Guinea Pig Glucagon Differs From Other Mammalian Glucagons

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SUMMARY

Mammalian glucagon is thought to be highly conserved. Glucagons from pig, cow, human, rat, and hamster have identical amino acid sequences, whereas the amino acid contents of rabbit and camel glucagons are consistent with this 29-amino acid sequence. It had earlier been reported that guinea pig (GP) glucagon contains 40 amino acids. In the current study, glucagon was purified from two GP pancreata by a series of three HPLC steps after acid-alcohol extraction and acetone precipitation. GP glucagon is a 29-amino acid peptide that differs from other mammalian glucagons by substitution of Gln for Asp in position 21, Leu for Val in position 23, Lys for Gln in position 24, Leu for Met in position 27, and Val for Thr in position 29. In view of the marked changes in the COOH-terminal of GP glucagon, receptor binding studies were performed using both rat and GP liver membranes. Labeled synthetic porcine glucagon has similar binding in the two systems and its binding is inhibited to a similar degree by synthetic porcine glucagon, whereas GP glucagon is 10-fold less potent at inhibiting binding in both systems. This suggests that glucagon receptor binding sites in the GP are evolutionarily more conserved than is GP glucagon. DIABETES 1986; 35:508–12.

The guinea pig (GP) gastroenteropancreatic axis appears to have a unique evolutionary separation from that of other mammals. Thus, while most mammalian insulins differ from each other at no more than four sites among the 51 amino acids in the A- and B-chains, GP insulin differs from pig insulin at 17 sites.[1] Recently, we reported that GP vasoactive intestinal peptide (VIP) differs at four sites from other mammalian VIPs,[2] which are identical among pig,[3] cow,[4] human,[5] and rat.[6] We have also observed[7] that GP cholecystokinin (CCK) COOH-terminal octapeptide differs from all other mammalian CCKs that have been sequenced thus far[8–10] by a substitution of valine for methionine at position 6 from the COOH-terminal. It had been reported that glucagon, a peptide that is usually highly conserved, is considerably different in the GP. Thus, whereas pig,[11] cow,[12] human,[13] rat,[14] and hamster[15] glucagons have identical amino acid sequences and the amino acid contents of rabbit[16] and camel[17] glucagons are consistent with this 29-amino acid sequence, Sundby[18] reported that GP glucagon has a distinctly different composition from other mammalian glucagons and contains 40 amino acids. In this report, we describe the purification and sequence of GP glucagon and demonstrate that, like other pancreatic glucagons, it is a 29-amino acid peptide but that it differs from other mammalian glucagons in five positions, all located in the COOH-terminal nonapeptide. We also show that GP glucagon is less potent than synthetic porcine glucagon in displacing labeled synthetic porcine glucagon in receptor binding studies employing both rat and GP liver membranes.

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MATERIALS AND METHODS

GP pancreata were purchased frozen from Pel-Freez (Rogers, Arkansas).

Radioimmunoassay (RIA). The GP antiserum generally employed in this laboratory for RIA of other mammalian glucagons did not detect GP pancreatic glucagon. Other antisera of lower sensitivity to mammalian glucagons were screened and the one most sensitive to GP glucagon, GP A-8-5, was employed in this study at a dilution of 1:10,000. Synthetic porcine glucagon was purchased from Peninsula Laboratories (Belmont, California). ^{125}I-synthetic porcine glucagon was used as tracer.

Extraction and purification. Two GP pancreata having a total weight of 1.8 g were extracted in 10 vol of acid-alcohol (0.2 M HCl in 75% ethanol). The extract was stored at −70°C for 2 days and then centrifuged. The supernatant was re-

moved and mixed with 8 vol of acetone. The mixture was stored overnight at −70°C. The precipitate that formed was collected by centrifugation and the supernatant was discarded. The precipitate was dissolved in 5 ml of 1% trifluoroacetic acid (TFA) containing 0.1% mercaptoethanol. The GP glucagon in this solution was further purified by a series of HPLC steps. Each step was run at 1 ml/min except the μBondapak step, which was at 2 ml/min. At each of the first two steps, individual fractions from the single peak of glucagon were separately purified. The three steps consisted of the following columns and elution conditions: (1) μBondapak C_{18} radial-pak cartridge (Waters Associates, Milford, Massachusetts), 0.13% heptafluorobutyric acid (HFBA)/20–80% acetonitrile (ACN); (2) Mono S HR5/5 strong cation exchange column (Pharmacia, Piscataway, New Jersey), 0.1% TFA containing 20% ACN/0–1.0 M NaCl; and (3) Nova C_{18} radial-pak cartridge (Waters Associates), 0.1% TFA/30–60% ACN.

Amino acid analysis and sequencing. Purified peptide was subjected to amino acid analysis and sequencing using 40 pmol and 640 pmol, respectively. Amino acid analysis was performed on a fluorescamine analyzer.[19] Automated amino acid sequencing was performed on a gas-phase sequencer.[20] Phenylthiohydantoin derivatives of amino acids were identified on an Ultrasphere C_{18} column (Beckman/Altex, Berkeley, California), as previously described.[21]

Because the intact peptide was incompletely sequenced, the C-terminal sequence of GP glucagon was verified by digesting 2 nmol of the peptide with Endoproteinase Lys-C (Boehringer-Mannheim, Indianapolis, Indiana). The peptide fragments were separated by HPLC on an Ultrasphere C_{18} column and the elution monitored with UV absorbance at 215 nm and 280 nm and with fluorescamine.[22] The three peptide fragments obtained (K_{1-3}) were further subjected to amino acid analysis and sequencing.

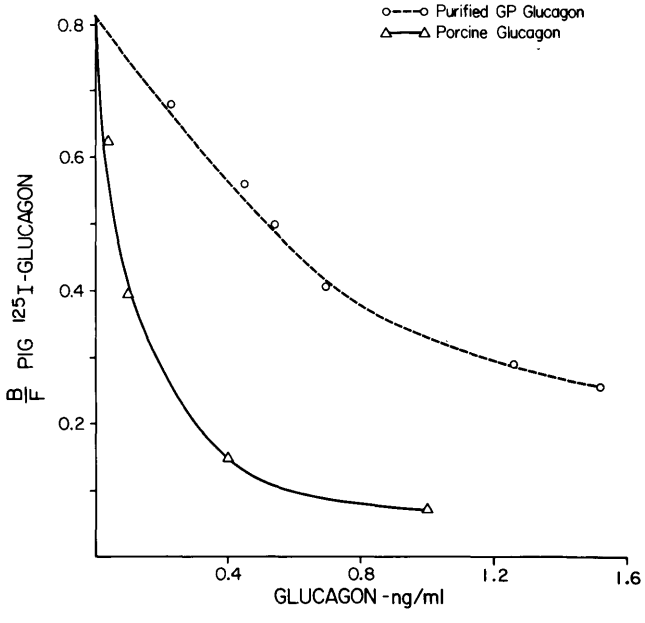

FIGURE 1. Standard curves for synthetic porcine and purified guinea pig glucagon using a guinea pig anti-porcine glucagon antiserum, A-8-5, at a dilution of 1:10,000 and ^{125}I-pig glucagon.

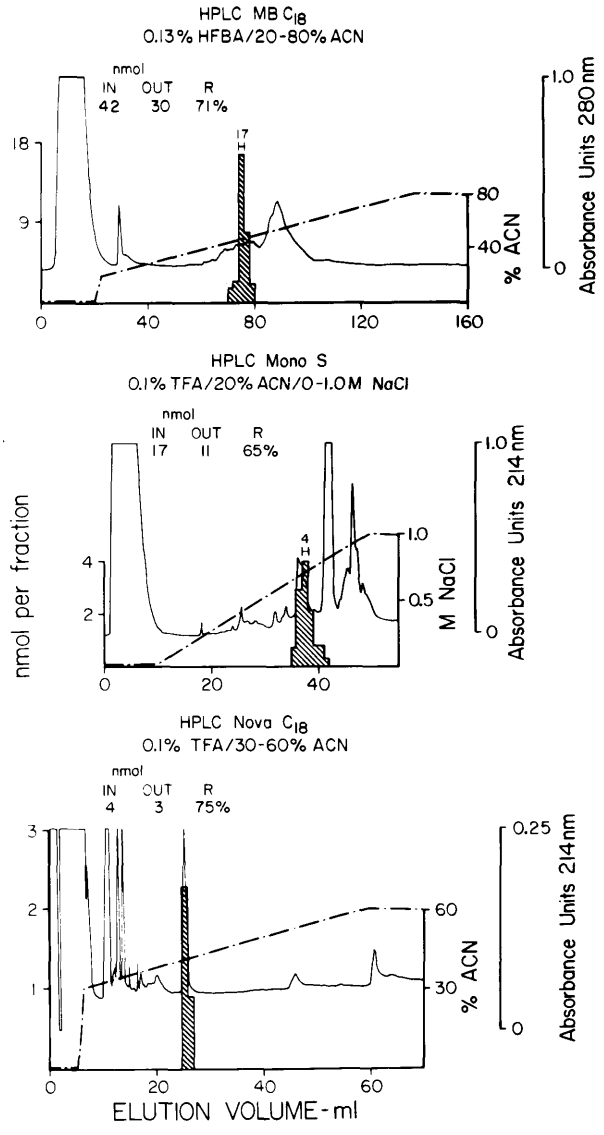

FIGURE 2. HPLC purification steps for guinea pig glucagon. R signifies recovery for each step. Fractions that were applied to the succeeding purification step are indicated by bars. The number above the bar indicates the nanomoles of glucagon in that fraction. The final peak was used for determination of amino acid content and sequencing.

Receptor assay. Liver membranes from rat and GP were prepared by differential centrifugation of homogenates in 0.25 M sucrose.[23] The final membrane pellet was resuspended in a volume equal to the original tissue weight. Receptor binding studies were performed in 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mg/ml bovine albumin, and 1 mg/ml bacitracin. Each assay tube contained 50 μl membrane suspension and 5 fmol ^{125}I-synthetic porcine glucagon in an incubation volume of 0.5 ml. The concentrations of added synthetic porcine glucagon ranged from 10^{-10} M to 10^{-6} M. Since the quantity of purified GP glucagon was limited, the maximum concentration employed was 10^{-7} M. The suspensions were incubated at 21°C for 2 h, the membranes were pelleted by centrifugation, the supernates were removed and discarded, and the pellets were counted. The presence of 1 μM synthetic glucagon was used to determine nonspecific binding.

TABLE 1
Amino acid compositions of pig glucagon, GP glucagon, and its Lys-C fragments

	Lys-C fragments			Pig		
	K1	K2	K3	GP	GP*	(Expected)
Asp (D)†	1.0(1)	1.1(1)	1.2(1)	2.7(3)	4	4
Thr (T)	1.5(2)			1.8(2)	2	3
Ser (S)	2.7(3)	1.1(1)		3.3(4)	4	4
Glu (E)†	0.9(1)	1.9(2)		3.0(3)	4	3
Gly (G)	0.9(1)			1.0(1)	2	1
Ala (A)		1.2(1)		1.1(1)	2	1
Val (V)			0.9(1)	1.1(1)	2	1
Met (M)						1
Ile (I)					1	
Leu (L)		1.8(2)	1.9(2)	4.2(4)	5	2
Tyr (Y)	1.3(1)	0.9(1)		2.4(2)	3	2
Phe (F)	1.3(1)	0.9(1)		1.9(2)	3	2
His (H)	1.1(1)			1.0(1)	1	1
Lys (K)	1.1(1)	1.1(1)		2.0(2)	3	1
Arg (R)		2.1(2)		2.4(2)	3	2
Trp (W)	ND	ND	ND	1.1(1)	1	1
Total residues				29	40	29

ND, not determined.
*Reported by Sundby.[18]
†Asn and Gln residues are included in Asp and Glu totals, respectively, due to conversion during acid hydrolysis.

RESULTS

A dilution curve of the extract of GP pancreata was not superimposable along a dilution curve of synthetic porcine glucagon. Therefore, recoveries were initially calculated on the basis of relative concentrations using a GP pancreatic extract as standard. After GP glucagon was purified, the true molar concentration of the extract was determined. Standard curves for synthetic porcine and GP glucagons are shown in Figure 1.

Total immunoreactive (IR) glucagon extracted from the two pancreata was 53 nmol. After acetone precipitation of the extract, 42 nmol were recovered. The successive steps of the HPLC purification are shown in Figure 2. The final peak shown was used for determination of amino acid composition, sequencing, and endopeptidase digestion. Additional side fractions were carried through similar HPLC steps so that final total recovery was 6 nmol.

The amino acid composition of GP glucagon is compared with that expected for pig glucagon and with the composition previously reported by Sundby[18] in Table 1. Shown in Figure 3 is the GP glucagon sequence as determined directly and from the three fragments generated by endoproteinase Lys-C digestion. The 20 NH_2-terminal amino acids are identical with the known sequences of other mammalian glucagons.[11-15] However, GP glucagon differs in 5 of the 9 COOH-terminal amino acids.

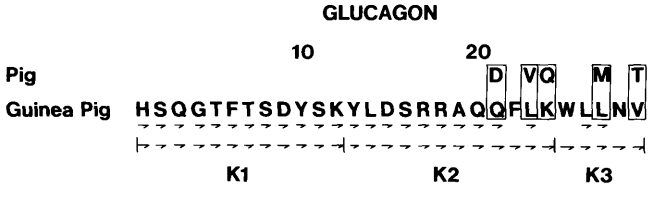

FIGURE 3. Amino acid sequences of pig and guinea pig glucagons. One-letter notation for amino acids is used (Table 1). In addition, N = Asn and Q = Gln. The amino acids shown in the boxes are the sites of differences identified by sequencing. K1-3 are the three fragments generated by endoproteinase Lys-C digestion.

The relative potency of GP glucagon compared to synthetic porcine glucagon in rat and GP liver membrane binding assays is shown in Figure 4. GP glucagon is 10-fold less potent than synthetic porcine glucagon in displacing ^{125}I-synthetic porcine glucagon in both systems. However, the binding and inhibition of binding of synthetic porcine glucagon are similar in both systems.

DISCUSSION

The glucagon content for freshly collected cow or pig pancreata averages about 1.5–3 nmol/g wet wt tissue.[24] In contrast, the glucagon content of the two GP pancreata averaged almost 30 nmol/g. It has long been known that insulin in the GP pancreas[25] is 5- to 10-fold higher than that in the cow or pig pancreas. It is of interest to speculate whether the concentration of other GP islet peptides are similarly elevated.

We are unable to account for the difference between the report by Sundby[18] that GP glucagon is a 40-amino acid peptide and our observation that GP glucagon is a 29-amino acid peptide. No experimental details were provided in the Sundby article. Since he reported that glucagon contained six basic amino acids compared with the four basic amino acids we found, it is possible that he observed a GP pancreatic proglucagon not detected with our antiserum.

All five amino acid substitutions are in the COOH-terminal nonapeptide. Two of the substitutions involve charged amino acids, which results in a more basic molecule at neutral pH. How should these substitutions affect glucagon-like bioactivity? It had earlier been reported that glucagon(1–21) is a full glucagon agonist both with regard to receptor binding and adenylate cyclase activation in rat liver plasma membranes[26] and with regard to glycogenolysis in isolated rat hepatocytes.[27] However, recent studies with synthetic and HPLC-purified glucagon(1–21) derived from the natural peptide demonstrate only negligible (<0.0001%) glucagon ag-

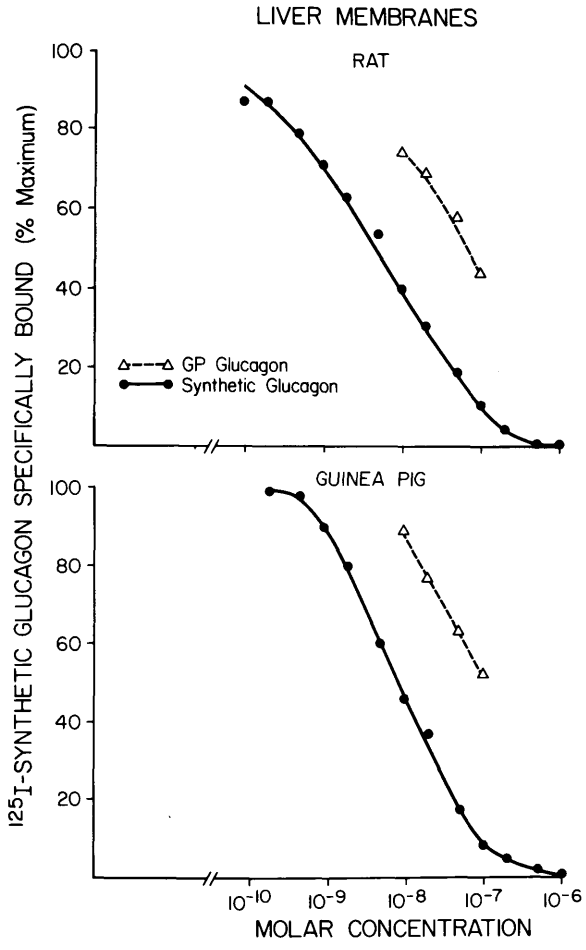

FIGURE 4. Inhibition of binding of ^{125}I-synthetic porcine glucagon to liver membrane preparations from rat (top) or GP (bottom) by synthetic porcine glucagon and GP glucagon. Nonspecific binding of ^{125}I-glucagon was 5% and 10% and specific binding was 5% and 7% of total counts for rat and GP liver membranes, respectively.

onist activity in these same test systems.[28] The earlier reports of considerably higher biopotency[26,27] were probably attributable to impurities in the glucagon(1–21) preparation. Our finding that GP glucagon has a 10-fold lower potency than mammalian glucagon in displacing labeled glucagon from rat and GP liver membrane binding sites serves as further evidence that the COOH-terminal has a significant role in receptor binding. At present, it is still not known whether the COOH-terminal of glucagon participates in bioactivation after receptor binding or is involved only in ensuring high affinity and specificity of binding to the receptor.

The finding that GP glucagon differs from other mammalian glucagons in five amino acids in the region involved in receptor binding does raise the question as to whether the GP glucagon receptor is the same as other mammalian glucagon receptors. Our observation that labeled porcine glucagon binds to and its binding is inhibited by porcine glucagon similarly in both the GP and rat systems suggests that the glucagon receptor is well conserved in mammals. The weaker inhibition of GP glucagon in both systems makes it quite likely that circulating glucagon as well as circulating insulin[29] is elevated in the GP. This would account for the high GP pancreatic glucagon content that we observed.

Initially, it was thought that insulin receptors remained unaltered despite evolutionary changes in the hormone.[30] It was later demonstrated that, although ^{125}I-beef insulin reacts virtually identically with GP and rat insulin receptors, the interaction of a modified insulin, $A_1B_1B_{30}$-triphthaloyl bovine insulin, with GP insulin receptors was distinguishable from insulin receptors in the mouse, rat, rabbit, and dog.[31] In view of the marked changes in the COOH-terminal of GP glucagon and its lower potency in our receptor binding studies, it is not unreasonable to anticipate that more sensitive tests of the GP glucagon receptor would reveal some difference between the usual mammalian and GP glucagon receptors. The decreased cross-reactivity of GP glucagon with GP and mammalian glucagon receptors suggests that GP glucagon, like GP insulin, will be less bioactive in vivo in other species.

NOTE ADDED IN PROOF

Conlon et al.[32] reported an identical sequence for GP glucagon.

ACKNOWLEDGMENTS

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