The growth hormone receptor interacts with its sheddase, the tumour necrosis factor-α-converting enzyme (TACE)

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Proteolysis of the GHR (growth hormone receptor) occurs at the cell surface and results in the release of its extracellular domain, the GHBP (growth hormone-binding protein). TACE (tumour necrosis factor-α-converting enzyme) has been identified as a putative protease responsible for GHR cleavage. However, GHR–TACE interaction has not been observed until now. Here, we identified TACE in Chinese hamster cells and confirmed processing and cell-surface expression. Interaction between GHR and TACE was only observed after growth hormone binding. As the growth hormone–GHR_2 complex is a poor substrate for TACE, we conclude that the GHR–TACE interaction precedes proteolysis, and is transient. Furthermore, we demonstrate that TACE is present in endosomes, where it partly co-localizes with endocytosed growth hormone ligand.

Key words: ADAM17, endocytosis, growth hormone receptor, protein–protein interaction, tumour necrosis factor-α, tumour necrosis factor-α-converting enzyme (TACE).

INTRODUCTION

Postnatal growth as well as lipid and carbohydrate metabolism is regulated by GH (growth hormone), a peptide hormone synthesized primarily by the anterior pituitary gland [1]. GH exerts its action on target cells through the GHR (GH receptor), which is ubiquitously expressed throughout the body with high levels in liver and adipose tissue [2]. In addition to effects on growth, up-regulated GH secretion results in impaired cardiovascular functions, and is associated with metabolic disorders like glucose intolerance and diabetes mellitus as well as certain types of cancer [3–5]. The effectiveness of a peptide hormone depends equally on its presence in the circulation as well as on the abundance of its receptor at the plasma membrane of its target cells. The latter depends on the one hand on the rate of synthesis in the endoplasmic reticulum (ER), and transport to the plasma membrane, and on the other hand on the rate of GHR removal from the cell surface. Two processes contribute to receptor removal: (i) internalization leads to degradation in the lysosome whereas (ii) proteolysis at the cell surface results in the release of the extracellular domain of the GHR referred to as GHBP (GH-binding protein) [6]. The latter process is also known as ectodomain shedding [7].

The physiological function of GHBP is unknown. Its abundance in the circulation probably reflects the GHR abundance in the system, with major contributions from liver [7]. GH can still bind to GHBP with high affinity. Depending on the species, formation of GHBP occurs through two distinct mechanisms. In rats or mice the GHBP is mainly secreted as an alternatively spliced product of the GHR gene [8,9]; however, rodent GHR is not completely resistant to proteolysis [10]. In rabbits and humans, GHBP results exclusively from proteolytic cleavage of the membrane anchored GHR [7]. In monkeys, and probably in rats and mice, both processes occur, but their relative contribution to GHBP production is unknown [11].

Shedding of the GHBP involves the action of the TACE (tumour necrosis factor-α-converting enzyme), a transmembrane metalloprotease [12]. Ectodomain shedding of the GHR can be promoted by phorbol esters presumably through a pathway involving the protein kinase Cα and downstream activation of mitogen-activated protein kinases [13]. However, GH binding inhibits GHR shedding probably by inducing a conformational change of the GHR, and thereby rendering the receptor inaccessible for TACE [6,14]. Cleavage of the GHBP most likely takes place at the plasma membrane, because a truncated form of the GHR (GHR 1–279) with a prolonged residence time at the cell surface is particularly prone for shedding [15,16]. At the same time inhibition of GHR endocytosis results in increased GHBP release in the medium [6]. The exact mechanism by which TACE recognizes the GHR is still unknown. Recently, a three-amino-acid juxta-membrane region in the extracellular domain of the GHR (Glu-242–Asp-244) has been reported to be a structural determinant required for shedding [17]. Wang et al. [18] identified the cleavage site in rabbit GHR after Pro-238, eight residues from the membrane in the proximal extracellular stem region.

In this study, we show that TACE is present in Chinese hamster cells, and can be processed and transported to the plasma membrane. Furthermore, we identified the mature, furin-cleaved species of TACE to be able to interact with the GHR in the presence of GH *in vitro*. We co-localized TACE and endocytosed GH to endosomes.

EXPERIMENTAL

Material

Chinese hamster lung ts20 cells stably transfected with rabbit GHR cDNA were used throughout this study as described previously [19,20]. Anti-GHR antibody was raised against amino acids 327–493 of the cytosolic tail of the GHR [20]; anti-$TACE_{cyto}$ antibody against the cytoplasmic domain of TACE and anti-EC antibody against the extracellular domain of TACE were generously given by Dr C. Blobel (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.) [21]. MG132

Abbreviations used: ER, endoplasmic reticulum; GH, growth hormone; GHBP, growth hormone-binding protein; GHR, growth hormone receptor, TACE, tumour necrosis factor-α-converting enzyme; Con A, concanavalin A.

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(carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.), and carboxybenzylleucyl-leucyl-leucinevinylsulphone was a gift from Dr H. Ploegh (Harvard University, New Haven, CT, U.S.A.). Human GH was kindly given by Eli Lilly Research Labs (Indianapolis, IN, U.S.A.). Culture media, foetal calf serum and antibiotics for tissue culture were purchased from Gibco (Invitrogen, Groningen, The Netherlands).

Construction of GHR(Δ E242-D244)

To obtain the Glu-242–Asp-244 deletion construct of the GHR, we performed two rounds of QuikChange site-directed mutagenesis (Stratagene, Cedar Creek, TX, U.S.A.) PCR using GHR in pcDNA3.1 as the template. In the first reaction we used primers A (5′-CAT TCA CAT GTG AAG TTC CGG TTT GCA TGG TTC TTA ATT ATT ATC-3′) and B (5′-GAT AAT AAT TAA GAA CCA TGC AAA CCG GAA CTT CAC ATG TGA ATG-3′) to delete the first 4 bp encoding Glu-242–Asp-244. Furthermore, we introduced a silent mutation to delete the *Nco*I site at position 794–801 bp for selection of the correct clones. In the second reaction we used primers C (5′-CCC ATT CAC ATG TTT CCG GTT TCC ATG GTT CTT AAT TAT TAT C-3′) and D (5′-GAT AAT AAT TAA GAA CCA TGG AAA CCG GAA ACA TGT GAA TGG G-3′) to delete the remaining 5 bp encoding Glu-242–Asp-244 and to re-introduce the *Nco*I site at position 794–801 bp for selection of the correct clones. The final construct GHR (Δ E242-D244) was verified by *in vitro* transcription translation.

Mammalian cells and transfection

Chinese hamster lung ts20 cells were propagated at 30 °C with MEMα supplemented with 4.5 g/l glucose, 10 % foetal calf serum, 10 units/ml penicillin and 100 μg/ml streptomycin. For transfection experiments ts20 cells were grown in 60 mm dishes to 30–40 % confluence before transfection with 5 μg of cDNA/dish, using Fugene (Roche, Mannheim, Germany) according to the manufacturer's protocol. Then, 32 h after transfection, cells were treated with 10 mM butyrate to enhance expression of cytomegalovirus-driven constructs. Cells were used for experiments 48 h after transfection.

Glycoprotein isolation and Western blotting

Chinese hamster lung ts20 cells were lysed on ice in buffer A (0.5 % Triton X-100, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF and 2 μM MG132). After clearing by centrifugation at 320 ***g*** and 4 °C, samples were precipitated using Con A (concanavalin A) beads (Amersham Biosciences, Uppsala, Sweden). Precipitated material was separated by SDS/PAGE together with total cell lysate, transferred to PVDF paper and immunoblotted with anti-$TACE_{cyto}$ antibody. After incubating the blots with Protein A conjugated to horseradish peroxidase, antigen–antibody complexes were visualized using the ECL system (Roche, Mannheim, Germany).

Cell-surface biotinylation

Chinese hamster lung ts20 cells were washed three times with ice-cold PBS, followed by a 30 min incubation on ice with 0.5 mg/ml Sulpho-NHS-SS-biotin (Pierce, Rockford, IL, U.S.A.). The cells were washed twice with ice-cold PBS and lysed in buffer B [0.5 % (w/v) Triton X-100, 50 mM NaF, 1mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF and 2 μM MG132 in PBS]. After clearing by centrifugation at 320 ***g*** and 4 °C, samples were precipitated using streptavidin beads (Pierce). Precipitated material was treated as above.

Co-immunoprecipitation

For co-immunoprecipitation, cells were grown in 6 cm dishes and, where indicated, incubated with 180 ng/ml human GH for 30 min at 30 °C. Cells were lysed on ice in buffer A. The lysates were cleared by centrifugation at 320 ***g*** and 4 °C. Supernatant was incubated with GHR antiserum for 2 h at 4 °C. Protein A–agarose beads (Repligen Co., Cambridge, MA, U.S.A.) were used to isolate the immunocomplexes at 4 °C. The immunoprecipitates were washed once with buffer A. Immunocomplexes were analysed as above. The blots were immunoblotted using either anti-GHR or anti-$TACE_{cyto}$ antibody. To ascertain the significance of the results, the experiments were repeated four or five times. The Figures show representative Western blot results.

Microscopy

Cy3-GH was prepared using a FluoroLink Cy3 label kit (Amersham Biosciences) according to the supplier's instructions. Chinese hamster lung ts20 cells transfected with wild-type GHR cDNA were grown on coverslips and incubated for 30 min with Cy3-GH (1μg/ml) in the absence or presence of MG132 (20 μM). Cells were washed with PBS to remove unbound label and fixed for 1 h at room temperature with 4 % paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Cells were permeabilized with 1 % Triton X-100 in PBS. As the anti-$TACE_{cyto}$ antibody did not work after fixation in the ts20 cells, the polyclonal anti-EC antiserum was used to visualize endogenous TACE, followed by Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands). Coverslips were embedded in Moviol containing 2.5 % 1,4-diazobicyclo-[2.2.2]-octane (Dabco) and studied by confocal scanning laser microscopy using a Leica TCS 4D system.

RESULTS AND DISCUSSION

Characterization of TACE in Chinese hamster lung ts20 cells

TACE is produced as an approx. 120 kDa pro-form, which is cleaved by a furin-like enzyme in the Golgi complex. Pulse-chase labelling in Cos-7 cells have shown that the half-life of the 120 kDa pro-form is approx. 10 h [21]. The cleaved, 100 kDa active form of TACE resides at the cell surface and is involved in the shedding of many substrates. To confirm the presence of TACE in Chinese hamster lung ts20 cells immunoblot analysis of cellular extracts was performed. Using an antibody directed against the cytosolic domain of TACE, we detected two bands both in cell lysate and after immunoprecipitations with anti-TACE antibody, at approx. 120 and 100 kDa, representing pro-TACE and the furin-cleaved active form of TACE, respectively (Figure 1, lanes 2 and 4). In cell lysates from ts20 cells two prominent high-molecular-mass bands were visible with an apparent molecular mass around 150000 Da (Figure 1, lane 4, and Figure 2A, left-hand panel), while in anti-TACE immunoprecipitation only the upper band was present (Figure 1, lane 2). These most probably represent non-specific bands due to the antiserum. As faint signals remained present after anti-GHR immunoprecipitation specific interaction of these proteins with the GHR cannot be excluded. To validate that the 120 kDa band is the high-mannose glycosylated pro-form

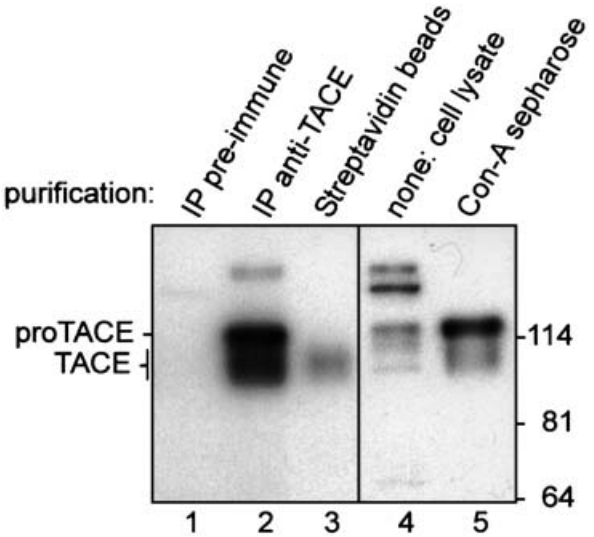

Figure 1 Characterization of TACE in Chinese hamster lung ts20 cells

Cells, grown in 6 cm dishes, were lysed and the lysate was detected either directly with anti-TACE (lane 4) or after immunoprecipitation with the same antiserum (lane 2). In lane 3 the cells were biotinylated with membrane-impermeable, activated biotin, and biotinylated proteins were isolated with streptavidin beads. In lane 5 the cell extract was incubated with Con A beads. In lane 1, immunoprecipitation was performed with pre-immune serum. After transfer to PVDF paper, proteins were analysed by Western blotting using anti-$TACE_{cyto}$ antibody. IP, immunoprecipitation.

of TACE, precipitation of total cell lysate with Con A-coated beads was performed. Con A binds mannose and glucose residues with high affinity, mainly present in high-mannose-containing proteins of ER and Golgi complex. Isolation with Con A beads resulted mostly in the isolation of the pro-form of TACE (Figure 1, lane 5). This conclusion is based on experimental data from Schlöndorff et al. [21] who have shown that the 120 kDa species is endoglycosidase H-sensitive, a general method to indicate ER- and *cis*-Golgi-located glycoproteins. Using cell biotinylation with membrane-impermeable, activated biotin, we characterized the cell-surface-resident form of TACE. Only the active, furin-cleaved 100 kDa form of TACE was detected at the cell surface (Figure 1, lane 3). The 100 kDa form of TACE at the cell surface represented only a small amount of total TACE in the cell. This is in agreement with studies on the localization of TACE indicating that the majority of TACE resides in endosomes and a minority at the cell surface (Figure 3 and [21]). Only active, furin-cleaved TACE is present at the cell surface [22].

Co-immunoprecipitation of mature, cell-surface-resident TACE with GHR

TACE has been previously shown to be involved in the proteolytic cleavage of the GHR extracellular domain thereby generating the GHBP [20]. van Kerkhof et al. [6] have shown that GHBP is released from Chinese hamster lung ts20 cells transfected with GHR cDNA. Inhibiting GHR internalization and thereby prolonging the residence time of the receptor at the cell surface further increases the amount of shed GHBP in the absence of ligand whereas the presence of ligand or the use of a matrix metalloprotease inhibitor results in a drastic decrease of GHBP shedding [23]. However, until now it was unknown whether TACE can bind the GHR in order to shed the extracellular domain of the receptor. For this reason we performed co-

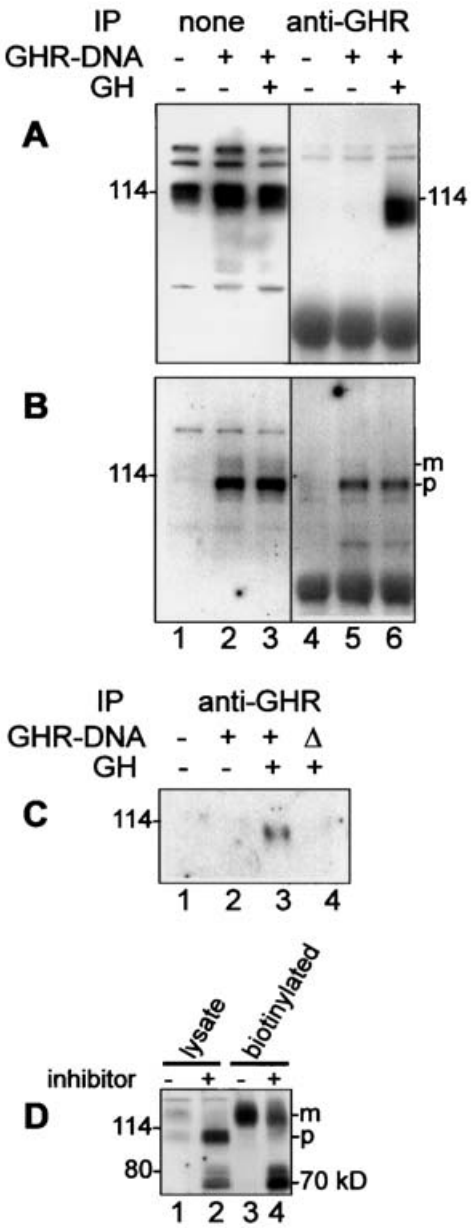

Figure 2 Co-immunoprecipitation of TACE with anti-GHR antiserum

(**A**) Cells, grown in 6 cm dishes, were transfected with either empty vector (lanes 1 and 4) or with wild-type GHR cDNA (all other lanes). In lanes 3 and 6, cells were incubated for 10 min at 30 °C with GH. After lysis, equal aliquots of the cell extracts were applied to SDS/PAGE (lanes 1–3), and the remaining extracts were immunoprecipitated with anti-GHR (lanes 4–6). Proteins were analysed by Western blotting using anti-$TACE_{cyto}$ antibody. IP, immunoprecipitation. (**B**) Re-blot of (**A**) probed for GHR. As seen in lanes 2 and 3, and 5 and 6, equal amounts of GHR species were expressed. p, precursor GHR (110 kDa); m, mature GHR (130 kDa). (**C**) The interaction between TACE and GHR(Δ E242-D244) was probed in the presence of GH (lane 4). Lanes 1–3 were as for lanes 4–6 in (**A**). Equal amounts of GHR were expressed in lanes 2–4 (results not shown). Δ, GHR(Δ E242-D244) cDNA. Proteins were analysed by Western blotting using anti-$TACE_{cyto}$ antibody. (**D**) Different GHR species were located using cell-surface biotinylation. Chinese hamster ovary ts20 cells were incubated overnight with or without the proteasomal inhibitor, carboxybenzylleucyl-leucyl-leucinevinylsulphone (20 μM), biotinylated on ice and lysed. Biotinylated proteins were purified using streptavidin beads. Cell lysates (lane 1 and 2) and the streptavidin-purified fractions (lane 3 and 4) were analysed by Western blotting using anti-GHR tail antibodies. Relative-molecular-mass standards (kDa) are indicated.

immunoprecipitation experiments using ts20 cells transiently transfected with either empty vector or wild-type GHR cDNA. Co-immunoprecipitations were carried out with an antibody directed

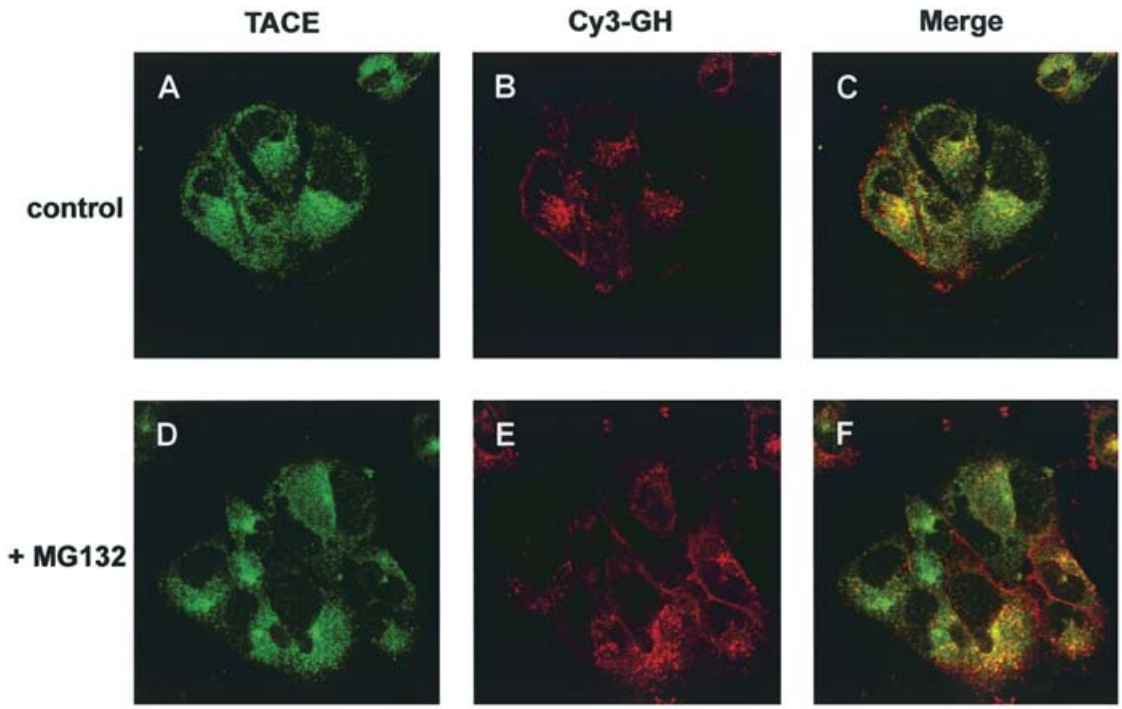

Figure 3 TACE and Cy3-GH localization

Cells, transfected with wild-type GHR cDNA, were pre-treated with MG132 (lower panels) and incubated for 30 min with Cy3-GH (1 μg/ml). After fixation and permeabilization endogenous TACE was visualized with the anti-EC antiserum followed by Alexa 488-conjugated goat anti-rabbit IgG (**A** and **D**). (**B**, **E**) Cy3-GH visualization. To control specificity of Cy3-GH an excess of unlabelled GH was added resulting in complete disappearance of the fluorescent Cy3 label (results not shown). Co-localization is depicted in the merged images (**C** and **F**).

against epitopes 300 amino acids downstream of the plasma membrane in the cytosolic tail of the GHR or the corresponding pre-immune serum. TACE co-immunoprecipitation with the GHR antibody was only possible if GH was present (Figure 2A, right-hand panel). In the absence of GH no TACE was detectable. Only the cleaved, plasma-membrane-resident form of TACE interacted with GHR. Pre-immune serum was not able to precipitate GHR nor could we detect non-specific binding of TACE under these conditions. In general, GHR occurs in cells as two proteins: a 110 kDa precursor and a 130 kDa mature receptor (apparent sizes on SDS gels). Only the mature GHR is present at the cell surface, while the 110 kDa band is in the ER and the Golgi complex [20]. In stably transfected cells both species are present in approximately equal amounts indicating a high turnover of the GHR. In transiently transfected cells, the 110 kDa precursor is in excess over the mature GHR. Cell lysates of cells transfected with GHR cDNA were detected with anti-GHR antibody, demonstrating equal loading of expressed GHR in these cells (Figure 2B, left-hand panel). Thus only the cell-surface-located form of TACE recognizes the mature GHR.

GH effectively protects the GHR against proteolytic degradation both by TACE and other proteases like proteinase K and trypsin [6,14]. This protective effect is probably due to a conformational change brought about by the formation of a ternary complex of two GHR molecules and one GH molecule. Therefore, our findings indicate that TACE still recognizes the GHR when GH is bound to the receptor. For that reason, the inhibitory effect of GH binding on shedding is not caused by an inhibition of TACE interaction with the receptor but rather an inhibition of enzyme function. Possibly, the conformational change in the receptor molecules results in the protection of the cleavage site in the GHR, which was proposed to be distinct from the site of interaction [24]. This is in agreement with our previous observation that dimerization of the GHR occurs in the ER and GH initiates the signal transduction by inducing a conformational change in the two GHR molecules [25]. It remains to be determined whether dimerization-defective GHRs are also substrates for TACE. As the cleavage site is close to the membrane and substrate recognition occurs through the extracellular TACE membrane-proximal domain, it is likely that substrate cleavage terminates TACE–GHR interaction. As we could only detect the interaction between the GHR and TACE if the cleavage was inhibited by GH, and not if the GHR was empty, substrate binding and subsequent substrate cleavage must be coupled events that happen within a short time frame, relative to the half-life of the mature receptor (50 min). Recently, three residues (Glu-242–Asp-244) located close to the transmembrane domain of the GHR have been identified as a structural pre-requisite for cleavage [17]. These amino acids do not represent the cleavage site for TACE, because mutation of these acidic residues into alanines does not alter the release of GHBP. However, deletion of these three residues abolishes GHBP shedding, probably by limiting the access of TACE to the cleavage site. Despite several attempts, we were not able to detect TACE binding to the GHR, in which the three juxta-membrane amino acids (Glu-242–Asp-244) were deleted (Figure 2C). As substrate interaction precedes cleavage, these results are in line with the findings of Conte et al. [17], suggesting that the juxta-membrane portion of the GHR constitutes or is part of the GHR–TACE interaction domain.

GHBP, resulting from TACE activity, is released into the culture medium or serum plasma. It is unknown whether TACE acts exclusively at the cell surface on the GHR. To examine this further, we biotinylated ts20 cells that were stably expressing GHR on ice and asked whether the GHR cytosolic tail (a 70 kDa

polypeptide remaining after removal of the GHBP) was detectable at the cell surface at steady state (Figure 2D). As expected the GHR precursor was not biotinylated (Figure 2D, compare lanes 2 and 4). As a positive control the cells were treated with proteasomal inhibitor to inhibit GHR endocytosis and, at the same time, stimulate TACE activity. As seen in Figure 2(D), lane 4, proteasome inhibition induced a clear band of the expected size whereas at steady state no 70 kDa band was visible (Figure 2D, lanes 1 and 3), indicating that compared with the uncleaved GHR very little cleaved product is present at the cell surface. Thus either the 70 kDa GHR tail is endocytosed very rapidly upon cleavage, or TACE also acts on the GHR in endosomes. As proteasomal inhibitors have two effects on GHR (they inhibit GHR endocytosis and they activate TACE [23]), it is currently impossible to determine the activity of TACE intracellularly.

Intracellular localization of TACE and the GH-GHR complex

Due to its broad specificity, TACE is involved in many regulatory processes targeting different receptors and other factors at the cell surface, reviewed in [26]. The location of both furin-cleaved TACE and the 130 kDa mature GHR is regulated: dependent on cell activation, TACE is distributed both at the cell surface and in endosomes [22], whereas the GHR is mainly at the cell surface and on route to the lysosomes. Both molecules have a high turnover rate, implicating that considerable amounts of intracellular epitopes are present in the ER. As the ER is distributed throughout the cellular cytoplasm, localization of GHR by immunofluorescence is complex. As the interaction between TACE and GHR is probably very transient and short, localization studies, even by electron microscopy, will probably not be able to prove and visualize the interaction at the molecular level. Therefore, we decided to co-localize the two proteins under different conditions by immunofluorescence using a confocal microscope. In order to compare TACE localization with that of surface and endocytosed GH, we marked the GHR with Cy3-GH. GH remains bound to its receptor all the way to lysosomes and at the same time it activates the signal transduction via Jak2. The cells were incubated for 30 min with Cy3-GH at 30 °C, fixed and labelled with an anti-TACE antibody directed against the extracellular domain of TACE (anti-EC). The major pool of TACE resided in intracellular perinuclear compartments, with some diffuse localization throughout the cell (Figure 3A), whereas GH showed some cell-surface labelling and an intensive punctate pattern in the cells, which corresponds with internalized GH in endosomes (Figure 3B). When comparing TACE and GH localization, the merged image showed little co-localization at the cell surface; however, TACE and GH did co-localize in the perinuclear area (Figure 3C). This further indicates that a substantial amount of TACE is present in endosomes and less at the cell surface. When the cells were treated with the proteasomal inhibitor MG132 to inhibit GHR endocytosis (Figure 3E), TACE localization stayed unchanged (Figures 3D and 3F), while the cell-surface labelling of Cy3-GH became prominent (Figures 3E and 3F). These findings indicate that TACE location is independent of GHR. However, subtle changes in localization might be difficult to observe due to the high amount of the precursor species of TACE present in the ER/Golgi region of the cells. As the blotting experiments of Figure 1 and Figure 2(A) show that a considerable amount of TACE in the Chinese hamster lung cells is present as precursor (ER- and Golgi-located, 120 kDa) species, it is obvious that part of the fluorescent labelling of TACE must originate from ER- and Golgi-located TACE, explaining the observation in Figure 3(C) that there is only a partial co-localization of endocytosed GH and TACE in the perinuclear region.

The data of Figures 2(D) and 3 are in favour of TACE acting both at the cell surface and in endosomes. In particular, the absence of detectable GHR cytosolic tail remnant is striking. Both the study of Schlöndorff et al. [21] and our immunolocalization study, combined with the finding that the majority of 100 kDa TACE is not reacting in the cell-surface biotinylation assay, indicate that TACE is present inside the cells. Endocytosis of TACE has been described as a (negative) controlling factor in TACE activity [22]. At present, there are no indications that TACE targets substrates intracellularly. However, the present experimental approaches do not exclude TACE activity in endosomal membranes. Further study is required to elucidate the turnover regulation of this enzyme.

In summary, we show that mature, furin-cleaved TACE interacts with the GHR, an interaction that we can only demonstrate if TACE action is inhibited by the presence of GH. The conformational change of the GHR molecules after GH binding results in an inaccessible cleavage site, thereby protecting the GHR molecules from shedding. Deletion of amino acids 242–244 in the extracellular domain of GHR probably restricts the contact of TACE with the interaction site, which might explain the protection from proteolytic cleavage in this mutant GHR.

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