Conserved and Non-Conserved Loci of the Glucagon Gene in Old World Ruminating Ungulates

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The homology and diversification of genomic sequence encoding glucagon gene among native Egyptian buffalos, camel and sheep were tested using cattle as model. Oligodeoxynucleotide primers designed from the available GenBank data were used for PCR probing of the glucagon gene encoding sequence at different loci. The DNA oligomer probes were constructed to flank either the whole gene encoding sequence or different intra-gene encoding sequences. The PCR products were visualized using agarose gel electrophoresis. All species showed a same size band of prepro-glucagon when PCR was used to amplify the whole gene encoding sequence. In contrary, amplifications of different intra-gene loci failed to give the same results. The results indicated variable degrees of diversity among old world ruminating ungulates in the glucagon gene encoding sequence. Compared with other ruminants, the variation appears predominantly in camel. Surprisingly, the similarity in size between both amplification products of whole gene encoding sequence and the proposed size of glucagon cDNA definitely excludes the possibility of large intervening introns spanning the genomic sequence of the glucagon gene in these species. This indicates that, in contrast to other tested mammals, the glucagon gene includes an essentially full-length copy of glucagon mRNA. The study revealed a possible new aspect of glucagon gene evolution in order to correlate its corresponding protein function among different ruminant species.

Key words: Glucagon, PCR, Ruminants

Introduction

Glucagon, a 29-amino acid polypeptide hormone of α cells of pancreatic islets (Sundby *et al.*, 1976), is originated, as many other hormones, from a larger precursor which is then enzymatically cleaved to the functional form (Steiner *et al.*, 1980). In mammals, the primary physiological effect of glucagon is in the liver, where it affects glucose metabolism by inhibiting glycogen synthesis, stimulating glycogenolysis and enhancing gluconeogenesis (Sokal, 1973; Hers, 1976). *In vitro* studies have shown that glucagon increases the mobilization of glucose, free fatty acids and ketone bodies (Lefebvre and Luychx, 1979). Moreover, molecules of various sizes, larger than glucagon, and displaying glucagon-like immunoreactivity have been isolated from the pancreatic tissues of many different species (Tager and Steiner, 1973). They all have originated from the same gene in many vertebrates (Irwin and Sivarajah, 2000) as well as mammals including ruminants (Lopez *et al.*, 1983). Beside glucagon, the mammalian proglucagon gene encodes other glucagon-like sequences, glucagon-like peptide I (GLP-I) and glucagon-like peptide II (GLP-II). Each of these three functionally distinct proglucagon-derived peptides has an unique, but related, function (Sivarajah *et al.*, 2001), *e.g.* GLP-I, a known stimulant of insulin secretion, controls feeding and drinking behavior (Yamamoto *et al.*, 2002).

Many of these glucagon-like peptides show varying degrees of sequence homology to mature pancreatic glucagon (Thulesen, 2004).

To the best of our knowledge there is no confirmed data concerning the glucagon gene in native Egyptian buffalo and camel breeds, which play considerable roles in economy in Egypt and many other neighbor countries. Although the role of glucagon has been fully studied in many animal species, little is known about its metabolic contribution in these two species. Nevertheless, carbohydrates metabolism in camel is still a matter of mystery. In spite of its digestive anatomy has a true

ruminant pattern, blood glucose level in camel simulates, for some extent, the mono-gastric animals (Abdel-Fattah *et al.*, 1999; Warda, 1998). Unlike camel, ungulates, like cattle, buffalos and sheep, proved to be less dependent on blood glucose as an energy determinant. Camel has a relatively higher blood glucose level than these species. The played role by glucagon hormone, as anti-insulin glycogenolytic hyperglycemic hormone, has been previously studied in camel (Abdel-Fattah *et al.*, 1999). In the previous study we found, in spite of rapid rate of elimination of injected glucagon from peripheral circulation, the level of glucose persisted significantly higher than that of pre-injection level (Abdel-Fattah *et al.*, 1999). This finding can not be attributed to a poor developed glucagon degrading enzyme system since there is a rapid elimination rate of glucagon from peripheral blood. Going further, the next step to disclose the role of this hormone in regulation of camel metabolism should aim at determining its structure, as a peptide, together with its related polypeptides. Therefore, a comparative study on glucagon peptide encoding sequence should be undertaken. This motivated us to probe the genomic sequences encoding glucagon and related peptides at different loci among different species.

In this study, different loci of genomic glucagon encoding sequences were PCR-probed, using cattle-based oligodeoxynucleotide primers (Accession No. 173916). Probing was done to test the sequence homology along the glucagon gene in different domestic ruminating ungulates (native breeds of cattle, sheep, buffalo and camel). Primers used flank either the whole gene encoding sequence or different intra-gene encoding loci.

Material and Methods

General

Deoxynucleotides (dNTP) were purchased from Amersham-Pharmacia, USA, *Taq* polymerase and polymerase 10X buffer from Finnzymes (Espoo, Finland) and RNase from Stratagene (USA). All other reagents used in DNA isolation, PCR, and electrophoresis were analytical grades. The bacteriophage Ø X 174 DNA-*Hae* III digest was used as DNA molecular size standard (Finnzymes). For PCR, a pTC-100 MJ-research Thermocycler was used.

Isolation of genomic DNA from leucocytes

The isolation of genomic DNA was performed after Sambrook *et al.* (1989). Briefly, citrated whole blood of normal adult cattle, buffalos, sheep and camel, after being collected from slaughter house, was centrifuged ($600 \times g$) at 10 °C for 20 min. The leucocytes-rich layer (buffy coat) was incubated with lysis buffer [10 mM 2-amino-2-hydroxy-methylaminomethane-HCl (Tris-HCl), pH 7.3, 0.3 M sucrose, 1% (v/v) Triton X-100, 5 mM MgCl₂] at 4 °C for 5 min. The nuclei were pelleted by centrifugation at $5,000 \times g$ for 20 min at 4 °C. 10% SDS and proteinase K (Sigma-Aldrich, USA) were added to the recovered pellet, then the mixture was incubated at 55 °C for 12 h. Proteins were then extracted, once with phenol and once with phenol/chloroform then with chloroform/isoamyl alcohol. To the final aqueous solution 3 M NaOAc was added. After a 10 min incubation on ice samples were microfuged at 4 °C for 10 min. To the supernatant, absolute ethanol was added stored at 20 °C overnight and DNA was recovered by centrifugation at $14,049 \times g$ (F1202 Fixed-angle Rotor, Beckman) at 4 °C for 20 min and the pellet was dried under vacuum (SpeedVac, USA). The pellet was dissolved in TE buffer, incubated with 10 mg/ml heat-treated *RNase A* for RNA digestion at 37 °C for 15 min. The quality of the recovered DNA was assayed by 0.8% agarose and the purity was estimated from the $OD_{260/280}$ ratio.

PCR

Oligodeoxynucleotide primers (MWG-Biotech AG, Ebersberg, Germany) were designed to anneal with different parts of glucagon gene encoding sequences of cattle. Primers used had the following sequences: primer # 1, 5′-GAA GGC AAA AAT GAA AAG C-3′; primer # 2, 5′-ACA CAC TTA CTT CCT GTC CAG-3′; primer # 3 5′-ACT CGC AGG GCA CAT TCA CCA-3′; primer # 4, 5′-CTT GGT ATT CAT CAA CCA CTG-3′.

Primers design

Based on GeneBank data (Accession No. 173916), the cDNA sequence is shown in Fig. 1. It encodes a 20-amino acid signal sequence of predominantly hydrophobic amino acids found in secretory proteins (91 to 150 base sequence). This is followed by glicentin, a 69-amino acid polypeptide containing an internal glucagon moiety. This glucagon moiety is encoded by the underlined sequence

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  1  aacagagctcaggacactgcacacccaaacgagggctcactctctcttcacctgctctgt

                      m        k   s   l   y   f   v   a   g   l
 61  tccacctcctggtgtcagaaggcagcaaaaatg     aaa agc ctt tac ttt gtg gct gga ttg

      f   v   m   l   v   q   g   s   w   q   r   s   l   q   n   t   e   e   k   s
121  ttt gta atg ctg gta caa ggc agc tgg caa cgt tcc ctt cag aac aca gag gag aaa tcc

      s   s   f   p   a   p   q   t   d   p   l   g   d   p   d   q   i   n   e   d
181  agt tca ttc cca gct ccc cag acc gac ccg ctc ggc gat cca gat cag atc aat gaa gat

      k   r   h   s   q   g   t   f   t   s   d   y   s   k   y   l   d   s   r   r
241  aag cgc cac tcg cag ggc aca ttc acc agt gac tac agc aag tac ctg gac tcc agg cgt

         Glucagon
      a   q   d   f   v   q   w   l   m   n   t   k   r   n   k   n   n   i   a   k
301  gcc cag gac ttc gtg cag tgg ttg atg aat acc aag aga aac aag aat aac att gcc aaa

         Glucagon-like I
      r   h   d   e   f   e   r   h   a   e   g   t   f   t   s   d   v   s   s   y
361  cgt cat gat gaa ttt gag aga cat gct gaa ggg acc ttt acc agt gat gta agt tct tat

      l   e   g   q   a   a   k   e   f   i   a   w   l   v   k   g   r   g   r
421  ttg gaa ggc caa gct gcc aag gaa ttc att gct tgg ctg gtg aaa ggc cga gga agg cga

      d   f   p   e   e   v   n   I   v   e   e   l   r   r   r   h   a   d   g   s
481  gat ttc cca gaa gaa gtc aac atc gtt gaa gaa ctc cgc cgc aga cac gcc gat ggc tct

         Glucagon-like II
      f   s   d   e   m   n   t   v   l   d   s   l   a   t   r   d   f   I   n   w
541  ttc tct gat gag atg aac act gtt ctc gat agt ctt gcc acc cga gac ttt ata aac tgg

      l   l   q   t   k   i   t   d   r   k   OC
601  ttg ctt cag acg aaa att act gac agg aag taa gtg tgt cat tca tta
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Fig. 1. cDNA nucleotide sequence of glucagon gene mRNA and the deduced amino acid sequence. The flanking underlined sequences are primers # 1 and 2. Primer 1 includes the start codon ATG (bold written). The glucagon encoding sequence is underlined. The glucagon sequence is flanked by primers # 3 and 4 (inclined underlined written sequences). Sequences of GLP-I and GLP-II are shown in bold. A one-letter symbol of each amino acid residue is written over its corresponding three-nucleotide codon.

in Fig. 1 (53–81 amino acid residues; nucleotide sequences from 247–333). Glicentin is followed by two additional glucagon-like peptides, *e.g.* GLP-I (the 1st bold written sequence in Fig. 1) from 92 to 120 amino acid residues and GLP-II (the 2nd bold written sequence) from 146 to 178 residues. These two peptides correspond to nucleotide sequences from 367 to 477 and from 532 to 630, respectively. Each of the peptides is flanked by paired basic amino acids (Lys, Arg) characteristic of prohormone processing (Blobel, 1980). Primers # 1 and 2 (underlined nucleotide sequences at the beginning and end of the whole sequence in Fig. 1) flank the whole glucagon gene encoding sequence. Primer # 1 flanks the beginning of the sequence including the methionine ATG start codon (indicated by bold letters). Primers # 3 and 4 are bold inclined sequences in the middle.

Three PCR runs were used. The first run was done by using primers # 1 and 2. In the 2nd run primers # 3 and 4 were used. The 3rd PCR run was performed using primers # 3 and 2. In all runs amplifications were carried out by preparing a 50 μl mix containing 150 μg DNA template, 1.0 μM of each primer, 200 μM of each dNTP, 10X PCR buffer (1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl, 8.3 pH), 1 unit *Taq* DNA polymerase and the reaction was processed by 35 cycles of denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, proceeded by initial denaturation at 95 °C for 3 min and terminated by a final extension step at 72 °C for 10 min. To exclude false results different runs of PCR were performed under different reaction conditions to confirm the obtained results.

Table I. Summarized results.

Primers	Fragment size [bp]	Amplification results in			
		cattle	buffalos	sheep	camel
# 1 and # 2	562	+ ve	+ ve	+ ve	+ ve
# 2 and # 3	392	+ ve	+ ve	+ ve	– ve
# 3 and # 4	89	+ ve	– ve	– ve	– ve

(+ ve) indicates the appearance and (– ve) indicates the absence of the corresponding band of known fragment size on agarose gel electrophoresis screening following PCR amplification.

Agarose gel electrophoresis

Amplicons of all PCR were tested using 1% agarose gel electrophoresis. The gels were ethidium bromide stained and UV-visualized. The band lengths were determined using the bacteriophage Ø X 174 DNA-*Hae* III digest DNA marker. The products exact sizes were determined against the DNA marker using Gel Pro software (San Diego, California, USA).

Results

The PCR product of each run was visualized using ethidium bromide stained agarose gel electrophoresis. The size of each amplicon was determined using a DNA marker. Negative control was run in parallel with samples in each gel. The exact fragment size was determined after gel scanning using Gel Pro software. In Table I the results of all PCR runs are summarized. PCR products using primers # 1 and 2 (Fig. 2) showed the same size band in all species examined. The size was determined to be 562 bp in all species. Primers # 3 and 4, that flank the glucagon hormone encoding sequence, explicit PCR amplification product of 89 bp (Fig. 3). This band appeared only in cattle samples rather than any other species examined. PCR products of primers # 2 and 3 gave a band of 392 bp which encode for the mature glucagon. Interestingly, this band appeared in all species but not in camel. Moreover, the most obvious part is that the band sizes in all amplifications are matching the predicted size of the corresponding segment in cDNA of glucagon gene (Accession No. 173916).
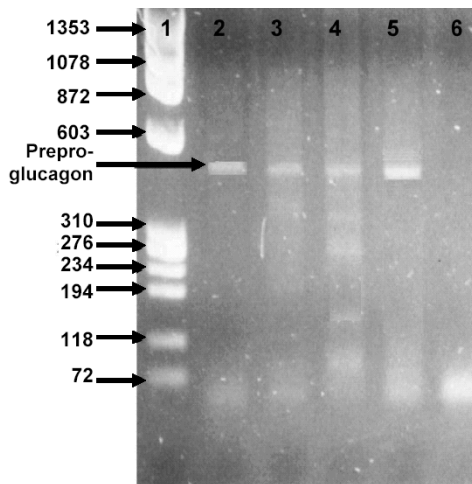

Fig. 2. Preproglucagon (562 bp): Lane 1 is the molecular weight marker, lanes 2, 3, 4 and 5 are cattle, buffalo, sheep and camel PCR amplicons. Lane 6 is the control negative of PCR. The primers used are primer # 1 and 2. It is clear that all species have the same segment of prepro-glucagons that show the band of 562 bp.
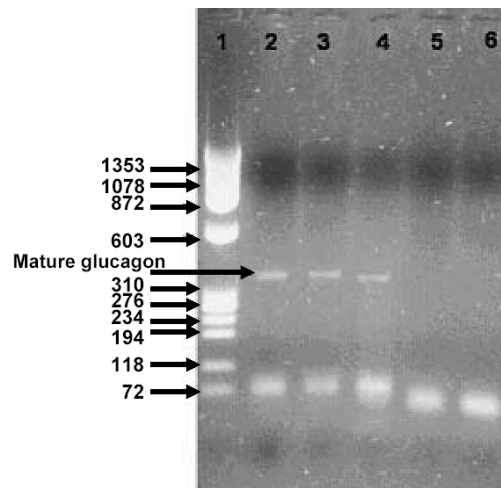

Fig. 3. Mature glucagon (392 bp): Lane 1 is the molecular weight marker, lanes 2, 3, 4 and 5 are cattle, buffalo, sheep and camel PCR amplicons. Lane 6 is the control negative of PCR using primer # 3 and primer # 2. The mature glucagon segment of 392 bp appears in case of cattle, buffalo and sheep; camel PCR, however, does not give such band.

Discussion

Glucagon is a pancreatic hormone of 29 amino acids that regulates carbohydrate metabolism and glicentin is an intestinal peptide of 69 amino acids that contains the sequence of glucagon flanked by peptide extensions at the amino and carboxy termini. Glucagon, however, is part of structurally related peptides that includes secretin (Mutt *et al.*, 1970), vasoactive intestinal peptide (Jornvall *et al.*, 1981), gastric inhibitory peptide, growth hormone-releasing factor (Spiess *et al.*, 1982), and prealbumin (Jornvall *et al.*, 1981). Our previous investigation provided evidence that the relative high blood sugar level in camel compared with other ruminants may be attributed to a higher plasma glucagon level in camel than that in other ruminants and men (Abdel-Fattah *et al.*, 1999). Recently, glucagon proved to increase membrane water permeability by inducing protein kinase A and microtubule-dependent translocation of water channels (Gradilone *et al.*, 2003). This may connect the high level of glucagon in camel with the possible role in osmoregulation in arid condition (Warda, 1998). Nevertheless, endocrine pancreas of the one-humped camel was found to be similar to many other mammalian species, including ruminants (Khatim *et al.*, 1985). The glucagon gene encodes a precursor containing glucagon and two additional, structurally related, glucagon-like peptides separated by an intervening peptide. These peptides were reported to be encoded in separate exons (Mojsov *et al.*, 1986).

In spite of the major regulatory role of glucagon in energy-dependent pathways involved in metabolism in different animals, little is known about its gene sequence homology in either Egyptian camel and buffalo when compared with other ruminants. To understand better the points of homology and diversification of genomic sequence encoding glucagon hormone at different loci, the size of amplified PCR products of genomic sequences at different loci along glucagon gene were compared in different native domestic ruminants taking cattle as reference.

The appearance of the same band corresponds to the whole preproglucagon encoding sequence in all species (Fig. 2) and the absence of other bands that represent the rest of intra-gene encoding sequences (Figs. 3 and 4) clearly indicates the conservative nature of the preproglucagon flanking sequences as single unit in these ruminant species. This conservative nature, however, might be
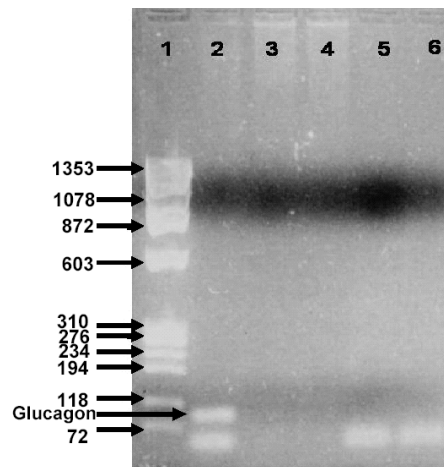

Fig. 4. Glucagon (89 bp): Lane 1 is the molecular weight marker, lanes 2, 3, 4 and 5 are products of PCR amplicons of cattle, buffalo, sheep and camel. Lane 6 is the control negative of PCR using primer # 3 and primer # 4. The primers used have matched only the cattle gene of glucagon.

subjected to different intra-gene modifications to meet varied metabolic requirements characteristic for each species. The structures of preproglucagon in different mammals are proved to be highly conserved (Lopez *et al.*, 1983; Irwin 2001). This agrees with our finding of preproglucagon encoding segments that appear in all species of examined ruminants. Moreover, Sivarajah *et al.* (2001) found that the diversification of the glucagon sequences encoded by the proglucagon gene (mature glucagon) is more common among species than the whole preproglucagon encoding sequence that tends to be conserved in our study. It is also reported that glucagon receptor binding sites in some animals were evolutionarily more conserved than that of the whole glucagon hormone (Huang *et al.*, 1986) with some degree of variability that noticed in amino acid contents of glucagon among the species.

More interesting is that the presence of the same band size (562 bp) of preproglucagon encoding sequence in all species examined (Fig. 2) that matches the same size of that sequence in cDNA of glucagon gene un-doubtly excludes any possibility of intervening introns that may span the genomic sequence of glucagon gene. This results however disagree with that reported in other mammals (Mojsov *et al.*, 1986). The presence of preproglucagon encoding sequence of 562 bp in

Egyptian camel and buffalo clearly indicates, like in many other mammals, that their glucagon gene encodes a precursor containing glucagon and two additional, structurally related, glucagon-like peptides (GLP-I and GLP-II) separated by an intervening peptide. In Fig. 3 the mature glucagon encoding sequence gives a band of 392 bp. This band appears in case of all ruminants but not in camel. Fig. 4 shows the result of amplification of the last PCR. The product of amplification using primers #3 and 4 flank glucagon peptide encoding sequence. Surprisingly, the agarose gel electrophoresis band of product amplification appears in case of cattle rather than other ruminants. The appearance of the mature glucagon encoding band (392 bp) in all examined ruminants but not camel (Fig. 3) and the absence of glucagon encoding band (89 bp) in all ruminants but not in the reference cattle model (Fig. 4) might speculate two degrees of diversity of glucagon gene among different examined ungulates. The first deviation was noticed in all ruminants that fail to explicit the 89 bp band corresponds to glucagon peptide. The second was observed only in camel, rather than other ruminants, as its PCR result indicates neither mature glucagon (392 bp band length) nor glucagon (89 bp band length) encoding sequences can be amplified. Generally, the deviations were noticed within the whole sequence but not in the flanking regions that indicate high degree of conservation between all species. This deviation not necessarily obligates encoding sequence difference in glucagon or its related peptides but more likely concerns the intervening peptides alterations to meet species-specific variations which occur at the level of post-translational processing.

It is worth noting that the unique divergent nature of camel metabolism might determine the pattern of forcing some of its genes to adopt their expressed polypeptide-specific functions to accommodate the creature to its habitats, *e.g.* in the proglucagon (mature glucagon) encoding sequence of camel, unlike many other mammalian vertebrates (Mojsov *et al.*, 1986), there is little or no sequence homology to the same loci that were found in related ruminant species.

In conclusion, in order to study the degree of homology of glucagon gene encoding sequences among different species of ruminating ungulates we probed different loci along the whole gene. PCR amplification results, using specific primers, indicate the conserved nature the preproglucagon fragment deduced from the identical fragment length of the resulting products in all species examined. The size of fragments is also matching the predicted size of corresponding cDNA sequences. This result excludes the possible intervening introns through the genomic sequence. The noticed intra-gene sequence diversities among ruminant species can be attributed to species-specific evolutionary adaptation that is markedly noticed in the one-humped camel.

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