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Processing of mutated proinsulin with tetrabasic cleavage sites to bioactive insulin in the non-endocrine cell line, COS-7

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The amino acid sequence, Arg^{-4}-X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$, is thought to be a concensus processing site for a constitutive secretory pathway in non-endocrine cells. We created a mutant proinsulin DNA with a peptide structure of B chain-Arg-Arg-Lys-Arg-C peptide-Arg-Arg-Lys-Arg-A chain, which compares to the native proinsulin structure of B chain-Arg-Arg-C peptide-Lys-Arg-A chain. When the mutant insulin was expressed in a monkey kidney-derived cell line, COS-7, approximately 60% of the total immunoreactive insulin appeared as mature insulin in the culture medium. This conversion to the mature form was strikingly facilitated by co-expressing the mutant proinsulin with furin, a homologue of the yeast endoprotease, Kex2.

Prohormone processing; Tetrabasic processing site; Proinsulin; Insulin; Furin; COS-7 cell

1. INTRODUCTION

Insulin is produced in pancreatic β-cells as the precursor, proinsulin, comprising three peptides linked by two pairs of basic residues in the following order: B chain-Arg-Arg-C peptide-Lys-Arg-A chain [1,2]. C peptide is cleaved off from proinsulin at the adjacent dibasic residues during its transport through the *trans*-Golgi networks to immature-type secretory vesicles [3,4]. The conversion from proinsulin to insulin is a unique function of endocrine cells [5]. A number of propeptide hormone cDNAs, including a human proinsulin cDNA, have been introduced into both endocrine and non-endocrine cells (reviewed in [6]), and those expressed in endocrine cells were generally processed correctly, while those expressed in non-endocrine cells were secreted constitutively as non-cleaved propeptides [5]. However, the inability of non-endocrine cells to convert proinsulin to insulin does not mean they are also unable to process other propeptides to smaller bioactive peptides. Non-endocrine cells, including fibroblasts, hepatocytes, and lymphocytes, produce biologically inactive propeptides and convert them to bioactive peptides by cleaving a unique consensus sequence, Arg^{-4}-X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$ [7]. In addition to this consensus sequence, a processing site with one more basic amino acid at position −3 was noted in several precursor proteins, including insulin receptor A chain and B chain, complement components C3 β- and α chain, C4 β- and α chain, and C4 α-and γ chain [8–10]. The proteolysis of the insulin receptor A chain-Arg^{-4}-Lys^{-3}-Arg^{-2}-Arg^{-1}-B chain is carried out at the tetrabasic residues, resulting in the functionally active receptor A chain-S-S-B chain. The insulin receptor with the mutated tetrabasic processing site, Arg^{-4}-Lys^{-3}-Arg^{-2}-Ser^{-1}, was identified in a diabetic patient with the type A syndrome of severe insulin resistance [11]. It was proved that the insulin binding affinity to this mutated receptor was markedly decreased, thus proteolytic processing at the tetrabasic residues is an absolute requirement for the attainment of full receptor function.

Proteolytic cleavage of the consensus sequence, Arg^{-4}- X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$, is catalyzed by the subtilisin-like endoprotease, furin [12,13]. Furin was originally identified as a homologue of the yeast propeptide-processing endoprotease, Kex2, and is thought to be a mammalian endoprotease in non-endocrine cells [14,15]. Furin has been demonstrated to be present in virtually all non- endocrine cells, including fibroblasts, epithelial cells and lymphocytes [15].

By utilizing the above information we constructed a mutant rat proinsulin DNA with a peptide structure of B chain-Arg-Arg-Lys-Arg-C peptide-Arg-Arg-Lys-Arg-A chain, with the expectation that a non-endocrine cell line, COS-7, would be able to convert this mutant proinsulin to mature bioactive insulin. In the event that the conversion to mature insulin was not sufficient, we planned to co-express a furin cDNA together with a mutant proinsulin DNA to boost the conversion. In this paper we demonstrated that the co-expression of both DNAs in COS-7 cells attained virtually 100% of conversion from proinsulin to insulin.

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2. MATERIALS AND METHODS

2.1. *Construction of the native and mutant insulin DNAs*

For the expression of native and mutant insulin, we utilized a rat insulin I gene [16], which comprises two exons, and one intron that is located 14 bp ahead of the initiation codon, ATG. This gene lacks a second intron in the middle of the DNA sequence corresponding to C peptide, unlike rat insulin gene II and insulin genes from other mammals [17]. The whole proinsulin-coding DNA sequence of 350 bp was obtained from this gene using a site-directed in vitro mutagenesis system kit (Takara, Kyoto) described as follows. At first a 1.1 kb *Pst*I–*Xba*I fragment of rat insulin I gene [16] was inserted into a M13 mp19 bacteriophage vector. The oligonucleotide primers used for the mutagenesis are depicted in Fig. 1. All oligonucleotides were synthesized using an Applied Biosystems Model 391 PCR-Mate DNA synthesizer. The *Bam*HI restriction sites were introduced 4 bp upstream of the initiation codon, ATG, and 7 bp downstream of the stop codon, TGA, adjacent to the insulin coding sequence. Mutant clones were identified directly by DNA sequencing with the dideoxynucleotide method. Double-stranded DNA was then prepared from a mutated M13 clone, and a 350 bp length of mutated rat insulin DNA was isolated with *Bam*HI digestion and cloned into the *Bam*HI site of the pGEM 3Zf(+) vector (Promega, Madison, WI). The direction of the insert was determined by DNA sequencing. The vector containing the insulin DNA in the sense direction was digested with *Pst*I and *Eco*RI, and subcloned into the *Pst*I and *Eco*RI sites of a pcDL-SRa296 expression vector [18]. Both native and mutant insulin expression vector constructs were purified twice by cesium chloride gradient centrifugation. The pSVDFur expression vector construct containing a furin cDNA [15] was prepared in the same manner.

2.2. *Cell culture and DNA transfection*

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37°C in 5% CO_2. Transfections of the expression vector constructs into the cells were carried out by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA). Cells were allowed to grow to near confluence in a 100 mm dish. The cells were washed once with PBS buffer (Ca^{2+}- and Mg^{2+} free), harvested with 0.05% trypsin/0.02% EDTA, and then resuspended in PBS buffer at a concentration of 2×10^7 cells/ml. A cell suspension of 0.8 ml with 15 mg of an expression vector construct was transferred to an electroporation cuvette (Bio-Rad). The cuvette was placed on ice for 5 min, and then electroporated at a setting of 300 V and 500 μF. The cells were allowed to recover on ice for 10 min and then divided into four 100 mm dishes containing the culture medium. 12 h after the electroporation, culture medium was removed and the cells were incubated in fresh medium with 5% fetal bovine serum for 72 h. Every 24 h the culture medium was collected for insulin radioimmunoassay. In the co-expression experiment the proinsulin DNA and the furin cDNA constructs were mixed at the same molar ratio, then the electroporation of the DNA mixture was carried out in the same manner.

2.3. *Radioimmunoassay*

Immunoreactive insulin in the culture medium and the solution fractionated by gel filtration chromatography was determined using an insulin immunoassay kit (Amersham Japan, Tokyo), according to the manufacturer's instruction.

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 50 mM sodium acetate elution buffer (pH 5.0) for analysis of the apparent size of insulin secreted into the culture medium. The medium was lyophilized and then dissolved in the elution buffer and then applied to the column. Fractions of 1.5 ml were routinely collected. Size calibration was carried out with blue dextran (V_o) and potassium ferricyanide (V_t).

2.5. *Glucose transport analysis*

Glucose transport activity of fat cells stimulated by insulin was assessed by measuring the rate of uptake of 3-*O*-methyl-D-glucose at 37°C according to the 3-s method of Whitesell and Gliemann, and modified by Toyoda et al. [19]. Adipocytes were isolated by collagenase digestion from a rat epididymal adipose tissue. Approximately 10 mg of adipocytes, measured by a hematocrit tube, were incubated with 100 μM 3-*O*-[^{3}H]methyl-D-glucose (10 mCi/ml) in the presence or absence of insulin for 3 s at 37°C in a total volume of 100 μl. The reaction was terminated by adding 300 μl of 1 mM phloretin, an inhibitor for glucose transport, and cells in 300 μl of the suspension were collected by the oil-flotation method of Gliemann et al. [20]. The amount of glucose in the extracellular space was determined by adding labeled 3-*O*-methyl-D-glucose after adding phloretin. The radioactivity in the cell fraction was determined in a liquid scintillation counter.

3. RESULTS

3.1. *Expression of the native and mutant proinsulin in COS-7 cells*

Each proinsulin DNA construct was transfected into COS-7 cells by the electroporation method. 12 h after the electroporation, the culture medium was removed and the cells were incubated in fresh medium. The culture medium was collected every 24 h from the point of this medium change up to 72 h. Comparing the immunoreactive insulin (IRI) secreted into the culture medium during the first 24 h from native and mutant proinsulin-expressed cells, one can note that IRI from the mutant proinsulin-expressed cells was almost half of that from the native proinsulin-expressed cells, as shown in Fig. 2. IRI in the second and third 24 h culture medium from the two cell groups showed exactly the same tendency. Interestingly we constantly observed the lower production of mutant proinsulin in other cell lines (data not shown). IRI from the mutant proinsulin-expressed cells, however, was still 5-fold higher than that from the rat insulinoma cell line, RINm5F [21], as shown in Fig. 2. A small amount of IRI was also detected in cell extracts of transfected COS cells. However, it was only 1–2% of the IRI in the culture medium, as was observed in the case of progastrin expressed in fibroblasts [22]. Since COS cells do not contain secretory granules, peptides must be released into culture medium as soon as they are synthesized.

3.2. *Processing of native and mutant proinsulin in COS-7 cells*

We next examined the extent of conversion from proinsulin to mature insulin in the native and mutant proinsulin-expressed cells. The culture medium from each cell group was subjected to gel filtration on a Sephadex G-50 column. When the culture medium of the native proinsulin cells was applied to this gel, IRI was eluted first at the proinsulin position, followed by a small peak corresponding to the mature insulin position, as depicted in Fig. 3a. This result was unexpected since Moore et al. [5] reported the production of human proinsulin without any processed insulin from a mouse

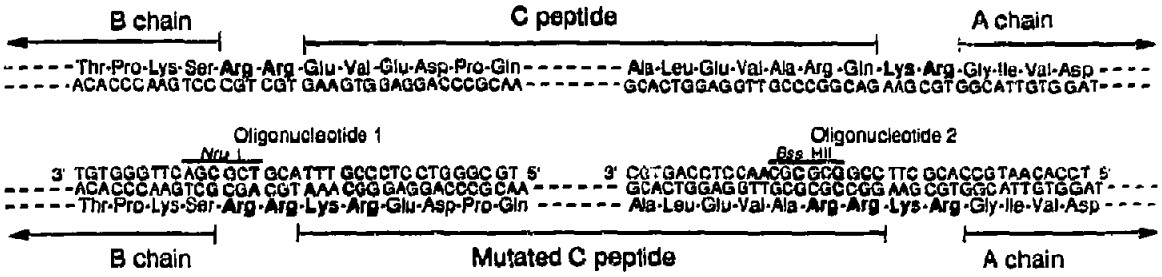

Fig. 1. The nucleotide and amino acid sequences across the two processing sites on native and mutant proinsulin. Upper sequences represent the nucleotide sequence and its corresponding amino acid sequence of native rat proinsulin. Lower sequences depict the two oligonucleotides utilized for the site-directed mutagenesis experiment, the nucleotide sequence and its corresponding amino acid sequence. The replaced nucleotides and basic residues in bold type. *Nru*I and *Bss*HII restriction sites were created to facilitate identification of the mutated proinsulin DNA.

fibroblast-derived cell line (Ltk$^-$ cells). At first we thought the IRI at the mature insulin position might be converted from proinsulin after the secretion into the culture medium. We pooled the proinsulin fraction and incubated it with non-transfected COS-7 cells over 24 h. Re-application of the culture medium onto the same column exhibited a single proinsulin peak, again without the appearance of a new IRI peak at the mature insulin position. We then noticed from amino acid sequence of the processing sites on rat proinsulin that both sites of rat proinsulin were somehow matched to the consensus proteolytic cleavage sequence of non-endocrine cells (Arg^{-4}-X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$), while one of the two on the human proinsulin sequence was not, as described in section 4 (Fig. 1).

The culture medium from the mutant proinsulin-expressed cells demonstrated two peaks on a Sephadex G-50 gel filtration chromatography, one at the proinsulin position and the other at the mature insulin position, as depicted in Fig. 3b. The ratio of mature insulin to total IRI was approximately 60%. Thus, we realized that the creation of the tetrabasic processing sites on the proinsulin sequence was not sufficient for full conversion of proinsulin to mature insulin. Thus, we co-expressed furin, which is thought to be a mammalian processing endoprotease in non-endocrine cells, together with native or mutant proinsulin. As depicted in Fig. 3d, co-expression of furin and mutant proinsulin resulted in the complete conversion of proinsulin to mature insulin, and did not leave any trace amount of IRI at the proinsulin position. Co-expression of furin together with native proinsulin also facilitated further conversion of proinsulin to mature insulin (Fig. 3c). In this case, a little over 50% of the total IRI appeared at the mature insulin position.

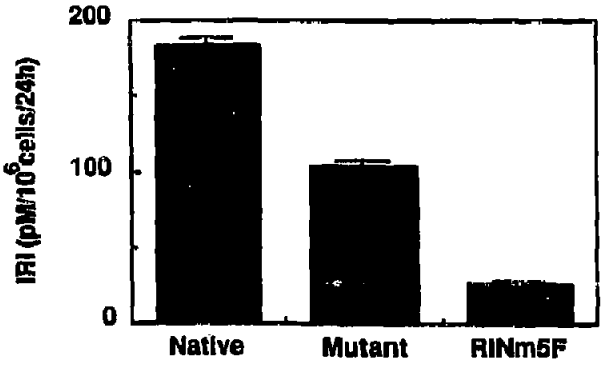

Fig. 2. Comparison of the immunoreactive insulin (IRI) production from the native and mutant proinsulin-expressed COS-7 cells, and from *RINm5F* cells. 12 h after electroporation, culture medium was collected every 24 h for 72 h for insulin radioimmunoassay. This figure depicts IRI in the first of the 24 h samples. The bars represent the mean ± S.D.

3.3. *Bioactivity of mature insulin from COS-7 cells*

We further examined its biological activity by measuring the incorporation of 3-*O*-[^{3}H]methylglucose to adipocytes. Adipocytes were collected after the digestion of epididymal adipose tissue with collagenase, and incubated with 3-*O*-[^{3}H]methylglucose for 3 s in the presence of insulin. As depicted in Fig. 4, insulin from the mutant proinsulin-expressed cells (filled bars) exhibited a similar biological activity to the synthetic human insulin (hatched bars) at two different insulin concentrations (25 and 90 pM). Therefore, the insulin from COS-7 cells was fully functional from the aspect of its glucose uptake capability.

4. DISCUSSION

A decade ago, Gruss and Khoury [23] expressed rat proinsulin in monkey kidney cells by inoculating the recombinant SV 40 DNA construct that replaced the rat insulin I gene in the late region of a SV-40 vector, with a temperature-sensitive helper virus. Lomedico [24] succeeded in producing rat proinsulin by transfecting a SV 40 early region-rat insulin II gene DNA construct to COS cells. Since then, many investigators have used this heterologous cell expression system for studying processing of peptide precursors [6]. Moore et al. [5] expressed human proinsulin in a mouse anterior pituitary-derived cell line, AtT20 cells, and in a mouse fibroblast-derived cell line, Ltk$^-$ cells. Proinsulin was correctly

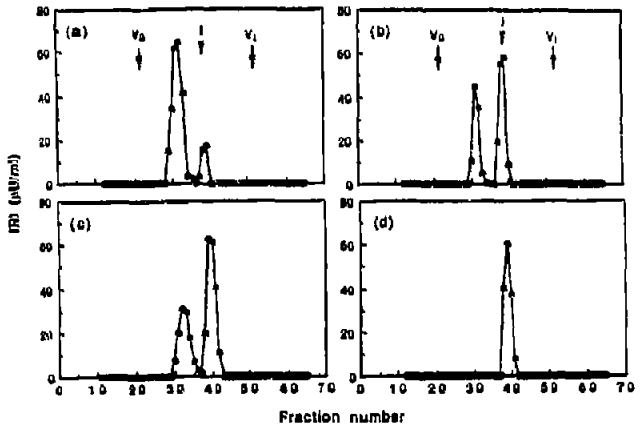

Fig. 3. Gel filtration profiles of IRI in the culture medium of native and mutant proinsulin-expressed COS cells. The concentrated media were applied to Sephadex G-50 superfine columns (1.0 × 120 cm) equilibrated in acetate buffer, pH 5.0. Fractions of 1.5 ml were collected and measured for IRI by radioimmunoassay. Molecular size was calibrated with blue dextran (V_0), potassium ferricyanide (V_t) and synthetic human insulin. Similar elution profiles were obtained in at least three other chromatographs for each experiment. (a) IRI in the medium from the native proinsulin-expressed cells; (b) IRI in the medium from the mutant proinsulin-expressed cells; (c) IRI in the medium from the native proinsulin and furin-co-expressed cells; (d) IRI in the medium from the mutant proinsulin and furin-co-expressed cells.

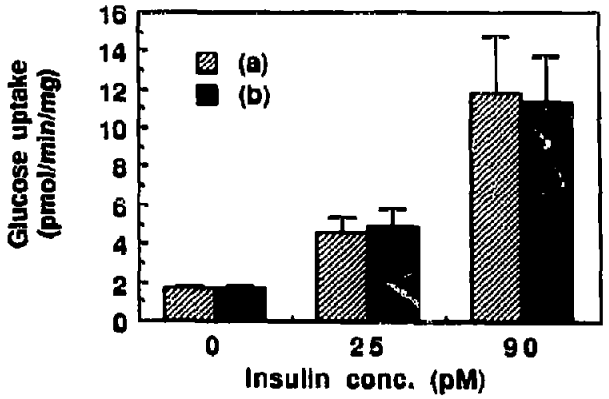

Fig. 4. Incorporation of 3-*O*-[^{3}H]methylglucose to adipocytes stimulated by mature insulin from the mutant proinsulin-expressed cells. (a, hatched bars) Synthetic human insulin; (b, filled bars) mature insulin fraction from the mutant proinsulin-expressed cells.

processed to mature insulin in the endocrine cell line, AtT20, while it remained as proinsulin in the fibroblast cell line, Ltk$^-$. Generally production of unprocessed propeptides has been observed when foreign prohormone genes are expressed in non-endocrine cell lines. However, Warren and Shields [25] and Sevarino et al. [26] observed correctly cleaved somatostatin in the non-endocrine cell lines, COS-7 and 3T3-Swiss-Albino, respectively, although the cleaved fraction was very small compared to the uncleaved somatostatin precursor fraction. Later, Stoller and Shields [27] altered the paired basic amino acid processing site, Arg-Lys, of prosomatostatin to Lys-Arg and Arg-Arg, and expressed the native and mutated precursors in the rat pituitary endocrine cell line, GH3, and in the mouse non-endocrine cell line, 3T3. The processing site of native prosomatostatin is composed of Arg^{-4}-Glu^{-3}-Arg^{-2}-Lys^{-1}, which shows only one amino acid difference at position −1 from the non-endocrine cell consensus processing site Arg^{-4}-X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$. Lysine at position −1 is also a basic amino acid, thus it is not surprising that native prosomatostatin was processed to somatostatin to some degree in COS and 3T3 cells. As expected, the mutated precursor with the non-endocrine cell consensus processing site, Arg^{-4}-Glu^{-3}-Lys^{-2}-Arg^{-1} or Arg^{-4}-Glu^{-3}-Arg^{-2}-Arg^{-1} was processed to the 14 amino acid somatostatin more efficiently than the native precursor in 3T3 cells. More recently partial cleavage of the neuropeptide Y precursor was reported in transfected Chinese hamster ovary cells [28]. This cleavage is also explained by the resemblance of the precursor processing site to the concensus processing site of non-endocrine cells.

As described above, Moore et al. [5] reported that human proinsulin was not processed in a mouse fibroblast-derived cell line, Ltk$^-$. In our experiment, rat native proinsulin was processed to a small extent to insulin in COS-7 cells (Fig. 3a). We do not think this is due to the difference of cell lines used in each experiment. By comparing the amino acid sequences of human and rat proinsulin processing sites in Fig. 1, we noted that the junction sequence between the C peptide and the A chain is Leu-Gln-Lys-Arg in human proinsulin and Arg-Gln-Lys-Arg in rat proinsulin I [29], which is perfectly matched to the non-endocrine cell consensus processing site, Arg^{-4}-X^{-3}-Lys/Arg^{-2}-$Arg^{-1} \downarrow X^{+1}$. Since the other junction sequence is Lys-Thr-Arg-Arg in human proinsulin and Lys-Ser-Arg-Arg in rat proinsulin I [29], and Lys at the −4 position is also a basic amino acid, it is not surprising to observe processed insulin in the rat proinsulin expression experiment, as is the case of prosomatostatin to somatostatin conversion in COS cells [25].

The cleavage of the consensus sequence is thought to be catalyzed by the subtilisin-like endoprotease furin. Furin was identified by homology to the yeast endoprotease, Kex2, and is believed to represent a mammalian cell processing enzyme [14,15]. All cell lines so far examined contain some amount of furin, although its content is different from cell to cell [15]. COS cells appeared to contain less furin compared with other cell lines by Northern blot analysis (unpublished data). Thus, expression of mutant proinsulin itself in COS cells might not attain complete conversion to mature insulin without a boost of furin co-expression. The mature insulin from COS cells presented a similar biological activity to the synthetic human insulin in terms of its [^{3}H]methylglucose incorporation ability to adipocytes. We are currently in the process of expressing insulin perma-

nently in other non-endocrine cells of different origin to investigate the processing efficiency of tetrabasic sites in these cells.

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