Shedding of growth hormone-binding protein is inhibited by hydroxamic acid-based protease inhibitors: proposed mechanism of activation of growth hormone-binding protein secretase

T Amit¹ ², Z Hochberg³, M Yogev-Falach¹, M B H Youdim¹ ² and R J Barkey¹

¹Department of Pharmacology, Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel
²Eve Topf and NPF Centers for Neurodegenerative Diseases, Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel
³Department of Pediatrics, Rambam Medical Center, Haifa, Israel

(Requests for offprints should be addressed to R J Barkey, Department of Pharmacology, Bruce Rappaport Faculty of Medicine, Technion, PO Box 9649, Haifa 31096, Israel; Email: rjb@tx.technion.ac.il)

Abstract

The present study describes events postulated to be involved in the regulated mechanism of proteolytic shedding of growth hormone (GH)-binding protein (GHBP). Using Chinese hamster ovary (CHO) cell lines stably transfected either with the full-length human GH receptor (hGHR) or with the cytoplasmic domain-truncated hGHR (hGHR-tr), we show that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), caused a rapid time- and dose-dependent increase in GHBP secretion, which, as expected, was matched by a corresponding decrease in cell-surface GHR. Furthermore, PMA equally enhanced GHBP release from CHO/hGHR-tr cells, suggesting that the cytoplasmic domain of hGHR is not essential for PMA-induced shedding. PMA is known to specifically activate protein kinase C and, indeed, the stimulatory effects of PMA in both cell lines were completely inhibited by the protein kinase inhibitor, staurosporine (100 nM), suggesting that activation of protein kinase C (PKC) may mediate PMA-induced GHBP shedding. Since proteolytic cleavage of several cell-surface proteins was shown to be stimulated by modulators of PKC activity and inhibited by metalloprotease inhibitors, we studied the effects of two hydroxamic acid-based inhibitors of zinc-dependent metalloproteases, BB-3103 and Ro31–9790, on GHBP proteolysis. Pretreatment of CHO/hGHR cells with both these inhibitors reduced PMA-enhanced shedding of GHBP, in a dose-dependent manner, with IC₅₀ values of ~0.41 µM for BB-3103 and ~0.97 µM for Ro31–9790. In addition, these inhibitors dose-dependently reduced the shedding enhanced by the sulfhydryl alkylator, N-ethylmaleimide (NEM), with IC₅₀ values of ~0.32 µM and ~0.58 µM for BB-3103 and Ro31-9790 respectively. It was of interest to find out that Ro31-9790 acted not only to modulate PMA- or NEM-induced shedding processes, but also markedly reduced the spontaneous, time-dependent accumulation of GHBP released from CHO/hGHR cells growing in serum-containing medium. Taken together, these results suggest that one or more zinc-dependent metalloprotease(s), acting at the cell surface, may be involved in GHBP secretase activity. A scheme is proposed whereby at least part of the regulated maturation and/or activation of the protease activity may involve a cysteine-switch mechanism and/or PKC-dependent phosphorylation. In the long run, specific inhibitors of these processes could be applied in the regulation of GHBP levels and, thus, of GH availability and/or activity.


Introduction

In the human and rabbit, growth hormone (GH) binding-protein (GHBP) is generated by proteolytic cleavage of the full-length transmembrane GH receptor (GHR) (Leung et al. 1987, Trivedi & Daughaday 1988, Sotiropoulos et al. 1993). Furthermore, an alternatively spliced form of human (h) GHR was demonstrated to encode a cytoplasmically truncated isoform of hGHR (hGHR-tr) and to regulate GHBP generation (Dastot et al. 1996, Amit et al. 1997, Ross et al. 1997). Functional studies confirmed that while hGHR-tr was inactive by itself, it could act as a dominant negative regulator of the full-length receptor (Amit et al. 1997, Ayling et al. 1997, Ross et al. 1997).

In spite of the current understanding of the generation of GHBP, the proteolytic mechanism involved is not clearly defined or understood and the protease activity is resistant to a broad panel of common protease inhibitors (Harrison et al. 1995). We and others have demonstrated that sulfhydryl-reactive agents markedly induced GHBP
release from IM-9 human lymphocytes (Trivedi & Daughaday 1988, Massa et al. 1993, Alele et al. 1998), Hep G2 human hepatoma cells (Amit et al. 1994, Harrison et al. 1995) and Chinese hamster ovary (CHO) cells transfected with rabbit or human GHR (hGHR) (Bick et al. 1995) and Chinese hamster ovary cells transfected with rabbit or human GHR (hGHR) (Bick et al. 1995) and Chinese hamster ovary cells transfected with rabbit or human GHR (hGHR) (Bick et al. 1995) and Chinese hamster ovary cells transfected with rabbit or human GHR (hGHR) (Bick et al. 1995) and Chinese hamster ovary cells transfected with rabbit or human GHR (hGHR) (Bick et al. 1995). We then suggested that the increased release of GHBP might be a consequence of alkylation of one or more free sulphydryl group(s) on an endopeptidase that apparently becomes activated to induce GHBP shedding (Amit et al. 1999). In support of this hypothesis, Alele et al. (1998) reported that in IM-9 cells the metalloprotease inhibitor, immunex compound 3 (IC3), blocked GHBP shedding induced by the alkylator N-ethylmaleimide (NEM), indicating that NEM may activate a GHBP-generating enzyme of the metalloprotease family.

Further studies have recently shown that phorbol ester increased human GHBP release in IM-9 cells (Alele et al. 1998, Saito et al. 1998) and suggested a pathway whereby phorbol ester activates intracellular protein kinase Ca (PKCa), which then activates an extracellular protease to cleave hGHR and form hGHBP (Saito et al. 1998, 1999). Activation of PKC by phorbol esters has frequently been shown to induce ectodomain shedding of a variety of cell-surface proteins (Hooper et al. 1997). In addition, metalloproteases have been implicated in the shedding or release of several different cell-surface proteins from the plasma membrane, including various cytokines, cytokine receptors, adhesion proteins and other proteins such as β-amyloid precursor protein (β-APP) (Arribas et al. 1996, Black et al. 1997, Blobel 1997, Hooper et al. 1997, Moss et al. 1997). In this vein, the recent demonstration that a metalloprotease, presumably tumor necrosis factor (TNF)-α converting enzyme (TACE)/α disintegrin and metalloprotease (ADAM)-17, is involved in membrane GHR cleavage (Alele et al. 1998, Zhang et al. 2000), supports the suggestion that this family of proteases might play a wider role in GHBP generation and secretion from different cells or tissues. In this study we have examined the role of the cytoplasmic domain of hGHR in the mechanism of phorbol 12-myristate 13-acetate (PMA)-induced shedding of GHBP and the involvement of metalloproteases in the spontaneous, as well as of NEM- or PMA-induced shedding, in CHO cells stably transfected with hGHR or hGHRtr, using two hydroxamic acid-based inhibitors, BB-3103 and Ro31–9790.

Materials and Methods

Cell culture and transfections

CHO cells stably expressing the full-length hGHR or its truncated isoform, hGHRtr, were kindly provided by S Amselem (INSERM, Creteil, France), who described in detail the plasmid construct, the transfection and selection procedures (Dastot et al. 1996). Stably transfected cells (designated CHO/hGHR and CHO/hGHRtr) were cultured in Ham’s F-12 medium supplemented with 10% (v/v) fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM l-glutamine, 10 mg/liter penicillin/streptomycin/nystatin and 10 mM HEPES buffer, pH 7.4. The sterile culture medium, FCS and antibiotic solutions were purchased from Biological Industries (Kibbutz Beit HaEmek, Israel). Cell cultures were incubated at 37 °C in humid 5% CO2 – 95% air environment. Stable transfectants were selected in 500 µg/ml G418 (neomycin; Life Technologies Inc., Grand Island, NY, USA).

Drug treatments

PMA and NEM were obtained from Sigma Chemical Co. (St Louis, MO, USA). The protein kinase inhibitor, staurosporine, was obtained from Calbiochem (San Diego, CA, USA). Ro31–9790 was a kind gift from Roche Discovery (Welwyn Garden City, Herts, UK) and BB–3103 was a kind gift from British Biotechnology Pharmaceuticals (Oxford, Oxon, UK).

Confluent cells trypsinized from growth flasks, were seeded in 6-well plates (~3 × 105 cells/well) and treated with PMA, NEM, staurosporine, Ro31–9790, BB–3103 or dimethylsulfoxide (DMSO)/vehicle control at 1:100 final dilution and incubated at 37 °C for the indicated times at the indicated concentrations. Whereas drug treatments were always conducted in serum-free medium, constitutive release of GHBP was studied in serum-containing medium.

Binding assays

Recombinant authentic hGH (a kind gift from Bio–Technology General, Rehovot, Israel) was radiolabeled with Na[125I] (Amersham Pharmacia Biotech UK Ltd, Amersham, Bucks, UK) using the Chloramine–T method (Greenwood et al. 1963) and chromatographed on a Sephadex G–100 column (45 × 1·5 cm) as previously described (Barkey et al. 1981). The specific activity of Na[125I] was 70–80 µCi/µg.

Confluent cells were incubated with 125I–hGH (2 ng) in the absence (total binding) or presence (nonspecific binding) of 2 µg hGH, in a final volume of 400 µl binding buffer, consisting of 10 mM phosphate buffer, 1% BSA, and 30 mM MgCl2, pH 7·4 for 20 h at 4 °C. After aspiration of the binding buffer, cell monolayers were washed three times with 1 ml ice-cold 10 mM PBS, pH 7·4 and lysed in 1 ml 10% sodium dodecyl sulfate (SDS) solution at 37 °C for 1 h. Cell-bound activity was measured in a multivit γ-counter. All determinations were carried out in triplicate. Specific binding was expressed as a percentage of the total radioactivity added, and data were normalized to 350 µg cellular protein, which was the average protein content/well. The protein


www.endocrinology.org

Downloaded from Bioscientifica.com at 10/19/2018 09:51:19PM via free access
concentration was determined by the method of Lowry et al. (1951).

**Determination of secreted GHBP**

Conditioned media of confluent cells were centrifuged at 3000 g (10 min, 4 °C) to remove cell debris and the cleared supernatants were concentrated tenfold by lyophilization. To ascertain that this procedure was sufficient to remove all cell debris, medium that was ultracentrifuged at 100 000 g (60 min, 4 °C) was shown to yield similar binding results (data not shown). GHBP release into the conditioned medium during incubation was assessed on the basis of specific binding of 125I-hGH, as previously described (Bick et al. 1996, Amit et al. 1999). Briefly, after incubation, bound and free hormones were separated by adding 1 ml dextran-coated charcoal (4% Norit-A, 0-4% dextran T-70) in 10 mM phosphate buffer, pH 7-4, followed by centrifugation and counting of the radioactivity in the supernatant. Specific binding was expressed as a percentage of the total radioactivity incubated, and data were normalized to 350 µg cellular protein.

**Affinity cross-linking**

Cross-linking studies with GHBP were performed in concentrated (×10) culture medium from confluent cells, as previously described (Amit et al. 1999). The protease inhibitors used were 1 mM EDTA, 3-2 µM aprotonin, 2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin and 10 mM benzamidine (Sigma Chemical Co.). The medium was incubated with 125I-hGH (10 ng) in the presence (nonspecific binding) or absence (total binding) of hGH (10 µg) at 4 °C for 20 h. Covalent cross-linking was then achieved by the addition of 1 mM disuccinimidyl suberate for 30 min at 4 °C. This was followed by immunoprecipitation, by the addition of monoclonal antibody (MAb) 263 or an unrelated MAb (anti-Brucella), kindly provided by Dr M J Waters (Queensland, Australia), at a 1:100 (v/v) final dilution. After incubation at 4 °C for 2 h, the immune complexes were collected on protein A-Sepharose beads, and the pellets were washed four times with 10 mM Tris buffer, pH 7-4. Samples were dissolved in an equal volume of twofold concentrated Laemmli sample buffer (Laemmli 1970), boiled for 3 min, and subjected to 10% acrylamide SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After drying the gels, autoradiography was performed using Kodak X-Omat AR film (Kodak Co., Rochester, NY, USA).

**Results**

Initial studies were performed to establish the effect of the phorbol ester PMA, a common specific activator of PKC, on GHBP proteolytic cleavage in CHO cells stably transfected with the full-length hGHR. Figure 1A clearly shows that treatment of CHO/hGHR cells with PMA (100 nM) for various times resulted in a rapid and marked enhancement in soluble GHBP released into the medium, compared with GHBP level in control, unstimulated cells. The accelerated GHBP generation observed following PMA treatment was accompanied by a time-dependent decrease in the level of cell-associated GHR (Fig. 1B). While the increased GHBP release was evident already after 15 min incubation with PMA and reached a plateau at 60 min, maximal GHR reduction was achieved at
CHO/hGHR cells indicated similar molecular weight values of approximately 80 kDa (data not shown). This value is consistent with an $M_r$ of approximately 60 kDa for GHBP, after accounting for the $M_r$ of hGH.

We have recently characterized a truncated isoform of hGHR (hGHRtr) and demonstrated that neither GHBP spontaneous generation nor GHBP shedding induced by the sulfhydryl-reactive agent, NEM, were affected by truncation of the intracellular domain of hGHR (Amit et al. 1997, 1999). Here, we further studied the regulatory mechanism of GHBP shedding and examined whether PMA also promotes the release of soluble GHBP from CHO/hGHRtr cells. We found that shedding of GHBP from the truncated receptor of CHO/hGHRtr cells was strongly stimulated, in a time- and dose-dependent manner, following PMA treatment to a level even higher (per 350 µg cellular protein) than that observed with the full-length receptor of CHO/hGHR cells (Figs 1 and 2). These findings provide further support for the notion that the cytoplasmic domain of hGHR does not appear to be required in order to induce GHBP release by PMA.

The involvement of protein kinase in the PMA-induced generation of GHBP was validated using the general protein kinase inhibitor, staurosporine (Ruegg & Burgess 1989), which was also shown to be one of the most potent inhibitors of PKC (Tamaoki et al. 1986). In both CHO/hGHR and CHO/hGHRtr cells, pretreatment with staurosporine (100 nM at 37°C for 15 min) completely prevented the PMA-induced elevation in GHBP shedding, as well as the PMA-induced GHR loss, maintaining their levels at their basal, control values. These findings suggest that activation of a protein kinase, presumably PKC, may mediate the PMA-induced GHBP shedding (Table 1). However, pretreatment with staurosporine had no effect on NEM-induced GHR cleavage (data not shown), confirming the involvement of different mechanisms in NEM- and PMA-induced GHBP generation.

Since in several cell-surface proteins proteolytic cleavage was shown to be stimulated by modulators of PKC activity and inhibited by metalloprotease inhibitors (Hooper et al. 1997), we studied the effects of two hydroxamic acid-based metalloprotease inhibitors, BB-3103 and Ro31–9790, on GHBP proteolysis. As shown in Fig. 3A, pretreatment of CHO/hGHR cells with both these inhibitors reduced PMA-enhanced shedding of GHBP in a dose-dependent manner. The IC$_{50}$ values of inhibition were $\sim 0.41$ µM for BB-3103 and $\sim 0.97$ µM for Ro31–9790. Addition of the inhibitors to the CHO/hGHR cells was also associated with a corresponding, dose-dependent inhibition of the PMA-induced reduction in cellular GHR (Fig. 3B), supporting the suggestion that the inhibition of PMA-induced GHBP shedding by these inhibitors may be a direct result of inhibition of proteolytic cleavage of GHR at the cell surface.

30 min. A similar pattern of PMA-induced GHR proteolysis was observed previously in IM-9 cells (Alele et al. 1998, Saito et al. 1998). The PMA-enhanced GHBP proteolysis was also dose-dependent; exposure of CHO/hGHR cells to increasing concentrations of PMA for 60 min resulted in a dose-dependent elevation in GHBP release (Fig. 2A), that was associated with a corresponding decrease in cellular GHR levels (Fig. 2B). Maximal stimulation of GHBP and reduction of GHR were noted at 100 nM PMA. The increase in levels of GHBP secreted was not related to a change in its molecular weight. Studies of affinity cross-linked $^{125}$I-hGH to conditioned media from control (unstimulated) and from PMA-treated CHO/hGHR cells indicated similar molecular weight values of approximately 80 kDa (data not shown). This value is consistent with an $M_r$ of approximately 60 kDa for GHBP, after accounting for the $M_r$ of hGH.

We have recently characterized a truncated isoform of hGHR (hGHRtr) and demonstrated that neither GHBP spontaneous generation nor GHBP shedding induced by the sulfhydryl-reactive agent, NEM, were affected by truncation of the intracellular domain of hGHR (Amit et al. 1997, 1999). Here, we further studied the regulatory mechanism of GHBP shedding and examined whether PMA also promotes the release of soluble GHBP from CHO/hGHRtr cells. We found that shedding of GHBP from the truncated receptor of CHO/hGHRtr cells was strongly stimulated, in a time- and dose-dependent manner, following PMA treatment to a level even higher (per 350 µg cellular protein) than that observed with the full-length receptor of CHO/hGHR cells (Figs 1 and 2). These findings provide further support for the notion that the cytoplasmic domain of hGHR does not appear to be required in order to induce GHBP release by PMA.

The involvement of protein kinase in the PMA-induced generation of GHBP was validated using the general protein kinase inhibitor, staurosporine (Ruegg & Burgess 1989), which was also shown to be one of the most potent inhibitors of PKC (Tamaoki et al. 1986). In both CHO/hGHR and CHO/hGHRtr cells, pretreatment with staurosporine (100 nM at 37°C for 15 min) completely prevented the PMA-induced elevation in GHBP shedding, as well as the PMA-induced GHR loss, maintaining their levels at their basal, control values. These findings suggest that activation of a protein kinase, presumably PKC, may mediate the PMA-induced GHBP shedding (Table 1). However, pretreatment with staurosporine had no effect on NEM-induced GHR cleavage (data not shown), confirming the involvement of different mechanisms in NEM- and PMA-induced GHBP generation.

Since in several cell-surface proteins proteolytic cleavage was shown to be stimulated by modulators of PKC activity and inhibited by metalloprotease inhibitors (Hooper et al. 1997), we studied the effects of two hydroxamic acid-based metalloprotease inhibitors, BB-3103 and Ro31–9790, on GHBP proteolysis. As shown in Fig. 3A, pretreatment of CHO/hGHR cells with both these inhibitors reduced PMA-enhanced shedding of GHBP in a dose-dependent manner. The IC$_{50}$ values of inhibition were $\sim 0.41$ µM for BB-3103 and $\sim 0.97$ µM for Ro31–9790. Addition of the inhibitors to the CHO/hGHR cells was also associated with a corresponding, dose-dependent inhibition of the PMA-induced reduction in cellular GHR (Fig. 3B), supporting the suggestion that the inhibition of PMA-induced GHBP shedding by these inhibitors may be a direct result of inhibition of proteolytic cleavage of GHR at the cell surface.

**Figure 2** Dose–response of PMA-induced shedding of GHBP from CHO/hGHR and CHO/hGHRtr cells. Confluent CHO/hGHR and CHO/hGHRtr cells were incubated without (control) or with various concentrations of PMA for 60 min at 37°C and $^{125}$I-hGH binding to soluble GHBP in the medium (A) and to cell-surface GHR (B) was determined. Binding data are expressed as described in Fig. 1. Results are means ± S.E. ($n$ = 3 independent experiments). The 100% value for control cell-surface hGHR = 29.6 ± 1.3%/350 µg protein and for hGHRtr = 37.6 ± 1.9%/350 µg protein.
Cell-permeable sulfhydryl-reactive alkylating reagents, such as iodoacetamide or NEM, were previously shown to promote proteolytic cleavage of GHBP (Trivedi & Daughaday 1988, Massa et al. 1993, Amit et al. 1994, 1999, Harrison et al. 1995, Bick et al. 1996, Alele et al. 1998). Thus, it was important to test whether BB-3103 and Ro31–9790 could also inhibit NEM-induced GHBP shedding from the CHO/hGHR cell line, since another metalloprotease inhibitor, IC3, was shown to inhibit GHBP shedding from IM-9 cells (Alele et al. 1998). Indeed, these inhibitors dose-dependently reduced NEM-enhanced GHBP shedding (Fig. 4A) as well as NEM-induced loss of cellular GHR (Fig. 4B). For both BB-3103 and Ro31–9790, the dose–response curves and the IC50 values for inhibition of NEM-induced GHBP release (∼0.32 µM and ∼0.58 µM respectively) were very similar to those observed for inhibition of PMA-induced GHBP release.

We further examined the effect of the Ro31–9790 metalloprotease inhibitor on the spontaneous release of GHBP, previously demonstrated to occur in CHO/hGHR cells maintained in medium containing 10% FCS (Amit et al. 1997). It was of interest to find out that Ro31–9790 markedly reduced the spontaneous, time-dependent accumulation of GHBP released from CHO/hGHR cells growing in serum-containing medium, as compared with control DMSO/vehicle-treated cells (Fig. 5), in addition to its modulation of PMA- and NEM-induced shedding processes.

**Discussion**

In this study, we performed a comprehensive examination of the mechanism of regulated events in the proteolytic shedding of GHBP from the hGHR. For that purpose we studied the effects of protein kinase C activation by the phorbol ester PMA, on GHBP cleavage from the full-length isoform, compared with that from the truncated, hGHRtr isoform. We also studied the effects of hydroxamic acid-based inhibitors of metalloproteases on the spontaneous shedding of GHBP, as well as on shedding induced by PMA-induced activation of protein kinase by and NEM-mediated sulfhydryl group alkylation.

In human and rabbit, GHBP generation is induced by proteolytic cleavage of the extracellular domain of GHR. GHBP was first shown to be released in vitro as a result of exposure to the cell-permeable sulfhydryl-reactive agents, iodoacetamide or NEM, from CHO cells transfected with rabbit or human GHR cDNA (Bick et al. 1996, Amit et al. 1999) as well as from IM-9 lymphocytes and from Hep G2 cells (Trivedi & Daughaday 1988, Massa et al. 1993, Amit et al. 1994, Harrison et al. 1995, Alele et al. 1998). We have recently suggested a role for sulfhydryl group-mediated activation of an endopeptidase in the proteolytic shedding of GHBP, based on studies with CHO cells stably transfected with either full length GHR or with hGHRtr (Amit et al. 1999). Using these transfected cells we now show that the phorbol ester, PMA, presumably by activation of PKC, caused a rapid dose- and time-dependent increase in GHBP secretion, which, as expected, was matched by a corresponding decrease in cell-surface GH receptors. Indeed, phorbol esters were shown in previous studies to down-regulate GHR in various cell lines, including IM-9 cells (Suzuki et al. 1990), 3T3–F442A fibroblasts, H35 rat hepatoma cells and CHO cells expressing rat GHR (King et al. 1996). In addition, PMA strongly induced GHBP release in HepG2 cells stably transfected with rabbit GHR, and in IM-9 cells (Harrison et al. 1995, Alele et al. 1998, Saito et al. 1998). Using anti-hGHR cytoplasmic domain antibody, it was demonstrated in IM-9 cells that the PMA-induced proteolytic cleavage of GHR also resulted in the formation of a cell-associated transmembrane/cytoplasmic domain remnant (Alele et al. 1998). The involvement of PKCα in phorbol ester–enhanced GHBP release was suggested on the basis of the use of several PKC-specific inhibitors in IM-9 cells (Saito et al. 1998). The work presented here further examines the role of the cytoplasmic domain of GHR in the mechanism of PMA-induced GHBP shedding.

**Table 1** Effect of staurosporine on PMA-induced shedding of GHBP from CHO/hGHR and CHO/hGHRtr cells. Confluent CHO/hGHR and CHO/hGHRtr cells were preincubated without or with staurosporine (100 nM) at 37 °C for 15 min, and then incubated without or with PMA (100 nM) for a further 60 min. 125I-hGH binding to soluble GHBP and to cell-surface GHR is expressed as a percentage of specific binding per 350 µg cellular protein. Results are means ± s.e. (n=3 independent experiments).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>PMA</th>
<th>Staurosporine/</th>
<th>Control</th>
<th>PMA</th>
<th>Staurosporine/</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PMA</td>
<td></td>
<td></td>
<td>PMA</td>
</tr>
<tr>
<td>CHO/hGHR</td>
<td>0.96 ± 0.2</td>
<td>6.43 ± 0.4**</td>
<td>1.35 ± 0.1</td>
<td>28.36 ± 2.1</td>
<td>7.45 ± 0.5*</td>
<td>28.07 ± 1.8</td>
</tr>
<tr>
<td>CHO/hGHRtr</td>
<td>1.53 ± 0.4</td>
<td>9.12 ± 1.1**</td>
<td>2.17 ± 0.7</td>
<td>35.65 ± 1.5</td>
<td>12.19 ± 1.7*</td>
<td>38.33 ± 2.9</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01 vs control.
In the current study, we demonstrated that PMA also markedly enhanced GHBP release from CHO/hGHR cells, to a similar or even to a larger extent than its effect in CHO/hGHR cells, indicating that the cytoplasmic domain of hGHR is not essential for the PMA-induced shedding of GHBP. This finding is in keeping with previous studies on the mechanism of PMA-inducible shedding of the receptors for p55 TNF (Brakebusch et al. 1992), for interleukin (IL)-6 (Mullberg et al. 1994) and for colony-stimulating factor 1 (CSF-1) (Downing et al. 1989), which demonstrated that, in most cases, ectodomain-induced shedding occurs even if the cytoplasmic domain of the substrate has been deleted entirely. Furthermore, others and ourselves have recently reported that truncation of the intracellular domain of hGHR does not preclude GHBP shedding from hGHRter-transfected COS-7 (Dastot et al. 1996) and 293 (Ross et al. 1997) cells and from hGHRter-stably transfected CHO cells (Amit et al. 1997), nor enhancement of shedding by the sulfhydryl-reactive agent NEM in hGHRter-stably transfected CHO cells (Amit et al. 1999).

The phorbol ester-induced activation of PKC has been shown to induce protein ectodomain shedding of a large variety of cell-surface proteins (Lantz et al. 1990, Pandiella & Massague 1991, Mullberg et al. 1993, Arribas et al. 1996, Black et al. 1997, Blobel 1997, Hooper et al. 1997, Moss et al. 1997). Despite the observation of nonconserved cleavage sites that may be adjacent to the membrane or further out on the molecule, there are clues that a common strategy may operate in membrane protein ectodomain

Figure 3 Inhibition of PMA-induced GHBP shedding by hydroxamic acid-based inhibitors. Confluent CHO/hGHR cells were preincubated without or with different concentrations of BB-3103 or Ro31–9790 for 15 min and then treated with PMA (100 nM) for 1 h. Control values for GHBP and GHR in unstimulated, DMSO-treated CHO/hGHR cells were 0.5 ± 0.2%/350 μg and 35.5 ± 1.4%/350 μg cellular protein respectively. 125I-hGH binding to soluble GHBP in the medium (A) and to cell-surface GHR (B) was expressed as described in Fig. 1. Results are means ± s.d. of 2 experiments.

Figure 4 Inhibition of NEM-induced GHBP shedding by hydroxamic acid-based inhibitors. CHO/hGHR cells were preincubated without or with increasing concentrations of BB-3103 or Ro31–9790 for 15 min and then treated with NEM (0.5 mM) for 30 min. Control values for GHBP and GHR in unstimulated (no NEM) CHO/hGHR cells treated with DMSO vehicle were 0.9 ± 0.2%/350 μg and 27.7 ± 2.1%/350 μg cellular protein respectively. 125I-hGH binding to soluble GHBP in the medium (A) and to cell-surface GHR (B) was expressed as described in Fig. 1. Results are means ± s.d. of 2 experiments.
shedding, involving one or several components sensitive to metalloprotease inhibitors (Arribas et al. 1996). Furthermore, while there are still no indications of the exact mechanisms involved in the activation of secretase activity, several possibilities have been raised; upon cell activation via protein kinases or other pathways, coclustering of the membrane-anchored secretase and substrate would facilitate their interaction; alternatively, the kinase might bring about conformational changes, either in the enzyme, increasing its activity, or in the substrate, increasing availability of cleavage sites (Werb & Yan 1998). It could reasonably be assumed that kinase-induced phosphorylation, whether of the enzyme or of the substrate, could mediate these changes and, indeed, the ADAM family member, MDC9, was recently shown to become phosphorylated following PMA stimulation (Roghani et al. 1999). Based on these findings, these authors suggested that the metalloprotease activity could be up-regulated by inside-out signaling via PKC-dependent phosphorylation of its cytoplasmic tail. Nevertheless, PMA stimulation can result in the phosphorylation of a variety of different proteins and thus the specificity of this effect remains to be determined. Interestingly, the recent demonstration that phorbol ester-induced ectodomain shedding is dependent on kinase activation and on the mitogen-activated protein kinase signaling cascade (Fan & Derynck 1999, Gechtman et al. 1999) adds another level of complexity in understanding the signaling mechanisms involved in the PMA-induced activation of the secretase.

Hydroxamic acid-based inhibitors, which bind the essential zinc ion at the active site of the protease, were originally designed as inhibitors of zinc-dependent matrix metalloproteases. In addition, these inhibitors have been shown to inhibit protein ectodomain shedding of several different types of membrane proteins including pro-TNF-α (Gearing et al. 1994, McGeohan et al. 1994, Mohler et al. 1994), pro-transforming growth factor (TGF)-α (Arribas et al. 1996), 80-kDa TNF-α receptor (Crowe et al. 1995), IL-6 receptor, p60 TNF receptor (Mullberg et al. 1995), thyrotrphin receptor (Couet et al. 1996), angiotensin converting enzyme, t-selectin (Feheman et al. 1996, Preece et al. 1996, Bolrand et al. 1999) and β-APP (Arribas et al. 1996). In this study, we used two such hydroxamic acid-based metalloprotease inhibitors, BB-3101 and Ro31–9790, to demonstrate the involvement of metalloproteases in both the constitutive/serum-induced shedding of GHBP, as well as in the induced process, in CHO/hGHR cells.

Our data demonstrate that the hydroxamic acid-based peptides, BB–3103 and Ro31–9790, dose-dependently inhibit both PMA- and NEM-induced GHBP shedding, further suggesting the involvement of a metalloprotease in the induced proteolytic cleavage of GHBP. Thus, it may be speculated that PMA and NEM promote GHBP shedding via the activation of the same or similar metalloprotease(s). Indeed, IC3, another metalloprotease inhibitor, was recently observed to induce a similar inhibition in IM-9 cells (Alele et al. 1998).

In addition, we examined whether these peptide hydroxamic acid compounds would also inhibit the constitutive/serum-induced GHBP proteolytic cleavage. We have previously shown in CHO/hGHR cells that, in the presence of FCS, large amounts of GHBP are constitutively secreted from these transfected cells over relatively short times (AmiT et al. 1997, 1999). We now report that in addition to inhibiting PMA- and NEM-enhanced GHBP release, the Ro31–9790 metalloprotease inhibitor also inhibited the more physiologically relevant, constitutive/serum-induced GHBP shedding from CHO/hGHR cells, further supporting the probability that a common mechanism underlies the constitutive and pharmacologically inducible GHBP proteolytic processes. This suggestion is in keeping with the demonstration that serum induces shedding of proTGF-α (Pandiella & Massague 1991) and the recent findings that addition of fresh FCS to serum-starved CHO cells expressing the heparin-binding epidermal-like growth factor transmembrane precursor (HB-EGF_TM) tagged with hemagglutinin and Myc epitopes, resulted in the rapid activation of p42 and p44 MAP kinases and shedding of HB-EGF_TM (Gechman et al. 1999). Furthermore, we have also variously observed that treatment of CHO/hGHR and CHO/hGHRα cells with the protein kinase inhibitor, staurosporine (100 nM, 3 h), caused a small (25–30%), though consistent, reduction in serum-induced GHBP shedding (unpublished observations). These findings all support the evolving conclusion that so far unidentified growth factors present in the serum could be constitutively activating the
secretases/sheddases, at least in part, through a protein kinase-dependent mechanism (Gechtman et al. 1999) and thus regulate the basal shedding of the soluble binding proteins. Consistent with this conclusion, a recently presented study further implicates growth factors in the proteolytic processing of the GHR (Guan et al. 2000).

Taken together these results suggest that GHBP secretase activity may involve a zinc-dependent metalloprotease. Indeed, recent characterization of the group of proteolytic enzymes, referred to collectively as secretases or sheddases, has revealed common features, particularly activation by sulfhydryl alkylating agents, up-regulation by PKC and inhibition by hydroxamic acid-based metalloprotease inhibitors (Black & White 1998, Werb & Yan 1998). Although the exact protease(s) responsible for GHR cleavage cannot be ascertained at this time, it may be a member of that family of secretases. The first such secretase characterized was TACE (Black et al. 1997, Moss et al. 1997), a member of the ADAM subgroup of the metzincin family of proteases which are closely related to the matrix-type metalloprotease family, and the evidence accumulated so far shows that the secretase involved in GHBP shedding shares similar characteristics.

Based on the above suggestion and on the results of this study, a model was adapted to describe the maturation and activation of the secretase responsible for GHBP generation and this is shown schematically in Fig. 6. The model invokes at least two steps in the maturation and regulation of a metalloprotease. A ‘cysteine-switch’ mechanism was

---

**Figure 6** Proposed scheme for activation of GHBP secretase. I. The secretase is said to be in the ‘off’ position when the prodomain cysteine ligation the catalytic Zn$^{2+}$, and is switched ‘on’ when the cysteine is dissociated. Activation may be induced by various stimuli affecting dissociation of the cysteine residue from the zinc atom. For example, alkylating agents such as NEM presumably alkylate the cysteine, causing its dissociation from the zinc atom, with concomitant exposure of the active site, leading to GHR proteolysis and GHBP shedding. II. Alternatively, secretase activity may be regulated by phosphorylation by PKC. Hydroxamic acid-based inhibitors block both NEM- and PKC-induced activation, whereas protein kinase inhibition will inhibit only PKC-induced activation. The interrelationship between stages I and II has not yet been clarified.
proposed to describe repression/activation of matrix-type metalloproteases (Springman et al. 1990, Van Wart & Birkedal-Hansen 1990). This mechanism was also shown for human ADAM 12 protease (Loechel et al. 1999) and for MDC9 and TACE (Roghani et al. 1999, Schlondorff et al. 2000); it is now thought to be related to the maturation of the pro-protein, in the Golgi secretory apparatus, through cleavage of the N-terminal prodomain, whose cysteine ligates the catalytic Zn$^{2+}$ and inhibits the protease. Dissociation of the cysteine is, therefore, a prerequisite for translocation to the cell membrane and for protease activity. Thus, activation may be induced by a variety of stimuli, all of which affect the dissociation of the cysteine residue from the zinc atom. For example, alkylating agents, such as NEM, will presumably alkylate the cysteine, causing its dissociation from the zinc atom and the concomitant exposure of the active site. The observation that the truncated GHR is known not to undergo internalization (Amit et al. 1997) and that it is activated by NEM to the same degree as the full length isoform (Amit et al. 1999), provides support for the involvement of the cysteine-switch mechanism in the translocation of the protease to the cell membrane. An additional mechanism to regulate protease activity may involve phosphorylation of its cytoplasmic tail by PKC. Hydroxamic acid-based inhibitors block protease activation induced both by NEM and by PMA-stimulated PKC activation, whereas a variety of PKC inhibitors (Alele et al. 1998, Saito et al. 1998) and the general protein kinase inhibitor staurosporine, will inhibit only PKC-induced protease activation. Since PMA increased GHBP generation to a similar degree in CHO/hGH and in CHO/hGH$_{tr}$ and since, as referred to above, GHR$_{tr}$ is not internalized, it may be postulated that such or most of the protease activity occurs at the cell surface. Since the hydroxamic acid-based Ro31–9790 metalloprotease inhibitor also inhibited constitutive or basal, FCS-dependent GHBP release, this may well suggest that a similar endogenous activation mechanism is operational in a physiological context.

In conclusion, it is likely that a closely related family of metalloproteases may control the surface expression of multiple integral membrane proteins, including GHR. It will be of particular interest to determine whether a common mechanism is responsible for the generation of GHBP and for the processing of other cytokine receptors.

Acknowledgements

We are most grateful to Roche Discovery (Welwyn Garden City, UK) for providing us with the Ro31–9790 compound and to British Biotech Pharmaceuticals Ltd (Oxford, UK) for their BB-3103 inhibitor. We thank Biotechnology General (Rehovot, Israel) for their gift of recombinant hGH, Dr M.J. Waters (Queensland, Australia) for providing us with the GHHR antibody and Dr S. Amsellem (Creteil, France) for the CHO/hGHHR and CHO/hGHHR$_{tr}$. The authors are grateful for the financial support of the Technion-Visc President for Research, Hedson Fund for Medical Research, of The Public Committee for Distribution of State Legacies, the Israel Ministry of Health (to R J B and T A) and of the Rappaport Family Research Institute (to M B H). This study is in partial fulfillment of the requirements of the Technion-Israel Institute of Technology for an MSc degree by M Y-F. We also thank Mrs Iris Fichmann for typing the manuscript.

References


Preece G, Murphy G & Ager A 1996 Metalloproteinase-mediated regulation of TGF-α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signalling cascades. EMBO Journal 18 6982–6972.

Young JR, Roussel MF & Sherr CJ 1989 Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. Molecular and Cellular Biology 9 2890–2896.

Fan H & Derynick R 1999 Ectodomain shedding of TGF-α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. EMBO Journal 18 6982–6972.


King APJ, Tseng MJ, Logsdon CD, Billestrup N & Carter-Su C 1996 Distinct cytoplasmic domains of the growth hormone receptor are required for glucocorticoid- and phosphol ester-induced decreases in growth hormone (GH) binding. These domains are different from that reported for GH-induced receptor. Journal of Biological Chemistry 271 18088–18094.


Loo SY, Sotiropoulos A, Goujon L, Simonin G, Kelly PA, Postel-Vinay MC & Finidori J 1993 Evidence for generation of the growth...
hormone-binding protein through proteolysis of the growth hormone membrane receptor. Endocrinology 132 1863–1865.

Received 4 January 2001
Accepted 12 January 2001