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Production of bioactive gastrin from the non-endocrine cell lines CHO and COS-7

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Abstract

We made a mutated progastrin cDNA construct that contains a cleavage site (-Arg^{-4}-Arg^{-3}-Lys^{-2} -Arg^{-1}) specific for the Kex2-like endoprotease furin, located ahead of the bioactive gastrin. For expressing the mutated progastrin cDNA, we used two non-endocrine cell lines, CHO and COS-7. CHO cells exhibit amidating enzyme activity and levels of amidation enzyme mRNA as high as those in the pituitary-derived endocrine cell line GH_3, whereas COS-7 cells have far less amidating activity and lower amounts of mRNA. Mutant progastrin-expressing CHO cells produced mostly amidated gastrin. Gel filtration showed the size of this gastrin corresponded to that of the synthetic human gastrin-17. In contrast, COS-7 cells produced glycine-extended gastrin and only a small amount of amidated gastrin. The difference in the amount of amidated gastrin products produced by the two non-endocrine cell lines is due to differing amounts of the amidation enzyme contained in each cell line.

Key words: Prohormone processing; Gastrin; Furin; PAM; CHO cell; COS-7 cell

1. Introduction

Gastrin is a polypeptide hormone with an amide moiety at its carboxyterminal end, and exhibits a number of bioactive forms including gastrin-71 (component I), gastrin-34, gastrin-17, and gastrin-14. This hormone is the primary regulator of gastric acid secretion and growth of the gastrointestinal mucosa. The amidated gastrin-17 (G17-NH_2) is the major molecular form in the antral endocrine cells of the stomach, where it is synthesized first as a precursor preprogastrin (Fig. 1a). This precursor requires a series of post-translational processing reactions to become bioactive gastrin [1]. The processing reactions include dibasic cleavage at paired basic residues, their subsequent removal by carboxypeptidase H, and formation of a carboxyl (C-) terminal amide moiety via the action of the amidation enzyme, peptidyl-glycine α-amidating mono-oxygenase (PAM). This enzyme reaction was recently found to be a two-step reaction involving the two enzymes peptidyl-glycine α-hydroxylating monooxygenase and peptidyl-α-hydroxy-glycine α-amidating lyase [2]. In this reaction, the glycine residue at the carboxyterminal end serves as the substrate for the amidation enzymes that leave the amide moiety with the gastrin peptide. The amidated gastrin (G17-NH_2) thus formed, exhibits gastric acid-secreting activity three orders of magnitude higher than does glycine-extended gastrin (G17-Gly) [3,4].

Non-endocrine cells, including fibroblasts, epithelial cells, and hepatocytes produce biologically inactive propeptides and convert them to bioactive peptides by cleaving a unique consensus sequence -Arg^{-4}-X^{-3}-Lys/Arg^{-2}-Arg^{-1} ↓ X^{+1}- [5]. This sequence contains an additional arginine at position −4 as compared to the common cleavage site of most propeptide hormones, -Lys/Arg^{-2}-Arg^{-1} ↓ X^{+1}-. Proteolytic cleavage of this consensus sequence is catalyzed by the subtilisin-like endoprotease named furin [6]. Furin is thought to be a mammalian endoprotease in non-endocrine cells. This endoprotease is present in virtually all non-endocrine cells, including fibroblasts, epithelial cells, and hepatocytes [7].

In order to explore the physiological role of gastrin, for example, to make hypergastrinemic animals, or to express gastrin in certain local tissues or cells, gastrin need to be expressed not only in endocrine cells but also in non-endocrine cells. To express gastrin in non-endocrine cells, at least two processing steps need to be overcome: cleavage and amidation. The mutated gastrin with the concensus cleavage site mentioned above, -Arg^{-4}-X^{-3}-Lys/Arg^{-2}-Arg^{-1}, should be cleaved in both non-endocrine and endocrine cells. Amidation has been thought to be a unique function of neuroendocrine cells, but this enzyme is distributed widely in almost every tissue, including exocrine organs and heart [8,9]. Re-

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cently, Johansen et al. reported the formation of amidated peptides in a non-endocrine cell line, Chinese hamster ovary (CHO), by showing the expression of amidated neuropeptide Y [10]. Moreover, Eipper et al. demonstrated the presence of the amidation enzyme mRNA in non-endocrine cell lines: Buffalo rat liver BRL 3A cells, mouse connective tissue L-M cells, mouse mammary tumor C127 cells, and mouse-embryo-derived fibroblast-like NIH3T3 cells [11]. Thus, it was thought possible to generate amidated gastrin from progastrin-expressing non-endocrine cells without the co-expression of amidation enzyme.

In this paper, we utilized cells from two contrasting non-endocrine cell lines, CHO and the African green monkey kidney-derived cell line COS-7. CHO cells contain substantial amounts of amidation enzyme mRNA and its enzyme activity, whereas COS-7 cells have very little mRNA, and the enzyme activity is much lower. We have constructed an expression vector containing a mutated gastrin cDNA with the above-stated cleavage site, -Arg^{-4}-X^{-3}-Lys/Arg^{-2}-Arg^{-1}, ahead of the amino terminus of gastrin-17. Mutant progastrin-expressing CHO cells produced mostly amidated gastrin. In contrast, mutant progastrin-expressing COS-7 cells produced correctly cleaved Gly-extended gastrin with only a little amidated gastrin in the culture medium.

2. Materials and methods

2.1. Mutagenesis of human gastrin cDNA and construction of the expression vectors

Human gastrin cDNA (Dr. E. Boel, Copenhagen, Denmark) [12] was modified to remove the 5'-poly(G) tail and the 3'-polyadenylation site, as described previously [13]. The DNA was subcloned into the *Bam*HI site of a M13 mp19 RF form. All oligonucleotides were synthesized using an Applied Biosystems Model 391 PCR-Mate DNA Synthesizer. The mutant DNA was generated by using a site-directed in vitro mutagenesis system kit (Takara, Kyoto, Japan). The progastrin sequence with or without mutation at the processing site was terminated after the Gly position of the G17-Gly. This truncation was carried out by inserting a stop codon after the Gly residue using a polymerase chain reaction (PCR) method [14]. An approximately 300 bp length of the truncated progastrin DNA was subcloned into the *Xho*I site of a pME18S expression vector (Dr. K. Maruyama, Institute of Medical Science, University of Tokyo). The promoter SRα of this vector is composed of the simian virus 40 early promoter, the R segment and part of the U5 sequence of the long terminal repeat of the human T-cell leukemia virus type I [15].

For amidating the C-terminal end of gastrin, we cloned a full sequence of PAM cDNA from a rat brain cDNA library using a partial cDNA clone for PAM (Dr. Betty A. Eipper, Johns Hopkins University, Baltimore) [16]. The full sequence of PAM cDNA was introduced into the *Eco*RI site of the pcDL-SRα vector equipped with a neomycin-resistant gene [15]. The two gastrin expression vectors, MGP1 (with -Pro-Ser-Lys^{-2}-Lys^{-1} at the cleavage site), and MGP2 (with -Arg^{-4}-Arg^{-3}-Lys^{-2}-Arg^{-1} at the cleavage site) (Fig. 1b), as well as the PAM expression vector, were purified twice by a cesium chloride gradient centrifugation.

2.2. Cell culture and DNA transfection by electroporation

A Ham's F12 medium, supplemented with 5% fetal bovine serum (FBS) (Gibco, Grand Island, NY), was used to culture the Chinese hamster ovary-derived CHO-K2 cells (ATCC CCL 61), and a Dulbecco's Modified Eagle's medium (Sigma, St Louis, MO) with 10% FBS was used to culture the African green monkey kidney-derived COS-7 cells (ATCC CRL 1651). Cells were placed in humidified, 5% CO_2 at 37°C. Transfection of DNA into the cells was carried out by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA) as described before [17]. Twenty-four hours after the electroporation, the culture medium was harvested and the cells were incubated in a fresh medium for another 24 h. These COS-7 cells were used for the transient expression of gastrin. The culture medium harvested after the second 24 h period was used for the analysis of immunoreactive gastrin (IRG). The CHO cells were made permanent by co-transfecting the MGP1 or the MGP2 DNA construct with a plasmid containing a neomycin-resistant gene, and maintaining the culture in a medium containing the neomycin analogue G418 (0.4 mg/ml). The culture medium harvested after the first 24 h subculture was used to analyze the gastrin from the CHO cells. The medium was stored at −20°C for later analysis.

The co-expression experiment was carried out using three different ratios of progastrin DNA to amidation enzyme cDNA (1:1/10, 1:1/3 and 1:1). These were mixed maintaining the MGP2 DNA at 15 μg/cuvette, and the electroporation was carried out in the same manner.

2.3. Radioimmunoassay of amidated gastrin and glycine-extended gastrin

G17-NH_2 (Peptide Institute, Osaka, Japan) was labeled with ^{125}I using the standard lactoperoxidase method, and a carboxyterminal hexagastrin plus Gly peptide (GL7) [18] was labeled using the standard chloramine-T method. The radiolabeled peptides were purified using high performance liquid chromatography. Radioimmunoassay (RIA) for amidated gastrin was carried out using an antibody to G17-NH_2 (Ab 5135) (Dr. John H. Walsh, UCLA Medical School). This antibody is specific for gastrin-17 with an amide moiety at its C-terminal end [18]. The assay for Gly-extended gastrin was done using the antibody Ab8237, which is specific for gastrin-17 with a Gly moiety at its C-terminal end [18]. Standard assay mixtures consisted of the ^{125}I-peptide, various amounts of a standard peptide or unknown sample, and an appropriate antibody (final dilution of 1:90,000 for Ab5135, and 1:30,000 for Ab8237) in 0.3 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 0.2% egg albumin and 0.3% bovine γ-globulin. After incubation for 1.5 h at 20°C, 1 ml of 21% polyethylene glycol in 20 mM Tris-HCl buffer (pH 8.0) was added. After mixing the reaction solution, free and bound radioactivity was separated by centrifugation at 3,000 rpm for 15 min, and the radioactivity was measured in a gamma counter.

2.4. Gel filtration

Gel filtration was performed with a 1.0 × 120 cm column (Bio-Rad) of a Sephadex G-50 superfine gel (Pharmacia LKB, Piscataway, NJ) equilibrated with a sodium barbital elution buffer (pH 8.6). Fractions of 1.5 ml were routinely collected. Size calibration was carried out with blue dextran (Vo), potassium ferricyanide (Vt), and standard G17-NH_2 and G34-NH_2 (Peptide Institute).

2.5. Amidation enzyme assay

The amidating activity of the cell lines was assayed by measuring the conversion of ^{125}I-labeled Ac-Tyr-Val-Gly to ^{125}I-labeled Ac-Tyr-Val-NH_2. Ac-Tyr-Val-Gly (Peptide Institute) was radioiodinated by iodobeads (Pierce, Rockford, IL). The standard assay protocol was as described by Mains et al. [19]. The enzyme source was untransfected cells which were scraped from the dish and suspended in 50 mM Tris-HCl (pH 7.0) with the protease inhibitor, aprotinin (100 units/ml). The protein content of the cell extracts was measured with a micro-BCA protein assay kit (Pierce). The specific activity of PAM was calculated by dividing the PAM enzyme activity by the protein content, and expressed as pmol/mg/h.

2.6. RNA isolation and Northern blot analysis

RNA was extracted and electrophoresed as described before [20]. Hybridization was performed with a probe of the amidation enzyme cDNA fragment (*Bam*HI–*Pst*I, 1.3 kb) labeled with [α-^{32}P]dCTP.

3. Results

3.1. Construction of gastrin expression vectors

We mutated a native gastrin cDNA (Fig. 1a) to replace the original cleavage site, -Pro^{-4}-Ser^{-3}-Lys^{-2}-Lys^{-1}, with a new cleavage site, -Arg^{-4}-Arg^{-3}-Lys^{-2}-Arg^{-1}. Then, to simplify the processing reaction, we truncated the C-terminal extension region of progastrin by inserting a stop codon just after G17-Gly (Fig. 1b). With Gly as the C-terminus, the truncated precursor will serve as a direct substrate for PAM without any cleavage reaction. The truncated gastrin precursor with the original cleavage site is named MGP1, the precursor with the new cleavage site is named MGP2 (Fig. 1b). Both precursor cDNAs were inserted into the mammalian expression vector pME18S.

3.2. Amidating activity and RNA analysis

We chose the two cell lines, CHO and COS-7 for this study. Johansen et al. found that CHO amidates exogenously expressed neuropeptide Y (NPY) [10], and Eipper et al. found that COS-7 contains the PAM mRNA [11]. Fig. 2a shows that the CHO cells exhibit enzyme activity comparable to the PAM activity of the rat pituitary endocrine cell line, GH_3, whereas the enzyme activity of the COS-7 cells is much lower.

Northern blots probed with the cDNA for PAM revealed a prominent band of hybridization with RNA from CHO cells, but only a barely visible band with that from COS-7 cells (Fig. 2b). The density of the hybridization band of the RNA from CHO is less than half of that from GH_3 cells, and did not reflect the comparable enzyme activities between CHO and GH_3 cells. Anyway, the content of PAM enzyme activity and the presence of RNA in the COS-7 and CHO cells is in marked contrast.

3.3. Expression of gastrin

Both CHO and COS-7 cells transfected with MGP1 or MGP2 DNA produced a substantial amount of gastrin products. As expected, the cells retained only 1 to 3% of the gastrin released into the culture medium. The same result has been observed in other non-endocrine cell lines: NIH3T3 cells, baby hamster kidney-derived fibroblast-like BHK cells, and rat hepatoma-derived Hepa1–6 cells [21]. The characteristics of gastrin products were monitored by two antibodies: C-terminal glycine-specific (Ab8237), and C-terminal amide-specific (Ab5135) [18].

When either MGP1 or MGP2 was expressed in CHO cells, a substantial amount of amidated gastrin was produced in the culture medium but only a trace of Gly-extended gastrin was produced (Table 1). The ratio of amidated gastrin to the total of Gly-extended gastrin plus amidated gastrin is almost 99% in both the MGP1 and MGP2-expressing CHO cell lines. This result is consistent with the data by Johansen et al., which demonstrated that CHO transfected with a NPY cDNA produced mostly amidated NPY in the culture medium [10]. In contrast, when MGP1 and MGP2 were expressed in COS-7 cells, Gly-extended gastrin was produced much more abundantly than amidated gastrin (Table 1). The ratio of amidated gastrin to the total of Gly-extended plus amidated gastrins was 27% in MGP1-expressing COS-7 cells and 12% in MGP2-expressing COS-7 cells.

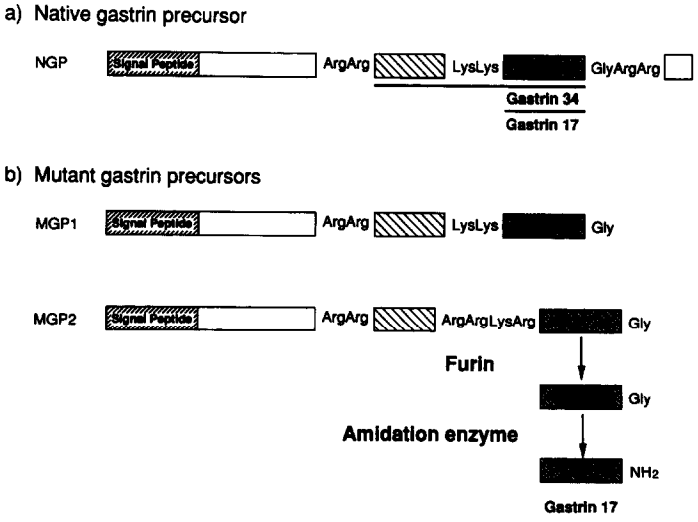

Fig. 1. Structures of gastrin precursor constructs. (a) Structure of a native gastrin precursor (NGP). The 101 amino acid human gastrin precursor is shown with the signal peptide and three dibasic cleavage sites Arg^{57}-Arg^{58}, Lys^{74}-Lys^{75}, Arg^{94}-Arg^{95}. The amino acid is numbered from the methionin residue at the amino terminus of the gastrin precursor. The cleavage of these dibasic residues leads to the formation of G17-NH_2 and G34-NH_2, which are underlined with a solid bar. (b) Structures of the truncated gastrin precursor with an original cleavage site, Lys^{-2}-Lys^{-1} (MGP1), and a tetrabasic cleavage site, Arg^{-4}-Arg^{-3}-Lys^{-2}-Arg^{-1} (MGP2). This amino acid is numbered from the last basic residue at the cleavage site. Both MGP1 and MGP2 have a glycine residue at the C-terminal end.

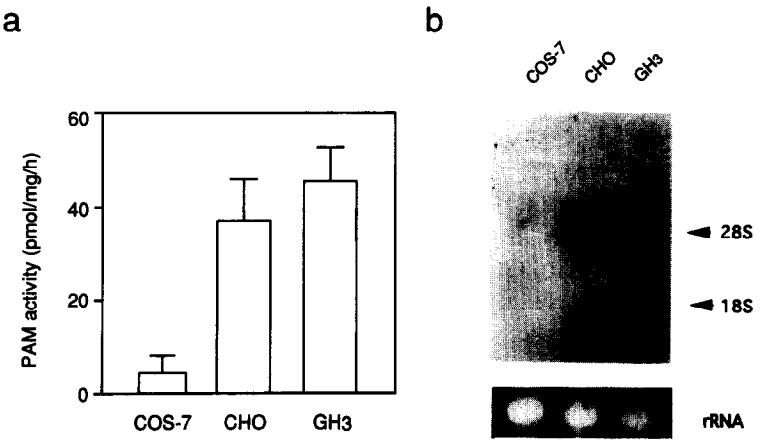

Fig. 2. Analysis of the amidation enzyme PAM in COS-7, CHO, and GH_3 cells. (a) Amidating enzyme activity in cultured cell lines in vitro. The assay was carried out at least three times for each cell line. The values are expressed as the mean ± SE. (b) Northern blot analysis of each cell line with a probe for PAM. Ribosomal RNA size markers electrophoresed on the same gel are indicated as 28S and 18S. The size of PAM RNA in each cell line was estimated to be approximately 4.0 kb.

3.4. Gel filtration

We further characterized the molecular size of the gastrins produced from the CHO and COS-7 cells using Sephadex G-50 superfine columns. The amidated gastrin products from the MGP1-expressing CHO cells were separated into two dominant sizes, shown as a large and a small peak (Fig. 3, left upper panel). The small peak corresponded to the G17-NH_2 standard. The large peak eluted just before the G34-NH_2 position. The immunoreactive gastrin (IRG) from the MGP2-expressing CHO cells also showed two peaks, but in this case the second peak (corresponding to the G17-NH_2 standard) was larger than the first (Fig. 3, left lower panel). Thus, the MGP2 gastrin precursor was cleaved much more efficiently than the MGP1 precursor by endogenous endoproteases.

Since the gastrin products from COS-7 cells are mostly Gly-extended gastrin with some of amidated gastrin, we analyzed the size of the Gly-extended gastrin. The gastrin products from the MGP1-expressing COS-7 cells showed two dominant molecular sizes, as did those from the CHO cells (Fig. 3, right upper panel). As from the CHO cells, the first large peak corresponded to molecules slightly larger than the G34-NH_2 standard, and the second peak corresponded to the G17-NH_2 standard. The MGP2-expressing COS-7 cells produced only a single peak, corresponding to the G17-NH_2 standard (Fig. 3, right lower panel). Thus, the cleavage of the mutated progastrin MGP2 is more efficient in COS-7 cells than in CHO cells.

Table 1
Production of Gly-extended and amidated gastrin in the MGP1 and MGP2-expressing COS-7 and CHO cells

Cell type	Transfected DNA	Gly-extended gastrin (fmol/ml/ 10^6 cells)	Amidated gastrin (fmol/ml/ 10^6 cells)
COS-7	MGP1	712.5 ± 165.8	257.1 ± 32.5
	MGP2	1,285.8 ± 483.7	176.2 ± 12.4
CHO	MGP1	6.0 ± 6.0	698.4 ± 133.9
	MGP2	5.8 ± 5.0	488.0 ± 71.3

Values are expressed as mean ± S.E.

3.5. Co-expression of gastrin with PAM in COS-7 cells

To obtain more amidated gastrin from the COS-7

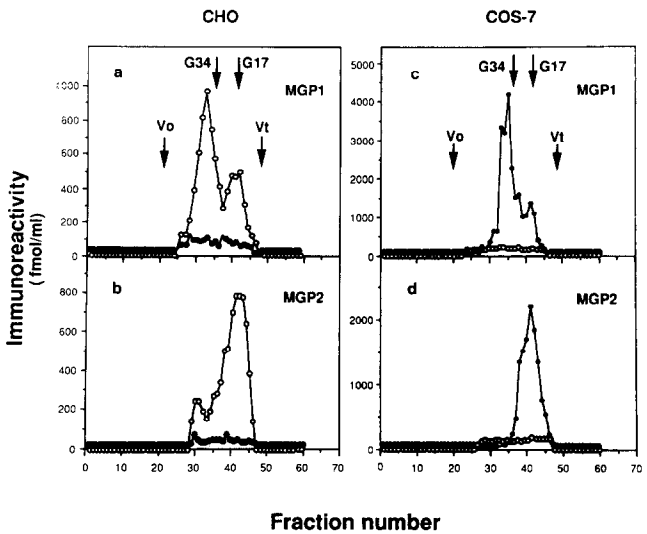

Fig. 3. Gel filtration of IRG in media from CHO and COS-7 cells transformed to express MGP1 and MGP2 DNA. (a) MGP1-expressing CHO cells; (b) MGP2-expressing CHO cells; (c) MGP1-expressing COS-7 cells; (d) MGP2-expressing COS-7 cells. IRG in eluted fractions was analyzed with the Ab5135 antibody (○) and Ab8237 antibody (●), as described in Section 2. Columns were calibrated with blue dextran (Vo), potassium ferricyanide (Vt), and standard G17-NH_2 and G34-NH_2. Similar chromatograms were obtained in at least three other experiments for each cell line.

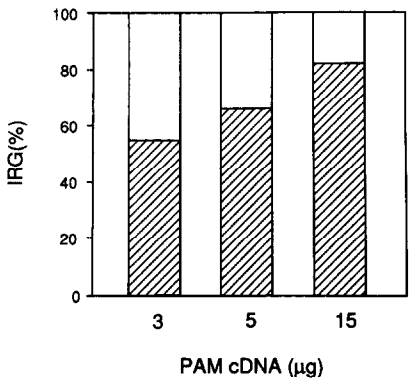

Fig. 4. The relative ratio of Gly-extended gastrin and amidated gastrin in the co-expression of MGP2 and PAM in COS-7 cells. 15 μg of MGP2 DNA was co-transfected to COS-7 cells with three different amounts of PAM cDNA (1.5 μg, 5.0 μg, and 15 μg). IRG was assayed with the antibodies Ab5135 and Ab8237 as described in Section 2. The amount of each gastrin form is expressed as the percentage of the total gastrin (Gly-extended gastrin plus amidated gastrin). The hatched column represents the percentage of amidated gastrin, and the open column does that of Gly-extended gastrin.

cells, we co-expressed the amidation enzyme cDNA together with the MGP2 DNA. The PAM cDNA-transfected COS-7 cells showed amidating enzyme activity as high as that of CHO cells. As expected, increased amounts of PAM cDNA caused increased amounts of amidated gastrin products, and correspondingly decreased amounts of Gly-extended gastrin (Fig. 4). Gly-extended gastrin must be utilized as a substrate for the amidation reaction.

4. Discussion

In this study we have shown that non-endocrine cells are able to produce correctly cleaved and amidated gastrin if the gastrin precursor is designed to undergo cleavage by the non-endocrine cell-specific endoprotease furin. The need for the expression of the amidation enzyme, PAM, depends on the level of the endogenous amidation enzyme activity in the non-endocrine cells.

It has been long understood that propeptide-expressing non-endocrine cells produce only non-processed propeptides, as demonstrated in a variety of non-endocrine cell lines, such as CHO, COS, and NIH3T3 [21–23]. However, in exceptional cases, some peptide precursors were correctly cleaved in non-endocrine cells. Warren and Shields [24] observed correctly cleaved somatostatin in non-endocrine cell line COS-7, as did Sevarino et al. [25] in the non-endocrine cell line 3T3-Swiss-Albino. In both cases, however, the cleaved fraction was small compared to the uncleaved somatostatin precursor. More recently, partial cleavage of the NPY precursor was reported in transfected CHO cells [26]. Partial cleavage was also observed in the pancreatic polypeptide (PP) precursor, a peptide hormone of the same family, by expressing its cDNA in NIH3T3, Hepa1–6, and BHK cells [21]. The cleavage of certain propeptide hormones is explained by the resemblance of processing sites of a precursor to the concensus processing sequence of non-endocrine cells, $-Arg^{-4}-X^{-3}-Lys/Arg^{-2}-Arg^{-1}$. The order of the basic amino acids in their cleavage sites, however, does not strictly adhere to this rule. This type of cleavage is useful for producing small, bioactive peptide hormones from non-endocrine cells [17,20].

One unexpected result was the appearance of an IRG peak close to the standard G34-NH_2 position. In the previous study [21], when the full sequence of native progastrin was expressed in non-endocrine cells (NIH3T3 derived-psi2, BHK, and Hepa1–6), the only large IRG peak appeared close to the void volume on a Sephadex G-50 gel filtration using an antibody that recognizes the arginin-extended progastrin, but not recognizes the glycine-extended or amidated gastrin. Since IRG was not examined with antibodies reactive to the glycine-extended or amidated gastrin, we did not know of the IRG peak close to the G34-NH_2 position in that study [21].

Amidation is the other important reaction for the processing of most peptide hormones to induce their full biological potency [3,4]. When peptide hormone precursors are expressed in endocrine cell lines such as GH_3 cells, mouse pituitary-derived AtT20 cells, rat insulinoma-derived RIN cells, or rat pheochromocytoma-derived PC12 cells, they are correctly cleaved and amidated [27]. In contrast to the processing in endocrine cells, the peptide hormone precursors in non-endocrine cells are not generally cleaved and the C-terminal glycine is not exposed for the amidation reaction [21]. Thus, the amidation capability in non-endocrine cells remained to be investigated. Recently, Johansen et al. demonstrated that 50 to 80% of the exogenously expressed NPY was amidated in CHO cells by using an antibody specific for the C-terminal amide moiety of NPY [10]. Dickinson et al. also demonstrated definite amidation of exogenously expressed PP in an exocrine pancreatic cell line, AR42J, which contains high levels of amidation enzyme mRNA comparable to that in CHO and GH_3 cells [21]. Thus, the amidation capability of non-endocrine cells appears to depend on the amidation enzyme activity of non-endocrine cell lines. This was exemplified by our present data using the two cell lines, CHO and COS-7, which had contrasting amounts of amidation enzyme. CHO essentially produced only amidated gastrin while COS-7 produced mostly non-amidated Gly-extended gastrin. Thus, in order to produce amidated, bioactive gastrin from cells with low amidating activity, co-expression of PAM is required.

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