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Blockade of Growth Hormone Receptor Shedding by a Metalloprotease Inhibitor*

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ABSTRACT

GH, an important growth-promoting and metabolic hormone, exerts its biological effects by interacting with cell surface GH receptors (GHRs). The GHR is a single membrane-spanning protein that binds GH via its extracellular domain. The high affinity GH-binding protein (GHBP), which corresponds to a soluble form of the GHR extracellular domain, carries a substantial fraction of the GH in the circulation of various species and probably has a role in modulation of the hormone's bioavailability. Although in rodents, it is believed that the GHBP is largely derived by translation of an alternatively spliced GHR messenger RNA, in humans and rabbits, proteolytic cleavage of the membrane-anchored receptor releases the GHR extracellular domain, which is believed to thereby become the GHBP. In this study, we used human IM-9 lymphocytes and GHR antibodies to study this proteolytic shedding of the GHBP. As determined by immunoblotting with anti-GHR cytoplasmic domain serum, addition of phorbol 12-myristate 13-acetate (PMA; 1 μg/ml) to serum-starved cells led to rapid loss (roughly 60% decline after 1 h; $t_{1/2}$ = ~5 min) of mature GHRs (115–140 kDa) from either total cell or detergent-soluble extracts. Loss of full-length GHRs was accompanied by accumulation of four proteins (65–68 kDa), each reactive with the cytoplasmically directed antiserum. The pattern of appearance of these GHR ctyoplasmic domain proteins, the electrophoretic and immunological characteristics of which are similar to those of a recombinant rabbit GHR mutant that lacks the extracellular domain, was such that progressively faster migrating forms were evident between 5–60 min of PMA exposure. Treatment with *N*-ethylmaleimide (NEM; 5 mM), an agent known to cause GHBP shedding from IM-9 cells, promoted a similar rapid loss of full-length GHRs and an accumulation of GHR cytoplasmic domain remnant proteins. PMA-induced, but not NEM-induced, GHR proteolysis was blocked by the protein kinase C inhibitor, GF109203X. Both PMA- and NEM-induced receptor proteolysis were, however, inhibited by the metalloprotease inhibitor, Immunex Compound 3 (minimum effective concentration, 10 μM). Notably, PMA and NEM also promoted shedding of GHBP into the conditioned medium of the cells, as determined by a chromatographic [^{125}I]human GH binding assay; this GHBP shedding was also inhibited by Immunex Compound 3. These results strongly implicate a member(s) of the metalloprotease family as a potential GHBP-generating enzyme. (*Endocrinology* **139:** 1927–1935, 1998)

GH IS AN important growth-promoting and metabolic hormone (1). GH initiates its intracellular signaling cascades by interaction with cell surface GH receptors (GHRs). The membrane-anchored surface GHR is a glycoprotein comprised of a ligand-binding extracellular domain, a transmembrane domain, and a 350-residue cytoplasmic domain, which is required for signaling (2, 3).

In various species, a substantial fraction of circulating GH is carried by a high affinity GH-binding protein (GHBP), the structure of which corresponds to the GHR extracellular domain (4–6). In rodents, it is believed that the GHBP is largely derived by translation of an alternatively spliced GHR messenger RNA encoding only the receptor extracellular domain and a hydrophilic tail, which replaces the transmembrane and cytoplasmic domains and facilitates the protein's secretion (7–9). In humans and rabbits, proteolytic cleavage of the membrane-anchored receptor [either the full-length GHR or recently described truncated GHR forms (10, 11)] releases the GHR extracellular domain, which thereby becomes the GHBP (4, 5).

The mechanistic details of proteolytic shedding of the human and rabbit GHRs have remained elusive despite the establishment of cell culture models to facilitate such studies (12–20). Constitutive shedding of both endogenous and transfected GHRs has been observed; some degree of inhibition of proteolytic release has been achieved with EDTA and certain protease inhibitors, and a divalent metal dependence for the proteolysis has been postulated (16). Treatment of cells with sulfhydryl alkylating reagents promotes GHR shedding, although the mechanism of this effect is not clear (12, 13, 15, 19).

Using antibodies directed against both the receptor's extracellular and cytoplasmic domains, we now demonstrate in human IM-9 cells [B lymphoblasts that homologously express human (h) GHRs] that pharmacological activation of protein kinase C by phorbol 12-myristate 13-acetate (PMA) promotes rapid proteolytic cleavage of the GHR that is sim-

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ilar to but distinguishable from that promoted by the sulfhydryl alkylator, *N*-ethylmaleimide (NEM). We also observe that constitutive, PMA-induced, and NEM-induced GHR proteolysis are all strongly inhibited by the protease inhibitor, Immunex Compound 3 (IC3). Notably, IC3 also inhibits NEM- and PMA-induced shedding of the GHBP into the conditioned medium of these cells. These results strongly implicate a member(s) of the metalloprotease family as a potential GHBP-generating enzyme.

Materials and Methods

Materials

PMA and NEM were purchased from Sigma Chemical Co. (St. Louis, MO) as were routine reagents, unless otherwise noted. The protein kinase C inhibitor, GF109203X, was purchased from Calbiochem (San Diego, CA). Routine molecular biology reagents were purchased from New England Biolabs (Beverly, MA). IC3, the same as Compound 2 (21) except that the naphthyl-alanine side-chain is replaced by a *tert*-butyl group, was produced by Immunex Corp. (Seattle, WA).

Cells, cell culture, and transient transfections

IM-9 and COS-7 cells were maintained as described previously (22). IM-9 cells were treated with PMA or NEM [or dimethylsulfoxide (DMSO) vehicle control] after serum starvation for 16–20 h. Serum starvation for both IM-9 and COS-7 cells was accomplished by substituting 0.5% (wt/vol) BSA (fraction V, Boehringer Mannheim, Indianapolis, IN) for FCS in the culture medium.

COS-7 cells (6×10^6/dish) were transiently transfected in 10 ml DMEM medium in 100×20-mm dishes (Falcon, Oxnard, CA) by the calcium phosphate precipitation method as described previously (23). Each dish was transfected with 30 μg pSX rGHR or pSX $rGHR_{del\ ext}$, which are described below, or (for a negative control) with the pSX vector only. Serum starvation was begun 24 h after transfection and continued for 18–20 h before harvesting and detergent extraction, as described below.

Antibodies

Polyclonal rabbit anti-GHR_{cyt} serum (24), directed at the residue 317–620 region of the human GHR cytoplasmic domain, has been described. The anti-GHR_{ext} mouse monoclonal antibody (IgG1 κ) was raised against a glutathione-*S*-transferase (GST) fusion protein incorporating residues 1–245 of the rabbit (r) GHR (2) [GST/rGHR-(1–245)] and screened by ELISA at the University of Alabama Multipurpose Arthritis Center Hybridoma Facility (Dr. M. Accaviti). [Induction and purification of the fusion protein have been described previously (25).] Anti-GHR_{ext} reacted by ELISA specifically with GST/rGHR-(1–245) and not with GST. Similarly, immunoblotting of thrombin-cleaved GST/rGHR-(1–245) and GST/hGHR-(271–620) fusions indicated that anti-GHR_{ext} reacted with rGHR-(1–245), but not with hGHR-(271–620) or GST; this pattern of reactivity was identical to that of mAb 263, a well characterized, commercially available (Agen) monoclonal antibody to the GHR extracellular domain (not shown). Anti-GHR_{ext} was purified from mouse ascites using the Affi-Gel Protein A MAPS II Kit (Bio-Rad, Richmond, CA) according to the manufacturer's suggestions.

Plasmid construction

The pSX plasmid (a gift from Dr. J. Bonifacino, NIH, and Dr. K. Arai, DNAX) drives eukaryotic protein expression from the SRα promoter, which is composed of the simian virus 40 early promoter and the R-U5 segment of the human T cell leukemia virus-1 long terminal repeat (26). The generation of pSX and the ligation into it at the *Xba*I and *Kpn*I sites of the rGHR complementary DNA (cDNA) (2) (pBS.RGHR1.1, a gift from Dr. W. Wood, Genentech, South San Francisco, CA) to generate the expression plasmid, pSX rGHR, has been previously described (25). The cDNA encoding the $rGHR_{del\ ext}$ mutant [in-frame internal deletion of residues 1–247 of the rGHR with preservation of the predicted signal sequence (residues −18 to −1)] was generated by PCR using the pBS.RGHR 1.1 plasmid as a template. The 5′-primer corresponded to a sequence in the Bluescript plasmid 5′ to the multiple cloning site. The 3′-primer was an oligonucleotide 36-mer, which 5′ to 3′ corresponded antisense to bases encoding residues 249 and 248 (a naturally occurring and unique *Nco*I restriction site) and −1 to −8 of the rabbit GHR (2). This PCR product was ligated into the *Not*I (from the pBS multiple cloning site) and *Nco*I sites of pBS.RGHR1.1 after removal from that plasmid of the *Not*I-*Nco*I fragment that encoded all rabbit GHR residues amino-terminal to residue 248. The resultant plasmid, pBS $rGHR_{del\ ext}$, was subjected to dideoxy-DNA sequencing to ensure the presence of the in-frame internal deletion mutation and the fidelity of the remaining sequences exposed to PCR amplification. The cDNA region encoding $rGHR_{del\ ext}$ was then ligated into pSX using the *Xba*I and *Kpn*I sites to generate the expression plasmid, pSX $rGHR_{del\ ext}$.

Cell stimulation, protein extraction, and immunoprecipitation

Serum-starved IM-9 cells were resuspended at $25–50 \times 10^6$ cells/ml in binding buffer [BB; consisting of 25 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.01% (wt/vol) BSA, and 1 mM dextrose]. After a 15-min preincubation at the specified temperature for equilibration, PMA, NEM, or their vehicle, DMSO (each added at a 1:100 to 1:200 dilution), was added at the indicated final concentrations. For time-course experiments, PMA, NEM, and DMSO were added such that all stimulations ended simultaneously. After gentle agitation to ensure adequate mixing, cells were incubated in a gently shaking water bath for the indicated durations. For inhibitor experiments, GF109203X or IC3 (each solubilized in DMSO) was added at a 1:100 dilution to the indicated final concentrations, generally during the 15-min preincubation period, unless otherwise indicated.

Stimulations were terminated, and IM-9 cells were collected by centrifugation ($800 \times g$ for 1 min at 4 C) and aspiration of the BB. Cell pellets were either processed directly or snap-frozen in liquid nitrogen and stored at −80 C before processing (no difference in results was noted regardless of whether cell pellets were first frozen). Detergent cell extracts were generated by resuspending the cell pellet at 100×10^6 cells/ml in a lysis buffer consisting of 1% (vol/vol) Triton X-100, 150 mM NaCl, 10% (vol/vol) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, and 10 μg/ml aprotinin and incubating for 15 min at 4 C. After centrifugation at $15{,}000 \times g$ for 15 min at 4 C, the detergent-soluble supernatant was collected and mixed with an equal volume of twice concentrated ($2 \times$) SDS sample buffer [$1 \times$ sample buffer contains 1% (wt/vol) SDS, 50 mM Tris-HCl (pH 6.8), 10% (wt/vol) glycerol, and 3% (vol/vol) 2-mercaptoethanol] and boiled before SDS-PAGE. Total cell extract was prepared as previously described (27); in brief, stimulation of cells in BB at 25×10^6 cells/ml was terminated by the addition of an equal volume of boiling $2 \times$ sample buffer. The resulting mixture was clarified by boiling and vortexing before SDS-PAGE. COS-7 detergent cell extracts were prepared as described for IM-9 cells, except that the cells were collected by scraping and centrifugation in ice-cold PBS before addition of the lysis buffer.

For immunoprecipitation of the hGHR with the monoclonal anti-GHR_{ext} antibody, 0.6 μg purified antibody was added to detergent cell extract and tumbled on a rotator at 4 C for 2 h before the addition of protein G-Sepharose (Pharmacia, Piscataway, NJ) to adsorb immune complexes. After extensive washing with lysis buffer, SDS sample buffer eluates were resolved by SDS-PAGE, as described below.

Electrophoresis and immunoblotting

Resolution of proteins in immunoprecipitates, detergent cell extracts, and total cell extracts (prepared as above) under reducing conditions by SDS-PAGE, Western transfer of proteins, and blocking of nitrocellulose membranes (Hybond-ECL, Amersham, Arlington Heights, IL) with 2% BSA were performed as previously described (22, 28). Anti-GHR_{cyt} serum was affinity purified (or used without purification (1:2000)) and used for immunoblotting as previously described (27). Horseradish-peroxidase-conjugated antirabbit secondary antibodies (1:1500) and ECL detection reagents (all from Amersham) were used for detection of specifically recognized hGHRs according to the manufacturer's suggestions.

GHBP assay

GHBP activity was measured in conditioned medium by a standardized GH binding assay (29). Briefly, conditioned medium from IM-9 cells treated as indicated in Fig. 6 was incubated with freshly labeled [^{125}I]hGH (~0.5 ng) for 45 min at 37 C. Bound GH was then immediately separated from free GH by gel chromatography on a Sephadex G-100 column at 4 C. The fraction of GH bound was determined by peak integration. Nonspecific binding under these conditions is about 1.5% and was subtracted. The coefficient of variation for the assay is 7.5%.

Data analysis

Densitometry of ECL immunoblots was performed using a solid state video camera (Sony 77, Sony Corp., Park Ridge, NJ) and a 28-mm MicroNikkor lens over a lightbox of variable intensity (Northern Light Precision 890, Imaging Research, Toronto, Canada). Quantification was performed using a Macintosh II-based image analysis program (Image 1.49, developed by W. S. Rasband, Research Services Branch, NIMH, Bethesda, MD). The fraction of full-length hGHRs or remnants remaining in extracts from hGH-treated cells was estimated by measuring by densitometry the intensity of the specifically detected hGHR or remnant signal relative to that signal present within the same experiment in extracts from unstimulated cells. As indicated when graphically shown, pooled data from numerous experiments are displayed as the mean ± SEM The significances of differences of pooled results are estimated by unpaired *t* tests.

Results

PMA promotes rapid proteolysis of GHRs in IM-9 cells

Phorbol esters have been shown in various systems to cause rapid down-regulation of cell surface GHRs (30, 31). In investigating this phenomenon, we used a newly prepared monoclonal antibody to the GHR extracellular domain (anti-GHR_{ext}) to immunoprecipitate detergent-soluble hGHRs from extracts of IM-9 cells treated with PMA or vehicle only (Fig. 1A). Full-length receptors present in these immunoprecipitates were detected after SDS-PAGE by immunoblotting with an antibody directed at the cytoplasmic domain (anti-GHR_{cyt}). Significant loss of full-length hGHRs was detected after as little as 5 min of treatment with PMA [1 μg/ml (1.6 μM) at 30 C].

Rapid PMA-induced disappearance of detergent-soluble full-length hGHRs was also observed if extracted proteins were resolved without prior immunoprecipitation and immunoblotted with anti-GHR_{cyt} (Fig. 1B). In addition, this analysis revealed concurrent PMA-induced accumulation of a group of anti-GHR_{cyt}-reactive proteins of 65–68 kDa (Fig. 1B, *arrowheads*) that were not detected in anti-GHR_{ext} precipitates (compare Fig. 1B with Fig. 1A). The pattern of appearance of these GHR cytoplasmic domain-containing proteins was such that progressively faster migrating forms were evident between 5–60 min of PMA exposure; the predominant (and fastest migrating) form present at 60 min migrated identically to a protein present, but less abundant, in the sample exposed to the DMSO vehicle only (compare the proteins indicated by the *lowest arrowhead* in lanes 1 and 4). Neither the hGHR nor the 65- to 68-kDa proteins were detected when a nonimmune serum was used for immunoblotting (data not shown).

The same pattern of acute PMA-induced receptor loss and corresponding appearance of 65- to 68-kDa anti-GHR_{cyt}-reactive proteins observed in detergent-soluble extracts was noted when cells were directly solubilized in boiling SDS-PAGE sample buffer (thus generating total cell extracts) before electrophoresis and anti-GHR_{cyt} immunoblotting (Fig. 1C). This finding indicated that PMA appears to cause a loss of total cell full-length hGHRs rather than a redistribution of receptors to a detergent-insoluble pool, such as we recently reported to occur in these cells in response to hGH (27). Quantitative analysis of the kinetics of this PMA-induced loss of total cell hGHRs is presented in Fig. 1D, in which the pooled results of several experiments such as that shown in Fig. 1C are displayed. At 37 C, 1 μg/ml PMA promoted loss of hGHRs with a $t_{1/2}$ of about 5 min. In separate experiments (data not shown), as little as 0.01–0.1 μg/ml PMA induced receptor loss and the corresponding appearance of 65- to 68-kDa anti-GHR_{cyt}-reactive proteins.

The apparent conversion of full-length GHRs into a discrete set of smaller proteins immunologically identified as including the receptor cytoplasmic domain made us consider that this process might represent a constitutive discrete proteolytic cleavage of the transmembrane-anchored receptor that is enhanced by PMA. Such a process would be analogous to that observed for some other receptors and procytokines (32, 33), in which the extracellular domain is released (shed) into the medium and a transmembrane/cytoplasmic domain remnant is thereby generated. As the constitutive proteolysis of the hGHR and rabbit GHR involved in GHBP generation is believed to occur at or near the receptor extracellular-transmembrane domain junction (13, 15), we tested whether a recombinantly derived rGHR remnant would share properties with the remnant generated by expression of the full-length rGHR.

As indicated in Fig. 2A, we generated a cDNA encoding a mutant receptor, designated $rGHR_{del\ ext}$, in which the rGHR extracellular domain is deleted, but the wild-type rGHR transmembrane and cytoplasmic domains remain. COS-7 cells were transiently transfected with expression plasmids encoding the wild-type rGHR, $rGHR_{del\ ext}$, or (as a negative control) no insert (Fig. 2B, lanes 1–3). Detergent extracts of each pool of transfected cells were resolved by SDS-PAGE and immunoblotted with anti-GHR_{cyt}. Bands at approximately 115 and 66 kDa were detected in the resolved extract of cells transfected with wild-type rGHR, but not in that of cells transfected with the empty vector (compare lanes 1 and 3). As we have previously observed (28), the 115-kDa band is consistent with the mature, fully glycosylated full-length rGHR, whereas the 66-kDa anti-GHR_{cyt}-reactive band, constitutively detected in these cells, is analogous to the protein(s) of similar M_r in the IM-9 cells described above. Notably, $rGHR_{del\ ext}$ (lane 2), which had the expected specific anti-GHR_{cyt} reactivity, migrated nearly identically to the 66-kDa protein constitutively generated in the rGHR-expressing cells. Thus, this 66-kDa protein and, by inference, those observed basally and in response to PMA in IM-9 cells, have electrophoretic and immunologic characteristics consistent with a GHR transmembrane/cytoplasmic domain remnant.

A protein kinase C inhibitor blocks PMA-enhanced, but not NEM-enhanced, hGHR proteolysis

Sulfhydryl-reactive alkylating reagents have been shown to enhance GHBP shedding from IM-9 cells (12, 13, 15).

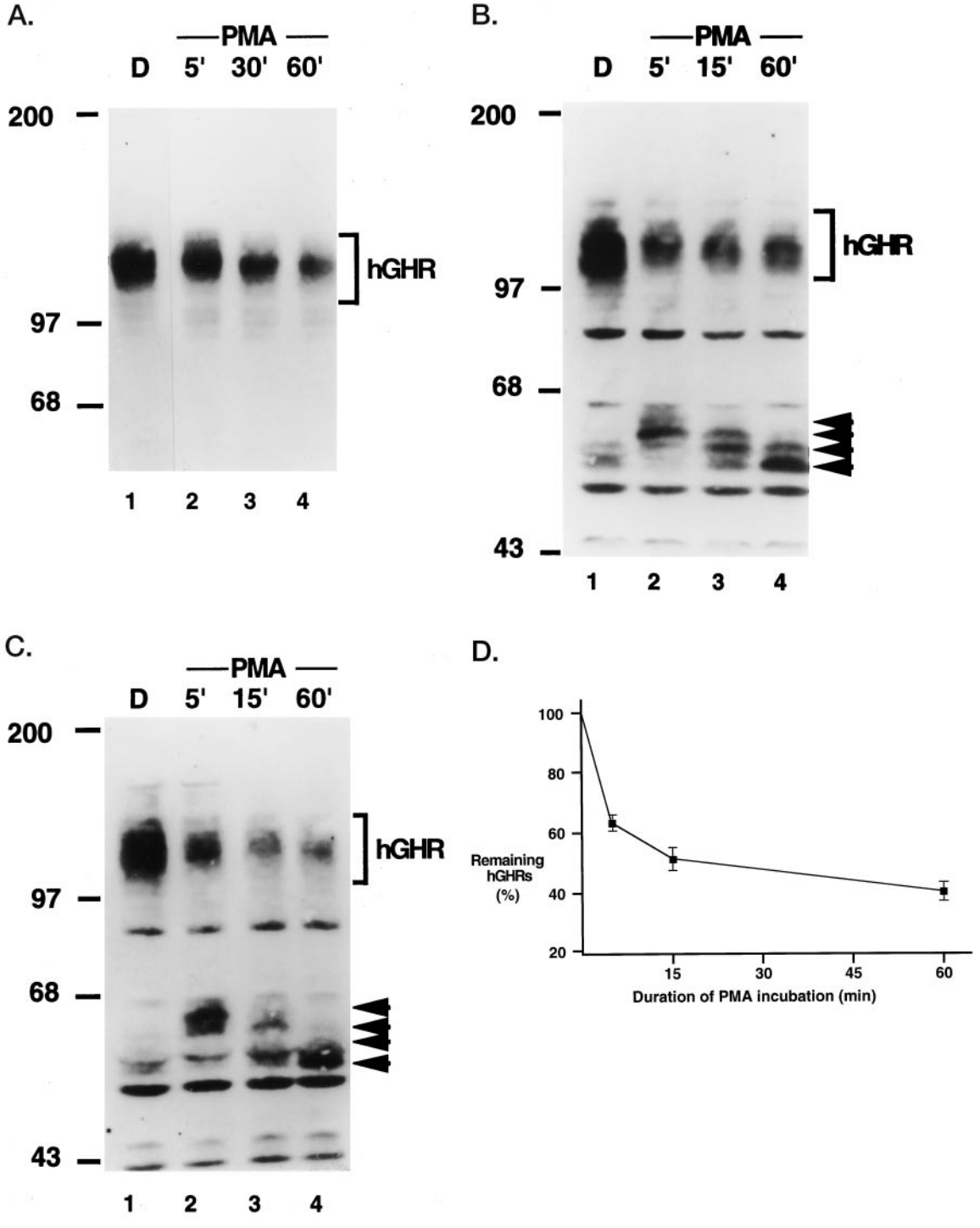

FIG. 1. PMA induces loss of IM-9 hGHRs and corresponding accumulation of 65- to 68-kDa anti-GHR_{cyt}-reactive proteins. A and B, PMA-induced proteolysis of detergent-soluble hGHRs. IM-9 cells (20 million/sample in A; 5 million/sample in B) were exposed to PMA (1 μg/ml) for the indicated durations or to the DMSO vehicle (D) as indicated at 30 C (A) or 37 C (B). Detergent extracts were prepared as described in *Materials and Methods* and were immunoprecipitated with anti-GHR_{ext} in A. Eluates (A) or unprecipitated extracts (B) were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR_{cyt}. In each case, the full-length hGHR (115–140 kDa) is indicated by a *bracket*, while the *arrowheads* in B indicate 65- to 68-kDa anti-GHR_{cyt}-reactive proteins appearing in unprecipitated extracts (but not in anti-GHR_{ext} precipitates) in response to PMA. C and D, PMA-induced proteolysis of total cell hGHRs. C, Cells were treated as described in A and B above. Total cell extracts (1.25 million/sample), prepared as described in *Materials and Methods*, were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR_{cyt}. As in B, the full-length hGHR and 65- to 68-kDa anti-GHR_{cyt}-reactive proteins appearing in response to PMA are indicated by a *bracket* and *arrowheads*, respectively. D, Densitometric analysis of the full-length total cell hGHRs remaining after various durations of PMA treatment, as assessed by immunoblotting with anti-GHR_{cyt}, in multiple experiments, such as in C. In each experiment, the amount of receptor present in DMSO-treated samples was considered 100%. The mean ± SEM are shown for several determinations (5 min, n = 5; 15 min, n = 4; 60 min, n = 4).

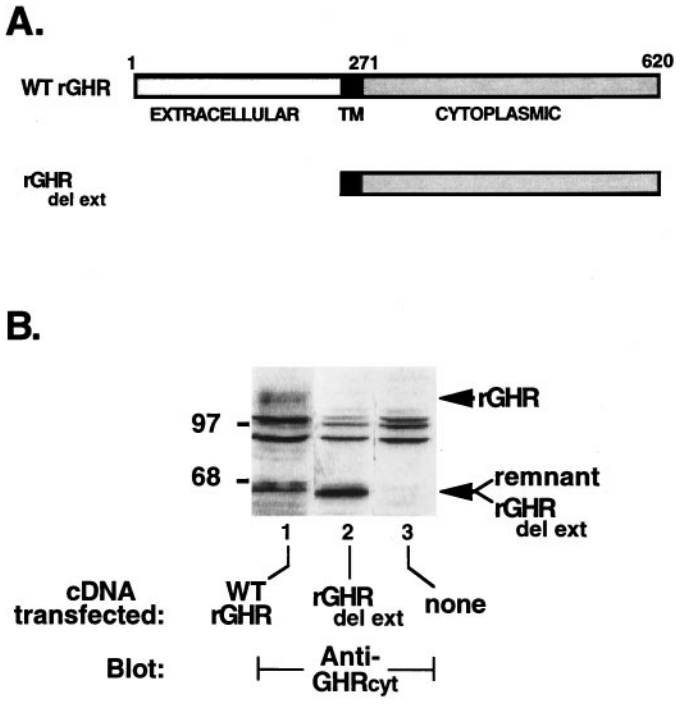

FIG. 2. Comigration and anti-GHR_{cyt} reactivity of a constitutively generated rGHR remnant and the recombinant rGHR mutant, $rGHR_{del\ ext}$. A, Diagram of the structure of the wild-type (WT) rGHR and the recombinant rGHR mutant, $rGHR_{del\ ext}$, generated as described in *Materials and Methods*. B, COS-7 cells were transiently transfected with expression plasmids encoding WT rGHR, $rGHR_{del\ ext}$, or, as a negative control, the empty vector only, as indicated. Detergent extracts of equal aliquots of the transfected cells were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR_{cyt}. The positions of the full-length rGHR and the constitutively generated and recombinantly produced remnant proteins, all specifically detected, are indicated. Note the equivalence of protein loading, as evidenced by the collection of nonspecific bands in the 97-kDa region.

Although thought to be proteolytic, the mechanism(s) accounting for this phenomenon is incompletely understood. We sought to determine whether the alkylator NEM would promote proteolytic cleavage of the hGHR in the same fashion as PMA. As shown in Fig. 3A, treatment with NEM (5 mM) for only 5 min induced the loss of full-length hGHRs detectable in total cell extracts and led to the appearance of a remnant similar to those observed with PMA treatment. In other experiments (not shown), the minimum effective NEM concentration to promote this processing was 0.5 mM.

As PMA can function as a pharmacological activator of protein kinase C, we tested whether PKC inhibition would block PMA- and/or NEM-induced hGHR proteolysis. As expected, when cells were pretreated with GF109203X, an inhibitor of PKC, PMA failed to promote either loss of total cell hGHRs or appearance of remnant proteins (Fig. 3B, compare lanes 5–8 with lanes 1–4). Notably, pretreatment with GF109203X under the same conditions had no inhibitory effect on NEM's ability to induce rapid receptor loss and remnant appearance (Fig. 3C, compare lanes 5–8 with lanes 1–4). Thus, although both PMA and NEM promote apparently similar hGHR proteolytic processing, NEM may be working through a PKC-independent pathway.

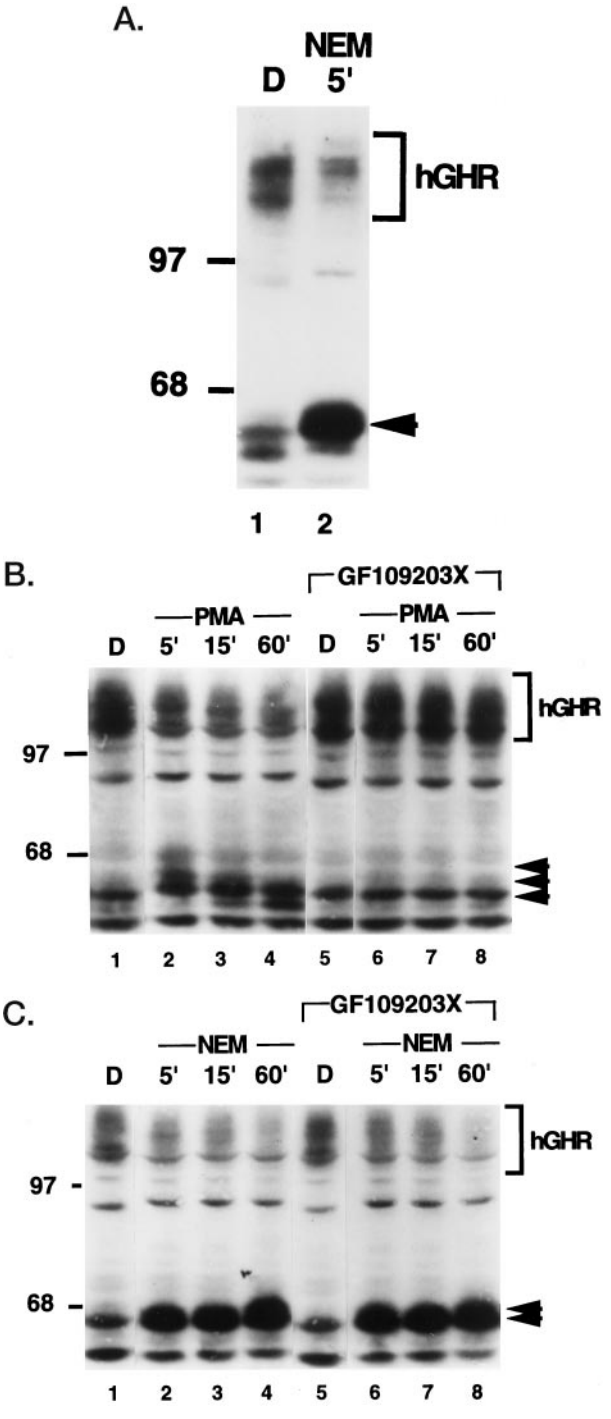

FIG. 3. A protein kinase C inhibitor blocks PMA-induced, but not NEM-induced, hGHR proteolysis. A, NEM-induced hGHR proteolysis. IM-9 cells (1.25 million/sample) were exposed to NEM (5 mM; 5 min) or DMSO at 37 C. Total cell extracts were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR_{cyt}. The full-length hGHR and hGHR remnant protein appearing in response to NEM are indicated by a *bracket* and an *arrowhead*, respectively. B and C, Cells (1.25 million/sample) pretreated with or without the PKC inhibitor, GF109203X (as described in *Materials and Methods*), were treated with DMSO or PMA (B) or NEM (C) for the indicated durations, as in Figs. 1C and 3A. Total cell extracts were resolved by SDS-PAGE and immunoblotted with anti-GHR_{cyt} (1:2000 dilution). The full-length hGHR and hGHR remnant proteins are indicated by a *bracket* and *arrowheads*, respectively.

The metalloprotease inhibitor, IC3, blocks PMA-enhanced, NEM-enhanced, and constitutive hGHR proteolysis

Previous reports indicated a possible divalent metal dependence for the constitutive GHBP shedding from IM-9 cells (16); additionally, it has recently been determined that the PMA-enhanced proteolytic shedding of the transmembrane form of the cytokine tumor necrosis factor-α (TNFα) that yields soluble TNFα is mediated by a metalloprotease activity (34, 35). Given these observations, we investigated whether the metalloprotease inhibitor, IC3, which blocks several metalloprotease-mediated shedding events (21, 36–40), would also be active in inhibiting the PMA- and/or NEM-enhanced hGHR proteolytic processing that we observed in IM-9 cells.

In the experiment shown in Fig. 4A, IM-9 cells were treated at 37 C with PMA for 5, 15, or 60 min in the presence of IC3 (50 μM; added 70, 60, or 15 min, respectively, before the addition of PMA) or in its absence (addition of the DMSO vehicle instead of IC3). Notably, anti-GHR$_{cyt}$ immunoblotting of resolved total cell extracts indicated that the presence of IC3 completely inhibited both the loss of full-length hGHR and the accumulation of remnants promoted by PMA at each time point (compare lanes 5–8 with lanes 1–4). In concentration dependence experiments (not shown), detectable PMA-enhanced proteolytic processing of the hGHR under these conditions was completely inhibited in the presence of as little as 10 μM IC3. Time-course experiments (not shown) also indicated that IC3 preincubation ($\geq$10 μM) for as little as 5 min blocked PMA-enhanced hGHR proteolysis. IC3 dramatically inhibited NEM-enhanced hGHR processing as well (Fig. 4B). Comparison of lanes 5–8 with lanes 1–4 shows marked diminution in both NEM-enhanced hGHR loss and remnant generation with IC3 treatment.

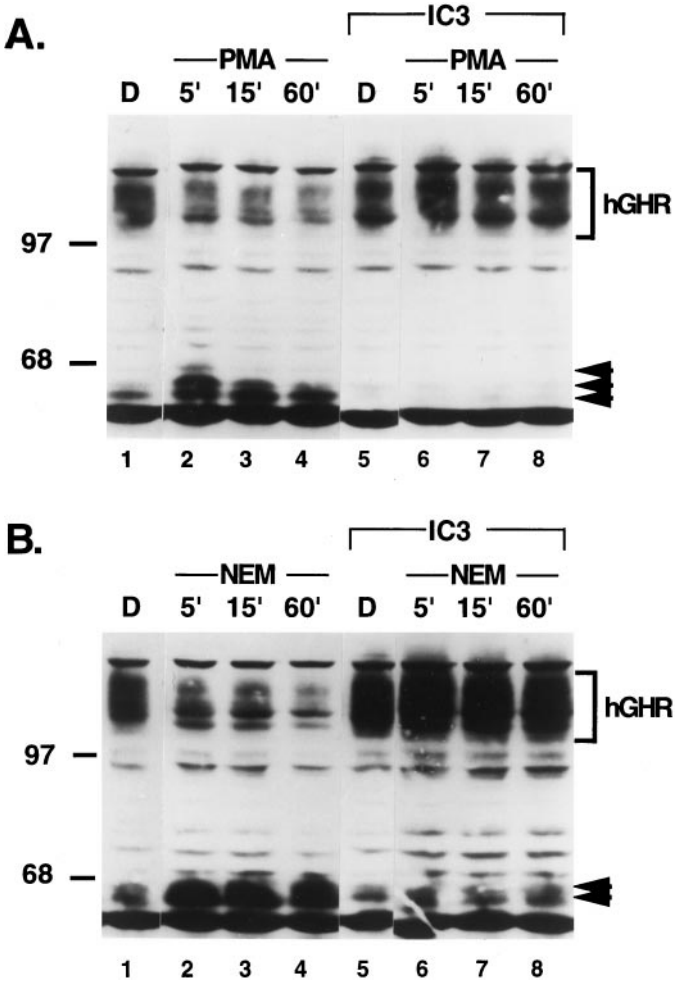

FIG. 4. The metalloprotease inhibitor, IC3, blocks PMA- and NEM-induced hGHR proteolysis. A and B, Cells (1.25 million/sample) pretreated with or without IC3 (as described in *Materials and Methods*), were treated with DMSO or PMA (A) or NEM (B) for the indicated durations, as described in Fig. 3, B and C. Total cell extracts were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR$_{cyt}$. The full-length hGHR and hGHR remnant proteins are indicated by a *bracket* and *arrowheads*, respectively.

Notably, comparison of lanes 1 and 5 in Fig. 4, A and B (samples in which the effect of IC3 alone, in the absence of NEM or PMA, can be observed) indicates that exposure of the cells to IC3 for 75 min at 37 C resulted in a greater abundance of total cell hGHRs and a decrease in an already low basal level of remnant(s). This effect of IC3 on constitutive GHR proteolysis is presented quantitatively in Fig. 5, in which the results of several such anti-GHR$_{cyt}$ immunoblotting determinations were densitometrically measured and expressed as the abundance of full-length GHR or remnant present after IC3 exposure relative to that present after exposure to the DMSO vehicle alone. IC3 treatment resulted, on the average, in a 26% increase in steady state, total cell, full-length GHRs and a 33% decline in the steady state remnant level. Thus, not only did IC3 inhibit PMA- and NEM-enhanced hGHR proteolytic processing; this metalloprotease inhibitor also inhibited a constitutive hGHR proteolysis that results in the loss of full-length receptor and the accumulation of the remnant protein. [Interestingly, similar analysis (not shown) of additional experiments such as those shown in Fig. 3 indicated that treatment with GF109203X alone for 75 min resulted in

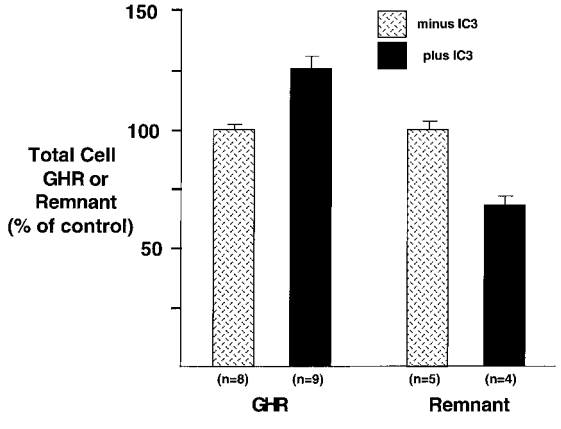

FIG. 5. IC3 inhibits constitutive hGHR proteolysis in IM-9 cells. Cells (1.25 million/sample) were treated with DMSO vehicle or IC3 (50 μM) for 75 min at 37 C. Total cell extracts were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-GHR$_{cyt}$. For multiple determinations (as indicated *under each bar*), the levels of full-length hGHRs or of hGHR remnant present in the IC3-treated samples were densitometrically compared with those present in the DMSO-treated samples. Pooled data for each are presented as the mean $\pm$ SEM. *P* values for IC3-induced accumulation of full-length hGHRs and decrease in hGHR remnant were 0.002 and 0.003, respectively.

a modest increase (16.4 ± 4.4%; $P < 0.01$) in GHR abundance and a decrease (16.2 ± 5.1%; $P < 0.05$) in remnant abundance compared with treatment with DMSO vehicle; thus, like PMA-induced receptor proteolysis, constitutive GHR proteolysis by an IC3-inhibitable protease may be at least in part mediated by PKC.]

IC3 inhibits GHBP shedding in IM-9 cells

Given the significant inhibition of constitutive and induced proteolytic processing of the hGHR by IC3 noted above (Figs. 4 and 5), we investigated whether this would be reflected by inhibition of GHBP shedding as well. IM-9 cells were incubated for 90 min at 37 C in the presence of PMA, NEM, or the DMSO vehicle, and the conditioned media of the cells were assayed for GHBP activity by a standardized GH binding assay (29), as described in *Materials and Methods*. As shown in Fig. 6A, PMA and NEM both induced significant elevations in GHBP in the cell supernatants (from 3% GH bound/350 μl conditioned medium for DMSO treatment (constitutive shedding under these circumstances) to 16% and 24% for PMA- and NEM-treated cells, respectively). Notably, inclusion of IC3 (50 μM) during the incubation eliminated (for PMA) or greatly reduced (for NEM) these PMA- and NEM-induced increases in GHBP activity in the conditioned medium (compare PMA- and NEM-treated samples to those treated with PMA plus IC3 and NEM plus IC3). The already low level of constitutively shed GHBP during this 2-h experiment was not detectably diminished by IC3 treatment (compare DMSO to DMSO plus IC3 samples). Qualitatively very similar results were obtained when the same experiment was performed at 30 C (Fig. 6B), except that the levels of GHBP shed in response to PMA and NEM were reduced at that lower temperature.

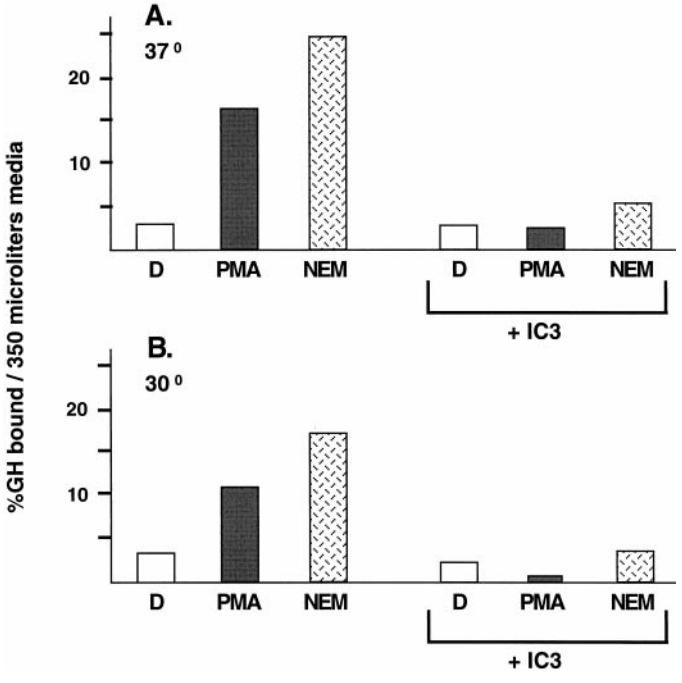

FIG. 6. IC3 inhibits PMA- and NEM-induced GHBP shedding from IM-9 cells. A and B, Cells (50 million/sample) were treated with DMSO vehicle or IC3 (50 μM) for 15 min before treatment with DMSO, PMA, or NEM for 90 min at 37 C (A) or 30 C (B). Conditioned medium (350 μl) from each sample was incubated with [^{125}I]hGH as described in *Materials and Methods*. The fraction of GH bound to high affinity GHBP was derived by peak integration. Data shown in A and B each represent the mean of two experiments.

Discussion

PMA-induced down-regulation of cell surface GHRs has previously been described (30, 31). In IM-9 cells, the receptor down-regulation was rapid [roughly half of surface GHRs lost by treatment at 37 C with PMA (≥1 μM) for 30 min] (30). It was concluded that the PMA-induced receptor down-regulation was related to acute internalization of the GHR, probably as a consequence of activation of protein kinase C. However, in that study the conditioned medium of the cells was not examined for the presence of GHBP-like activity, nor was the presence of a smaller cell-associated receptor remnant sought. Intriguingly, however, a 55-kDa phosphoprotein (thought to be receptor associated rather than a GHR fragment *per se*) was observed to inducibly copurify with the IM-9 receptor in response to PMA (30).

Our findings correspond to some aspects of the above observations and differ in other respects. The time course of the PMA-induced loss of full-length hGHRs that we observed by anti-GHR_{cyt} immunoblotting of anti-GHR_{ext} immunoprecipitates, detergent extracts, or total cell extracts of IM-9 cells corresponds well to that seen for PMA-induced receptor down-regulation. However, our studies do not support intracellular redistribution as the sole explanation for PMA-induced receptor down-regulation, as the loss of hGHRs we detected was observed even in total cell extracts (cellular proteins extracted by directly boiling the cells in SDS sample buffer). [This point is bolstered by our observation that although we detected a hGH-induced detergent-insoluble pool of hGHRs in IM-9 cells, we did not detect any such accumulation of detergent-insoluble receptors in response to PMA (27).] The enhanced appearance in response to PMA of anti-GHR_{cyt}-reactive protein(s), the electrophoretic and immunoreactivity characteristics of which are consistent with their being transmembrane/cytoplasmic domain remnants, supports the idea that a significant component of PMA-induced hGHR loss in IM-9 cells is due to rapid and discrete proteolysis. The complete inhibition of PMA-induced receptor loss by the metalloprotease inhibitor IC3 furthers this conclusion.

Our studies have particular relevance with regard to understanding the mechanism(s) of GHBP generation. Although its physiological role is not known with certainty, it has been estimated that up to 50% of serum GH in the human circulation is carried by this high affinity binding protein (6). Experimental evidence exists in support of both an inhibitory and a potentiating role for the GHBP in GH signaling (41–44). Evidence regarding the physiological regulation of GHBP levels is also inconclusive (4, 5). As mentioned above, the rodent GHBP is believed to be largely derived by translation of an alternatively spliced GHR message that encodes the receptor external domain, but in which the transmembrane and cytoplasmic domains are replaced by a short hydrophilic sequence (7–9). By contrast, human and rabbit GHBPs are

believed to be largely derived by proteolytic shedding of the external domain of membrane-anchored GHRs, although recent studies indicate that splice variants of the hGHR message that encode membrane-anchored receptors that are prematurely truncated in the proximal cytoplasmic domain may account for some of the receptors that are shed (10, 11). It is not known whether this proteolysis occurs at the plasma membrane or at intracellular membranes.

Using the IM-9 cell, a well established cell culture model system for hGHR shedding, we observed that PMA promoted rapid proteolytic hGHR cleavage, with the resultant formation of a cell-associated transmembrane/cytoplasmic domain remnant and release into the conditioned medium of significant GHBP activity. We do not yet know whether the multiple forms of the remnant that appear with a reproducible time course during PMA treatment reflect proteolysis at several discrete, closely spaced sites or a reversible modification (such as phosphorylation) that occurs to the remnant during or just after its generation. The fact that the sulfhydryl alkylator, NEM, promoted generation of a similar remnant(s) in the process of shedding functional GHBP activity is consistent with the findings of Trivedi and Daughaday (13). We note that PKC inhibition prevented PMA-induced receptor proteolysis, whereas NEM's effect was insensitive to this inhibition. This interesting distinction may prove useful in further understanding the nature of the enzymatic activity(s) that mediates GHBP shedding. Although we do not yet know the identity of the cleaving enzyme(s), the finding that IC3, a well characterized metalloprotease inhibitor, inhibited both PMA- and NEM-induced proteolysis and GHBP shedding may indicate that the pathways or proteins affected by these two distinct reagents may ultimately lead to activation of the same or a similar GHBP-generating enzyme(s).

The potency of IC3 with regard to inhibition of PMA-induced hGHR proteolysis in our experiments was similar to that published for other IC3-inhibited shedding events (*e.g.* TNFα, interleukin-6 receptor α, and transforming growth factor-α) that have been intensively studied (21, 35–40). We find it particularly notable that constitutive hGHR proteolytic processing, in addition to that induced by PMA, was also IC3 sensitive in IM-9 cells. Our inability to clearly detect an inhibitory effect of IC3 on constitutive shedding similar to that observed for its effect on constitutive proteolysis probably reflects the limit of the sensitivity of the GHBP activity assay in discriminating a change in the already low level of GHBP constitutively generated during the 105-min incubation period. Nonetheless, the biochemical findings highlight the possibility that the same enzyme(s) might be responsible for both the dramatic pharmacological (PMA-induced) hGHR shedding and the more subtle, but probably more physiologically relevant, consititutive shedding exhibited by these (and perhaps other) cells. This avenue is currently being actively pursued. We are also intrigued by the potential functional relevance of the basally detected and PMA- and NEM-augmented remnant protein. Given our previous findings (22) of GH-independent association of the GHR cytoplasmic domain with JAK2 (and, therefore, possibly with other JAK2-associated signaling molecules), we raise the possibility that the remnant we have identified may be capable either of GHR-specific signaling by itself or of modulation of full-length GHR signaling in cells in which the GHR and remnant are both present. We are actively investigating these possibilities as well.

The recent molecular characterizations (34, 35) of an IC3-sensitive enzyme responsible for the processing of the membrane-anchored precursor form of TNFα into the soluble TNFα [the so-called TNFα-converting enzyme (TACE)] may be enlightening in understanding the mechanism(s) of GHR shedding. TACE, a member of the ADAM subgroup of the metzincin family of proteases (45), is a transmembrane molecule with a zinc-dependent protease domain and a regulatory cysteine switch in its extracellular domain (46). Although the physiological mechanism(s) of regulation of TACE is as yet incompletely understood, activation by sulfhydryl alkylating reagents, such as NEM, is a common feature of this class of metalloproteases. Our findings in IM-9 cells that PMA- and NEM-induced as well as constitutive, proteolytic cleavage of the hGHR are inhibitable by IC3 raise the possibility that a TACE-like enzyme might be responsible for generation of the GHBP and suggest strategies that might be employed to identify such an enzyme.

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