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# Effects of Carboxypeptidase E Overexpression on Insulin mRNA Levels, Regulated Insulin Secretion, and Proinsulin Processing of Pituitary GH3 Cells Transfected With a Furin-Cleavable Human Proinsulin cDNA

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We recently developed two rat pituitary GH3 cell clones engineered to secrete human insulin (InsGH3). InsGH3 cells convert proinsulin into mature insulin, which is partially stored into a readily releasable pool of secretory granules. The efficiency of these processes, however, is relatively low in these cells, either in vitro or in vivo. This study was aimed at determining whether carboxypeptidase E (Cpe) overexpression can increase proinsulin processing and regulated secretion by InsGH3 clones. Indeed, in its membrane-bound form Cpe works as sorting receptor for the regulated secretory pathway of many hormones while, in its soluble form, Cpe takes part to the late step of insulin maturation. We obtained two Cpe-overexpressing cell lines from two different InsGH3 clones (InsGH3/C1 and C7). In the Cpe-overexpressing cell lines, derived from InsGH3 of clone 1 (InsGH3/C1-HACpe), in which the membrane-bound form of exogenous Cpe is accounted for by 90% of total Cpe immunoreactivity, we observed an increase in proinsulin gene expression, and in basal and stimulated insulin secretion compared with the original clone. In contrast, in the Cpe-overexpressing cell line derived from InsGH3 of clone 7 (InsGH3/C7-HACpe), where the exogenous membrane-bound form was only 60% of total Cpe, we detected a decrease in basal insulin release and a modest, albeit significant, increase in intracellular proinsulin processing. In conclusion, Cpe overexpression can increase regulated insulin secretion and proinsulin processing in InsGH3 cells; however, such improvements appear quantitatively and qualitatively modest.

Key words: Carboxypeptidase E; Insulin secretion; Bioenegineering; Pituitary cells

## INTRODUCTION

We recently proposed bioengineered insulin-secreting pituitary cells as potential β-cell surrogates for encapsulation-based diabetes cell therapy (5,16). Pituitary cells are interesting because they have a regulated secretory pathway (RSP) of protein secretion (21) and, in contrast to pancreatic β-cells (22,24), are not recognized by cytotoxic antibodies present in sera of diabetic NOD mice (20). Moreover, insulin-secreting pituitary cells transplanted into spontaneously diabetic NOD mice escape the recurrence of autoimmune destruction and survive indefinitely after implantation (14).

We previously developed two insulin-secreting GH3 cell clones (InsGH3/C1 and InsGH3/C7) stably transfected with a modified furin-cleavable human proinsulin cDNA (7) placed under the control of the prolactin promoter (5,16). We showed that InsGH3 cells are more resistant than pancreatic $\beta TC_3$ cells to different cytotoxic agents and can survive in vitro at extremely high density. This peculiar resistance to cell death signals has been related to their naturally high levels of antiapoptotic Bcl-2 protein (12,16). Although proinsulin is processed to mature insulin by InsGH3 cells, this process is not particularly efficient and mature insulin accounts for only 40% of total released proinsulin forms. Targeting of proinsulin transgene products to the RSP was confirmed by immunofluorescence that showed the colocalization of insulin with secretogranin II (a marker of regulated secretion), and by the ability of InsGH3 cells to increase insulin release in response to an acute stimulation. Moreover, implantation of InsGH3 cells into streptozotocin-induced diabetic mice reversed hyperglycemia and induced the appearance of circulating human c-peptide, proinsulin, and insulin levels of about 9, 32, and 35 pmol/L, respectively. However, uncontrolled graft growth and elevate constitutive insulin release by transplanted InsGH3 cells induced hypoglycemia in the long term.

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Encapsulation, which represents a physical barrier against uncontrolled cell growth, may control tumorigenicity but the only way to increase proinsulin processing and secretagogue-induced (regulated) secretion is to perform further engineering of InsGH3 cells.

Insulin is synthesized into the rough endoplasmic reticulum as a larger precursor (preproinsulin); preproinsulin is then transported to the trans-Golgi, where it segregates into the vesicles of the RSP and where the endoproteases PC1 and PC3 transform proinsulin into mature insulin. Finally, secretory granules rich of mature insulin (>90%) are released by a $Ca^{2+}$-dependent exocytotic process (1,15,23). Protein aggregation and the presence of a sorting receptor are the two models proposed to explain how proteins are selectively targeted to the RSP (1,15). The latter hypothesis is supported by the identification of a specific sorting signal motif in many prohormones (2,3), and by the demonstration that the membrane-bound form of carboxypeptidase E (Cpe) acts as a sorting receptor (4). Moreover, in its soluble form, Cpe participates to the last step of insulin processing by removing basic residues from the C-terminal region of the proinsulin molecule (15).

Therefore, we here explored whether Cpe overexpression may enhance regulated insulin secretion and proinsulin processing by InsGH3/C1 and C7 cell clones. Our data indicate that when overexpression occurs by 90% in its membrane-bound form, exogenous Cpe can actually increase secretagogue-induced (regulated) insulin secretion. Conversely, when exogenous Cpe expression is only 60% in its membrane-bound form, the increase in regulated insulin secretion does not occur but proinsulin processing to mature insulin is slightly increased.

## MATERIALS AND METHODS

### *Cells and Cell Culture*

Rat pituitary GH3 (American Type Culture Collection) and InsGH3 clones were cultured in DMEM tissue culture medium 25 mmol/L glucose supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, and 100 IU/ml streptomycin/penicillin (DMEM-c). Cultures were performed under standard humidified culture conditions of 5% $CO_2$ and 95% air at 37°C.

### *Expression Vector and Transfection*

The rat carboxypeptidase E cDNA, fused in the N-terminal region to the HA epitope tag of the human influenza virus (HACpe), was kindly provided by Dr. Lloyd Friker (Albert Einstein College of Medicine, Bronx, NY). HACpe was cloned into pCEP4 expression vector, containing the hygromycin resistance gene, under the control of the CMV promoter. About $5 \times 10^5$ InsGH3/C1 and C7 cells were plated onto six-well plates and, 24 h later, the cells were transfected with the plasmid by using Lipofectin (GIBCO/BRL) following the manufacturer's instructions. After 3 weeks of culture, cells resistant to 0.25 mg/ml of hygromycin (GIBCO/BRL) were trypsinized, collected, and expanded as cell lines (InsGH3/C1-HACpe and InsGH3/C7-HACpe). All experiments were performed between passages 2 and 15.

### *Northern Blot Analysis*

The expression of human proinsulin, Cpe, and ribosomal 18S genes was analyzed in InsGH3 and InsGH3/HACpe cells. Total cellular RNA was prepared with the RNAFAST isolation system (Biotecx Laboratories Inc., Houston, TX) and Northern blot analysis was performed. Twenty micrograms of total RNA from each sample was denaturated with formaldehyde, electrophoresed on 1% denaturing agarose gel, and blotted to a Hybond nylon membrane. Blots were hybridized with $^{32}$P-labeled human modified insulin, ribosomal 18S, and *Hin*dIII/*Xho*II fragment of rat Cpe cDNA (1.2 kb) probes. Relative expression levels of proinsulin, Cpe, and ribosomal 18S mRNA were determined by densitometric analysis.

### *Western Blotting*

Membrane-bound and soluble HACpe protein levels were measured in InsGH3/C1-HACpe and InsGH3/C7-HACpe cells by Western blotting by using anti-HA antibody (clone 12CA5, rabbit anti-mouse 1:1000, Roche Diagnostics Corp. Indianapolis, IN). Membrane-bound and soluble forms were identified by the molecular weight (19).

### *Insulin Assays*

Mature insulin content and release was measured by IRI IMX kit (Abbot Laboratories, Tokyo, Japan), which recognized only mature insulin. Proinsulin and the other split forms presented in cell lysates and released in the media were measured by DAKO TOTAL proinsulin kit (DAKO Diagnostics Ltd., Ely).

### *Secretagogue-Induced Insulin Release*

Secretagogue-induced mature insulin release by InsGH3 and InsGH3/HACpe cells was studied by static incubation. Cells were plated ($1.6 \times 10^5$ cells per well) into 24-well plates and cultured overnight in standard tissue culture medium. The following day, cells were washed in phosphate-buffered saline (PBS) and then incubated for 60 min in plain medium (without FCS), with the addition of 10 μmol/L forskolin (FSK). After incubation, supernatants were collected and stored at −20°C until assayed for mature insulin as described. Cell lysates were prepared for the measurement of total protein and insulin content, and insulin release was expressed in terms of protein content.

*Immunocytochemistry*

InsGH3 and Cpe-overexpressing cells were fixed in 4% paraformaldehyde in PBS for 10 min. Cells were blocked for 20 min in 20% normal goat serum in PBS (PBS/NGS), then permeabilized for 10 min with 0.15% Triton X-100 in PBS/NGS and incubated into anti-insulin (mouse anti-human 1:100, Signet Laboratoires Inc., Dedham, MA) at 37°C for 1.5 h. After three washes with PBS, cells were incubated with secondary antibody (1:2000) at room temperature for 1 h, washed three times with PBS, and nuclei were marked with DAPI (Sigma, St. Louis, MO, dil. 1:50 from 1 μg/ml in ethanol) for 10 min. All experiments were analyzed with a fluorescence microscope.

*Statistical Analysis*

Experiments were repeated at least three times. Data were expressed as means ± SE. Statistical analysis was performed using the unpaired Student's *t*-test. Statistical significance was considered at $p < 0.05$.

## RESULTS

*Development of InsGH3/HACpe Lines and HACpe Distribution*

After transfection and selection, hygromicin-resistent cells of clone 1 and clone 7 were pooled to develop the InsGH3/C1-HACpe and InsGH3/C7-HACpe lines. As shown in Figure 1, Cpe overexpression in these lines was confirmed at different passages by Northern Blot analysis. Compared with clones 1 and 7, which showed a similar level of endogenous Cpe (Fig. 1), InsGH3/C1-HACpe and InsGH3/C7-HACpe showed a significant increase in Cpe mRNA levels of about three- and sixfold compared with untrasfected cells, respectively. The presence of HACpe in transfected cells was confirmed also with Western blot experiments (Fig. 2, upper panel). Two bands of approximate molecular weight of 50 and 55 kDa were identified. Accordingly with the previous findings (19), these bands corresponded to the full-length membrane-bound (upper band) and truncated soluble (lower band) forms of Cpe. To assess the relative distribution of exogenous Cpe in its two form, the percentage of membrane-bound versus soluble form was analyzed at the densitometer (Fig. 2, lower panel). In InsGH3/C1-HACpe, more than 90% of HACpe immunoreactivity was localized in the membrane-bound form, while in Ins GH3/C7-HACpe cells exogenous Cpe was localized to about 60% in the membrane-bound form and 40% in the soluble form.

*Basal Insulin Content and Release in Cpe-Overexpressing Lines*

As shown in Table 1, InsGH3/C7-HACpe showed a significant decrease in mature insulin content when compared with untrasfected clone 7. Conversely, no differences were found after Cpe overexpression in clone 1.

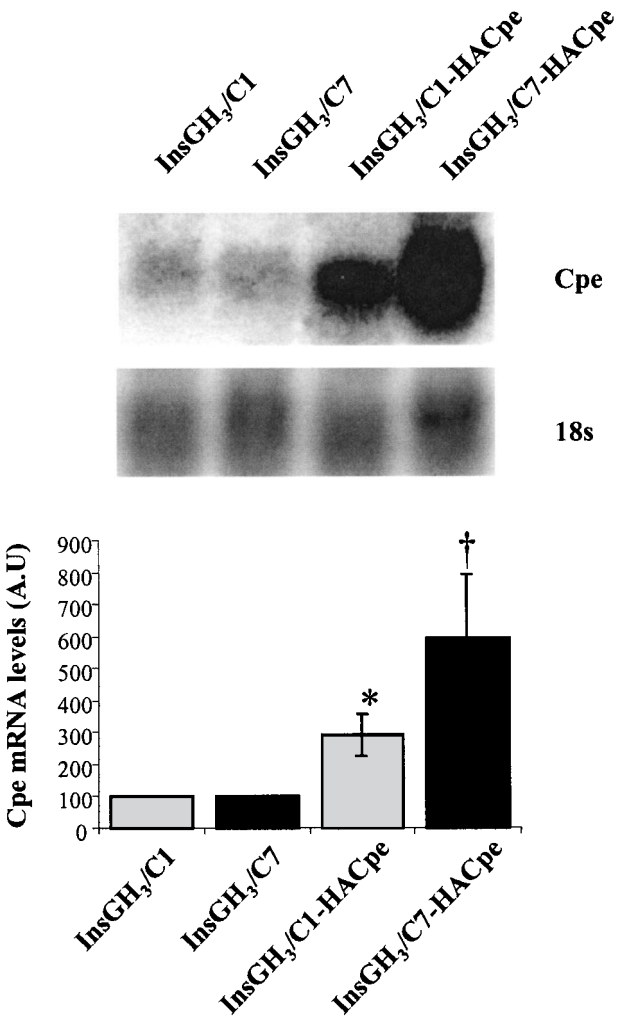


**Figure 1.** (Top) A representative Northern Blot showing carboxypeptidase E gene expression in InsGH3/HACpe of both lines compared with their original InsGH3 cell clones. (Bottom) Densitometric analysis of five different experiments: Cpe mRNA levels were threefold higher in InsGH3/C1-HACpe than in InsGH3/C1 and sixfold higher in InsGH3/C7-HACpe than in InsGH3/C7. Data are means of at least three different experiments ± SE. *$p < 0.05$. †$p < 0.02$.

InsGH3/C1-HACpe and InsGH3/C7-HACpe revealed a diverse response in basal insulin release after Cpe overexpression. Indeed, release of mature insulin was markedly increased in clone 1 (about 195% of untransfected cells), while it decreased (57% of untransfected cells) in clone 7 (Table 1).

*(Pro)Insulin Processing*

The percentage of mature insulin in media and extract of InsGH3/HACpe lines was measured and compared

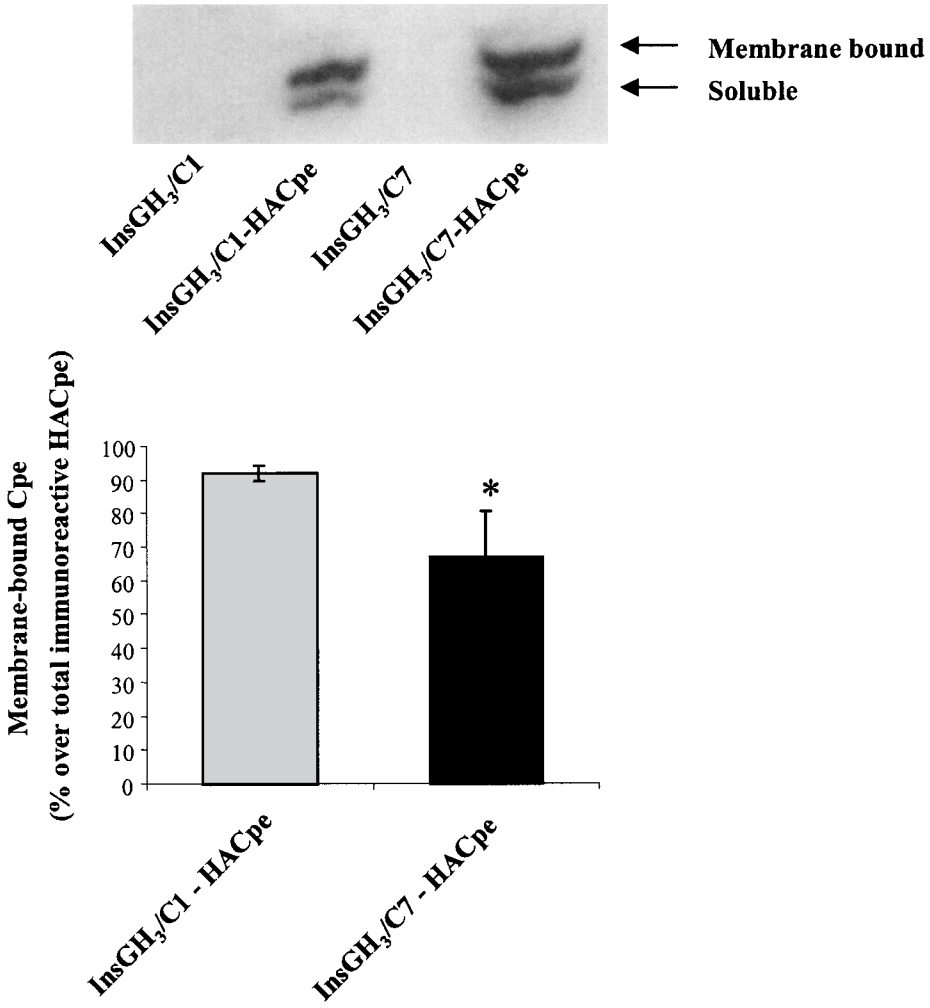


**Figure 2.** (Top) a representative Western blot where cell proteins were immunoblotted with an antibody against HA. Transfected cells showed two bands corresponding to full-length membrane-bound (upper band, molecular weight ~55 kDa) and truncated soluble form (lower band, molecular weight ~50 kDa) of Cpe, respectively. (Bottom) In InsGH3/C1-HACpe more than 90% of total HACpe immunoreactivity was in the membrane-bound form. Conversely, only 60% of exogenous Cpe was membrane bound in InsGH3/C7-HACpe cells. Data are means ± SE of three independent experiments. $*p < 0.05$.

with those of original clones (Table 2). A significant increase in mature insulin was detected in InsGH3/C7-HACpe extract (from 51% to 66%). In contrast, no differences were observed in the percentage of mature insulin secretion by Cpe-overexpressing InsGH3 cells when compared with untransfected controls.

*Forskolin-Induced Insulin Release*

In InsGH3 cells of both clones, FSK induced a dose-dependent increase in insulin secretion that was maximal at 10 μmol/L (16). As shown in Figure 3, after Cpe overexpression the insulin response to FSK increased further in InsGH3/C1-HACpe (about 50% increase when compared with untransfected InsGH3/C1). Conversely, FSK-stimulated insulin release did not change in InsGH3/C7-HACpe.

*Immunocytochemistry Analysis*

InsGH3/C1 cells transfected with Cpe showed a cytoplasmic pattern of insulin immunoreactivity slightly different from control cells (Fig. 4). Indeed, insulin immunoreactivity in InsGH3/C1-HACpe appeared more granular and mainly localized under the plasma membrane than in InsGH3/C1 cells. No difference was noted between InsGH3/C7 and InsGH3/C7-HACpe cells (data not shown).

**Table 1.** Mature Insulin Content and Basal Insulin Release in InsGH3 and InsGH3/HACpe Cells, Expressed in Terms of Protein Content and as Percentage of Untransfected Control Cells (Arbitrarily Assigned at 100%)

| | Content | | Release | |
|---|---|---|---|---|
| | μU/μg of Protein | % of Untransfected Cells | μU/μg of Protein | % of Untransfected Cells |
| InsGH3/C1 | 0.17 ± 0.01 | 100 ± 0 | 3.2 ± 0.3 | 100 ± 0 |
| InsGH3/C1-HACpe | 0.14 ± 0.01 | 84 ± 11 | 6.0 ± 1.1† | 195 ± 19† |
| InsGH3/C7 | 0.73 ± 0.06 | 100 ± 0 | 4.0 ± 0.2 | 100 ± 0 |
| InsGH3/C7-HACpe | 0.47 ± 0.05* | 64 ± 11* | 2.3 ± 0.08† | 57 ± 3† |

Insulin content was significantly lower in InsGH3/C7-HACpe than in untransfected InsGH3/C7 cells, while in InsGH3/C1-HACpe cells it remained similar to their control cells (InsGH3/C1). Basal insulin release increased significantly in Cpe-overexpressing cells of clone 1 (InsGH3/C1-HACpe) when compared with their control cells. In contrast, InsGH3/C7-HACpe cells showed a significant decrease in insulin secretion. Data are means ± SE of 7 experiments.
*$p < 0.05$ vs. control.
†$p < 0.01$ vs. control.

*Human Proinsulin mRNA Levels*

Cpe overexpression did not induced any significant modification in proinsulin mRNA levels of Cpe-overexpressing cells derived from clone 7. In contrast, proinsulin mRNA levels were significantly higher in InsGH3/C1-HACpe cells than in their untransfected control cells (Fig. 5).

## DISCUSSION

In β-cells and neuroendocrine cells, Cpe is present in a membrane-bound form, localized on the trans-Golgi and secretory vesicle membranes, and in a soluble form. The latter derives from the proteolytic cleavage of the C-terminal region, which is essential for the binding of Cpe to the membranes (15,17). The soluble form of Cpe is an enzymatically active endoprotease (11,15,23), while the membrane-bound form does not have enzymatic activity and binds prohormomes such as proinsulin, proenkephalin, and POMC in a first-order kinetics similar to that of enzyme–substrate interaction (4). The prohormone binding domain is distinct from the catalytic domain and becomes accessible only when Cpe is bound to the membranes of the trans-Golgi network or the secretory vesicles (26). Moreover, the binding of Cpe with the cholesterol-glycolipid raft is necessary for its sorting receptor function (6).

**Table 2.** Percentage of Mature Over Total Immunoreactive Insulin in Extracts and Media of InsGH3 and InsGH3/HACpe Cells

| | Extracts | Media |
|---|---|---|
| InsGH3/C1 | 52 ± 7 | 30 ± 7 |
| InsGH3/C1-HACpe | 47 ± 6 | 30 ± 9 |
| InsGH3/C7 | 51 ± 5 | 26 ± 6 |
| InsGH3/C7-HACpe | 66 ± 9* | 33 ± 10 |

A significant increase in mature insulin content was detected in InsGH3/C7-HACpe cells, while the content of mature insulin released in the media remained unchanged. No differences were found in InsGH3/C1 clone before and after Cpe overexpression. Data are means ± SE of 5 independent experiments.
*$p < 0.05$ vs. control.

Previous reports have clearly shown the fundamental role of Cpe in the processing of the prehormones in their active forms. Neuro-2a cells, in which Cpe expression was completely abolished by antisense technology (Neuro-2a $Cpe^{-/-}$ cells), secrete completely unprocessed proenkephalin via the constitutive pathway (4,19). Lack of Cpe activity was studied also in $Cpe^{fat}$ mice, which have a single point mutation in the Cpe gene that leads to the transcription of an enzymatically inactive protein. The phenotype of these mice is characterized by obesity, infertility, and a diabetic syndrome consequent to the release of unprocessed proinsulin, which has a poor biological activity (18). However, the observation that chromogranin A is correctly sorted into the regulated secretory pathway (RSP) in Neuro-2a $Cpe^{-/-}$ cells (19), and that β-cell lines derived from $Cpe^{fat}$ mice secrete unprocessed proinsulin but in a regulated manner (25), suggest that Cpe may be not strictly necessary for hormone sorting to the RSP.

We developed two Cpe-overexpressing lines, derived from the InsGH3/C1 and InsGH3/C7 clones. In resultant InsGH3/C1-HACpe and InsGH3/C7-HACpe cell lines, Cpe mRNA levels were about three- and sixfold higher than in their original untransfected clones, respectively

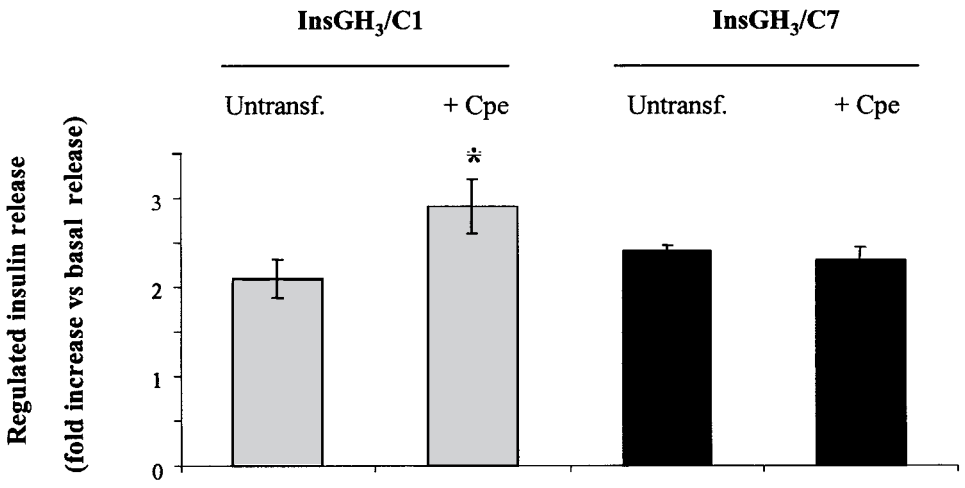


**Figure 3.** Secretagogue-induced (regulated) insulin release (normalized in terms of insulin content and expressed as percentage of basal release) by untransfected and Cpe-overexpressing InsGH3 cells in response to an acute stimulation with FSK. After 1-h incubation, insulin response to FSK was significantly higher in InsGH3/C1-HACpe than InsGH3/C1 cells ($*p < 0.02$), while no differences were found in the InsGH3/C7 cells.

(Fig. 1). As previously described to occur for the proinsulin gene (16), exogenous Cpe gene was transcribed more efficiently in InsGH3/C7 than in InsGH3/C1.

The effect of Cpe overexpression in InsGH3 cells varied depending on the relative distribution of its soluble and membrane-bound form of protein. In InsGH3/C7-HACpe cells, 60% of total Cpe immunoreactivity was detected in its membrane-bound form and only 40% in its soluble form (Fig. 2). This situation resembles the endogenous distribution of Cpe in AtT20 cells (9), and suggests that the membrane-bound/soluble Cpe ratio remained unaffected in InsGH3/C7-HACpe cells. Perhaps this is the reason why sixfold higher Cpe mRNA levels did not induce any changes in regulated insulin secretion by these cells. Instead, InsGH3/C7-HACpe cells showed a significant increase in intracellular proinsulin processing (Table 2) compared with the original. Indeed, it should be noted that all the other forms of unprocessed proinsulin decreased more dramatically in InsGH3/C7-HACpe, because the ratio between mature insulin over total immunoreactive insulin increased significantly in this cells line (Table 2). Nevertheless, the increase in percentage of mature insulin in extract of InsGH3/C7-HACpe indicates that a modest increase in proinsulin processing must have occurred in these cells. It should be noted, however, that the efficiency of proinsulin processing was significantly higher in wild-type GH3 cells cotransfected with proinsulin and the β-cell-specific endoproteases PC1 and PC3 (13).

The decrease in total immunoreactive insulin forms cannot be explained by a decrease in proinsulin mRNA levels, which were similar in InsGH3/C7 cells before and after Cpe overexpression. Therefore, reduced insulin biosynthesis must have occurred at the posttranscriptional level, probably as a result of the increased carboxypeptidase activity present in the cell. Indeed, the extremely high levels of enzymatically active form of Cpe present in these cells could react with other molecules and interfere with the normal proinsulin biosynthesis.

In the other Cpe-overexpressing cell line (InsGH3/C1-HACpe), exogenous Cpe was prevalently expressed in its membrane-bound form (90% of total HACpe immunoreactivity) (Fig. 2). Proinsulin processing was unchanged in InsGH3/C1-HACpe (Table 2), which showed a significant increase in basal (Table 1) and in regulated (FSK-induced) mature insulin release (Fig. 3). The high basal release of insulin by InsGH3/C1-HACpe cell line might be partially explained by the higher level of expression of the proinsulin gene, observed in these cells, compared with their untransfected controls (Fig. 5). As already stated, FSK-induced (regulated) insulin release also increased significantly in InsGH3/C1-HACpe (Fig. 3). Perhaps in these cells, Cpe overexpression, by increasing its membrane-bound form, has more efficiently targeted insulin into the RSP and in the secretory granules. The evidence that in InsGH3/C1-HACpe cells insulin immunoreactivity appears more granular compared with their original clone (Fig. 4) supports this hypothesis. However, ultrastructural studies showed that the pool of secretory granules was not quantitatively increased in InsGH3/C1-HACpe (data not shown). Immunoelec-

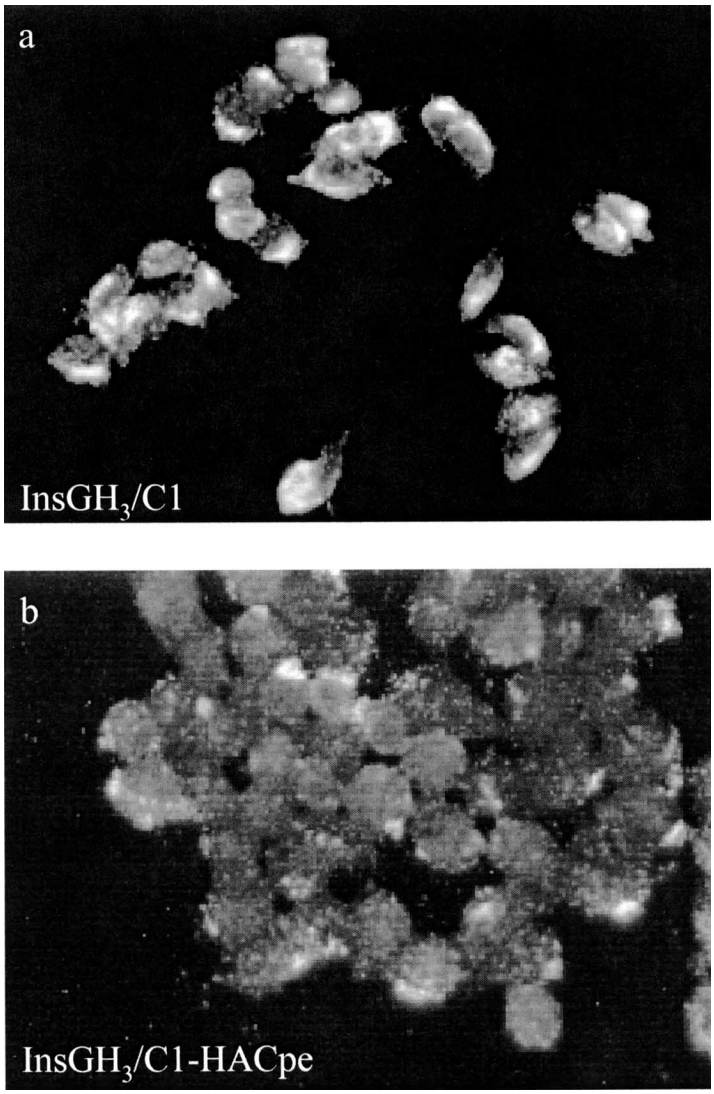


**Figure 4.** Insulin immunostaining in InsGH3/C1 and InsGH3/C1-HACpe cells. Compared with InsGH3/C1, in Cpe-overexpressing cells insulin imunoreactivity appears more granular and less abundant.

tromicroscopy with antibodies against insulin (or proinsulin) was not performed in this study. Therefore, we cannot exclude that an increase in mature insulin content would have occurred in the granules of these cells. It is also possible that exogenous membrane-bound Cpe, by facilitating the translocation of insulin from the trans-Golgi to the granules (accordingly to the receptor-mediated sorting model), might accelerate the process of granule formation and fusion to the plasma membrane, thereby decreasing the half-life of the granules in the cells.

In conclusion, Cpe overexpression into insulin-secreting pituitary GH3 cells (InsGH3) caused an increase in: i) insulin mRNA levels, ii) basal and regulated insulin secretion, and iii) proinsulin processing. The effects of Cpe overexpression were related to the subcellular localization of the exogenous Cpe protein. The increase in the membrane-bound form augmented regulated secretion, while the increase in the soluble form improved proinsulin processing to mature insulin. However, albeit statistically significant, these effects were of small magnitude, probably because endogenous Cpe is already abundantly expressed in neuroendocrine cells. Other strategies might therefore be explored to render the insulin secretory machinery of pituitary GH3 cells more similar to that of the pancreatic β-cell.

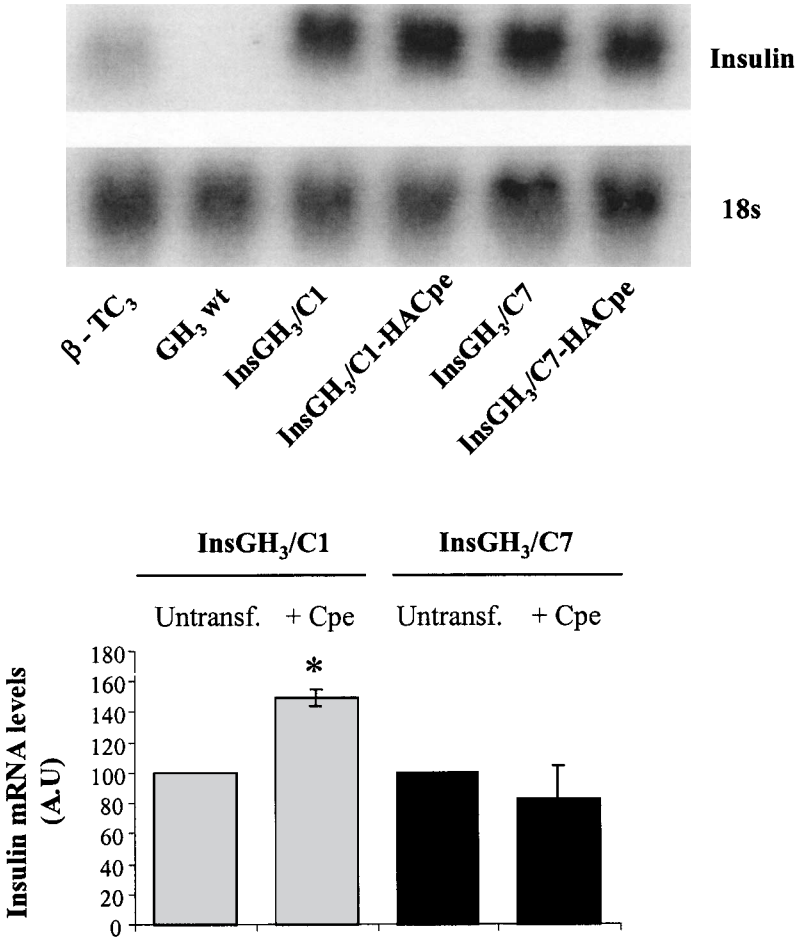


**Figure 5.** (Top) A representative Northern blot that depicts proinsulin gene expression in mouse insulinoma βTC3, wild-type (WT) GH3 cells, InsGH3 clone 1 and 7, and the respective InsGH3/HACpe cell lines. Proinsulin mRNA was higher in InsGH3 and in InsGH3/HACpe than in βTC3 cells and was absent in GH3 WT. (Bottom) Densitometric analysis of four different experiments (insulin mRNA level of untransfected clones were arbitrary assigned to 100%). InsGH3/C1-HACpe cells show a significant increase in insulin gene expression compared with untransfected InsGH3/C1 cells (*$p < 0.01$). In contrast, no differences were detected between InsGH3/C7 and InsGH3/C7-HACpe cells.

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