold of the juxtamembrane domains of

Figure 4 Fibronectin III domains of gp130 and LIFR

(A) The sequential alignment of the juxtamembrane fibronectin III domains of gp130 (gp130_D6), LIFR (LIFR_D8) and the template structure (1fna) that was used for building the molecular models. Residues conserved are marked by an asterisk (*); similar residues are indicated by # symbols; β -strands are underlined. (B) Ribbon representation of the molecular models of the juxtamembrane fibronectin III domains of gp130 (left panel) and LIFR (right panel). The juxtamembrane peripheral strands are in light grey. In the case of gp130 the distance to the transmembrane (TM) domain is spanned by one amino acid and for LIFR by three amino acids.

gp130 and LIFR was predicted to be similar to that in fibronectin type III [44,45], which we confirmed with fold recognition methods (see the Experimental section). Interestingly, the structure of the cytokine-binding module of gp130, consisting of two fibronectin III domains, has been solved by X-ray crystallography [46] and the structure of the C-terminal domain of the cytokine-binding module has been solved by NMR [47]. We built molecular models of the two juxtamembrane domains of gp130 and LIFR by using the structure of the tenth domain of fibronectin as a template. A sequence alignment of these domains of gp130, LIFR and the tenth domain of fibronectin is shown in Figure 4(A).

In Figure 4(B), the molecular models of these domains of gp130 (left) and LIFR (right) are shown and the sequences of the C-terminal β -strands of gp130 and LIFR are indicated. We reasoned that the exchange of the entire β -strand from LIFR to gp130 should not lead to gross structural alterations in the fibronectin III domain and that the different sequences introduced into the chimaeric molecules could be accommodated in the structure because (1) this strand is a peripheral one in the β -sheet and (2) it has only a few side-chain contacts that contribute to the core of the protein. We therefore generated chimaeric proteins in which the juxtamembrane seven residues of gp130 (TTPKFAQ; single-letter amino acid codes) had been replaced by the corresponding seven-residue region of LIFR (SMYVVTKE; gpSLIFR130) or nine-residue region of LIFR (SMYVVTKE; gpLLIFR130) (Figure 5A).

Pulse-chase experiments performed with these chimaeras revealed that both molecules were shed from the cell membrane after induction with PMA (Figure 5B) and a concomitant marked increase in soluble gp130 chimaeras (lanes 4, 6 and 8). However, both gpSLIFR130 and gpLLIFR130 showed little spontaneous shedding (Figure 5B, lanes 5 and 7), similar to that of gpIL130 (lane 3). It should be noted that gpLLIFR130 (lane 7) showed a weaker expression than that of gpSLIFR130 (lane 5), explaining the lower amount of protein detected in the medium. The intact signal transduction cascade after stimulation with IL-6 (Figure 5C) showed that both molecules were functional and were therefore structurally intact.

DISCUSSION

The extracellular domains of approx. 2% of membrane proteins can be released from the cell membrane by shedding, which seems to be regulated by a general mechanism. Shedding of most proteins is strongly induced by phorbol esters and inhibited by hydroxamates [1,3,48]. For several proteins it has been shown that the proteases involved in shedding belong to the ADAM family (reviewed in [3]).

Arribas et al. [28] inserted the TGF- α and β -APP cleavage site into the protein β -glycan, which is not a substrate of the shedding system. Replacement of the 14 extracellular amino acids adjacent to the transmembrane region of β -glycan with the corresponding regions of TGF- α or β -APP rendered β -glycan susceptible to shedding. From these results, the authors concluded that despite a lack of sequence similarity, the extracellular regions of pro-TGF- α and β -APP immediately preceding their transmembrane domains are key determinants of ectodomain shedding [28]. Consistent with these findings is our observation that insertion of the IL-6R cleavage-site peptide into gp130 rendered gp130 susceptible to shedding. The same observation was made after inserting the cleavage-site peptide sequences of TNF- α and TGF- α into gp130. Together these studies strongly suggest that juxtamembrane cleavage sites exist that determine the shedding of cell-surface proteins and are exchangeable between proteins.

Recently, however, we showed that the introduction of an IL-6R cleavage-site peptide into pro-TNF- α made this protein resistant to shedding [27]. Moreover, we show here that exchanging a non-cleavable sequence of the LIFR for the corresponding amino acids of gp130 resulted in a cleavable gp130 chimaera. A similar observation was made when the stalk region of angiotensin-converting enzyme was replaced by sequences similar to those in epidermal growth factor [49]. The observations that a cleavable sequence becomes uncleavable in a different protein context and, conversely, that a non-cleavable sequence can be cleaved in a different protein raise some doubt about the existence of a single defined cleavage site.

Shedding of the IL-6R is strongly stimulated after treatment of cells with PMA [6], with cleavage occurring at a single site, Gln³⁵⁷-Asp³⁵⁸ [23]. However, shedding of the IL-6R induced by bacterial toxins led to cleavage at a different position approx. 10 residues upstream of the Gln³⁵⁷-Asp³⁵⁸ cleavage site [50]. Interestingly, bacterial proteases such as the *Serratia marcescens* metalloproteinase cleaved the IL-6R at a different site again [51]. Nevertheless, all cleaved IL-6R species showed biological activity [23,50,51]. These studies suggested that different cleavage sites might exist within a single protein. It remains unclear whether cleavage at different sites is performed by one protease or by different members of the growing ADAM family of metalloproteases [3].

Figure 5 Effect of replacement of gp130 by corresponding LIFR sequences

(A) Schematic representation of chimaeric proteins containing short peptides of the juxtamembrane region of LIFR. Abbreviation: TM, transmembrane domain. (B) The proteins shown in (A) were expressed in transiently transfected COS-7 cells; 2×10^6 cells were metabolically labelled with $50 \mu\text{Ci}$ of Tran^{35}S -label for 2 h. Cells were incubated for 1 h in the presence or absence of PMA (10 ng/ml). gp130 proteins were immunoprecipitated from cell lysates and culture medium and analysed by SDS/PAGE and fluorography. (C) Reconstitution of the IL-6R/gp130/STAT3 signalling system in COS-7 cells. Tyrosine phosphorylation of gp130, chimaeric proteins and STAT3 was measured after transfection with human cDNA species for IL-6R, STAT3 and gp130 or the chimaeric gp130 proteins after stimulation with 50 ng/ml IL-6. Upper panel: tyrosine phosphorylation of gp130. Lower panel: tyrosine phosphorylation of STAT3. Abbreviations: α -gp130, anti-gp130; α P-Tyr, anti-phosphotyrosine; α STAT3, anti-STAT3; IP, immunoprecipitation; WB, Western blotting. In (B) and (C) the positions of molecular mass markers are indicated (in kDa) at the left.

Interestingly, the chimaeric proteins used in this study, which were all susceptible to shedding, differed in their spontaneous release in the absence of stimulation by PMA. A recent study on the mechanism of shedding activation of TGF- α and other proteins has revealed the involvement of different MAP kinase signalling cascades in the regulation of spontaneous and PMA-inducible shedding. These studies demonstrated that spontaneous

and PMA-inducible shedding are differentially regulated processes [52]. Our results imply that the sequence environment of the cleavage site contributes to the extent of spontaneous shedding.

When five residues upstream or downstream of the cleavage site were deleted from IL-6R, PMA-induced shedding was almost abolished [23]. However, from the present study it seems that the

length of the cleavage sequence does not determine cleavability. Replacement of seven residues of gp130 with 12 residues of IL-6R, TNF- α or TGF- α led to cleavable chimaeras. Subsequent truncation of the transferred cleavage site of the IL-6R did not interfere with shedding even when the originally defined cleavage sequence [23] was removed. Moreover, the replacement of the seven residues of gp130 with seven or nine residues of the LIFR protein did not change the cleavage pattern of the chimaeric proteins.

Point mutations around the cleavage site of IL-6R and TNF- α resulted in only minor changes of cleavage pattern of these membrane proteins [23,26], suggesting that there is no strict consensus sequence of a cleavage site environment. These findings are consistent with the fact that the point mutation Q615S introduced into the non-cleavable gp130 protein did not render gp130 susceptible to cleavage by the shedding machinery.

A study on the shedding of membrane-bound angiotensin-converting enzyme was performed to clarify the role of the so-called stalk region of this protein. This region is located between the extracellular domain and the transmembrane region [9]. The authors performed stalk truncations and concluded that cleavage required an accessible stalk and a minimum distance of the cleavage site from both the membrane and the proximal extracellular domain. Furthermore, the authors postulated that the protease positions itself primarily with respect to the proximal extracellular domain [9]. However, in our view an alternative interpretation of these results is that folded domains are not substrates of the shedding enzymes, presumably because the proteases do not have access to cleavable sequences.

How can the divergent results of this and other studies be synthesized into a single model of the mechanism of sheddases? The molecular models presented in Figure 4(B) suggest that there is no stalk region between the extracellular domain and the transmembrane region. The length of the juxtamembrane β -sheet of the fibronectin III domain (Figure 4) had no discernible influence on sheddability because the shedding patterns of gpSLIFR130 and gpLLIFR130 were identical. Shedding of gp130 might therefore occur by destabilization of the juxtamembrane fibronectin III domain. However, all cleavable and uncleavable gp130 mutants analysed in this study were functionally intact as demonstrated by tyrosine phosphorylation of gp130 and STAT3 activation after stimulation with cytokine. This argues against gross structural disturbances of the gp130 chimaeric proteins. We therefore suggest that alterations in the molecular dynamics of the juxtamembrane fibronectin III domain are responsible for cleavability of the gp130 chimaeras. This model assumes that the average time during which the juxtamembrane β -sheet of the fibronectin III domain of mutant gp130 proteins is disordered and accessible for shedding enzymes is increased in comparison with that of unaltered gp130. Our results strongly argue against the existence of a cleavage sequence itself in transmembrane proteins and suggest a model in which the structural integrity of the extracellular domain determines shedding behaviour.

A conclusion from our model would be that the more disordered the juxtamembrane extracellular domain of a membrane protein is, the more the protein becomes a substrate of shedding proteases. Such a model could be tested by careful measurements of shedding kinetics of several mutated proteins together with a structural analysis of their extracellular domain integrity.

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