Prohormone convertases PC2 and PC3, yeast Kex2-family endoproteases specific to the regulated secretory pathway, cleave proinsulin to insulin in the secretory granules of pancreatic β cells. The well-differentiated β cell line MIN6 expresses PC2 and PC3 and another regulated secretory pathway-specific protein chromogranin A. Furin, another yeast Kex2 endopeptidase, exists in the trans-Golgi networks of many cell types. The β cell line RINm5F (a cell line that is less differentiated than the MIN6 cell line) does not express the regulated pathway-specific proteins, but strongly expresses furin. We suspected that furin expression may cause the decrease of regulated secretory pathway-specific proteins. To test this hypothesis, we expressed a furin cDNA with a metallothionein promoter in MIN6 cells. With Zn2+ stimulation of furin expression, the messages of PC2, PC3, and chromogranin A decreased, and the processing of proinsulin to mature insulin became less efficient. The furin-expressing MIN6 cells exhibited less insulin content and weakened insulin secretion in response to a high glucose concentration. The conditioned medium from furin-expressing MIN6 cells also exerted a decrease of PC2 and PC3 expression in unaltered MIN6 cells. Thus, proteins cleaved by furin inside the cells or by truncated furin shed into the culture medium appear to cause decreased PC2 and PC3 expression, insulin content, and glucose-responsive insulin secretion in MIN6 cells.

Pancreatic β cells possess well-differentiated characteristics, such as the formation of insulin secretory granules, the processing of proinsulin to mature insulin, and the glucose-stimulated secretion of insulin. β cells synthesize insulin as proinsulin, a precursor peptide, at the rough endoplasmic reticulum, from which transport vesicles carry the proinsulin to the trans-Golgi networks. At the trans-Golgi networks, proinsulin is sorted to a secretory pathway that is well regulated by an extracellular stimulus, such as glucose (1). Through this pathway, proinsulin (B chain-Arg-Arg-C peptide-Lys-Lys-A chain) is packaged into immature granules and processed to mature insulin (B chain-S-S-A chain), which is then stored in dense-core granules (2). When this secretory system is stimulated, insulin molecules stored in the dense-core granules are secreted into the extracellular space. These granules contain at least three distinct groups of processing enzymes: the prohormone convertases PC2 and PC3 (also called PC1), carboxypeptidase H, and amidation enzymes (3–5). Processing of proinsulin into mature insulin requires cleavage by PC2 and PC3 at the paired basic residues and their subsequent removal by carboxypeptidase H (3, 4, 6).

In addition to the regulated secretory pathway, β cells are equipped with a secretory pathway that is not regulated by an extracellular stimulus. Along this constitutive secretory pathway, proteins are transported from the trans-Golgi networks to plasma membranes by small transport vesicles (1, 7). Furin, from the family of yeast Kex2 endopeptidases to which PC2 and PC3 also belong (8, 9), is located at the trans-Golgi network (10). While PC2 and PC3 are uniquely expressed in the regulated pathway of neuroendocrine cells, furin is thought to be present in the constitutive pathway of virtually all cell types (11). Furin is specific for the unique cleavage sequence -Arg^-4, X^-3,Lys/Arg^-2^-Arg^-1 (RX(K/R))R (12, 13). This furin-specific RX(K/R)R motif is found in a number of growth factor precursors (12), including nerve growth factor, platelet-derived growth factor, transforming growth factor β, activin A, the adhesion molecule cadherin, metalloproteinasess such as stromelysin 3 (14), and matrix metalloproteinase X1 (15), and, in some growth factor proreceptors (12) including those for insulin receptor, insulin-like growth factor 1 receptor, and hepatocyte growth factor receptor (oncoprotein MET). Several reports indicate that furin is responsible for the formation of bioactive proteins such as transforming growth factor β1, stromelysin 3 (14), and insulin receptor (17). We previously found that when mutant proinsulin with furin cleavage sites was expressed in the mouse fibroblast-like cell line NIH3T3, containing high levels of furin, and the Chinese hamster ovary-derived cell line CHO which expressed low levels of furin, NIH3T3 cells produced more bioactive insulin than did CHO cells (18). Thus, while furin is localized in virtually all cell types (11), increased expression of furin may generate more bioactive proteins from their precursors, and the increased protein levels may induce a change in cell growth as well as differentiation of cell functions. Furin expression in mature β cells may induce notable changes in their highly differentiated characteristics, such as the formation of insulin secretory granules, the processing of proinsulin into mature insulin, and glucose-stimulated secretion of insulin.

To investigate this possibility, we chose one well-differentiated β cell line, MIN6 (19, 20), and two less-differentiated cell lines, HIT-T15 (21) and RINm5F (22). We compared the ex-

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pression of the prohormone convertases PC2 and PC3, the regulatory pathway marker protein chromogranin A (23), and the constitutive pathway enzyme furin among the three β cell lines. Since furin expression appears to be correlated with an undifferentiated state of β cell function, we prepared a furin-expressing MIN6 cell line, MIN6-mf, under the control of a metallothionein promoter. The present study investigates the decrement of the differentiated characteristics of β cells using the MIN6-mf cell line.

**MATERIALS AND METHODS**

**Cell Culture**—We used three lines of β cells: MIN6 (passages 16–35), established from insulinomas derived from transgenic mice carrying a hybrid insulin-promoted SV40 (simian virus 40) tumor antigen gene (19, 20); HIT-T15 (American Type Culture Collection CRL 1777) (passages 56–62), established by SV40 transformation of Syrian hamster pancreatic islet cells (21); and RINm5F (number of passages unknown), established from a serially transplantable, radiation-induced rat islet tumor (22). MIN6 was cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 15% fetal bovine serum (FBS) (Life Technology, Inc.) at 37°C in 5% CO2. HIT-T15 was cultured in the same conditions except with 10% FBS. RINm5F was cultured in a RPMI 1640 medium supplemented with 10% FBS at 37°C in 5% CO2. All media contained 25 μg/ml geneticin unless stated otherwise.

**Radioimmunoassay**—Insulin was measured using an insulin immunoassay kit (Amersham Japan, Tokyo), according to the manufacturer’s instruction. The antibody in this kit is specific to both proinsulin and mature insulin equally on a molar basis. Assay samples were prepared by collecting the culture medium, obtaining acid-ethanol extracts from the islets or cells, and fractionating the solutions by gel filtration chromatography.

**Northern Blot Analysis**—Total cellular RNA was extracted from the islets or cells, treated with RNAase, dissolved in 0.1M guanidinium thiocyanate hot phenol method, as described previously (18). For Northern blot analysis, the RNA was fractionated on a 1.0% agarose gel containing 1 μg of 28S and 18S ribosomal RNA as standards. The RNA was transferred to a nylon membrane (Hybond N, Amersham Japan) and hybridized with the specific cDNA probe. The membranes were washed twice with 10× saline sodium citrate (SSC) and once with 2× SSC, and exposed to X-ray film for autoradiography.

**RESULTS**

**Insulin Content in Each β Cell Line**—The IRI content was highest in the MIN6 cells, followed by the HIT-T15 and RINm5F cells (Table I). The insulin content of β cells in rat islets has been reported by other researchers to be approximately 8 nmol/10^6 cells (29, 30), and we have previously found it to be 5.2 ± 0.3 nmol/10^6 cells. In the present study, the insulin content of the MIN6 cells (320 ± 10^5 cells) was one order of magnitude lower than that found in β cells of the rat islet. This difference is consistent with studies using the electron microscope that indicate that although MIN6 cells contain a fair number of dense-core granules, mature islet β cells contain far more dense-core granules than do MIN6 cells (19). No secretory granules were observed in HIT-T15 and RINm5F cells (data not shown). Their insulin content was three orders of magnitude lower than that of MIN6 cells (Table I).

**Conversion Ratio of Proinsulin to Insulin**—The conversion ratio of proinsulin to insulin was measured using an insulin immunoassay kit. The values represent the mean ± S.D.

| Cell line | IRI content (pmol/10^6 cells) | ± S.D. |
|-----------|-------------------------------|-------|
| MIN6      | 320.0                         | 19.7  |
| HIT-T15   | 0.610                         | 0.013 |
| RINm5F    | 0.430                         | 0.006 |

The abbreviations used are: FBS, fetal bovine serum; IRI, immunoreactive insulin; bp, base pair(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin.

1 The abbreviations used are: FBS, fetal bovine serum; IRI, immunoreactive insulin; bp, base pair(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin.
Furin Decreases Regulatory Pathway Proteins in β Cells

TABLE II

| Cell       | Proinsulin/total IRI | n |
|------------|----------------------|---|
| Islets     | 12.3 ± 1.9           | 3 |
| MIN6       | 12.7 ± 3.3           | 3 |
| HIT-T15    | 44.8 ± 5.8           | 3 |
| RINm5F     | 64.6 ± 1.7           | 3 |

The ratio of proinsulin to total IRI expressed as a percentage. Insulin in the culture medium of each β cell line was separated by gel filtration on a Sephadex G-50 column, and the ratio of area proinsulin/area insulin × 100 was calculated.

Characteristics of MIN6-mf Cells—Since MIN6 cells exhibit the dual secretory pathway, we compared insulin content and glucose-responsive insulin secretion between the MIN6 cells with the passage numbers 16 and 35. Insulin content was almost 2-fold higher in the cells at passage 16 than in the cells at passage 35 (Table III). Insulin secretion in response to 1 mM and 5 mM glucose was similar in MIN6 cells at 16 and 35 passages. However, when the glucose concentration was raised form 5 mM to 25 mM, the cells at passage 16 showed nearly a 2-fold increase in insulin secretion, while the cells at passage 35 showed an increase of only 1.2-fold (Fig. 4). Thus, PC2 and PC3 expression, insulin content, and glucose-responsive insulin secretion decreased with increasing passage number, whereas furin expression increased.

Characteristics of MIN6-mf Cells—Since MIN6 cells exhibit...
Fig. 4. Glucose-responsive insulin secretion in MIN6 cell lines at passages 16 and 35. Glucose-responsive insulin secretion from MIN6 cells was assessed at 1, 5, and 25 mM glucose concentrations. Insulin secretion stimulated by 1 mM glucose was expressed as 1, then that at 5 and 25 mM glucose was plotted as fold increase. The cells at passage 35 showed less insulin secretion than those at passage 16 in response to 25 mM glucose. Bars represent the mean ± S.D.

Table III

Comparison of IRI content between MIN6 cell lines at passages 16 and 35.

| Passage | IRI content (pmol/10^6 cells) | ± S.D. |
|---------|--------------------------------|--------|
| 16      | 320.0                          | 19.7   |
| 35      | 171.7                          | 9.2    |

Fig. 5. Expression of furin in Zn^{2+}-stimulated MIN6-mf cells. A, MIN6-mf cells were cultured for 0, 24, 48, and 72 h in the medium containing 100 μM Zn^{2+}. Isolated RNA (10 μg) was electrophoresed on an agarose gel prior to Northern blot analysis. β-Actin message was used to measure blotting efficiency. B, MIN6-cnls with the empty expression vector pMEP4 (MIN6-0 cells) were prepared and cultured for 0, 24, 48, and 72 h in the medium containing 100 μM Zn^{2+}. RNA isolation, electrophoresis, and Northern blot analysis were carried out as in A. A blotted membrane was probed for the expression of furin, PC2, and PC3.

Fig. 6. Immunocytochemical staining of furin in MIN6-mf cells cultured in the presence or absence of Zn^{2+}. Immunocytochemical staining of furin was performed on cells cultured for 48 h in the absence of 100 μM Zn^{2+} (A) and in the presence of Zn^{2+} (B). The Zn^{2+}-stimulated cells showed a strong accumulation of stained granular dots in the perinuclear region (B). Unstimulated cells showed fewer granular dots scattered in the cytoplasm (A).
With the decrement of regulated pathway-specific proteins, glucose-responsive insulin secretion was diminished. The MIN6 cells at passage 16 secreted a 2.0-fold higher level of insulin in a 25 mM glucose medium than in a 1 mM glucose medium (Fig. 4). This glucose-dependent increase became only 1.6-fold in MIN6-mf cells without Zn\(^{2+}\) stimulation, probably due to the increase in cell line passage (passage 26) (Fig. 9). The glucose-induced secretion was further reduced when the cells were stimulated with 100 \(\mu M\) Zn\(^{2+}\) for 48 h. Zn\(^{2+}\)-stimulated MIN6-mf cells exhibited only a 1.2-fold increase in insulin secretion when the glucose concentration was raised from 5 mM to 25 mM (Fig. 9). However, the increase from 1 mM to 5 mM glucose resulted in similar increases in furin expression (2.1-to 2.3-fold) in all three cell conditions, MIN6 cells at passage 16 and MIN6-mf cells with and without Zn\(^{2+}\) stimulation. Thus, the greatest change in glucose-responsive insulin secretion was a marked decrease observed in Zn\(^{2+}\)-stimulated MIN6-mf cells when glucose concentrations were shifted from 5 mM to 25 mM.

Effect of Conditioned Medium on PC2 and PC3 Expression—Furin generates bioactive proteins from their precursor forms which then may be released into the extracellular space. We investigated the possibility that furin-expressing MIN6-mf cells secrete these proteins into the culture medium. To address this question, we used MIN6-mf cells to prepare a conditioned medium and tested for the presence of such extracellular proteins.

**Table IV**

Comparison of IRI content between MIN6-mf cell lines cultured in the presence and absence of Zn\(^{2+}\).

| Zn\(^{2+}\) | Time | IRI content | ± S.D. |
|---|---|---|---|
| + | 0 | 234.0 | 6.7 |
| + | 72 | 178.0 | 13.6 |
| - | 72 | 254.0 | 14.8 |

With the decrement of regulated pathway-specific proteins, glucose-responsive insulin secretion in MIN6-mf cell lines with or without stimulation of Zn\(^{2+}\). Glucose-responsive insulin secretion in MIN6-mf cells was assessed at 1, 5, and 25 mM glucose concentrations. MIN6-mf cells were cultured for 48 h in the presence or absence of 100 \(\mu M\) Zn\(^{2+}\), then processed for the insulin secretion assay. Insulin secretion at 1 mM glucose was expressed as 1, then that at 5 and 25 mM glucose was plotted as fold increase. MIN6-mf cells cultured in the presence of 100 \(\mu M\) Zn\(^{2+}\) showed less insulin secretion than those cells cultured in the absence of Zn\(^{2+}\). Bars represent the mean ± S.D. of insulin secretion (folds).

**Fig. 7.** Expression of PC2, PC3, and chromogranin A mRNAs in Zn\(^{2+}\)-stimulated MIN6-mf cells. The same RNA isolated for Northern blot analysis was assessed for PC2, PC3, and chromogranin A (CgA) expression. PC2 and PC3 messages were decreased in the MIN6-mf cells cultured for 48 and 72 h in the presence of 100 \(\mu M\) Zn\(^{2+}\). Cells cultured for 72 h also showed a marked decrease in chromogranin A message.

**Fig. 8.** Gel filtration profiles of IRI in the culture medium of MIN6-mf cells with and without Zn\(^{2+}\) stimulation. MIN6-mf cells were cultured for 72 h in the presence or absence of 100 \(\mu M\) Zn\(^{2+}\). Culture medium was collected every 24 h. IRI in the culture medium collected between 48 and 72 h was separated with a Sephadex G-50 superfine gel column. The proinsulin peak of MIN6-mf cells stimulated by Zn\(^{2+}\) was larger than that of unstimulated MIN6-mf cells. Molecular size was calibrated with blue dextran (V₀), potassium ferricyanide (V₁), and synthetic human insulin. Similar elution profiles were obtained in three other chromatography tests.

**Fig. 9.** Glucose-responsive insulin secretion in MIN6-mf cell lines with or without stimulation of Zn\(^{2+}\). Glucose-responsive insulin secretion in MIN6-mf cells was assessed at 1, 5, and 25 mM glucose concentrations. MIN6-mf cells were cultured for 48 h in the presence or absence of 100 \(\mu M\) Zn\(^{2+}\), then processed for the insulin secretion assay. Insulin secretion at 1 mM glucose was expressed as 1, then that at 5 and 25 mM glucose was plotted as fold increase. MIN6-mf cells cultured in the presence of 100 \(\mu M\) Zn\(^{2+}\) showed less insulin secretion than those cells cultured in the absence of Zn\(^{2+}\). Bars represent the mean ± S.D. of insulin secretion (folds).

**DISCUSSION**

The present results demonstrate that when furin is induced in the pancreatic \(\beta\) cell line MIN6, expression of the regulatory pathway-specific prohormone convertases PC2 and PC3 and the secretory granule-specific protein chromogranin A, insulin content, and glucose-stimulated insulin secretion are decreased. MIN6 cells exhibited typical characteristics of mature \(\beta\) cells, including the presence of the three regulatory pathway-
specific proteins PC2, PC3, and chromogranin A, and a conversion ratio of proinsulin to insulin similar to that found in mature islet β cells. The cells also retain an insulin secretion capacity that can be regulated by a physiological range of glucose concentrations (19, 20). A previous study observed that pancreatic β cells purified by a fluorescence-activated cell-sorting system exhibited only 1.5- to 2.0-fold insulin secretion when the glucose concentration was raised from 3 mM to 20 mM, with insulin secretion being lower than that of pancreatic β cells exhibited in the islet (36). In contrast, RINm5F cells illustrated characteristics opposite to those of the well-differentiated β cells. These characteristics included a low level of insulin, virtually no regulated secretory pathway protein messages, no glucose-responsive insulin secretion (35). Thus, the glucose-responsive insulin-secreting capacity of MIN6 cells is well-maintained, although it is considerably lower than that of pancreatic β cells exhibited in the islet (36).

In contrast, RINm5F cells illustrated characteristics opposite to those of the well-differentiated β cells. These characteristics included a low level of insulin, virtually no regulated secretory pathway protein messages, no glucose-responsive insulin secretion (35), and an especially high level of furin expression. The presence of furin in RINm5F suggests that this enzyme may be expressed in the less differentiated state of β cells. Indeed, rat fetal and infant β cells showed strong furin immunostaining (data not shown), but adult β cells stained only faintly (38).

Since furin appeared to be expressed in less-differentiated β cells, we examined the furin expression in MIN6 cells of different passage numbers. For studies such as these, MIN6 is the β cell line of choice because the cells retain their glucose-responsive insulin-secreting capacity (19, 20). In many well-differentiated β cell lines, this glucose-responsive insulin secretion is known to be gradually diminished with the increase of cell line passages (39, 40), as seen in MIN6. With increasing MIN6 cell line passages and the resulting decline in secretion capacity, PC2 and PC3 messages also decreased and insulin content was diminished. In contrast, furin expression increased with increasing cell passages.

Proinsulin is converted to mature insulin through the regulated secretory pathway (1). On the other hand, furin is thought to cleave precursor proteins that are secreted through a constitutive secretory pathway. Thus, furin probably has a minor role in processing proinsulin. Furin cleaves the consensus processing site Arg-4X-3(Lys/Arg)-2-Arg-1 (12, 13). For cleavage to occur properly, more basic amino acids are required ahead of the general cleavage site of most propeptide hormones, -(Lys/Arg)-2-Arg-1. If furin is highly expressed in β cells, the bioactive proteins cleaved from their precursors would increase in concentration. To examine whether furin is a primary cause for the decline of PC2 and PC3 expression and for the diminished glucose-responsive insulin secretion, we developed a new cell line MIN6-mf with a furin expression system that was regulated by Zn²⁺. With an increase in furin expression, all three regulatory pathway-specific proteins (PC2, PC3, and chromogranin A) were diminished. The MIN6-mf cells stimulated with Zn²⁺ generated a larger proportion of proinsulin to total IRI than did MIN6-mf cells without Zn²⁺ stimulation. Two reasons may be considered for the increase in proinsulin proportion. First, the decrease of prohormone convertases PC2 and PC3 may have increased the proportion of unprocessed proinsulin. Second, the decrease of regulated pathway proteins may have eventually driven proinsulin to a constitutive pathway. Since furin is known to recycle between the trans-Golgi network and plasma membrane (33, 41), the furin recycling may increase a protein flow of the constitutive pathway, which results in an augmented transport of proinsulin molecules outside the cells. In a mouse pituitary tumor-derived cell line AT20, which carries both regulatory and constitutive pathways, a part of human proinsulin expressed in this cell line was sorted to a constitutive pathway where proinsulin is processed to an intermediate form des-31,32-split proinsulin (42). In the MIN6-mf cell line, it remains to be investigated whether proinsulin is processed to this intermediate form.

Insulin content and glucose-stimulated insulin secretion were also diminished in Zn²⁺-stimulated MIN6-mf cells. Proinsulin may have escaped by way of a rapid secretion of newly formed immature granules or through the constitutive pathway to the culture medium, which may result in the decrease of insulin content in MIN6 cells. The resulting diminished insulin content may lead to the decrease of insulin secretion in response to glucose. In addition to this possibility, we need to assess the impairment of the glucose-sensing system, namely the decreased expression of type 2 glucose transporter and glucokinase in furin-expressing MIN6-mf cells.

Furin generates secretory peptides such as platelet-derived growth factor, transforming growth factor β, and activin A as well as membrane-bound proteins such as cadherin and insulin receptor (12, 14–17). To examine if extracellular peptides cleaved by furin cause the loss of differentiation in MIN6 cells, we prepared the conditioned medium from the Zn²⁺-stimulated and unstimulated MIN6-mf cells. The conditioned medium from the Zn²⁺-stimulated cells induced the decrement of PC2 and PC3 expression in the genetically unaltered MIN6 cells. Thus, we can say at least that some extracellular peptides cleaved by furin must have exerted the loss of differentiation in β cells, although we cannot say whether furin cleaved precursor peptides intracellularly or extracellularly.

Activation of the constitutive secretory pathway appears to be correlated with furin expression. We do not know what factors are involved in the activation of the constitutive pathway, but once furin is induced, furin-cleaved bioactive proteins including a number of growth factors may lead well-differentiated β cells to an undifferentiated state. Since the decline of β cell functions are often observed in insulinomas (43, 44) and non-insulin-dependent diabetes mellitus (45), understanding the physiological switching from a regulated secretory pathway to a constitutive pathway may aid in understanding the onset of such disorders.

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Proprotein-processing Endoprotease Furin Decreases Regulated Secretory Pathway-specific Proteins in the Pancreatic Cell Line MIN6
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