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Molecular characterization of insulin from squamate reptiles reveals sequence diversity and possible adaptive evolution

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ABSTRACT

The Squamata are the most adaptive and prosperous group among ectothermic amniotes, reptiles, due to their species-richness and geographically wide habitat. Although the molecular mechanisms underlying their prosperity remain largely unknown, unique features have been reported from hormones that regulate energy metabolism. Insulin, a central anabolic hormone, is one such hormone, as its roles and effectiveness in regulation of blood glucose levels remain to be examined in squamates. In the present study, cDNAs coding for insulin were isolated from multiple species that represent various groups of squamates. The deduced amino acid sequences showed a high degree of divergence, with four lineages showing obviously higher number of amino acid substitutions than most of vertebrates, from teleosts to mammals. Among 18 sites presented to comprise the two receptor binding surfaces (one with 12 sites and the other with 6 sites), substitutions were observed in 13 sites. Among them was the substitution of $_{His}$B10, which results in the loss of the ability to hexamerize. Furthermore, three of these substitutions were reported to increase mitogenicity in human analogues. These substitutions were also reported from insulin of hystricomorph rodents and agnathan fishes, whose mitogenic potency have been shown to be increased.

The estimated value of the non-synonymous-to-synonymous substitution ratio (ω) for the Squamata clade was larger than those of the other reptiles and aves. Even higher values were estimated for several lineages among squamates. These results, together with the regulatory mechanisms of digestion and nutrient assimilation in squamates, suggested a possible adaptive process through the molecular evolution of squamate *INS*. Further studies on the roles of insulin, in relation to the physiological and ecological traits of squamate species, will provide an insight into the molecular mechanisms that have led to the adaptivity and prosperity of squamates.

1. Introduction

In contrast to mammals and aves, both of which are endothermic groups of amniotes, ectothermic amniotes are generally termed reptiles. The order Squamata, a group to which lizards and snakes belong, is recognized as the most successful group among reptiles. Squamate species have survived climatic changes several times since their first appearance in the Early Jurassic period (Jones et al., 2013), including that in the Late Cretaceous period which put an end to "the Age of Reptiles". More than 9000 squamate species are now extant worldwide, from subarctic to tropical zones (Herczeg et al., 2003; Utez, 2015; Vitt and Caldwell, 2013).

The species diversity of the Squamata alone is superior to that of mammals (approximately 5500 species), and is similar to that of aves (approximately 10,000 species) (Baillie et al., 2010). This is in contrast with the other orders of reptilian groups; only 367 species have been reported in Testudines, Crocodilia, and Rhynchocephalia (Utez, 2015). The molecular mechanisms responsible for the significant success of the Squamata among the reptiles are our research interest, as they are the key to achieving prosperity with an ectothermic system, and will provide novel insights into the adaptation of amniotes to terrestrial environments.

Since ectotherms do not constantly generate body heat for the regulation of body temperature, their metabolism is affected by

Abbreviations: aa, amino acid(s); BEB, Bayes Empirical Bayes; bp, base pair(s); cDNA, DNA complementary to RNA; dCTP, cytidine 5′-triphosphate; dN, number of nonsynonymous substitutions; dNTP, deoxyribonucleoside 5′-triphosphate; dS, number of synonymous substitutions; IGF, insulin-like growth factor; *INS*, insulin gene; LRT, log likelihood ratio test; mRNA, messenger RNA; ω, non-synonymous-to-synonymous substitution ratio; ORF, open reading frame; PCR, polymerase chain reaction; PSS, positively selected site(s); RACE, rapid amplification of cDNA ends; RT, reverse transcription.

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ambient temperature fluctuations (Skoczylas, 1970; Stevenson et al., 1985; Vitt and Caldwell, 2013). Therefore, the activity of ectotherms is restricted by an ambient climate. On the other hand, a lower basal metabolic rate enables them to live on a small intake of food. These disadvantages and advantages of the ectothermic systems indicate that ectotherms can minimize energy expenditure by downregulating their metabolic rate when the climate is unfavorable for their activity. However, when the climate becomes more favorable, they need to upregulate metabolic rate in order to capitalize on the environment for their various activities, such as foraging and reproduction. Such shift has been reported from squamate species, whose metabolic state changes dramatically in accordance with the feeding state. For example, oxygen consumption, together with the weights of the digestive and endocrinal organs, increase several-fold (or more) after feeding (Christel et al., 2007; Secor et al., 1994; Secor, 2008). An apoptotic or proliferative process was also shown to occur at the cellular level (Buono et al., 2006). For squamates, especially those with long feeding intervals as a result of sit-and-wait foraging (Secor, 2001; Wall and Shine, 2013) or seasonal variation in food availability (Buono et al., 2006; Bustard, 1968; Naya et al., 2010), this up- and down-regulation of the digestive system is favorable for reducing energy expenditure, as maintenance of digestive performance is metabolically costly (Andrade et al., 2005; Secor, 2001). One group of Squamata that appears to have benefited from this regulation is the infraorder Gekkota. The basal metabolic rate of the species in this group was reported to be lower than other squamates (Andrews and Pough, 1985; Brown and Nagy, 2007; Vidal and Hedges, 2005). This feature enables them to more efficiently reduce energy expenditure than the other groups, because they consume less energy in a resting state. This, in turn, results in a lower requirement for food, an advantageous feature to survive environment or period with poor food availability (King, 1996). The apparent species-richness and worldwide habitat of Gekkota may reflect their environmental adaptivity: approximately 1500 Gekkota species inhabit almost all over the world. This number amounts to 25% of squamates other than Serpentes (Gamble et al., 2012).

For the elucidation of the molecular basis underlying the regulatory mechanisms of squamate metabolism, insulin is interesting topic for examination as it plays central roles in anabolic regulation of vertebrates. Vertebrate insulin is generally released from pancreatic islets (β-cells) in response to food intake, and promotes the anabolism of substrates such as carbohydrates, lipids, and amino acids. However, the role that insulin plays in the physiology of squamates currently remains unclear. Although controversy exists (Sidorkiewicz and Skoczylas, 1974), several studies have suggested that the role of insulin in squamate species is unique among reptiles, with only a negligible effect on blood glucose regulation (Dessauer, 1970; Matty, 1966).

Therefore, we firstly isolated cDNA coding for insulin from two evolutionary divergent Gekkota species, the leopard gecko (*Eublepharis macularius*) and Japanese gecko (*Gekko japonicus*) in order to establish the molecular basis for further studies. Leopard geckos are ideal experimental animals as they are easy to keep and breed in the laboratory. Japanese geckos can be easily collected from several fields in Tokyo metropolitan area. Thus, they are good experimental subjects when focusing on physiological regulation under natural habitat. The cDNA sequences of preproinsulin, the primary transcript of *INS* (insulin gene), was isolated from pancreas of these two Gekkota species. Preproinsulin contains four regions: signal peptide, B-chain, C-peptide, and A-chain. Signal peptide is removed upon translocation of the product into endoplasmic reticulum (ER). The remaining polypeptide, comprising B-chain, C-peptide, and A-chain, is called proinsulin. In ER, two inter-chain disulfide bonds are formed between B7–A7 and B19–A20 to link B- and A-chain. An intra-chain bond is also formed between A6–A11. Proinsulin is transported into secretory granule, where cleavage of C-peptide takes place by prohormone convertase (PC) -1/3 and PC-2. Through these processes, mature insulin ("insulin" designates this form in this article), comprising B-chain and A-chain connected by two disulfide bonds, is formed (Rhodes et al., 2004). The amino acid sequence of the leopard gecko insulin resembled those of other amniotes. However, the Japanese gecko insulin showed the accumulation of multiple amino acid substitutions. Such variation of insulin sequences had never been reported from reptilian species. Moreover, some of the substitutions were located at sites that were widely conserved among other vertebrates, from teleosts to mammals. To examine the origin of this diversity in the insulin sequences, we herein isolated proinsulin cDNA from species that represent various groups of Squamata. The deduced amino acid sequences of squamate insulin isolated in this study (27 species in total), together with those of two species obtained from the GenBank Database (the green anole and king cobra) were compared with representative species of amniotes. The results suggested that amino acid sequences of squamate insulin were more divergent than those of the other amniotes. Further investigations into each substitution and evolutionary process suggested that squamate *INS* had undergone a distinct evolutionary process among vertebrates, which may have been advantageous for their survival.

2. Materials and methods

2.1. Animals

For the isolation of cDNAs coding for insulin, living samples of 27 squamate species (listed in Table 1) were obtained. Japanese geckos, Japanese five-lined skinks (*Plestiodon finitimus*), and Japanese grass lizards (*Takydromus tachydromoides*) were collected from a field in the Tokyo metropolitan area, Japan. Leopard geckos were bred over several generations in the laboratory. Other species of squamates were purchased from local Pet shops. All animals used in this research were treated according to the guidelines of the Bioscience Committee at the University of Tokyo. Animals were anesthetized by an injection of sodium pentobarbital at a dose of 25 μg/g body weight, and were killed by decapitation and exsanguination. Tissues were collected and frozen in nitrogen liquid. Frozen tissues were stored at −80 °C until used for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the pancreas using ISOGEN (NIPPON GENE, Tokyo). For the construction of cDNA templates, 1–3 μg of total RNA was used. cDNA templates for 3′-RACE were constructed as follows; denatured total RNA was reverse-transcribed using an oligo (dT)-adaptor primer and M-MLV Reverse Transcriptase (Promega, Madison, WI). The reaction volume contained oligo (dT)-adaptor primer at 5 pM, each dNTP at 2 mM, 200 units of M-MLV Reverse Transcriptase and 1 × M-MLV buffer (Promega). The volume was brought up to 20 μl with Nuclease-Free Water (Quiagen, Tokyo, Japan). The reaction was performed under 42 °C, 90 min and then 70 °C, 15 min. In some cases, ThermoScript Reverse Transcriptase (Invitrogen, Tokyo) was used, instead. The reaction volume contained oligo (dT)-adaptor primer at 5 pM, each dNTP at 2 mM, 15 units of ThermoScript Reverse Transcriptase and 1 × cDNA Synthesis Buffer (Invitrogen). The volume was brought up to 20 μl with Nuclease-Free Water (Quiagen). The reaction condition for this enzyme was 42 °C, 30 min, 55 °C, 45 min, and then 70 °C, 15 min. cDNA templates for 5′-RACE were constructed according to the following methods. Messenger RNA was purified from total RNA using Dynabeads Oligo (dT) 25 (Invitrogen) and then reverse transcribed using SuperScript III