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*-Lys/Arg*3-Arg*1 | X*1, is thought to be a consensus 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-C peptide-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.

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*-Lys/Arg*3-Arg*1 | 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*3-Lys-Arg*3-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*3-Lys-Arg*3-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*-Lys/Arg*3-Arg*1 | 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.
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 II gene 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 construct were purified twice by cesium chloride gradient centrifugation. 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 BamHI 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, was obtained from this gene using a site-directed in vitro mutagenesis kit (Takara, Kyoto) described as follows. At first a 1.1 kb PolI-Xbal fragment of rat insulin I gene [16] was inserted into a M13 mp19 bacteriophage vector. The oligonucleotide primers used for the mutagenesis were synthesized using an Applied Biosystems Model 391 PCR-Mate DNA synthesizer. The BamHI 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 prepared from a mutated M13 clone and a 350 bp length of mutated rat insulin DNA was isolated with BamHI digestion and cloned into the BamHI 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 PstI and EcoRI, and subcloned into the PolI and EcoRI sites of a pCDL-SRα29S 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 insert was determined by DNA sequencing. The vector containing the insulin insert was digested with PstI and EcoRI. The direction of the insert was determined by DNA sequencing.

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% CO2. Transfections of the expression vector constructs were carried out by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA). Cells were allowed to recover on ice for 10 min and then divided into four 100 mm dishes containing 10 ml of fresh medium. The culture medium was collected every 24 h from the point of the 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.

2.3. Glucose transport analysis

Glucose transport activity of fat cells stimulated by insulin was assessed by measuring the rate of uptake of 3-O-methyl-[3H]-glucose at 37°C according to the 3.5 method of Whitesell and Gliemann, and modified by Toyoda et al. [19]. Adipocytes were extracted by collagenase digestion from a rat epididymal adipose tissue. Approximately 10 mg of adipocytes, measured by a hemocytometer tube, were incubated with 100 μM 3-O-methyl-[3H]-glucose (10 mCi/mM) 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 M phloretin, an inhibitor for glucose transport, and cells in 300 μl of the suspension were collected by the oil-filtration method of Gliemann et al. [20]. The amount of glucose in the extracellular space was determined by adding labeled 3-O-methyl-[3H]-glucose after adding phloretin. The radioactivity in the cell fraction was determined in a liquid scintillation counter.

2.4. Gel filtration

Gel filtration was performed with a 10.0 x 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 (Vd) and potassium ferricyanide (Vv).
column exhibited a single proinsulin peak, again with-
out the appearance of a new IRI peak at the mature
insulin position. We then noticed from amino acid se-
quence of the processing sites on native rat proinsulin.

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 nucleotide sequence and its corresponding amino acid sequence of native rat proinsulin. The replaced nucleotides and basic residues in bold type.

The culture medium from the mutant proinsulin-ex-
pressed cells demonstrated two peaks on a Sephadex
G-50 gel filtration chromatography, one at the proin-
sulin position and the other at the mature insulin posi-
tion. Together with native or mutant proinsulin also facilitated further 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.

3.3. Bioactivity of mature insulin from COS-7 cells

We further examined its biological activity by meas-
uring the incorporation of 3-O-[3H]methylglucoside to adipocytes. Adipocytes were collected after the digestion of epididymal adipose tissue with collagenase, and incubated with 3-O-[3H]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 up-
take 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] ex-
pressed human proinsulin in a mouse anterior pituitary-
derived cell line, ART20 cells, and in a mouse fibroblast-
derived cell line, Ltk− cells. Proinsulin was correctly
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 medium from the native proinsulin and furin-co-expressed cells; (d) medium from the native proinsulin expressed cells; (b) IRI in the least three other chromatographs for each experiment. (a) IRI in the medium from the native proinsulin expressed cells; (c) IRI in the medium from the mutant proinsulin expressed cells; (d) IRI in the medium from the mutant proinsulin and furin-co-expressed 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 Lys-Ser-Arg-Arg in rat proinsulin [29], which is perfectly matched to the non-endocrine cell consensus processing site, Arg-X2-Lys/Arg-X2-Arg1 LX'1. Since the other junction sequence is Lys-Thr-Arg-Arg in human proinsulin and Lys-Ser-Arg-Arg in rat proinsulin [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 ease 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 [3H]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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