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Inhibitory Effect of the α_1-Antitrypsin Pittsburgh Type-Mutant (α_1-PIM/R) on Proinsulin Processing in the Regulated Secretory Pathway of the Pancreatic β-Cell Line MIN6

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Abstract. To elucidate its effect on proinsulin processing, we introduced the expression of a Pittsburgh type-mutant, α_1-protease inhibitor M/R (α_1-PIM/R) and its chimera protein with growth hormone (GH) (GHα_1-PIM/R) into MIN6 cells. In metabolic labeling and chasing experiments with [^{3}H]-Leu and [^{35}S]-Met, proinsulin appeared in the medium during stimulatory secretion only from MIN6 clones expressing GHα_1-PIM/R and, surprisingly, α_1-PIM/R, but not from the clones of either the control or α_1-PI. The major part of α_1-PIM/R was secreted through the constitutive pathway and about 10% of total secreted α_1-PIM/R in the chase periods entered the regulated pathway. On the other hand, GHα_1-PIM/R was mainly transported to the secretory granules and about 80% of the total secreted GHα_1-PIM/R in the chase periods was secreted during stimulatory secretion. In the first 3 h chase periods without stimulation, only α_1-PIM/R and no GHα_1-PIM/R appeared in the medium, thus suggesting that α_1-PIM/R might be transported through a constitutive-like pathway for those periods. The α_1-PI, which had no inhibitory effect on proinsulin processing, showed similar secretion pathways to those of α_1-PIM/R. This implies that some part of α_1-PIM/R and α_1-PI entered the regulated pathway, not due to any specific interaction between the processing endoproteases and serine protease inhibitors, but due to some type of passive transport in a nonselective manner. The inhibitory effect of α_1-PIM/R in the regulated secretory pathway was slightly but clearly evident when it was expressed in MIN6 β-cells.

Key words: Proinsulin, Processing, Regulated pathway, α_1-Antitrypsin Pittsburgh, MIN6

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MANY proteins and peptide hormones are first synthesized as large precursors and then undergo endoproteolytic cleavage and the amino-terminal and/or carboxy-terminal modifications by specific endoproteases and modifying enzymes in order to attain their mature forms. These proproteins may be transported through either a constitutive secretory pathway or a regulated secretory pathway [1, 2].

The constitutive pathway is probably the most common one for most cells from various organs, and in this pathway, proproteins are transported constitutively from the *trans*-Golgi network (TGN) to plasma membranes [3, 4]. The regulated pathway specifically exists in endocrine and neuroendocrine cells, which have secretory granules containing peptide hormones or neurotransmitters and these cells secrete them in response to extracellular stimulation [5]. During the transportation along the regulated pathway, propeptide hormones are cleaved at consensus amino acid sequence RR or KR by specific endoprotease PC2 and/or PC3 (also called PC1) in many cases and then are stored within the secretory granules [6]. In insulin-producing pancreatic β cells, proinsulin is thought to be transported from TGN to

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the immature granules where it is processed into a mature form, insulin by PC2 and PC3, followed by aggregation and crystallization in the mature granules [7–9] (Table 1).

The MIN6 cell line was established from insulinomas obtained by large T antigen gene-transgenic mice and predominantly secretes mature insulin, which indicates that it might maintain the regulated secretory pathway as a major route for insulin secretion, while it also has a potent processing capability due to the efficient expression of PC2 and PC3 [10, 11].

A genetic variant of α_1-antitrypsin, designated α_1-antitrypsin Pittsburgh was found in a patient associated with a fatal bleeding disorder [12, 13]. It has a single substitution, Arg for Met^{358} in the reactive site of human α_1-antitrypsin, resulting in its acquisition of the antithrombin III activity [13, 14] (Table 2). In addition, the mutant α_1-antitrypsin has been shown to inhibit the yeast Kex2 protease [15], furin [16] and the proinsulin-processing enzymes in β-cell granules [17]. We prepared the expression plasmid vectors of the α_1-antitrypsin Pittsburgh type-mutant (α_1-PIM/R) [18] constituted from rat α_1-antitrypsin (α_1-protease inhibitor designated as α_1-PI) cDNA and its chimera protein with growth hormone ($GH\alpha_1$-PIM/R). We hypothesized that α_1PIM/R might be transported from TGN to the plasma membrane along the constitutive pathway, while $GH\alpha_1$-PIM/R might be sorted to the secretory granules in the regulated pathway in a manner similar to that of other chimeric hormones and zymogens, which have been shown to be effectively transported and accumulated when expressed in endocrine cells [19, 20]. They were introduced into MIN6 cells, to elucidate any differences in their secretory pathways and effects on proinsulin processing during transportation from TGN either to plasma membrane or to immature granules and thereafter to mature granules.

Table 1. Processing sites of mouse and human proinsulins.

No. of amino acids	27	28	29	30	31	32▼[a]	33
Mouse I	Thr	Pro	Lys	Ser	**Arg**	**Arg**	Glu
Mouse II	—	—	Met	—	**Arg**	**Arg**	—
Human	—	—	Lys	Thr	**Arg**	**Arg**	—
No. of amino acids	60	61	62	63	64	65▼[a]	66
Mouse I	Val	Ala	Arg	Gln	**Lys**	**Arg**	Gly
Mouse II	—	—	Gln	—	**Lys**	**Arg**	—
Human	Gly	Ser	Leu	—	**Lys**	**Arg**	—

[a] The arrows indicate the cleavage sites by PC3 (at $Arg^{31}Arg^{32}$) and PC2 (at $Lys^{64}Arg^{65}$).

Materials and Methods

Materials

L-[3,4,5-^{3}H(N)]leucine (180 Ci/mmol) and L-[^{35}S] methionine (1175 Ci/mmol), were purchased from DuPont-NEN (Boston, MA). [^{32}P]dCTP (3 mCi /mmol) and Protein A sepharose CL-4B were from Amersham Pharmacia Biotech (Newark, NJ). Rabbit polyclonal anti-rat α_1-antitrypsin antiserum was made as previously described [18]. Guinea pig anti-human insulin antiserum was obtained from Linco (St Louis, MO). Rat pituitary cDNA library was purchased form Clontech (Palo Alto, CA) and human growth hormone cDNA was from ATCC (#40011). N-[1-(2,3-dioleyloxy)propyl]-n,n,n,-trimethylammonium chloride (lipofectin) and G418 were from GIBCO (Grand Island, NY).

Table 2. Amino acid sequences at the reactive sites of α_1-PI and α_1-PIM/R.

Inhibitor	Reactive Site						Target Enzyme
	P3	P2	P1	P1'	P2'	P3'	
α_1 PI (human)	Ile	Pro	**Met** ↓	**Ser**	Ile	Pro	Elastase, Trypsin
α_1 PI/Pittsburgh (human)			**Arg**				Thrombin, a)
AT III (human)	Ala	Gly	**Arg**	**Ser**	Leu	Asn	Thrombin
α_1 PI (rat)	Val	Pro	**Met** ↓	**Ser**	Leu	Pro	Elastase, Trypsin
α_1 PIM/R (rat)			**Arg**				a)

a): Inhibitory activity for proprotein convertases.

Cell culture

MIN6 cells [10] were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 25 mM glucose and 0.39 mmol/l mercaptoethanol, supplemented with 15% fetal bovine serum (FBS), 75 U/ml penicillin sulfate and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Plasmid construction and the generation of stable cell lines

The cDNA of rat growth hormone was cloned by screening the rat pituitary cDNA library with human growth hormone cDNA (ATCC#40011) as a probe. The α_1-PIM/R was constructed to incorporate a substitution of methionine 352 to arginine in the rat α_1-antitrypsin by site-directed mutagenesis as reported previously [18]. A *Nsp* V recognition site was introduced by site-directed mutagenesis at the 3′end of coding sequences in rat GH gene, and at the sequences of coding region immediately after the signal peptide of α_1-PIM/R. After the introduction of the *Nsp* V, two kinds of cDNA in the plasmid vectors were digested by *Nsp* V and also *Bam* H I in the multiple cloning region, and then were joined together resulting in the chimera gene of GH and α_1-PIM/R (GHα_1-PIM/R). Each of the genes of rat α_1-PI, α_1-PIM/R and GHα_1-PIM/R, was then inserted into the expression vector pCXN [21], which was based on the chicken β-actin promoter plus the cytomegalovirus immediate early region enhancer. They were transfected into MIN6 cells (passage 17) by a liposome method using lipofectin. As a control, MIN6 cells were also transfected with the empty vector pCXN alone. Stable transformants were selected by maintaining the culture in a medium containing 600 μg/ml of the neomycin analog G418.

Western blotting

The supernatants of the overnight culture of the stable expression MIN6 clones in serum-free culture medium containing 25 mM glucose were precipitated with 10% [wt/vol] trichloroacetic acid (TCA), washed with acetone and dried. The precipitated specimens were solubilized in phosphate-buffered saline (PBS), and the protein concentrations in the samples were determined by the method of Lowry *et al.* [22] using bovine serum albumin (BSA; fraction 5, Sigma) as a standard. Three μg protein of each sample were electrophoresed on a 9% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were washed with TBST buffer (20 mM Tris-buffered saline, pH 7.5, 150 mM NaCl, 0.05% [vol/vol] Tween) and then incubated in blocking buffer (TBST containing 3% [wt/vol] non-fat dry milk) for 1 h. The membranes were incubated in TBST buffer containing 1% [wt/vol] BSA with rabbit anti-rat α_1-antitrypsin (1 : 1,000 dilution) for 2 h. After washing with TBST buffer, the membranes were incubated with TBST buffer containing 1% BSA with peroxidase-conjugated anti-rabbit IgG (1 : 5,000 dilution) for 1 h, and washed three times with TBST and twice with TBS (TBST without Tween). Peroxidase was then developed with a solution consisting of 50 mM Tris-buffered saline, pH 7.5, 0.01% H_2O_2 and 3,3′-diaminobenzidine (0.2 mg/dl). For an analysis of proinsulin and proinsulin processing products, MIN6 clones were cultured overnight in DMEM containing 5.5 mM glucose supplemented with 15% FBS. The medium was then changed to DMEM containing 25 mM glucose without FBS and the incubation was continued for a further 2 h. The culture supernatants were precipitated with 10% TCA as described above. Three μg protein of each sample were electrophoresed on a 16.5% Tricine-buffered polyacrylamide gel to analyze proinsulin and proinsulin processing products, and were followed by Western blotting using guinea pig anti-insulin antiserum (1 : 1,000 dilution) as the first antibody and peroxidase-conjugated anti-guinea pig IgG (1 : 5,000 dilution) as the second antibody.

Metabolic labeling of MIN6 cells expressing α_1-PI, α_1-PIM/R or GHα_1-PIM/R

Four kinds of MIN6 clones, control (mock), α_1-PI, α_1-PIM/R and GHα_1-PIM/R, were cultured in DMEM containing 600 μg/ml G418, supplemented with 15% FBS. For metabolic labeling of proinsulin, cells were cultured in 60-mm plastic dishes with DMEM and incubated with leucine-free MEM containing 25 mM glucose for 2 h. The cells were then labeled by incubation in leucine-free MEM containing 5.5 mM glucose, 10% dialyzed FBS and 0.25 mCi of [^{3}H]-Leu at 37°C for 16 h, and chased in DMEM containing

1.67 mM glucose for the first 3 h to allow only the constitutive pathway to work, and then 25.0 mM glucose for the second 3 h in order to stimulate secretion. Media were collected three times at intervals of one hour in the first 3 h of chase period and once at the end of the second 3 h of chase period. The collected samples were then centrifuged to remove any cellular debris, followed by the addition of protease inhibitors and stored at −80°C until immunoprecipitation. For metabolic labeling of α_1-PI, α_1-PIM/R and GHα_1-PIM/R, cells were preincubated in methionine-free MEM containing 25 mM glucose for 2 h, and then were labeled by incubation in methionine-free MEM containing 5.5 mM glucose, 10% dialyzed FBS and 0.5 mCi of [^{35}S]-Met at 37°C for 16 h. The cells were then incubated according to the same protocol as [^{3}H]-Leu labeling, and media were collected at the same intervals and stored at −80°C with protease inhibitors.

Immunoprecipitation

Immunoprecipitation was performed on 1.5 ml of media. The samples were precleared with 7.5 μl of non-immune serum and 25 μl of protein A sepharose. After 1 h of incubation, the suspension was centrifuged, and then the supernatant was subjected to immunoprecipitation with antisera to insulin or α_1-PI, as previously described [18, 23]. Immunoprecipitates were washed, solubilized in Laemmli buffer containing 2% 2-mercaptoethanol, and boiled at 95°C for 5 min. Samples were electrophoresed on a 16.5% Tricine-buffered polyacrylamide gel for the analysis of proinsulin and proinsulin processing products or a 9% SDS-polyacrylamide gel for α_1-PI and α_1-PI mutants. Gels were fixed in 10% TCA, 10% acetic acid and 30% methyl alcohol for 1 h and soaked in a fluorographic solution for 30 min. The gels were then dried and exposed to X-ray film with an intensifying screen for 3–7 days. Densitometric analyses of the fluorographic films were carried out using a personal scanning imager PDSI (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis

MIN6 cells cultured in DMEM were washed twice with PBS to remove any detached cells. Total RNA of each clone was extracted using the acid guanidium thiocyanate/phenol/chloroform extraction method [24]. Ten μg of each sample were electrophoresed using a gel consisting of 0.8% [wt/vol] agarose, 6.6% [vol/vol] formaldehyde/MOPS (3-[N-morpholino]propanesulfonic acid; Dojindo Kumamoto, Japan) and then were transferred to a nylon membrane (Hybond N$^+$; Amersham). Hybridizations were carried out using mouse probes corresponding to the 3′ end of furin [25], PC2 [26] and PC3 [27]. The probes were radiolabeled with [α-^{32}P]-dCTP and hybridized at 65°C overnight. After washing at 65°C, the membranes were exposed to X-ray film and then underwent autoradiography at −80°C.

Immunocytochemistry

The cells of four kinds of stably-transfected clones were cultured on glass coverslips for 2 to 3 days, and fixed with 4% [wt/vol] paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4, for 1 h at 4°C. The cells were made permeable by treatment with 0.1% [wt/vol] saponin in PBS and blocked with 0.1% [wt/vol] BSA and 0.02% [vol/vol] Tween in PBS. The cells were then incubated with rabbit anti-α_1-PI antiserum diluted 1 : 100 in PBS containing 0.1% saponin for 10 min, and then rhodamin-labeled goat anti-rabbit IgG for 10 min. For double immunostaining, the cells were then incubated with guinea pig anti-insulin antiserum diluted 1 : 200 for 10 min, followed by incubation for 10 min with FITC-conjugated goat anti-guinea pig IgG. The specimens were examined through an Olympus BX50 microscope equipped with an incident illuminator BX-FLA.

Results

Effects of α_1-PIM/R and GHα_1-PIM/R on proinsulin processing in MIN6 cells

The MIN6 cells stably-transfected with the expression vector of α_1-PI, α_1-PIM/R or GHα_1-PIM/R secreted into the medium, 56 kD, 56 kD or 76 kD protein, respectively, which were all detected by anti-rat α_1-PI anti-serum (Fig. 1). The 76 kD protein was compatible with the chimeric protein of GH and α_1-PIM/R. The proinsulin and proinsulin-processing products in the medium of these MIN6 clones were analyzed by Western blotting using anti-insulin anti-

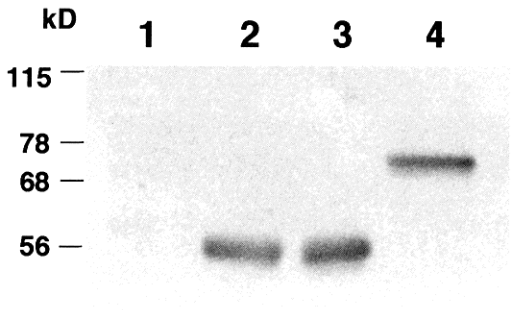

Fig. 1. A Western blot analysis of α_1-PI, α_1-PIM/R and GHα_1-PIM/R in the secretion media of the stable MIN6 clones.
MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R were incubated in serum-free DMEM for 24 h. Aliquots of the secretion media were precipitated with 10% of TCA at the final concentration, and solubilized with PBS (pH 7.4). Three μg of each sample was analyzed by 9% SDS-polyacrylamide gel electrophoresis and Western blot methods as described in the "Materials and Methods" section. lane 1; control vector, lane 2; α_1-PI, lane 3; α_1-PIM/R, lane 4; GHα_1-PIM/R

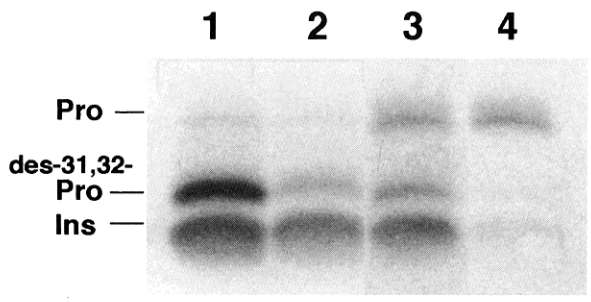

Fig. 2. A Western blot analysis of proinsulin processing in the secretion media of MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R.
MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R were incubated in the medium containing 5.6 mM of glucose overnight and then 25.0 mM of glucose for 2 h, and media were collected, precipitated with 10% of TCA at a final concentration and solubilized with PBS (pH 7.4). Three μg of each sample was electrophoresed on a 16.5% Tricine-buffered polyacrylamide gel and then were analyzed by Western blot methods as described in the "Materials and Methods" section. Pro, des-31,32-Pro and Ins represent proinsulin, des-31,32-proinsulin and insulin, respectively. lane 1; control vector, lane 2; α_1-PI, lane 3; α_1-PIM/R, lane 4; GHα_1-PIM/R

serum (Fig. 2). In the control and α_1-PI-transfected cells, the major products secreted into the medium were insulin and des-31,32-proinsulin, and both of their percentages of proinsulin per total of proinsulin, des-31,32-proinsulin and insulin were 3.9%. Contrary to our expectations, α_1-PIM/R inhibited proinsulin processing even though it was only partially complete and, as a result, proinsulin comprised 22.6% of total of proinsulin, des-31,32-proinsulin and insulin. GHα_1-PIM/R also inhibited the processing of proinsulin, and showed an intensive inhibitory effect, which caused a 64.0% proinsulin secretion in the medium.

Since these samples used in the Western blotting analyses, contained the peptides secreted from both the constitutive and regulated pathways during the incubated period, we decided to perform metabolic labeling to elucidate the secretory pathway of proinsulin, particularly to determine which one suffer the inhibition of processing by α_1-PIM/R and GHα_1-PIM/R. As shown in Fig. 3, during the first 1 h of the first chase period, all four clones secreted proinsulin and insulin at various percentages. In the first 1 h, both the control and the cells expressing α_1-PI secreted insulin predominantly and proinsulin in a very small percentage (1.0% and 4.2%, respectively), and also secreted only insulin in the following 2 h under low glucose concentration. In 3 h of the second chase period, the amount of insulin secreted from these clones increased due to the stimulation of a high concentration of glucose, and proinsulin secretion was hardly observed. On the other hand, the cells expressing α_1-PIM/R and GHα_1-PIM/R secreted proinsulin in a larger proportion than control and the cells expressing α_1-PI, during the first 1 h of the first chase period (37.6% and 23.5%, respectively) and continued to secrete proinsulin during the following 2 h of the first chase period. They also secreted proinsulin in addition to insulin, in the second chase period due to the stimulation of a high concentration of glucose, thus indicating that a considerable amount of proinsulin, without any further processing of insulin, existed in the secretory granules. Although α_1-PIM/R itself seems to be secreted through the con-

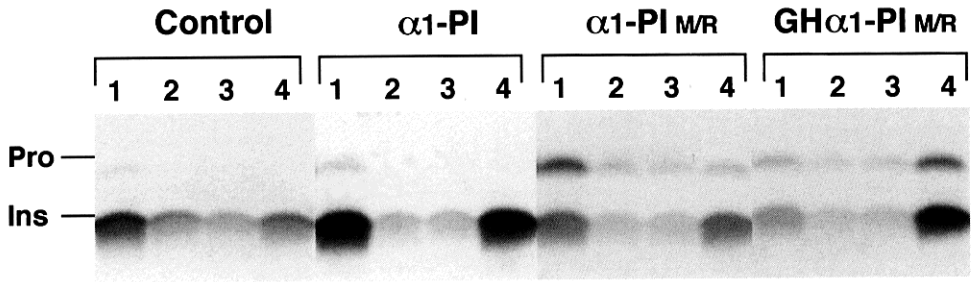

Fig. 3. Metabolic labeling of proinsulin in MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R.
MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R were labeled with 1.0 mCi of [^{3}H]-Leu for 16 h and chased for 3 h in the medium containing 1.7 mM glucose, and then for an additional 3 h in the medium containing 25.0 mM glucose. The media were collected at the end of each 1 h of the first chase period (lanes 1, 2, 3) and the second chase period (lane 4), and were immunoprecipitated with anti-insulin antiserum. Each aliquot of sample was electrophoresed on a 16.5% Tricine-buffered polyacrylamide gel followed by fluorography. "Pro" and "Ins" represent proinsulin and insulin, respectively.

stitutive pathway, this result suggests that a portion of α_1-PIM/R was secreted through the processing site of the proinsulin.

The secretory pathways of α_1-PIM/R and GHα_1-PIM/R

A metabolic labeling experiment using [^{35}S]-Met was employed to analyze the secretory pathways of α_1-PI, α_1-PIM/R and GHα_1-PIM/R. As shown in Fig. 4A, major proportions of the newly synthesized α_1-PI and α_1-PIM/R were secreted during the first 1 h of the first chase period (79.8% and 90.2% respectively) (Fig. 4B), and the remaining small proportions of α_1-PI and α_1-PIM/R (13.9% and 7.1%) were secreted in the second and the third 1 h of the first chase period. Faint bands of the 56 kD protein were seen in the media of these cells during stimulatory secretion, thus indicating that very small proportions of α_1-PI and α_1-PIM/R (6.1% and 2.7%) were transported to the secretory granules. In contrast, only 16.5% of GHα_1-PIM/R was secreted for the first 1 h of the first chase period and most of the radioactivity of GHα_1-PIM/R (82.1%) was detected in the medium from the second chase period. These results indicate that large proportions of the α_1-PI and α_1-PIM/R were secreted through the constitutive pathway and very small proportions were secreted through the regulated pathway, while GHα_1-PIM/R mainly targeted the secretory granules and appeared in the medium due to the regulated secretion. In addition, small proportions of α_1-PI and α_1-PIM/R (13.9% and 7.1%) were secreted in the second and the third 1 h of the first chase period, while no GHα_1-PIM/R was secreted during those chase periods. These differences might indicate that in the first chase period, small proportions of α_1-PI and α_1-PIM/R were discharged from the secretory granules, while GHα_1-PIM/R was not discharged but instead was retained in the secretory granules.

Northern blot analysis of PC2, PC1/3 and furin

All the clones showed major expressions of PC2 and PC3 (Fig. 5). The expression of a very small amount of furin was observed in all clones only after a long exposure, although no differences in the level of expression were observed among these clones.

Immunocytochemical analysis for the localization of α_1-PI, α_1-PIM/R and GHα_1-PIM/R within the MIN6 cells

The immunoreactivities of α_1-PI, α_1-PIM/R and GHα_1-PIM/R in MIN6 cells were analyzed by conventional immunofluorescence method using both anti-insulin antiserum and anti-α_1-PI antiserum (Fig. 6). The results of immunostaining for inhibitors revealed the presence of fluorescence in perinuclear structures resembling Golgi apparatus and granular structures scattered around the cytoplasm of MIN6 cells expressing α_1-PI, α_1-PIM/R and GHα_1-PIM/R (Fig. 6d, f, h). In cells expressing GHα_1-PIM/R, the signals of cytoplasmic granular pattern were more

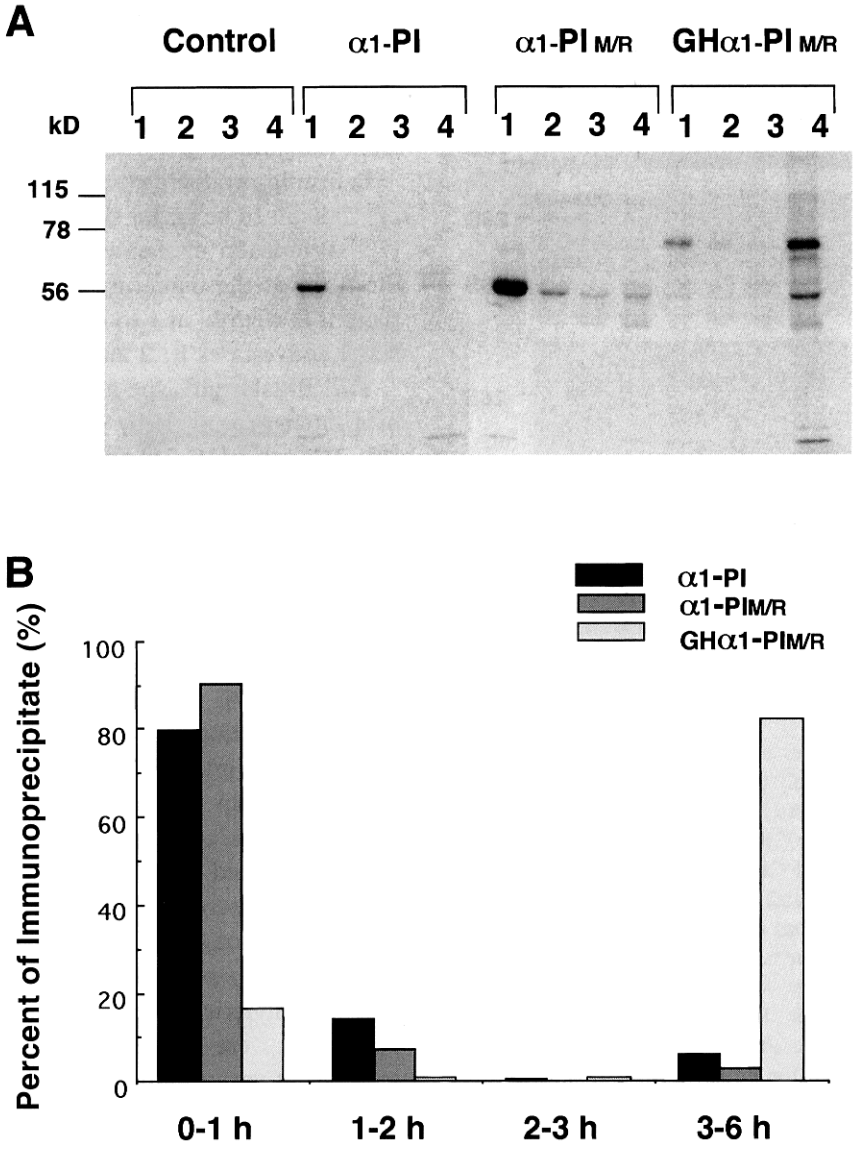

Fig. 4. Metabolic labeling of α_1-PI, α_1-PIM/R and GHα_1-PIM/R.
A: MIN6 cells expressing α_1-PI, α_1-PIM/R or GHα_1-PIM/R were labeled with [^{35}S]-Met for 16 h and chased for 3 h in the medium containing 1.7 mM glucose and for an additional 3 h in the medium containing 25.0 mM glucose. The media were collected at the end of each 1 h of the first chase period (lanes 1, 2, 3) and the second chase period (lane 4), immunoprecipitated with anti-α_1-PI anti-serum and electrophoresed on a 9% SDS-polyacrylamide gel followed by fluorography. B: The quantities of α_1-PI, α_1-PIM/R or GHα_1-PIM/R were assessed by densitometric analyses of the above films. All data are expressed as means of two independent experiments. The graph depicts the percentages of α_1-PI, α_1-PIM/R or GHα_1-PIM/R, secreted during the indicated chase periods to all the secreted α_1-PI-related materials during the total chase periods.

intensive than in the cells expressing α_1-PI and α_1-PIM/R, and these granular structures coincided approximately with the secretory granules including insulin, as compared with the staining patterns with anti-insulin antiserum (Fig. 6c, e, g). There was basically no difference in the localization of α_1-PI, α_1-PIM/R and GHα_1-PIM/R, although the distribution of the major intensity of fluorescence was perinuclear in α_1-PI and α_1-PIM/R, while it was in the granular structures in GHα_1-PIM/R.

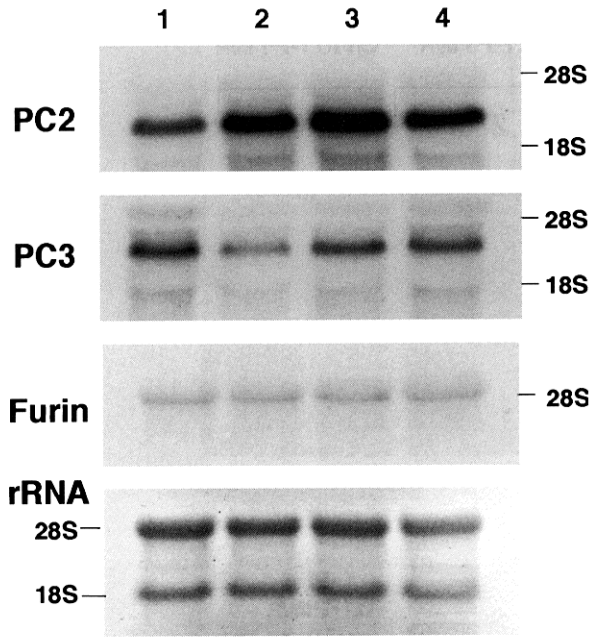

Fig. 5. A Northern blot analysis of proprotein convertases in MIN6 cells expressing α_1-PI, α_1-PIM/R or GHα_1-PIM/R.
Total RNA was extracted from 1×10^7 cells of each MIN6 clone stably-transfected with control vector (lane 1), the expression vector of α_1-PI (lane 2), α_1-PIM/R (lane 3) or GHα_1-PIM/R (lane 4). Each 10 μg of samples was electrophoresed, transferred to a nylon membrane, and the membrane was hybridized with mouse probes corresponding to the 3′ end of PC2, PC3 and furin. The expressions of PC2, PC3 and furin messages were assessed by Northern blotting as described in the "Materials and Methods" section. The exposure times on X-ray film were 30 min for PC2 and 3 h for PC3 and furin at −80°C. 28S and 18S represent ribosomal RNAs with ethidium bromide staining.

Discussion

Newly synthesized proteins are thought to be transported along two major routes from TGN: the constitutive secretory pathway from TGN to the cell surface, and the regulated secretory pathway existing in both endocrine and neuroendocrine cells. In the cytoplasm of such cells, mature secretory granules with a dense core are formed, accumulated and fated for the regulated exocytosis. However, recent studies suggest that protein secretion involves a more complicated process with numerous trafficking routes than previously thought [6]. One of them may be a constitutive-like pathway [28], which is supposed to exist in islet β-cells, because a small quantity of C-peptide cleaved from proinsulin is continuously discharged from the immature granules and therefore the slight excess of insulin in a molar basis is secreted from the mature granules by stimulation.

In insulin-producing β-cells, the immature granules are thought to be major sites of proinsulin processing [7]. Proinsulin may enter the regulated pathway in the form of the immature granules from TGN, and there it is cleaved at two dibasic sites by the prohormone convertases PC2 and PC3.

The Pittsburgh type-mutant α_1-PI inhibited the proprotein processing by yeast Kex-2 protease (kexin) [15, 34], furin [16, 35] and proinsulin-processing enzymes in the secretory granules [17]. This mutant has been used to confirm the characteristics of proprotein-processing enzymes purified from various organs as a specific inhibitor, before and after the identification of furin and the other prohormone convertases by means of molecular clonings of their genes [17, 34, 36, 37].

By introducing the expression of α_1-PIM/R and GHα_1-PIM/R into MIN6, we expected to elucidate the relationship between proinsulin processing and targeting to the regulated pathway. The original α_1-PI was secreted from the hepatocytes through the constitutive secretory pathway [38], while GHα_1-PIM/R was thought to be secreted through the regulated secretory pathway, based on previous reports that the chimeric proteins with peptide hormones or zymogens, were always transported to the secretory granules at various efficiencies [19, 20]. We thus expected that α_1-PIM/R, if it was transfected into MIN6 cells, could not inhibit proinsulin processing, while GHα_1-PIM/R could. Contrary to such expectations, the inhibition of proinsulin processing in MIN6 cells by α_1-PIM/R was clearly demonstrated, though not as effectively as that by GHα_1-PIM/R, probably because α_1-PIM/R was transported to the secretory granules less efficiently than GHα_1-PIM/R as shown in the [^{35}S]-Met labeling experiment. α_1-PIM/R was largely secreted through the constitutive pathway (90.2%) and only a small proportion entered the regulated pathway, some of which (7.1%) was secreted continuously, presumably through the constitutive-like pathway during granule maturation, while a small remaining portion (2.7%) was stored in the mature granules and secreted in response to stimulation. On the other hand, GHα_1-PIM/R was mainly transported

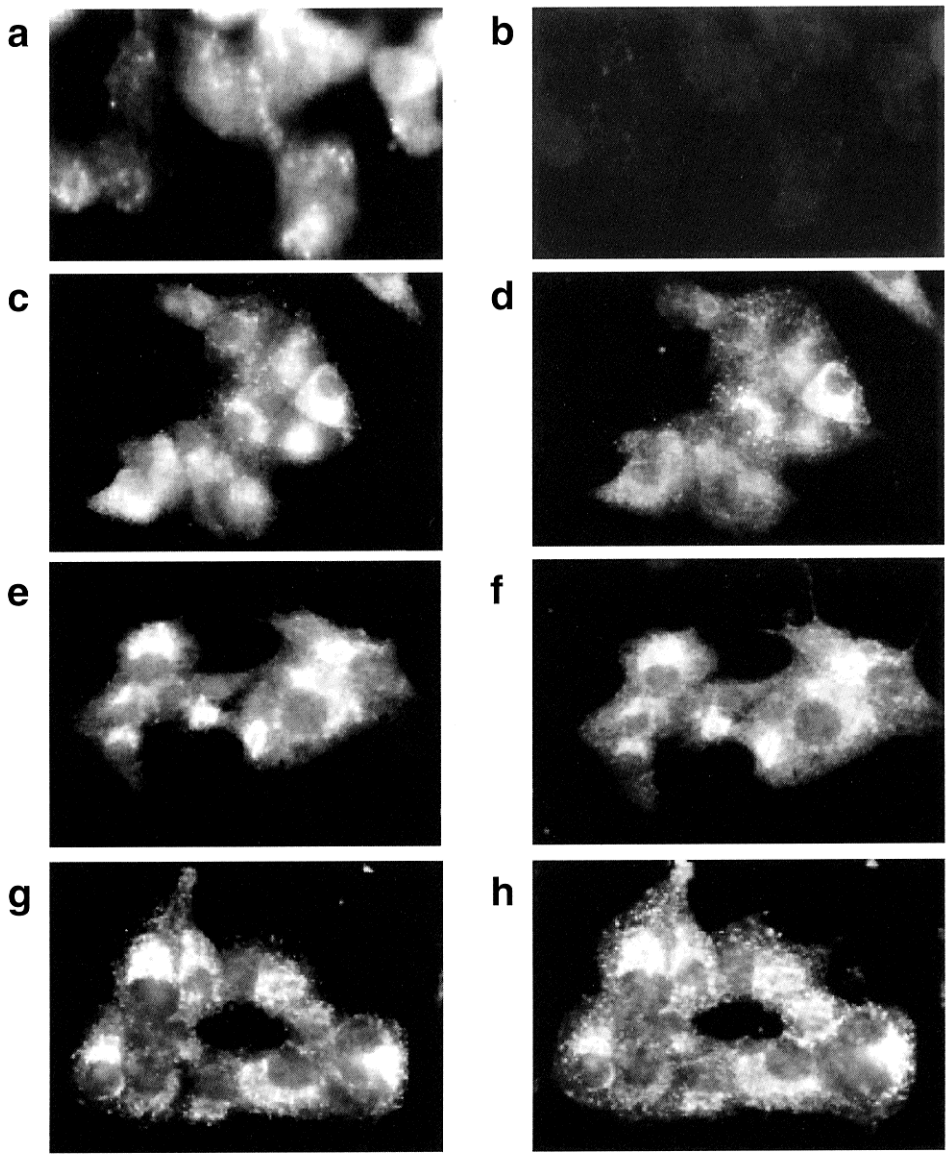

Fig. 6. Double immunocytochemical staining of insulin and protease inhibitors in the stably-transfetced MIN6 clones. Double immunocytochemical staining of insulin (a, c, e, g) and each inhibitor (b, d, f, h) was performed on the MIN6 clones stably expressing α_1-PI (c, d), α_1-PIM/R (e, f), GHα_1-PIM/R (g, h) and control cells transfected with vector only (a, b), as described in the "Materials and Methods" section.

to the secretory granules and underwent the regulated secretion.

The members of the kexin-like endoprotease family, including furin, PC2 and PC3, are also initially synthesized as large precursors and then are converted into mature forms after cleavage of the propeptides at the C-terminal of the sequential basic amino acids. α_1-PIM/R may also possibly affect such activational cleavage of kexin-like endoproteases. We could not clarify this point in our present experiments; however, the conversion of profurin, proPC3 and proPC2 was not affected in the experiments using AtT20 cells transfected with either the α_1-antitrypsin Pittsburgh or the α_1-antitrypsin Portland (α_1-PDX), a potent inhibitor for furin [39]. Furthermore, α_1-antitrypsin Pittsburgh did not show any inhibitory effect for the processing of pro7B2.

Exactly how α_1-PIM/R is transported to the granules remains to be elucidated. One possibility is that α_1-PIM/R might be drawn into the regulated pathway

by the interaction with PC3 or PC2. In contrast with the facts that α_1-antitrypsin and other homologous serine protease inhibitors form 1 : 1 covalent complexes with their target proteases, the reversible interaction between α_1-PIM/R and proprotein-processing enzymes [34] could make it possible that α_1-PIM/R might be secreted at a continuous and slow rate through the constitutive-like pathway. However, the fact that a small proportion of α_1-PI, which has no inhibitory effect on processing, also existed in the secretory granules suggested that α_1-PI and α_1-PIM/R were sorted to the regulated pathway not by any specific interaction between the processing endoproteases and serine protease inhibitors, but by some type of passive transport in a nonselective manner.

The exact cellular site where α_1-PIM/R or GHα_1-PIM/R inhibits proinsulin processing remains to be elucidated. Double immunocytochemical staining of insulin and each inhibitor showed basically no difference between the localizations of α_1-PI, α_1-PIM/R and GHα_1-PIM/R (Fig. 6). From the results of [^{35}S]-Met biolabeling, we speculate that α_1-PIM/R may mainly inhibit the processing within the clathrin-coated immature granules, while GHα_1-PIM/R may inhibit it at sites from the immature granules to the dense-core secretory granules. Further immunocytological dissections using electron microscopy are proposed.

In conclusion, both α_1-PIM/R and GHα_1-PIM/R inhibited the processing of proinsulin when they were expressed in MIN6 cells. α_1-PIM/R was secreted through the constitutive secretory pathway in high proportion; however, a small proportion of α_1-PIM/R was secreted through the constitutive-like pathway and the rest was targeted to the secretory granules. On the other hand, GHα_1-PIM/R was mainly transported to the secretory granules, and showed a greater inhibitory effect on proinsulin processing than α_1-PIM/R. α_1-PIM/R may be transported in the same way as α_1-PI, which has no inhibitory effect on processing, suggesting that they may enter the regulated pathway by a passive means of transport in a nonselective manner and may be discharged through a constitutive-like pathway, while a very small proportion remains in the secretory granules. The inhibitory effect on proinsulin processing, as shown by α_1-PIM/R in regulated secretion, was slightly, but clearly evident when it was introduced into MIN6 cells, and this effect was confirmed to be caused by transporting a part of α_1-PIM/R from TGN to the secretory granules through the regulated secretory pathway.

References

1. Halban PA, Irminger J-C (1994) Sorting and processing of secretory proteins. *Biochem J* 299: 1–18.
2. Traub LM, Kornfeld S (1997) The *trans*-Golgi network: a late secretory sorting station. *Curr Opin Cell Biol* 9: 527–533.
3. Steiner DF (1998) The proprotein convertases. *Curr Opin Chem Biol* 2: 31–39.
4. Seidah NG, Day R, Marcinkiewcz M, Chretien M (1998) Precursor convertases: An evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. *Ann N Y Acad Sci* 839: 9–24.
5. Gumbiner B, Kelly RB (1982) Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell* 28: 51–59.
6. Seidah NG, Chretien M (1997) Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr Opin Biotech* 8: 602–607.
7. Orci L, Ravazzolq M, Amherdt M, Madsen O, Vassalli J-D, Perrelet A (1985) Direct identification of prohormone conversion site in insulin-secreting cells. *Cell* 42: 671–681.
8. Steiner DF, Michael J, Houghten R, Mathieu M, Gardner PR, Ravazzola M, Orci L (1987) Use of a synthetic peptide antigen to generate antisera reactive with a proteolytic processing site in native human proinsulin: Demonstration of cleavage within clathrin-coated (pro)secretory vesicles. *Proc Natl Acad Sci* 84: 6184–6188.
9. Orci L, Ravazzola M, Amherdt M, Perrelet A, Powell SK, Quinn DL, Moore H-PH (1987) The *trans*-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. *Cell* 51: 1039–1051.
10. Miyazaki J-I, Araki K, Yamamoto E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K (1990) Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127: 126–132.
11. Kayo T, Sawada Y, Suzuki Y, Suda M, Tanaka S,

Konda Y, Miyazaki J-I, Takeuchi T (1996) Proprotein-processing endoprotease furin decreases regulated secretory pathway-specific proteins in the pancreatic β cell line MIN6. *J Biol Chem* 271: 10731–10737.

12. Lewis JH, Iammarino RM, Spero JA, Hasiba U (1978) Antithrombin Pittsburgh: An α_1-antitrypsin variant causing hemorrhagic disease. *Blood* 51: 129–137.
13. Owen MC, Brennan SO, Lewis JH, Carrell RW (1983) Mutation of antitrypsin to antithrombin: α_1-antitrypsin Pittsburgh (358 Met→Arg), a fatal bleeding disorder. *N Engl J Med* 12: 694–698.
14. Courtney M, Jallat S, Tessier L-H, Benavente A, Crystal RG, Lecocq J-P (1985) Synthesis in *E. coli* of α_1-antitrypsin variants of therapeutic potential for emphysema and thrombosis. *Nature* 313: 149–151.
15. Bathurst IC, Brennan SO, Carrell RW, Cousens LS, Brake AJ, Barr PJ (1987) Yeast KEX2 protease has the properties of a human proalbumin converting enzyme. *Science* 235: 348–350.
16. Misumi Y, Oda K, Fujiwara T, Takami N, Tashiro K, Ikehara Y (1991) Functional expression of furin demonstrating its intracellular localization and endoprotease activity for processing of proalbumin and complement pro-C3. *J Biol Chem* 266: 16954–16959.
17. Rhodes CJ, Brennan SO, Hutton JC (1989) Proalbumin to albumin conversion by a proinsulin processing endopeptidase of insulin secretory granules. *J Biol Chem* 264: 14240–14245.
18. Misumi Y, Ohkubo K, Sohda M, Takami N, Oda K, Ikehara Y (1990) Intracellular processing of complement pro-C3 and proalbumin is inhibited by rat α_1-protease inhibitor variant (Met^{352}→Arg) in transfected cell. *Biochem Biophys Res Commun* 171: 236–242.
19. Moore H-PH, Kelly RG (1986) Re-routing of a secretory protein by fusion with human growth hormone sequences. *Nature* 321: 443–446.
20. Seethaler G, Chaminade M, Vlasak R, Ericsson M, Griffiths G, Toffoletto O, Rossier J, Stunnenberg HG, Kreil G (1991) Targeting of frog prodermorphin to the regulated secretory pathway by fusion to proenkephalin. *J Cell Biol* 114: 1125–1133.
21. Niwa H, Yamamura K-I, Miyazaki J-I (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–200.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265–275.
23. Oda K, Misumi Y, Sohda M, Takami N, Sakaki Y, Ikehara Y (1991) Selective processing of proalbumin determined by site-specific mutagenesis. *Biochem Biophys Res Commun* 175: 690–696.
24. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159.
25. Hatsuzawa K, Hosaka M, Nakagawa T, Nagase M, Shoda A, Murakami K, Nakayama K (1990) Structure and expression of mouse furin, a yeast Kex2-related protease. *J Biol Chem* 265: 22075–22078.
26. Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M, Chretien M (1990) cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: Tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol* 9: 415–424.
27. Smeekens SP, Avruch AS, LaMendola J, Chan SJ, Steiner DF (1991) Identification of cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc Natl Acad Sci USA* 88: 340–344.
28. Kuliawat R, Arvan P (1992) Protein targeting via the "constitutive-like" secretory pathway in isolated pancreatic islets: Passive sorting in the immature granule compartment. *J Cell Biol* 118: 521–529.
29. Smeekens SP, Montag AG, Thomas G, Albiges-Rizo C, Carroll R, Benig M, Phillips LA, Martin S, Ohagi S, Gardner P, Swift HH, Steiner DF (1992) Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2 and PC3. *Proc Natl Acad Sci USA* 89: 8822–8826.
30. Rhodes CJ, Alarcon C (1994) What β-cell defect could lead to hyperproinsulinemia in IDDM? Some clues from recent advances made in understanding the proinsulin-processing mechanism. *Diabetes* 43: 511–517.
31. Bailyes EM, Shennan KIJ, Seal AJ (1992) A member of the eukaryotic subtilisin family (PC3) has the enzymatic properties of the type 1 proinsulin-converting endopeptidase. *Biochem J* 285: 391–394.
32. Bennett DL, Bailyes EM, Nielsen E, Guest PC, Rutherford NG, Arden SD, Hutton JC (1992) Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family. *J Biol Chem* 267: 15229–15236.
33. Rhodes CJ, Lincoln B, Shoelson SE (1992) Preferential cleavage of des-31,32-proinsulin over intact proinsulin by the insulin secretory granule type II endopeptidase. *J Biol Chem* 267: 22719–22727.
34. Brennan SO, Peach RJ, Bathurst IC (1990) Specificity of yeast KEX2 protease for variant human proalbumins is identical to the in vivo specificity of the hepatic proalbumin convertase. *J Biol Chem* 265: 21494–21497.
35. Nagahama M, Ikezumi J, Misumi Y, Ikehara Y, Murakami K, Nakayama K (1991) Evidence that differentiates between precursor cleavages at dibasic and Arg-X-Lys/Arg-Arg sites. *J Biochem* 110: 806–811.
36. Brennan SO, Peach RJ (1988) Calcium-dependent

KEX2-like protease found in hepatic secretory vesicles converts proalbumin to albumin. *FEBS Lett* 229: 167–170.

37. Brennan SO, Peach RJ (1991) The processing of human proinsulin and chicken proalbumin by hepatic vesicles suggests a convertase specific for *X*-*Y* Arg-Arg or Arg-*X*-*Y*-Arg sequences. *J Biol Chem* 266: 21504–21508.
38. Le A, Graham KS, Sifers RN (1990) Intracellular degradation of the transport-impaired human PiZ α_1-antitrypsin variant. *J Biol Chem* 265: 14001–14007.
39. Benjannet S, Savaria D, Laslop A, Munzer JS, Chretien M, Marcinkiewicz, Seidah NG (1997) α_1-Antitrypsin Portland inhibits processing of precursors mediated by proprotein convertases primarily within the constitutive secretory pathway. *J Biol Chem* 272: 26210–26218.