

Primary Structures and Multiplicity of Pancreatic Hormone in Fish

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Key Words: insulin, glucagon, multiplicity, primary structure, function,
immunoreactivity

Abstract

The pancreas contains several peptide hormones including insulin and glucagon which regulate nutrition metabolism and consequently growth. The primary structures and biochemical characters of these hormones are important information for the artificial synthesis of these hormones and the development of the immunoassay system. In fish, several species possess plural molecular forms of insulin and glucagons, and this phenomenon complicates the understanding of the physiological function of these hormones. The present study aimed to review the current state of knowledge concerning biochemical characters of those hormones and the systems producing multiple molecular forms.

The production systems of multiple molecular forms of insulin and glucagon are different among species. Purifying the hormones and analyzing of primary structures and gene structures elucidated this phenomenon. The production systems are classified into the following types: (1) arising from a single prohormone by proteolytic cleavage at different sites of the signal peptide (flounder insulins) or (2) C-terminus regions (ratfish insulins, barfin flounder glucagons), (3) arising from two distinct genes amplified by chromosome duplication (kaluga sturgeon insulins, paddlefish insulins and glucagons) or (4) irrespective of chromosome duplication (bonito insulins, kaluga sturgeon glucagons).

It is not clear whether there are any functional differences between each molecular form in fish. However, immunoreactive differences have been demonstrated in insulins from flounders. These differences are significant, but both molecular forms are crossreactive to the antibody. This suggests that it is difficult to measure their blood levels distinctly by immunological assays in each fish species. Thus, multiplicity of insulin and glucagon is an obstacle to develop measurement techniques for these hormones due to immunological similarities between each molecular form, but science needs to overcome this obstacle to understand the physiological function of each hormone.

Introduction

Insulin and glucagon are key hormones regulating nutrition metabolism in fish like in mammals. These hormones are contained mainly in B-cells and A-cells of the endocrine pancreas at high concentrations. Insulin is a two-chained polypeptide hormone consisting of A- and B-chains and derived from preproinsulin by proteolytic removal of a signal peptide and a peptide called C-peptide which connects A- and B-chains. Insulin secretion is induced by a feeding stimulus (Mommsen and

Plisetskaya, 1991; Navarro *et al.*, 1993; Andoh and Nagasawa, 2002) and accelerates nutrition anabolism (Inui *et al.*, 1978; Plisetskaya *et al.*, 1984; Mommsen and Plisetskaya, 1991). Furthermore, insulin possesses the growth promotion function in fish. Insulin promotes sulfation uptake in gill cartilage of fish at the physiological concentration (Duan and Hirano, 1992; Duan *et al.* 1992; Plisetskaya, 1998). Plasma insulin levels in salmonids correlated with body weight significantly and rainbow trout from fast-growing families showed significantly higher plasma insulin levels than did fish from slow-growing families (Sundby *et al.*, 1991).

Glucagon is a single polypeptide chained hormone. The proglucagon gene encodes glucagon and related peptides including glucagon-like peptide, oxyntomodulin and glicentin. These hormones are cleaved proteolytically from proglucagon. Glucagon shows a hyperglycemic effect and a depletion of liver glycogen at least in short-term experiments using fish (e.g. Ince and Thorpe, 1977; Ottolenghi *et al.*, 1989, Plisetskaya and Mommsen, 1996).

Primary structures of insulins from over 30 species (Conlon, 2001) and glucagons from over 20 species (Irwin, 2001) have been established in fish. Several of these papers showed that some fish species possessed plural molecular forms of these hormones. However, primary structure analyses of those hormones are not completely established except for limited species.

When there are plural molecular hormone forms, the following differences should be examined to understand the physiological functions, such as biological activities, immunoreactivities, and the production system (plural distinct genes or a single gene). Purifications and the establishment of primary structures of each molecular form are base information for these points.

The present study aimed to review the current state of knowledge concerning biochemical characteristics of insulin and glucagon, their systems producing multiple molecular forms and prospective way to move on to the next phase in fish.

Multiplicity of Insulin in Fish

Barfin flounder (*Verasper moseri*) possesses two molecular forms of insulin. A- and B-chains of insulin-I consist of 21 and 30 amino acid residues, respectively (**Fig.1**). Both chains of Insulin-II are 21 and 32 amino acid residues, respectively. Amino acid sequences of both insulins are completely identical to each other except for two amino acid residue extension of B-chain N-terminus of insulin-II. These characteristics and a result of Southern blot analysis suggested that these molecular forms were derived from a single preproinsulin and cleaved at different sites in the signal peptide regions (Andoh and Nagasawa, 1998a). Starry flounder (*Platichthys stellatus*) and stone flounder (*Kareius bicoloratus*) also possess two molecular forms of insulin in the same manner as in barfin flounder (Andoh and Nagasawa, 1998b, c).

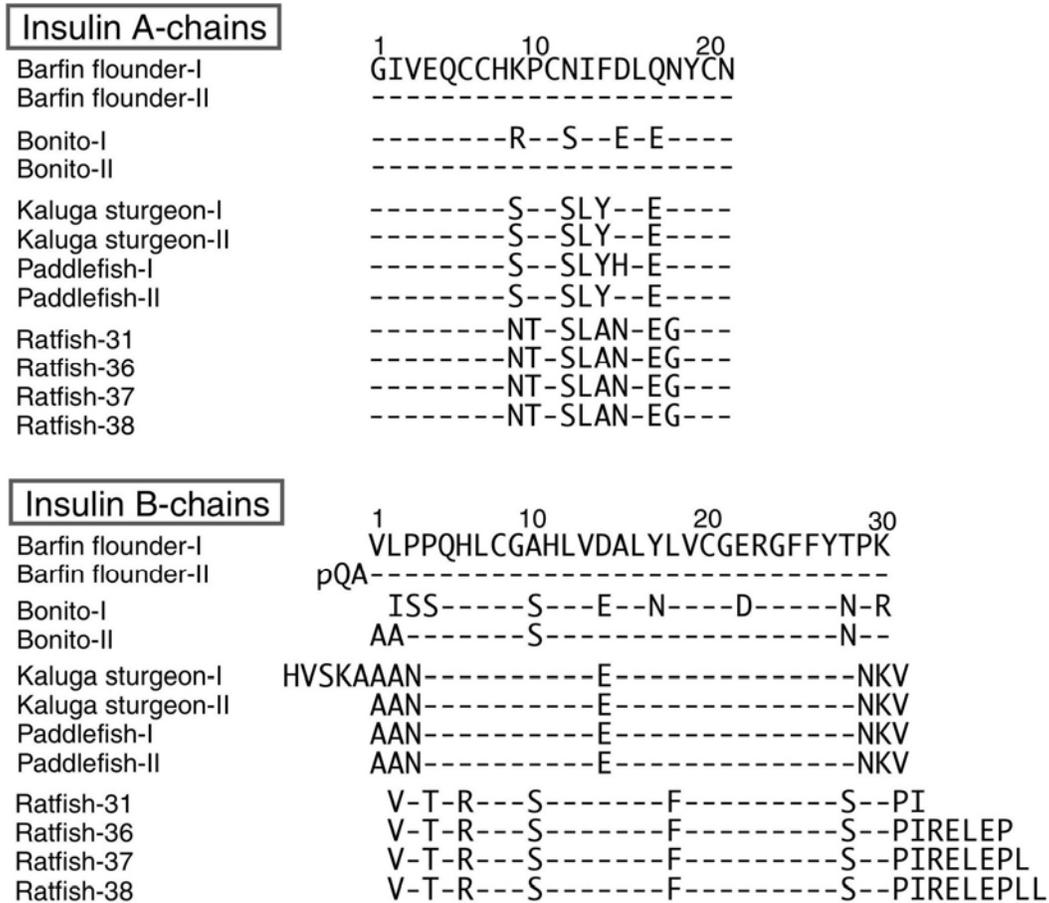


Fig. 1
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Figure 1. Comparison of the primary structures of insulin in fish. Hyphens indicate residues identical to those of barfin flounder insulin-I in other species.

Sturgeons (paddlefish, *Polyodon spathula*, Nguyen *et al.*, 1994; kaluga sturgeon, *Huso dauricus*, Andoh *et al.*, 2000) possess two molecular forms of insulin (insulin-I and -II). Those primary structures are identical to one another except for one internal amino acid residue (paddlefish insulin- I) or N-terminal five amino acid residues

extension (kaluga sturgeon insulin-I) (**Fig. 1**). Two molecular forms of insulin in these chondrosteans appear to be encoded by two distinct genes amplified chromosome duplication, because these species are tetraploid species (Dingerkus and Howell, 1976; Birstein *et al.*, 1993). Two molecular forms of toadfish which is a tetraploid teleost also appear to be produced in the same manner as sturgeons.

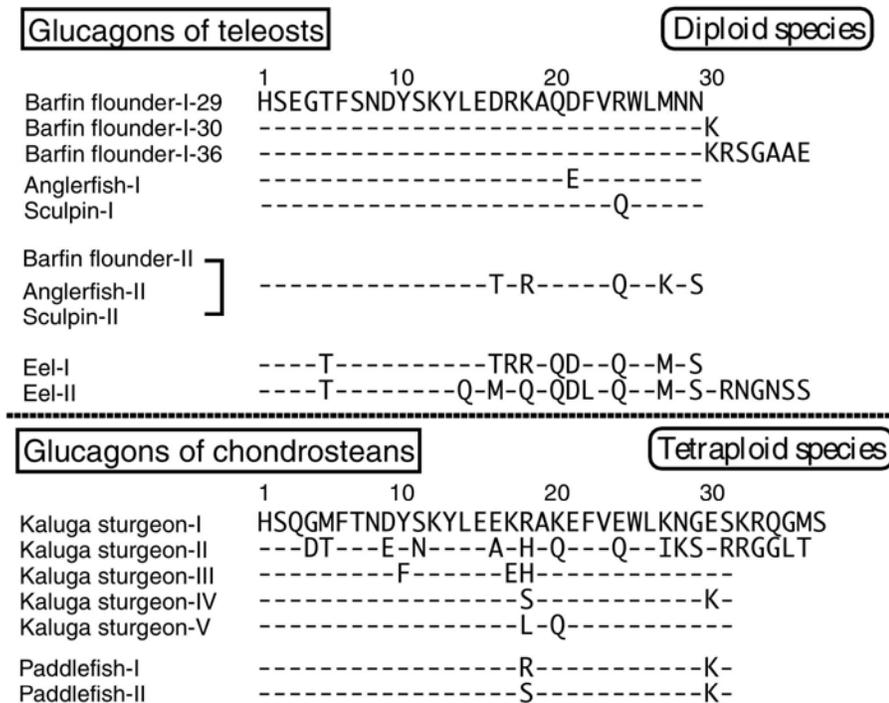
Kotaki and his colleagues (Yamamoto *et al.*, 1960; Kotaki, 1961; Kotaki, 1962; Kotaki *et al.*, 1962; Kotaki, 1963) demonstrated that bonito (*Katsuwonus pelamis*) possessed two molecular forms of insulin including internal substitutions of amino acid residues. However, the establishment of these primary structures were partially limited in parts of the molecular forms. We purified two molecular forms of insulin from bonito again, established amino acid sequences and confirmed the sequences by cloning two preproinsulin genes and coding both molecular forms (**Fig.1**, Andoh, *et al.*, unpublished data). Amino acid sequences established by protein sequencing were identical with those deduced by gene cloning, but were different from the results by Kotaki's group in several amino acid sequence residues. However, it was confirmed that bonito possessed two molecular forms of insulin containing internal substitutions of amino acid residues. Bonito is a diploid species confirmed by DNA content analysis (Hinegardner and Rosen, 1972) and chromosome number analysis of related species (Ida *et al.*, 1978). This suggests that two preproinsulin genes are amplified irrespective of chromosome duplication in bonito unlike in the tetraploid species. Two molecular forms of insulin found in rats were generated by an RNA-mediated duplication-transposition event by retroposon, and rat preproinsulin-I lost one of the two introns present in preproinsulin-II, possibly during the transposition event (Soares *et al.*, 1985). Both preproinsulin genes of bonito possess two introns, and this suggests the possibility that two preproinsulin genes of this species are not generated by an event of retroposon.

Conlon *et al.* (1989) showed that Pacific ratfish (*Hydrolagus colliei*) possessed four molecular forms of insulin, such as I-31, I-36, I-37 and I-38 (Fig. 1). Primary structure differences among these molecular forms are limited in C-terminus of B-chain followed by C-peptide region. The numbers of the abbreviated names of each molecular form indicate the residue length of B-chain. Conlon *et al.* (1989) proposed that these four insulins arose from a single proinsulin by proteolytic cleavages at different sites within the C-peptide region.

Multiplicity of Glucagon in Fish

Several teleosts possess two molecular forms of glucagon (I and II) containing internal sequence residues substitutions (**Fig. 2**). Amino acid sequence similarities between glucagon-II's from sculpin (*Cottus scorpius*, Conlon *et al.*, 1987), anglerfish

(*Lophius americanus*, Lund *et al.*, 1983; Nichols *et al.*, 1988), barfin flounder (Andoh, unpublished data) and tilapia (*Oreochromis niloticus*, Nguyen *et al.*, 1995) are higher than those between glucagon-I and -II in each species. Similarities between glucagon-I's are lower than glucagon-II's, but are also higher than those between glucagon-I and -II. Diploidy of these species is supported by the chromosome number analyses and DNA contents of these species or relatives (Hinegardner and Rosen, 1972). These suggest that two glucagon genes from sculpin, anglerfish and flounder were amplified irrespective of chromosome duplication and amplified before divergence of these fish species.



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Fig.2

Figure 2. Comparison of the primary structures of glucagons in teleosts and chondrosteans. Teleosts and chondrosteans indicated in this figure are diploid and tetraploid species, respectively. Hyphens indicate residues identical to those of barfin flounder glucagon-I-29 or kaluga sturgeon glucagon-I in other species.

On the other hand, eel (*Anguilla anguilla*) glucagon-I gene appeared to be originated from eel glucagon-II gene irrespective of chromosome duplication and by the mechanism which is different from those of other teleosts. Eel (Park and Grimm,

1981) is also a diploid species like the above teleosts. Eel glucagon-I shows a high degree of similarity with glucagon-II of other teleosts, while eel glucagon-II shows a low degree of similarity with glucagon-II of other teleosts. This suggests that eel glucagon-I and glucagon-II of those teleosts are more ancient and eel glucagon-II gene were generated independently by amplification of eel glucagon-I gene or its ancestral gene in eel.

Sturgeons are polyploid species. Paddlefish, a tetraploid species, possesses two molecular forms of glucagon. The number of substituted amino acid sequence is only one internal residue, suggesting a possibility that two glucagon genes were amplified by chromosome duplication. Kaluga sturgeon is also a tetraploid species like the paddlefish (Birstein *et al.*, 1993), but five molecular forms of glucagon were purified from pancreas of this species (**Fig. 2**, Andoh *et al.*, 2000). Up until present, more than two molecular forms of glucagon have not been characterized in a singular species. Numbers of substituted amino acid sequence between each molecular form varies from 2 to 12 residues and glucagon-I and -II possess 7 and 6 residues extension at the C-terminus, respectively. These suggest that glucagon genes in kaluga sturgeon were amplified both by chromosome duplication and by a mechanism irrespective of chromosome duplication.

Barfin flounder possesses two molecular forms of glucagon (glucagon-I-29 and -II) like other teleosts, but additional two molecular forms of glucagon-I were purified from the Brockmann body and were established the amino acid sequences recently (**Fig.2**, Andoh, unpublished data). Glucagon-I-30 and -36 possess a C-terminal extension consisting of one and seven amino acid residues, respectively, and other amino acid sequence residues were completely identical with that of glucagon-I. These characters of the sequences suggest a possibility that three molecular forms of glucagon-I are originated from a single proglucagon at different cleavage sites of C-terminal region. In mammals, glucagon-37 involved a complete sequence of glucagon-29 and was encoded by the same gene with glucagon-29. Glucagon-37 is called oxyntomodulin which shows an inhibitory effect on stomach acid secretion. Glucagon-I-36 in barfin flounder corresponds possibly to glucagon-37 in mammals. Gene cloning and physiological investigation of the glucagons will confirm this possibility.

Points to Analyze in Each Species

The above section indicated that the production system of multiple molecular forms of insulin and glucagon were different between each fish species. Those are classified into the following four types (**Fig. 3, 4**): (1) arising from a single prohormone by proteolytic cleavage at different sites of the signal peptide (barfin flounder insulins) or (2) the C-terminus regions (ratfish insulins, barfin flounder glucagons), (3) arising

from two distinct genes amplified by chromosome duplication (kaluga sturgeon insulins, toadfish insulins, paddlefish insulin and glucagons) or (4) irrespective of chromosome duplication (bonito insulins, eel glucagons, kaluga sturgeon glucagons). This complication of multiplicity is an obstacle to understanding the secretion regulation system of insulin and glucagon in fish due to the difficulty of developing a measurement system for quantification. It is necessary to simplify the analyses to solve the following four points. These points have not been analyzed until now except for some cases in a few species, but the solutions will show the way to the goal.

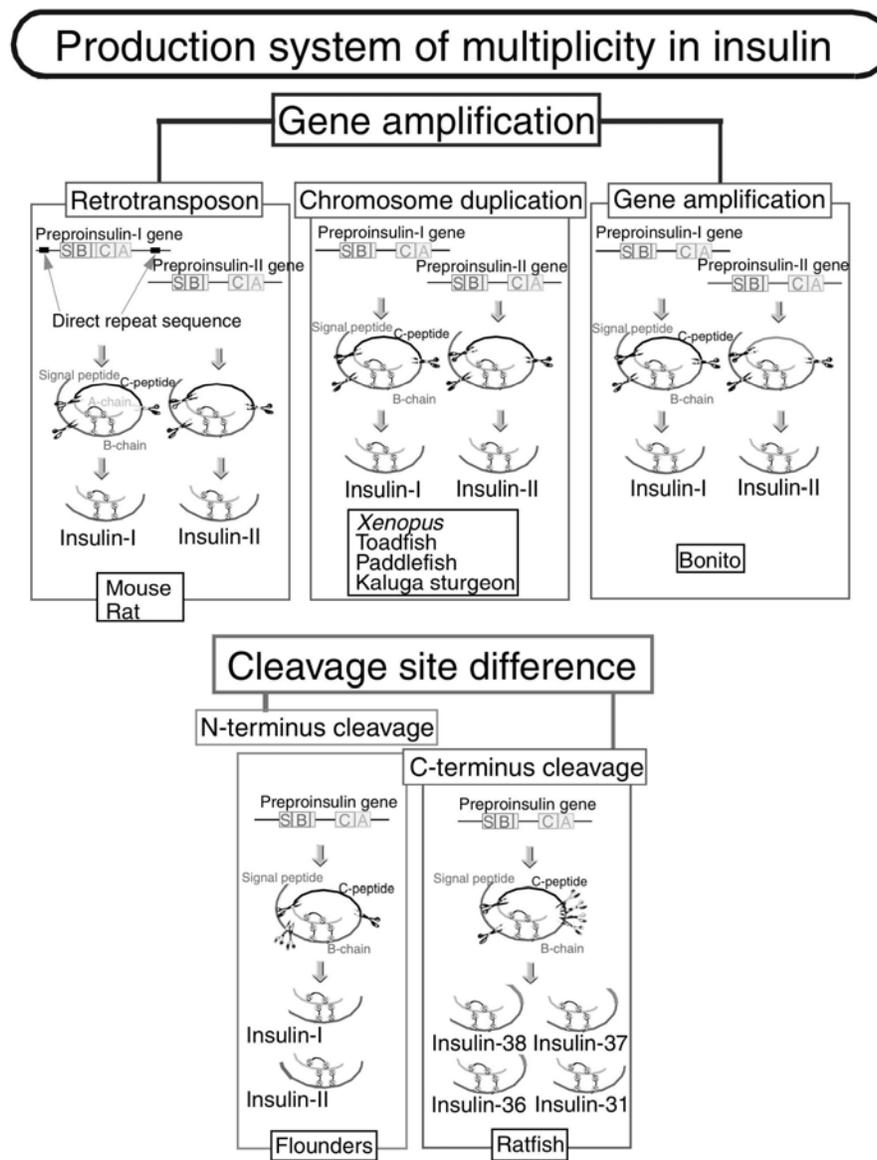


Figure 3. Production system of multiplicity of insulin in vertebrates. Production system of multiplicity of insulin in vertebrates is divided to two major categories,

such as system types by gene amplification and by cleavage site difference. Furthermore, those are divided to minor categories. The former are systems delineated by retrotransposon, by chromosome duplication and by gene amplification irrespective by chromosome duplication. The latter are divided into system types by N-terminus cleavage and by C-terminus cleavage.

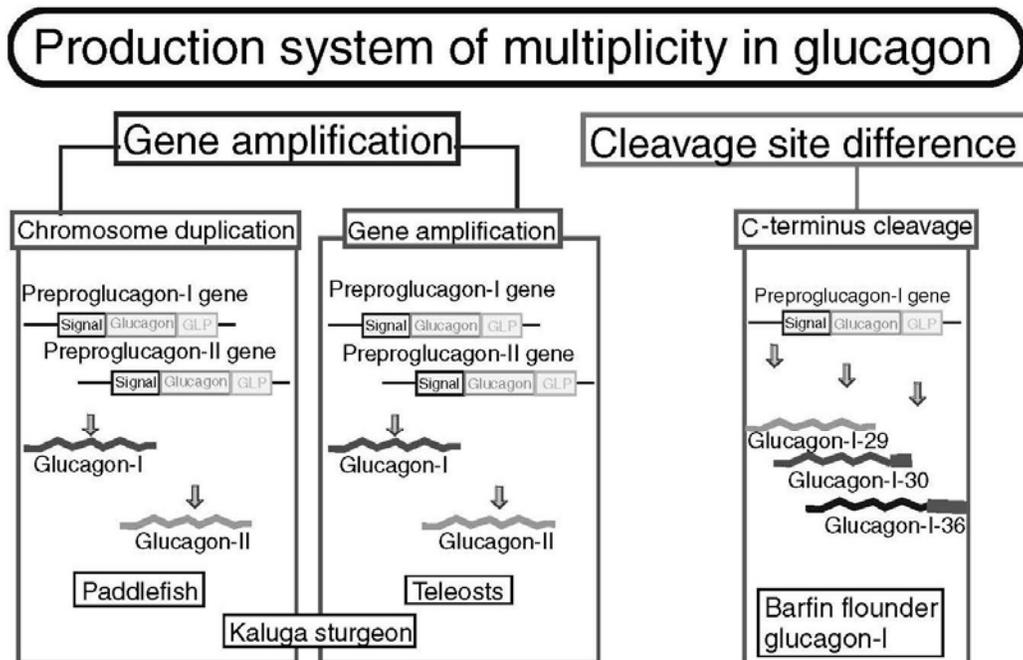


Fig. 4
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Figure 4. Production system of multiplicity of glucagon in fish. Production system of multiplicity of glucagon in fish are divided to two major categories, such as system types by gene amplification and by cleavage site difference. Furthermore, the former are divided into system types by chromosome duplication and by gene amplification irrespective of gene amplification. The latter is a type by C-terminus cleavage site difference.

1. How Many Molecular Forms Does the Species Possess?

Gene structure analysis and cloning are an important approach for estimating the number of molecular forms, but these methods sometimes give underestimated numbers due to the dependence on sequences of probes and primers. Therefore, purification from the Brockmann body is better a approach than gene cloning, because it reflects

generally on the existence ratio in the organ. The Brockmann body in fish is suitable tissue for purification of peptides unlike pancreas in other vertebrate because the Brockmann body is a pure endocrine tissue and does not contain exopancreatic enzymes. This characteristic enables one to purify peptides easily using combination of ethanol-ether precipitation or SepPak cartridge (Millipore, Milford, MA) and reverse-phase HPLC without degradation. Furthermore, biochemical characterization of each molecular form enables the determination of retention time for each molecular form in blood samples on chromatographic separation.

2. Is the Molecular Form Secreted Into the Blood?

The next point is to identify molecules in the blood. It is essential to know whether the molecule processing is intermediate before maturation or not. Realistic identification of the molecules in the blood accomplished by using a combination of separation by reverse-phase HPLC and immunoassay or receptor binding assay after peptide extraction of the blood. Each molecule in the blood is identified by retention time and the activities. For example, reverse-phase HPLC separates only a single amino acid residue difference between glucagon-I-29 and -30 in barfin flounder. This identification procedure needs a high specificity to the molecules of the assay system and standard peptides for measurement. Differences between the binding activity of the antibody and the receptor between the molecular forms are not a big problem in most cases.

3. Is There Functional Difference Between Molecular Forms?

It is not clear whether there are any functional differences between fish insulin and glucagon molecular forms. This is the most important problem when examining the multiplicity of insulin and glucagon in fish.

Receptor binding assay for insulin have been developed in several fish using mammalian insulin labeled radioisotope. However, differences among the receptor binding activity of each molecular form have not been examined in fish yet. A tetraploid amphibian, *Xenopus laevis*, possesses two molecular forms of insulin like several of the above mentioned fish. These two insulins were expressed differentially during neurulation (Shuldiner, *et al.*, 1991), although these genes coordinate under several different environments in adults (Celi *et al.*, 1994). This suggests that two molecular forms of insulin play differential roles at the larval stage in *Xenopus*. Receptor binding activity and glucose oxidation in rat adipocyte were compared between *Xenopus* insulin-I and porcine insulin in the paper while the comparisons of insulin-II were not performed due to its lower yield. Purification of the native peptide is required for comparing molecular forms. In fish, insulin and glucagon are purified

easily at high yield as described above. In addition, glucagon can be synthesized artificially. Insulin can be also synthesized artificially, but artificial synthesis of this hormone needs special techniques.

4. Is There Immunological Differences Between Molecular Forms?

This point is not as important for understanding differences of physiological functions. However, an immunological detection system is the most reliable and sensitive method and does not tend to be influenced by sample components for measurement of the hormone molecule compared with receptor assay. Therefore, the immunoreactive difference between the molecular forms is sometimes an important problem to investigating secretion dynamics. Immunoreactive differences between molecular forms of insulin have been demonstrated in barfin flounder (Andoh and Nagasawa, 2002), starry flounder (Andoh and Nagasawa, 1998c) and bonito (Andoh *et al.*, unpublished data). Studies that investigate the secretion dynamics of those molecular forms needs distinct specific immunoassay systems because there may be distinct functions between molecular forms.

Practical Example in Barfin Flounder Insulin

Results of analyses of the primary structure and Southern blot showed that two molecular forms of insulin from barfin flounder arose from a single preproinsulin by proteolytic cleavage at different site of the signal peptide region (Andoh and Nagasawa, 1998a). Both insulins secreted into the blood by feeding stimulation at approximately the same ratio as that of the quantities of both insulins harbored in the Brockmann body. This indicates that the half life of both molecular forms are the same level in the blood (Andoh and Nagasawa, 2002). Furthermore, receptor binding activities of both insulins are completely same as the barfin flounder insulin receptor (Andoh and Matsubara, unpublished data). These suggest that there are no functional differences between the two molecular forms of insulin in barfin flounder and that two distinct immunoassay systems recognizing each molecular form are not required to measure the absolute blood level of insulin. Considering these points, we improved our assay system to create new immunoassay using B1 monobiotinylated barfin flounder insulin-I which was synthesized chemically for measurement of insulin concentration as total absolute values. Our previous assay system (Andoh and Nagasawa, 2002) used tribiotinylated barfin flounder insulin-II which was not labeled B1 amino acid residue. The B1 residue consists of an important antigenic site of insulin and only a different site between two molecular forms found in barfin flounder. Therefore, B1 labeling was expected to decrease crossreactive difference between the two insulins. The binding inhibition curves of insulin-I and -II in the previous assay system were different, but

are improved in the new system (Fig. 5; Andoh, 2004). Thus, this process constructed a firm methodological basis for physiological analyses of two molecular forms of insulin in barfin flounder.

Conclusions and Perspectives

The present study reviewed primary structures, multiplicity and their production systems, and introduced a way to analyses of physiological functions of peptide hormones when plural molecular forms exist. A practical example was demonstrated for the barfin flounder. It is expected that a more comprehensive investigation of the Brockmann body of teleosts will show the existence of other molecular forms of pancreatic peptide hormones, especially glucagon-like peptide and somatostatin are also complicated due to plural molecular forms and differences among physiological functions.

Complete sequence analyses of the genomes have been finished in a lot of organism species including several fish species because of the human genome project. Recent projects are changing to focus on proteome and peptidome. Proteomics and peptidomics perform comprehensive profiling of all proteins and peptides in an organ or whole body of a species by amino acid sequence establishment.

The Brockmann body, which corresponds to islets of Langerhans in mammalian pancreas, consists of pure endocrine tissue including insulin, glucagon and other pancreatic peptide hormones in teleosts. Moreover, the Brockmann body does not contain proteases and nucleases originated from exocrine pancreatic tissue. This segregation facilitates the isolation and characterization of peptide hormones and mRNA encoding the precursors of the hormones by fewer purification steps under the inhibition of degradation unlike the mammalian pancreas. Actually, teleost is the greatest group which established primary structure of insulin and glucagon, and the final yields of insulin and glucagon of barfin flounder possessing the Brockmann body are apparently higher than those of kaluga sturgeon possessing a mammalian type pancreas. In addition, easy purification of mRNA in the Brockmann body is more advantageous to confirm of the amino acid sequences by deduction from nucleotide sequences and in quantification of transcripts. Therefore, the Brockmann body of teleosts is a suitable organ for proteomic and peptidomic analyses and the analyses will not only clarify multiple molecular forms and a possibility of existence of novel molecular forms, but also give information about the level of transcripts, translated products and the production system of each molecular form in the species. Furthermore, this analysis is a good substitute for the mammalian pancreas as a model for analysis of the insulin secretion mechanism.

Acknowledgments

The author would like to thank Prof. H. Nagasawa, the University of Tokyo, and Dr. T. Matsubara, Hokkaido National Fisheries Research Institute, Fisheries Research Agency, for their helpful suggestions and supports.

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