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Carboxypeptidase E, Identified As a Direct Interactor of Growth Hormone, Is Important for Efficient Secretion of the Hormone

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We have identified 88 interactor candidates for human growth hormone (GH) by the yeast two-hybrid assay. Among those, we focused our efforts on carboxypeptidase E (CPE), which has been thought to play a key role in sorting prohormones, such as pro-opiomelanocortin (POMC), to regulated secretory vesicles. We found that CPE colocalizes with and interacts with GH in AtT20 pituitary cells. Downregulation of CPE led to decreased levels of GH secretion, consistent with involvement of CPE in GH sorting/secretion. Our binding assay *in vitro* with bacterially expressed proteins suggested that GH directly interacts with CPE but in a manner different from POMC.

INTRODUCTION

Endocrine cells have specialized features to secrete peptide hormones. It has been known that these cells have two distinct pathways for hormone secretion: the constitutive and regulated pathway (Burgess and Kelly, 1987). These pathways have been extensively characterized for prohormones (hormones) such as pro-opiomelanocortin (POMC) and insulin (Moore et al., 2002). Their precursors are first synthesized in endoplasmic reticulum, transferred to the Golgi and packaged into either constitutive or regulated secretory vesicles (RSV), the latter of which are unique to endocrine/exocrine cells and neurons.One of the key factors of the regulated secretary pathway of hormones is the sorting receptor protein, which assists prohormones sorting to and retention at RSV by interacting with prohormones. A candidate for such a receptor molecule was first identified for POMC to be carboxypeptidase E (Cool et al., 1997). Carboxypeptidase E (CPE) is also known to be associated with prohormone proteolytic processing (Hook and Loh, 1984). CPE therefore contributes to both POMC processing and to POMC sorting to and retention at RSV. CPE is thought to function as the sorting receptor by directly interacting with the sorting motif of POMC (Cool and Loh, 1998). Some of the experiments using CPE mutant mice and CPE-knockdown cells have provided evidences consistent with the role of CPE as the sorting receptor (Cool et al., 1997; Normant and Loh, 1998; Shen and Loh, 1997).

The structures reminiscent of the POMC-sorting motif have been identified for other prohormones or propeptides, including proenkephalin, proinsulin and pro-BDNF (Cool et al., 1995; Lou et al., 2005; Zhang et al., 1999). In the case of glucagon, however, it has been reported that CPE is involved in sorting yet the POMC-type sorting motif does not appear to be present (McGirr et al., 2013). There are, furthermore, many other prohormones/propeptides that apparently do not have the POMC-type sorting motif, and it is not understood how they become sorted to RSV. For example, human growth hormone (GH), which is one of the classical and extensively studied hormones, does not require proteolytic activity of CPE for processing. GH is nevertheless sorted to dense-core RSV, similarly to POMC. It has been described that aggregation plays a role in GH sorting (Dannies, 2002). On the other hand, no direct interactor of GH that is involved in sorting and/or secretion has been identified.

In the present work, we searched for interactors of human GH by the yeast two-hybrid method, and identified a number of interactor candidates, including carboxypeptidase E. We then showed that GH and CPE, expressed in AtT20 mouse pituitary cells or in *E. coli*, interact with each other. Finally, with siRNA against CPE, we presented evidence that CPE is involved in efficient GH secretion in AtT20 cells, suggesting its role in GH sorting/secretion.

MATERIALS AND METHODS

Plasmids and yeast strains

For yeast two-hybrid screening, we used bait and prey vectors that we constructed ourselves (A. Mizutani et al., unpublished results). In brief, the bait vector contained two expression cassettes: the bait cassette and the other for expressing monomer red fluorescent protein mCherry (Shaner et al., 2008). The bait cassette contained human GH segment (amino acid 1-217). The prey vector also contained two expression cassettes: the prey cassette and the other for expressing monomer cyan fluorescent protein mCFP. The prey cassette contained a synthetic

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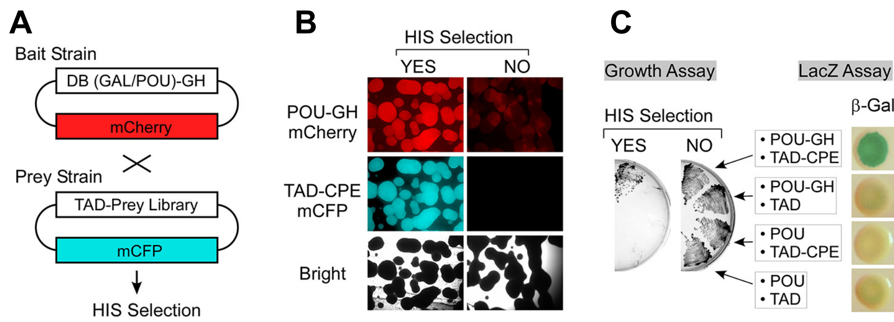

Fig. 1. Yeast two-hybrid screening. (A), the bait strain contained the bait plasmid, composed of the expression cassette for GH bait, fused to the DNA-binding domain (DB) of either GAL4 (GAL) or POU2F2 (POU), and the mCherry expression cassette. The prey strain contained the prey plasmid, composed of the expression cassette for prey cDNA library fused to transcriptional activation domain (TAD), and the mCFP expression cassette. (B), true-positive diploid cells that contained the POU-GH mCherry bait plasmid and the TAD-CPE mCFP prey plasmid were grown with or without his selection, and pictures of grown colonies were taken under a fluorescent microscope. (C), diploid clones, containing either of the four bait-prey combinations as indicated, were grown with or without his selection (left panel), or were subjected to β-galactosidase (β-Gal) assay with X-gal.

transcriptional activating sequence (Tanaka, 1996), to which approximately 3 millions multiple-tissue normalized cDNAs (Clontech) were fused. The reporter yeast strain contained the HIS3 and LacZ reporter cassettes. For mammalian transient expression experiments, pCG (Tanaka and Herr, 1990)-based CMV expression vectors were used. In those constructs, fluorescent or epitope tag was placed at the C-termini of fusion proteins. For *E. coli* protein expression, pET49b (Novagen)-based vectors, containing Strep-TagII, were used. A more detailed description of the plasmids and yeast strains is given in Supplementary Materials and Methods.

Yeast two-hybrid screening

Yeast cells containing the GH bait plasmid and the prey library yeast cells were mated, and were plated on his-selective medium. Note that on this selective medium, only His-plus diploid cells can grow irrespective of the presence of the bait and prey plasmid. The His-plus colonies were observed under a microscope (BX61; Olympus Co.) for red and cyanic fluorescence, and only entirely red and cyanic colonies (i.e., colonies that grew on the selective plate dependently on both the bait and prey plasmids) were picked. The picked cells were successively streaked for three times on the same histidine-minus selective medium and also on the non-selective medium, containing histidine, to verify bait- and prey-dependency. The clones that went through the procedure were subjected to PCR amplification and sequence determination.

Cell culture and microscopy

AtT20 cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% horse serum and 2.5% fetal bovine serum. For transient expression, plasmids were transfected with Lipofectamine 2000 (Invitrogen). Cells at 36-h and 40-h post transfection were used for microscopic and immunoprecipitation analyses, respectively. The cells for microscopic observation were fixed with 4% paraformaldehyde prior to acquiring images on a BZ-9000 microscope (KEYENCE). For RNA interference experiments, siRNA against mouse CPE or a control siRNA (Bonac Co.) was transfected into AtT20 cells with Lipofectamine 2000 at a final concentration of 75 nM. At 18 hours post siRNA transfection, cells were transfected by the GH-HA expression plasmid. At 28 h post GH-HA transfection, the media was changed to fresh media, the cells were incubated for 3 h, and the media and cells were harvested for protein analyses.

Biochemical analyses of proteins

For immunoprecipitation with transfected AtT20 cells, equal amounts of protein extracts were incubated with anti-HA beads (Roche Diagnostics) in a Tris-based pH7.5 buffer, composed of 50 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 15% Glycerol, 0.1% IGEPAL CA-630, 1 mM dithiothreitol and Complete protease inhibitor (Roche Diagnostics) at 4°C for 2 h. Immune complexes were fractionated on a SDS-polyacrylamide gel, followed by immunoblotting. The blots were probed with anti-Flag antibody (Sigma-Aldrich Corp.). The signals were detected by chemiluminescence (ECL-Plus and ECL-prime; GE Healthcare) and LAS1000 imager (Fujifilm). For bacterial expression of proteins, BL21 (DE3) was used. The GH and CPE proteins were purified with Strep-Tactin Sepharose (Qiagen). Degrees of purity of different proteins can be seen in Supplementary Fig. B. For interaction assays, purified GST-GH or control GST proteins were incubated with glutathione Sepharose (GE Healthcare) for 1 h at 4°C, and the beads were washed by a MES-based pH5.5 buffer, composed of 50 mM MES (pH5.5), 120 mM NaCl, 5 mM KCl and 0.1% IGEPAL CA-630 (Cool et al., 1997). The CPE proteins, mixed with 10-times volume of the pH5.5 buffer, were added to the washed glutathione beads, incubated at 23°C for 30 min and then at 4°C for 1 h. The bound proteins were analyzed by Western blot with anti-Flag antibody or by SYPRO Ruby staining, following SDS-polyacrylamide gel electrophoresis. Detailed procedures are described in Supplementary Materials and Methods. Note that immunoprecipitation and GST pull-down experiments were performed multiple times, generating consistent results.

RESULTS

Screening of growth hormone interactor candidates by the yeast two-hybrid assay

We screened human growth hormone (GH) interaction partners by the yeast two-hybrid screening system, which we developed in our laboratory (A. Mizutani et al.; a through description of the system to be published elsewhere). The human GH coding sequence, fused to the DNA-binding domain of GAL4 or POU2F2, was used as bait (Fig. 1A). Bait cells and prey library cells were mated, and were plated on a selective medium, where only His+ diploid cells can grow. On the his selective

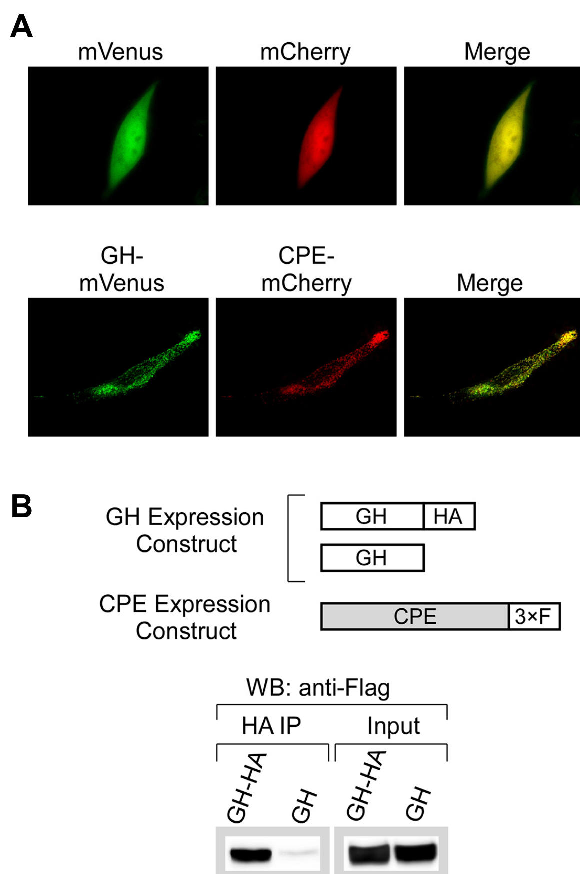

Fig. 2. Co-localization and co-immunoprecipitation of GH and CPE expressed in AtT20 cells. (A) mVenus and mCherry fluorescent protein, or those fused to the GH or CPE coding sequence as indicated, were transiently co-expressed in AtT20 cells, and were observed under a fluorescent microscope. (B) GH-HA or non-tag GH control was co-expressed with CPE-3×F (3×Flag) in AtT20 cells. The protein extracts were subjected to HA immunoprecipitation (HA IP). The immunoprecipitates and input extracts were analyzed by Western-blot analysis (WB) with anti-Flag antibody.

media, true positive colonies, growth of which should depend on the presence of both bait and prey plasmid, are expected to be entirely red and cyanic (see an example of positive cells shown in Fig. 1B). We were, therefore, able to identify rare red/cyanic positive colonies on the primary selective plates, where most colonies were not entirely red or entirely cyanic and were judged to be false positives.

We screened ten million of mated cells for GAL-GH and for POU-GH bait, and analyzed 135 red/cyanic positive clones in total. Sequence analyses of these clones led to identification of 88 yeast two-hybrid GH interactors (Supplementary Table). Two factors, OTUB1 and PDHB, were previously identified as GH interactors (Ewing et al., 2007). There are, furthermore, a number of interactor candidates that attracted our attention (see Discussion). In the present work, we chose carboxypeptidase E (CPE) for further analyses, because it has been reported that CPE is the RSV sorting receptor of some other hormones (Cawley et al., 2012), and it may also function in GH sorting and secretion (Shen and Loh, 1997).

We first confirmed the GH-CPE interaction in our two-hybrid assay. As shown in Fig 1B, the fluorescent assay indicated that essentially all the colonies exhibited red and cyanic fluorescence when grown on the his-selective medium, showing that His-plus phenotype relies on both the POU-GH and TAD-CPE plasmids. The results were further confirmed by a more conventional growth assay and a LacZ assay, depicted in Fig. 1C, showing that growth under his selection and induction of *lacZ*-reporter expression depend on both the GH and CPE plasmids.

Localization of GH and CPE in pituitary cells

We analyzed intracellular localization of GH and CPE in AtT20 mouse pituitary cells. mVenus and mCherry proteins when expressed in AtT20 were distributed throughout the entire cells (Fig. 2A; top panels). In contrast, when fused to GH and CPE, mVenus and mCherry fluorescence showed punctuated distribution and were mostly co-localized densely at the tips of cell processes (Fig. 2A; bottom panels). These localization patterns are indicative of GH- and CPE-fusions localizing to RSV (Rivas and Moore, 1989).

CPE and GH, expressed in AtT20 cells, can interact with each other

We tested if CPE can interact with GH in immunoprecipitation assay using AtT20 cells. The 3×Flag-tagged CPE was co-expressed with HA-tagged or non-tag GH, and the cell extracts were subjected to immunoprecipitation with anti-HA antibody beads. The results of Western blot analyses of input extracts and immunoprecipitates are shown in Fig. 2B. The results showed that a significant amount of CPE-3×Flag was co-immunoprecipitated with GH-HA (Fig. 2B; 1st lane). When non-tag GH was subjected to the same analysis, only a small amount of CPE-3×Flag was detected (Fig. 2B; 2nd lane). Western blot analyses of input extracts showed that expression levels of CPE-3×Flag are similar between the GH-HA and non-tag GH control sample (Fig. 2B; see panel on the right). The results indicated that CPE and GH, when ectopically expressed in AtT20 cells, interact with each other. Because we were not able to reproduce CPE-GH interaction by mixing them *in vitro* after expressing them separately (data not shown), the CPE-GH interaction observed here is likely to reflect the interaction in cells.

Direct interaction between CPE and GH

E. coli-expressed CPE and GH proteins were tested for interaction. We first tested pH dependence of the interaction and found that CPE binds to GH-GST more efficiently at lower pHs, down to pH5 (Supplementary Fig. A), reminiscent of the POMC-CPE interaction (e.g., Cool et al., 1997). We observed, however, that CPE aggregated at pH5 (Supplementary Fig. B). We therefore analyzed interaction between CPE and GH at pH5.5, where none of CPE truncations aggregated (Supplementary Fig. B). The results depicted in figure 3 showed that full-length CPE (1-434) and a C-terminal truncation (1-310) can bind to GH-GST more efficiently than to GST control (Fig. 3A; 1st and 2nd panels on the top). The amounts of bead-bound GH-GST and negative-control GST were all equivalent (Fig. 3A; bottom panel on the left). In addition to the full-length protein marked by open arrowheads, we detected CPE truncations of various sizes, most of which also bound to GH. However, the small truncation indicated by a filled arrowhead did not bind as efficiently, underlying the selectivity of the assay. Both the N-terminal (1-217) and C-terminal (218-434) segments of CPE

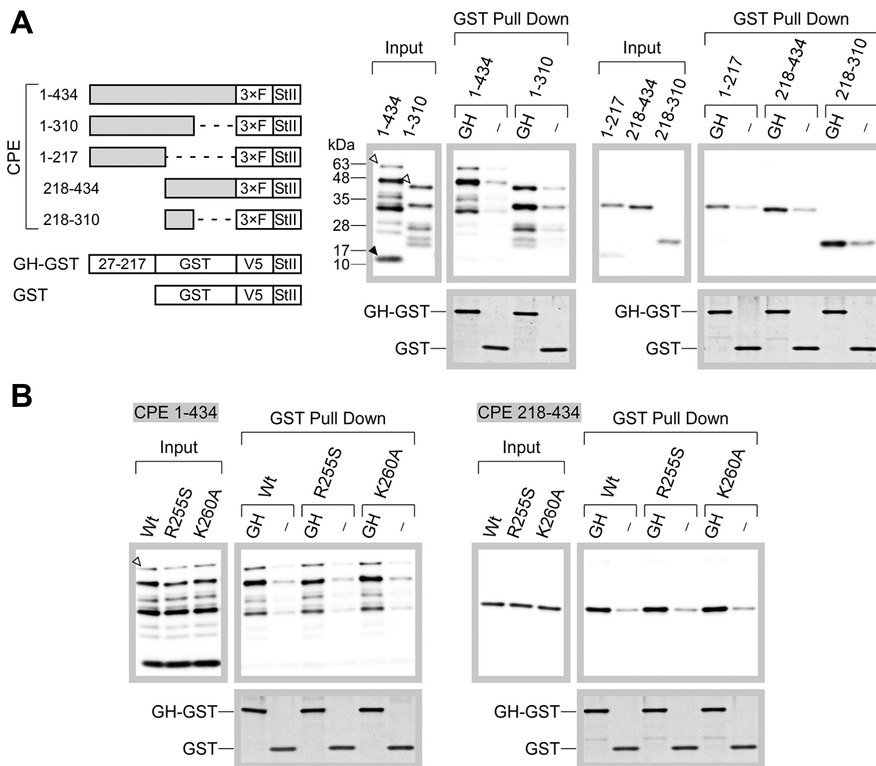

Fig. 3. Interaction of *E. coli*-expressed CPE and GH protein. (A) GH-GST or GST was first bound to glutathione Sepharose beads, and 3×Flag-tagged CPE truncations, displayed to the left, were added to examine their binding to GH. The bead-bound proteins and input proteins were analyzed by anti-Flag Western blot (panels on the top). The bead-bound proteins were also analyzed by SYPRO Ruby staining (panels on the bottom). The results with CPE 1-434/1-310 and those with CPE 1-217/218-434/218-310 are shown to the left and to the right, respectively. (B) R255S and K260A mutations were introduced into CPE1-434 (shown to the left) and 218-434 (shown to the right), and their binding to GH was examined. Note that input proteins shown in the same panel were diluted equally for comparison.

showed significant binding to GH (Fig. 3A; two panels on the right), implying that CPE has at least two segments, which can separately bind to GH. The C-terminal 218-434 segment was further delineated to a small 92-amino acid segment encompassing amino acid 218-310 (Fig. 3A).

It has been previously reported that some substitution mutations of CPE are detrimental to its ability to bind to POMC (Zhang et al., 1999). Two of these mutations (i.e., R255S and K260A), however, did not show any appreciable effect on the ability of CPE to bind to GH (see the results for CPE 1-434 and CPE 218-434, shown to the left and to the right, respectively, in Fig. 3B). The result suggested that GH interacts with CPE in a different manner than POMC.

CPE plays a role in GH secretion in AtT20 pituitary cells

We used siRNA against CPE to test the effect of acute down-regulation of CPE on GH expression and/or secretion in AtT20 cells. The cells were sequentially transfected by anti-CPE siRNA (or by a control siRNA) and then by the GH-HA plasmid. The amounts of GH secreted in the media were analyzed by Western blot analyses, together with cellular levels of CPE, GH and beta-actin (Fig. 4A). The results showed that anti-CPE siRNA significantly reduced CPE expression and also the amounts of GH secreted in the media. In contrast, effects of the siRNA were very limited, if any, on the levels of cellular GH. In AtT20 cells under these conditions, therefore, CPE plays a critical role in the GH sorting/secretion process. Because the presence of serum results in induction of GH secretion by several folds (Fig. 4B), we assume that most of the siRNA effects that we observed are due to decreased induction of GH secretion by serum.

DISCUSSION

We identified 88 GH interactor candidates in the two-hybrid assay. Some of them are supposed to be localized to nucleus or to mitochondria, and they are presumed to be false positives; they do not interact with GH in mammalian cells in spite of that they do so in the yeast two-hybrid assay. We focused our efforts on CPE in the present work. There are, however, other interactor candidates that attracted our attention. In addition to OTUB1 and PDHB, which were previously identified as GH interactors in a high-throughput biochemical screening (Ewing et al., 2007), those included factors related to the Golgi (e.g., CLASP2 and COPB1) and/or protein trafficking (e.g., SNXs and SCFD1). In particular, it is intriguing that, as direct interactor candidates of GH, we identified DCTN2 (Park et al., 2008), MYO5B (Hales et al., 2001; Khvotchev et al., 2003) and VPS41 (Shu et al., 2013), which might be involved in the regulated secretory pathway. Although interaction of GH with these factors has yet to be verified, our identification of them could become a step towards further elucidating how GH, by interacting with various factors, including CPE, becomes sorted and secreted. Another intriguing interactor candidate would be IL6, because it might explain, at least in part, how IL6 may affect the action of GH under some situations (Ballinger et al., 2001).

Carboxypeptidase activity of CPE is often associated with proteolytic processing of prohormones such as POMC and proinsulin (Davidson and Hutton, 1987; Hook and Loh, 1984). The CPE protein, furthermore, is thought to play an important role in targeting prohormones to RSV as a receptor, presumably through direct interaction with sorting motifs of prohormones (Cool et al., 1997; Zhang et al., 1999). On the other hand, GH

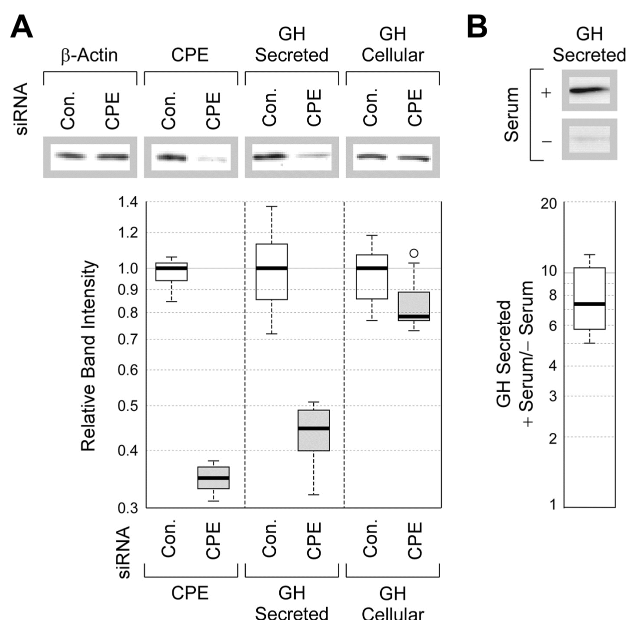

Fig. 4. Effects of siRNA against CPE on GH secretion in AtT20 cells. (A) AtT20 cells were transfected by siRNA and then by the GH-HA expression plasmid, and the media and cell extracts were subjected to Western-blot analyses. Images of Western blots of a representative assay are shown to the top, and quantitations of nine independent assays are shown to the bottom. Band-intensity ratios against the β-actin bands were quantified from Western blots, and the relative values compared to the medians for control siRNA samples are plotted on the y-axis of the graph. Center lines depict the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers show the minimum and the maximum values excluding an outlier, which is represented by an open circle. (B) AtT20 cells, transfected with control siRNA and then by the GH-HA expression plasmid, were incubated in the media with or without serum for three hours, and the levels of GH secreted in the media were analyzed by Western-blot analyses. Images of a Western blot of a representative assay are shown to the top, and quantitations of six independent assays are shown to the bottom.

does not require proteolytic activity of CPE for its processing. We have shown here, nevertheless, that CPE co-localizes with and interacts with GH in AtT20 cells. The interaction was also reproduced *in vitro* with *E. coli*-expressed and purified proteins.

In our GH-CPE binding assay *in vitro*, we tested mutations of CPE (R255S and K260A) that are reported to be deleterious to CPE-POMC interaction (Zhang et al., 1999). These mutations, however, did not show any appreciable effect on GH-CPE interaction in our assay. The result suggested that GH interacts with CPE differently from others, including POMC, proinsulin and BDNF, which share the CPE-interacting sorting motif of similar structures (Lou et al., 2005; Zhang et al., 1999). Interestingly, Wang et al. have reported identification of a motif reminiscent of the POMC-type sorting motif at the C-terminal region of GH (Wang et al., 2005). This putative "GH sorting motif", important for GH trafficking (Wang et al., 2005), contains evolutionarily conserved two acidic amino-acid residues, E174 and E186. The structure of the putative GH-sorting motif, however, appears to be different from those of POMC-type, which contain surface-exposed two acidic amino acid residues. For example, E174 of GH does not appear to be exposed to the surface, and, furthermore, the distance between E174 and E186 of GH is near 20Å and may be significantly larger than the POMC-type cases (PDB code: 1HGU). It is therefore possible that if this putative GH sorting motif functions by interacting with CPE, it may interact differently from the POMC-type sorting motifs do, and this could be why either the R255S- or K260A-mutation of CPE did not show an appreciable effect on the GH-CPE interaction.

The role of CPE in the hormone sorting and secretion pathway has been intensively studied for POMC and proinsulin, using CPE-mutant mice, or using pituitary or neuroblast cells with siRNA against CPE (Cawley et al., 2012 and references therein). With some complications due to residual activity of the CPE missense mutant and a mechanism compensatory to CPE defects, for example (Cawley et al., 2003; Hosaka et al., 2005; Irminger et al., 1997; Kemppainen and Behrend, 2010), the results have been mostly consistent with the role of CPE as an RSV sorting receptor. In the present work, we have shown with anti-CPE siRNA that acute downregulation of CPE leads to decreased secretion of GH in AtT20 cells. It has been previously reported that GH as well as POMC are miss-sorted to the constitutive secretory pathway in pituitary cells of CPE mutant mice (Shen and Loh, 1997). It may not be appropriate to directly compare these results because of differences in the cell type used, how GH was analyzed, and how CPE was deteriorated. Both of these results nevertheless are consistent with that CPE plays critical roles in GH sorting and secretion through direct interaction, which we have shown. In the current study, however, we have not determined separately how CPE is involved in the constitutive and regulated secretion of GH nor have we directly investigated if CPE contributes to sorting of GH, for example, as an RSV receptor, as reported for the POMC-type prohormones (Cool et al., 1997). The clarification of these issues would be of interest, particularly because GH appears to interact with CPE differently from the POMC-type prohormones, and this GH-type CPE-interacting motif might be unique to GH or might represent non-POMC type motifs, which may be shared among other prohormones and propeptides.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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