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Positively Charged Residues at Positions 12, 17, and 18 of Glucagon Ensure Maximum Biological Potency*

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Cecilia G. Unson‡, Cui-Rong Wu, Connie P. Cheung, and R. B. Merrifield

From the Rockefeller University, New York, New York 10021

Glucagon is a peptide hormone that plays a central role in the maintenance of normal circulating glucose levels. Structure-activity studies have previously demonstrated the importance of histidine at position 1 and the absolute requirement for aspartic acid at position 9 for transduction of the hormonal signal. Site-directed mutagenesis of the receptor protein identified Asp^{64} on the extracellular N-terminal tail to be crucial for the recognition function of the receptor. In addition, antibodies generated against aspartic acid-rich epitopes from the extracellular region competed effectively with glucagon for receptor sites, which suggested that negative charges may line the putative glucagon binding pocket in the receptor. These observations led to the idea that positively charged residues on the hormone may act as counterions to these sites. Based on these initial findings, we synthesized glucagon analogs in which basic residues at positions 12, 17, and 18 were replaced with neutral or acidic residues to examine the effect of altering the positive charge on those sites on binding and adenylyl cyclase activity.

The results indicate that unlike N-terminal histidine, Lys^{12}, Arg^{17}, and Arg^{18} of glucagon have very large effects on receptor binding and transduction of the hormonal signal, although they are not absolutely critical. They contribute strongly to the stabilization of the binding interaction with the glucagon receptor that leads to maximum biological potency.

Glucagon is a polypeptide hormone that consists of 29 amino acid residues and is a member of a highly homologous family of biologically active peptides. Secreted by pancreatic A cells, its primary target organ is the liver where, together with insulin, it plays a central role in the maintenance of normal circulating glucose levels critical to the survival of the organism. The initial event in glucagon action is binding to its receptor on the surface of liver cells. The binding message constitutes the signal that is transmitted across the membrane to guanine nucleotide binding protein-linked intracellular effectors that are ultimately responsible for glucose production.

The glucagon receptor is a member of a unique branch of the G protein-coupled receptor superfamily that has highly homologous sequences but shares very few of the conserved structural features found within the rest of the G protein-coupled receptor family (1, 2). Members of this receptor group include receptors for the glucagon family of hormones, glucagon-like peptide 1 (GLP-1)[1] (3), secretin (4), gastrin inhibitory peptide (5), and vasoactive intestinal peptide (6). The receptor has a relatively large extracellular N terminus thought to be involved in hormone-receptor interaction, followed by hydrophobic helical segments postulated to span the membrane seven times and a cytoplasmic C-terminal domain (Fig. 1). Signal transduction is thought to proceed upon binding of the hormone with the extracellular region of the receptor. The mechanism by which the signal is conveyed from the cell surface across the transmembrane helical network to activate G protein-coupled effectors on the surface of cytoplasm is not understood.

Extensive structure-function studies of glucagon have afforded some insight into the understanding of its mechanism of action. The general picture that has emerged is that the active pharmacophore is dispersed throughout the glucagon molecule and that the intact hormone is necessary for the expression of full hormonal activity. Nevertheless, specific active site residues responsible for either high affinity binding or activation have been singled out. Electrostatic interactions of the negatively charged side chain of aspartic acid 9, 15, and 21 were shown to be essential in glucagon function (7, 8). Activity was lost when Asp^9 was deleted or replaced by any other amino acid.

An important early finding from pioneering structure-activity studies established that the N-terminal histidine which is strictly conserved within the glucagon peptide family was essential for receptor activation and less so for binding and implied that the deletion of histidine would produce a glucagon antagonist (9). Indeed, the first partial antagonists that were developed were des-His^1 derivatives or glucagon analogs that had modified histidines at the N terminus (10–11). Further studies demonstrated that the imidazole ring of histidine at position one of the hormone furnishes determinants for both receptor binding affinity and activity (12).

Serine residues at positions 2, 8, and 16 were also shown to play prominent roles in glucagon action (13). The apparent connection of His^1, Asp^9, and Ser^{16} residues led to the hypothesis that a catalytic triad resembling that of a serine protease might be involved in the mechanism of glucagon signal transduction (13).

The glucagon binding cavity on the receptor is most likely a discontinuous domain that involves contributions from the long N-terminal extension as well as from the three extracellular loops that connect the seven transmembrane helices (14). Information about the complementary peptide and protein interactions that dictate the binding phenomenon is central to the design of antagonists of the hormone that might be clinically relevant. To investigate the molecular mechanism of hormone-receptor interaction and of receptor activation by site-directed

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‡ To whom correspondence should be addressed: The Rockefeller University, 1230 York Ave., New York, New York 10021. Tel.: 212-327-8239; Fax: 212-327-8245.

[1] The abbreviation used is: GLP-1, glucagon-like peptide 1.

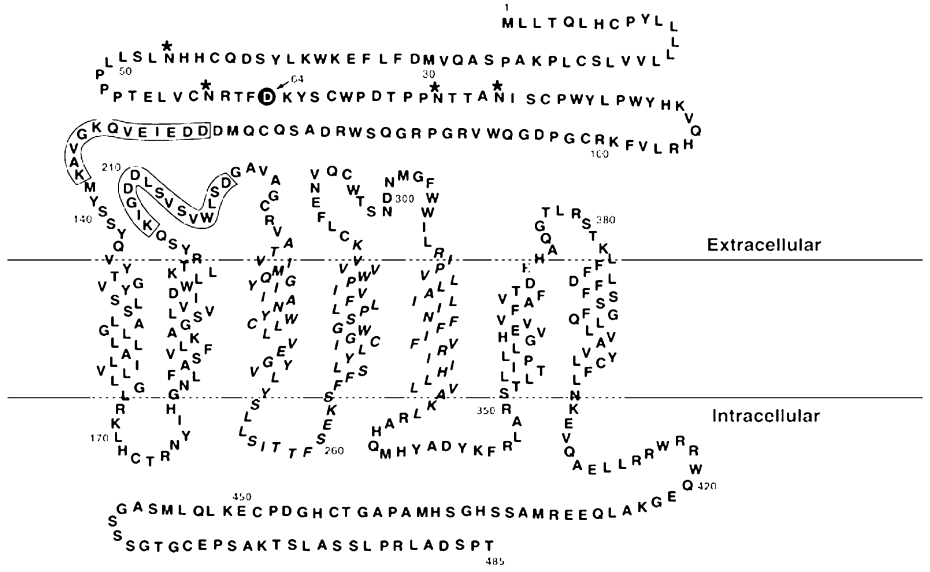

FIG. 1. **Schematic representation of the rat glucagon receptor primary and secondary structure.** Seven putative transmembrane helices based on previous models of G protein-coupled receptors are shown. The N terminus and extracellular surface is toward the *top*, and the C terminus and cytoplasmic surface is toward the *bottom* of the figure. Asp^{64}, which was studied by site-specific mutagenesis, is *numbered* and labeled with an *arrow* (15). The locations of sequences from the extracellular domain that were used as the peptides for anti-receptor anti-peptide antibody production are *boxed*.

mutagenesis, we have synthesized a gene for the rat glucagon receptor (15, 16).

The earliest information to come from site-directed mutagenesis of the glucagon receptor protein identified Asp^{64} in the extracellular N-terminal tail to be absolutely required for the recognition function of the receptor (15). Recent studies have also implicated areas in the membrane proximal portion of the N terminus and the first extracellular loop to be part of the hormone binding site (17). More importantly, antibodies raised against peptides representing sequences from these regions were inhibitors of glucagon binding to the receptor (18). These peptide epitopes contained clusters of negatively charged residues, which suggested that an electrostatic association with a complementary positively charged residue on the ligand might occur at these receptor sites.

To test this possibility, we assessed the contribution of the positively charged groups at positions 12, 17, and 18, to glucagon receptor recognition, which to date has not been clearly established. We synthesized glucagon analogs containing substitutions of Lys^{12}, Arg^{17}, and Arg^{18} of glucagon with neutral or negatively charged residues to examine the roles of the positive charge on those sites on binding and adenylyl cyclase activity. It was reported in an earlier study of the C-terminal region of the hormone that the analog [Lys^{17},Lys^{18},Glu^{21}]glucagon exhibited enhanced receptor binding and was a superagonist (19). This behavior was attributed to an increased α-helical content and the possible formation of an intramolecular salt bridge between charged side chains at positions 18 and 21 (20, 21). Our results demonstrate that glucagon binding and activity are not dictated solely by electrostatic interactions but include the interactions of hydrophobic side chains with the receptor.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Thirty three analogs of glucagon with replacements at positions 12, 17, and 18 were assembled by the solid-phase method (22, 23), on an Applied Biosystems 430A peptide synthesizer, using procedures previously described for the synthesis of glucagon analogs (24). Briefly, the peptide analogs with C-terminal amides were prepared on *p*-methylbenzhydrylamine-resin (Peptides International, 0.66 mmol/g) using N^{α}-Boc [*tert*-butyloxycarbonyl] protection chemistry. N^{α}-Boc-protected amino acids were purchased from Peptide Institute. Side chain protection was Arg(Tos), Asp(OcHx), Glu(OcHx), His(Tos), Lys[Z(Cl)], Ser(Bzl), Thr(Bzl), Trp(For), and Tyr[Z(Br)] (where Tos is tosyl; cHx is cyclohexyl; Z(Cl) is 2-chlorobenzyloxycarbonyl; Bzl is benzyl; and For is formyl). Standard protocol for double couplings with preformed symmetric anhydrides in dimethylformamide were used routinely, except for arginine, asparagine, and glutamine which were coupled as N^{1}-hydroxybenzotriazole esters (25). The N^{im}-formyl group on tryptophan was removed with 50% piperidine in dimethylformamide, prior to HF treatment. After cleavage by anhydrous HF, the crude peptides were purified by preparative low pressure reverse-phase liquid chromatography on octadecyl-silica (Vydac C18, Separations Group). The peptides were eluted by applying a linear gradient of 25–40% acetonitrile in 0.05% trifluoroacetic acid. Purity of the lyophilized product was evaluated by analytical high pressure liquid chromatography (Vydac 218TP54) in at least two different solvent systems and mass spectral analysis by the electrospray method identified the expected $(M + H)^{+}$ peaks within ± 0.3 Da. Amino acid analysis yielded amino acid compositions consistent with theory.

Receptor Binding Assay—Liver plasma membranes were prepared from rat liver (Sprague-Dawley, 100–150 g, Charles River) following the method of Neville with modifications described by Pohl (26). Membrane aliquots were stored in liquid nitrogen and used within 4–6 months. The receptor binding assay was done according to Wright and Rodbell (27), in which competition for glucagon receptors in 10 μg of liver membrane protein, between ^{125}I-labeled glucagon (NEN Life Science Products) (1.6 nM) and the synthetic analogs in concentrations ranging from 10^{-11} to 10^{-5} M, was measured. Assay suspensions were filtered on Durapore membrane filters (0.45 μm) using a vacuum filtration manifold (Millipore). Binding affinity (percent) is calculated as the ratio of the concentration of glucagon that inhibits 50% of the binding of tracer (IC_{50}) to that of peptide analog $\times$ 100. Duplicate determinations were made for each concentration point, and each assay was run at least twice. Nonspecific binding, determined in the presence of 10^{-5} M unlabeled glucagon, was typically 10% of total binding.

Adenylyl Cyclase Assay—Adenylyl cyclase activity was measured according to the procedure described by Salomon *et al.* (28). cAMP released was determined with a commercially available assay kit, from Amersham Pharmacia Biotech, in which unlabeled cAMP produced competes with [8-^{3}H]cAMP for a cAMP-binding protein. Data for stimulation of adenylyl cyclase are expressed as picomoles of cAMP produced per mg of membrane protein per min and plotted against the logarithm of peptide analog concentration. Maximum activity (percent) of an analog is the percentage of maximum stimulation of cAMP production above basal by glucagon. Relative potency (percent) is the ratio of the concentration of natural glucagon that elicits 50% maximum production of cAMP (EC_{50}) to that concentration of peptide analog $\times$ 100. Inhibition of cAMP production was determined in a similar assay in which a constant amount of glucagon is allowed to compete with increasing concentrations of analog. The inhibition index $(I/A)_{50}$ is defined as the ratio of the concentrations of inhibitor to agonist when the response is reduced to 50% of the response to agonist in the absence of inhibitor. Analogs were tested for inhibitory properties if they had relative potencies of $\leq$1% and a binding affinity of $\geq$1%. The pA_2 value, calculated by the method of Arunlakshana and Schild (29), is the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response produced by 0.5 unit of agonist. Duplicate determinations were made for each concentration point, and each experiment was carried out at least twice.

TABLE I
Glucagon analogs with neutral residue replacements at positions 12, 17, and 18

Analog of glucagon amide[a]	Binding affinity[b]	Adenylyl cyclase activity			
		Maximum activity[c]	Relative potency[d]	$(I/A)_{50}$[e]	pA_2[f]
	%	%	%		
Glucagon amide	100	100	15		
1. Ala^{12}	17.3 ± 0.2	59.7	15.8		
2. des-His^1,Ala^{12}	0.91	12.5	0.15		
3. Gly^{12}	11.4	85.1	13.8		
4. des-His^1,Gly^{12}	0.58	19.4	0.13		
5. N^{ϵ}-acetyl-Lys^{12}	47 ± 1	90.5	31.6		
6. Ala^{17}	38	29	0.013		
7. des-His^1,Ala^{17}	2.3	28	0.28	43.7	7.0
8. Leu^{17}	30 ± 1.8	88 ± 2	37.1 ± 1.7		
9. des-His^1,Leu^{17}	9.3	23	2.13	34.7	7.5
10. Ala^{18}	13	94.4	70.8		
11. des-His^1,Ala^{18}	3.1	14	0.14	43.7	7.3
12. Leu^{18}	56 ± 1.5	95 ± 3	45.7 ± 1.6		
13. des-His^1,Leu^{18}	3.6	22.5	1.5	85.1	7.0
14. Ala^{17},Ala^{18}	8	97	27.5		
15. des-His^1,Ala^{17},Ala^{18}	0.32	10	0.19		
16. Leu^{17},Leu^{18}	7 ± 0.1	85.4 ± 0.8	52.5 ± 1.2		
17. des-His^1,Leu^{17},Leu^{18}	1	17	1.15	43.7	7.1
18. Ala^{12},Ala^{17},Ala^{18}	0.08	62.8	1.3		

[a] Analogs of glucagon amide were assayed using native glucagon as the standard, in both membrane binding and adenylyl cyclase activity. [des-His^1]Glucagon amide had a binding affinity of 63% and a relative potency of 0.16% in the adenylyl cyclase assay.
[b] Binding affinity (%) is the ratio of agonist concentration to analog concentration at 50% receptor occupancy (IC_{50}) × 100.
[c] Maximum activity (%) is the percentage of maximum glucagon stimulation of cAMP production above basal.
[d] Relative potency (%) is the ratio of glucagon concentration to analog concentration at 50% response (EC_{50}) × 100.
[e] The inhibition index $(I/A)_{50}$ is the ratio of peptide inhibitor concentration to glucagon concentration when the response is reduced to 50% of the response of agonist in the absence of inhibitor.
[f] The pA_2 value is the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

RESULTS

Thirty three glucagon analogs have been synthesized to assess the roles of the positively charged basic residues at positions 12, 17, and 18 of glucagon, in receptor binding affinity as well as in adenylyl cyclase activation. Our initial approach was to examine the effect of neutralizing the positive charge by substituting uncharged amino acids at positions 12, 17, and 18 (Table I). Replacing Lys^{12} with neutral residues in the analogs Ala^{12} and Gly^{12} glucagon amides (analogs 1 and 3) resulted in an 80–90% reduction in binding affinity relative to glucagon for the glucagon receptor in rat liver membranes. However, both analogs were still capable of a full agonist response, with reduced potency. Similarly, acetylation of the ϵ-amino group of Lys^{12} provided [N^{ϵ}-acetyl-Lys^{12}]glucagon amide (analog 5), which bound with 47% affinity and elicited 90% maximum adenylyl cyclase stimulation. These results were consistent with an earlier observation that N^{ϵ}-acylated derivatives of glucagon were full agonists (30) and further implicated a preference for a hydrophobic functional group at position 12. An exchange of Arg^{17} for alanine or leucine in analogs 6 and 8 (Table I) effected a loss of 60–70% binding affinity. Ala^{17} was a weak agonist, whereas Leu^{17} stimulated adenylyl cyclase with an 88% maximum activity. Replacing Arg^{18} with alanine (analog 10) led to an 87% loss in binding, whereas a more hydrophobic leucine substitution (analog 12) suffered a smaller loss of 44% affinity. Both analogs were capable of a full agonist response. Substitution of both sequential arginines with a neutral amino acid in Ala^{17},Ala^{18} (analog 14) and Leu^{17},Leu^{18} (analog 16) resulted in a 90% loss of binding, which in the case of Leu^{17},Leu^{18} appeared to be additive. The concurrent loss of both positive charges did not influence the ability to activate adenylyl cyclase since the doubly substituted analogs were full agonists but with lowered potency. In contrast, the exchange of all three basic residues with alanines in [Ala^{12},Ala^{17},Ala^{18}]glucagon amide induced almost complete loss of binding and resulted in a very weak partial agonist. Deletion of His^1 from some of the analogs that retained good binding affinities (analogs 6, 8, 10, 12, and 16), produced the corresponding des-His^1 derivatives (analogs 7, 9, 11, 13, and 17) that exhibited lowered potencies and measurable antagonist properties, which is consistent with the established role of histidine in glucagon (9–12). Because these des-His^1 analogs retained the capacity to induce low levels of cAMP, they were only partial glucagon antagonists (31).

Aside from neutral amino acids, positive residues at positions 12, 17, and 18 were each replaced with an aspartic or glutamic acid to examine the effect of a reversal of charge. Asp^{12} and Glu^{12} (analogs 19 and 20, Table II) displayed poor binding affinities of 0.6 and 1%, respectively. Likewise, substitution by aspartic acid at positions 17 or 18 as in [Asp^{17}]- and [Asp^{18}]glucagon amides (analogs 22 and 26) led to 99% loss of binding. Interestingly, glutamic acid was better tolerated at these positions, with Glu^{17} and Glu^{18} glucagon amides (analogs 24 and 28) exhibiting a retention of 21 and 6% binding affinity, respectively, and full stimulation of adenylyl cyclase. Despite reduced binding affinities a reversal of charge at all positions produced glucagon analogs that elicited substantial agonist responses although with reduced potencies. Unlike Ala^{17},Ala^{18}, however, a double replacement with acidic residues in [Asp^{17},Asp^{18}]- and in [Glu^{17},Glu^{18}]glucagon amides (analogs 30 and 32) rendered the peptides incapable of receptor recognition. Since it is acknowledged that an intact N-terminal histidine provides determinants for both the binding and activation function of glucagon, the des-histidine derivative of every position −12, −17, and −18 replacement analog predictably lost additional receptor binding affinity and potency of adenylyl cyclase activation.

DISCUSSION

There is renewed interest in the peptide glucagon because of its role in diabetes mellitus. Despite considerable positive evidence, the participation of glucagon is still somewhat controversial and further evidence for its role is needed. It has been

TABLE II
Glucagon analogs with acidic residue replacements at positions 12, 17, and 18

Analog of glucagon amide[a]	Binding affinity[b]	Adenylyl cyclase activity			
		Maximum activity[c]	Relative potency[d]	$(I/A)_{50}$[e]	pA_2[f]
	%	%	%		
Glucagon amide	100	100	15		
19. Asp^{12}	0.6	78.4	10		
20. Glu^{12}	1	80.4	50.1		
21. des-His^1,Glu^{12}	0.11	28	0.22		
22. Asp^{17}	1.4	82.4	4.4		
23. des-His^1,Asp^{17}	0.1	11.5	0.08		
24. Glu^{17}	21.3 ± 0.5	94.8 ± 0.2	40.7 ± 3		
25. des-His^1,Glu^{17}	1.7	21.5	1.0	57.5	6.4
26. Asp^{18}	0.22	69.2	0.24		
27. des-His^1,Asp^{18}	<0.038				
28. Glu^{18}	6.2 ± 0.2	100 ± 2	3.3 ± 0.3		
29. des-His^1,Glu^{18}	0.44	18	0.24		
30. Asp^{17},Asp^{18}	<0.032				
31. des-His^1,Asp^{17},Asp^{18}	<0.032				
32. Glu^{17},Glu^{18}	0.036	100	1.2		
33. des-His^1,Glu^{17},Glu^{18}	<0.050				

[a] Analogs of glucagon amide were assayed using native glucagon as the standard, in both membrane binding and adenylyl cyclase activity. [des-His^1]Glucagon amide had a binding affinity of 63% and a relative potency of 0.16% in the adenylyl cyclase assay.
[b] Binding affinity (%) is the ratio of agonist concentration to analog concentration at 50% receptor occupancy (IC_{50}) × 100.
[c] Maximum activity (%) is the percentage of maximum glucagon stimulation of cAMP production above basal.
[d] Relative potency (%) is the ratio of glucagon concentration to analog concentration at 50% response (EC_{50}) × 100.
[e] The inhibition index $(I/A)_{50}$ is the ratio of peptide inhibitor concentration to glucagon concentration when the response is reduced to 50% of the response of agonist in the absence of inhibitor.
[f] The pA_2 value is the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

observed that overproduction of glucose by elevated circulating levels of glucagon may be a contributing factor to hyperglycemia and ketoacidosis that is characteristic of the disease (32, 33). It was reasonable to assume that antagonists of the hormone that are able to inhibit the actions of this endogenous glucagon by competing for the same binding cavity in the glucagon receptor could have clinical potential in the management of diabetic complications (9, 32, 33). Indeed, several peptide analogs have been developed that have been shown to effectively inhibit the effects of glucagon both *in vitro* and *in vivo* (24, 31, 34–38). Continued efforts in the study of glucagon are spurred by the idea that the ability to single out specific contact points between the peptide ligand and its receptor protein would serve as a basis for the rational design of analogs that bind yet do not activate adenylyl cyclase.

This study of the electrostatic interaction of the basic residues Lys^{12}, Arg^{17}, and Arg^{18} of glucagon with acidic residues of the glucagon receptor is based on the supposition that any one or all of these groups may provide a counterion to a specific aspartic acid residue on the extracellular domain of the receptor that has been shown in recent mutagenesis studies to be critical for ligand recognition (15, 18). The importance of histidine at position 1 to both receptor binding and activation has been firmly established (9–12). Removal of the histidine group afforded an analog that retained affinity for the receptor but, more importantly, appeared to partially inhibit glucagon-stimulated adenylyl cyclase (9). Although the N-terminal histidine is strictly conserved within the glucagon family of peptide hormones, Lys^{12}, Arg^{17}, and Arg^{18} are relatively unique at these positions in glucagon and might also serve as determinants of receptor specificity.

The results of our study reveal that while neutral residue scanning of positions 12, 17, or 18 of glucagon strongly attenuated receptor binding, most of the resulting analogs were weak but full agonists, suggesting that a positive charge at these particular positions was not absolutely critical for activity. That no positively charged amino acids of glucagon, with the exception of histidine 1, are critical for the activation function is indicated by the observation that none of the replacement analogs in Table I behaved as antagonists. However, some of the corresponding des-His^1 derivatives of positions 17 and 18 replacement analogs displayed partial antagonist properties, which was therefore associated with the deletion of position 1 histidine (31). These were in the $(I/A)_{50}$ range of 34–85, whereas the most potent glucagon antagonist reported to date was 0.85 (36).

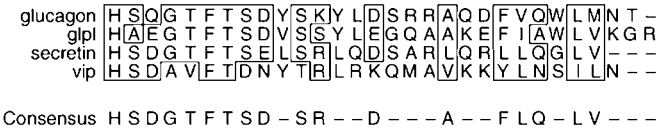

FIG. 2. **Sequence alignment of peptides of the glucagon family that have close homology.** Residues that are conserved in either charge or hydrophobic character are *boxed*.

A negatively charged acidic amino acid was, however, less tolerated at these positions and impaired receptor binding by a hundred-fold or more. Strongly reduced binding affinities were coupled with adenylyl cyclase responses with much weakened potencies. Thus, a positive charge at these positions is necessary for optimal hormonal function.

Our previous findings established the roles of the aspartic acid residues at positions 9, 15, and 21 of glucagon. Asp^9 is critical for transduction but not for binding (7), whereas the negative charge at Asp^{15} is absolutely essential for binding (8). In contrast, the positively charged residues in the central region of the hormone have a specific role in achieving optimal, or even significant, binding and maximal biological potency. An alteration in the charge distribution along the molecule clearly results in decreased binding of the derivatives. Unlike positions 9 and 15 where the precise location of an aspartic acid residue is critical to hormone-receptor interaction, it appears that glucagon binding affinity is not regulated by the topographic location of a specific positive charge but by a net positive charge. An overall positively charged molecule definitely enhances the affinity for its membrane-bound receptor protein. A single replacement in glucagon amide with an uncharged residue did not adequately alter the overall charge to reduce

binding and activity, but one substitution with a negatively charged residue resulted in a neutral molecule.

Secretin, GLP-1, and vasoactive intestinal peptide, glucagon's closest relatives within the family of peptide hormones, share 50% sequence homology mostly at the N-terminal half of the molecule (Fig. 2). With the exception of His^1, the positions of basic residues scattered primarily along the C-terminal part of these sequences are not well conserved. In an earlier report, a glucagon-GLP-1 chimeric peptide, in which the first 14 residues of glucagon were combined with the last 16 residues of GLP-1, bound to both glucagon and GLP-1 receptors (39). The normal peptides only bind weakly to each other's receptor. Interestingly, what appears to be preserved is an overall positive charge, suggesting that a positively charged ligand may be one requisite feature common to members of this G protein-coupled receptor sub-group and therefore not a strict determinant of specificity.

The study also reveals a hydrophobicity component of the binding interaction. The basic amino acids arginine and lysine can contribute not only a charged group but also an aliphatic component to the polar and non-polar interface of the ligand binding pocket. The proposed ligand binding site should lie within a hydrophobic core of the receptor where nonspecific hydrophobic interactions between the hormone and its receptor embedded in the membrane bilayer augment binding affinity. This explains why substitution with a neutral yet hydrophobic molecule like alanine or leucine was well tolerated despite the loss of a positive charge and could sustain 30–60% of the affinity for the receptor. A reversal of charge on the other hand adversely altered the polar character of the peptide and led to a greater loss of receptor recognition (Table II). Nevertheless, an increased hydrophobic contribution from a glutamic acid side chain probably compensated for the reversal of charge and accounted for the retention of substantial binding affinity and potency of the Glu^{17} and Glu^{18} replacement analogs (analogs 24 and 28, Table II). In addition, des-His^1,Glu^{17} (analog 25, Table II), which retained 2% binding and a weakened potency due to deletion of histidine, was still a partial antagonist. The analog [Lys^{17},Lys^{18},Glu^{21}]glucagon has been reported to be a superagonist which bound 5-fold better than the natural hormone and had a higher potency (19, 20). A recent x-ray crystal structure for [Lys^{17},Lys^{18},Glu^{21}]glucagon suggested that the formation of a salt bridge between the ϵ-amino group of Lys^{18} and the carboxyl of Glu^{21} may stabilize the turn of a putative α-helix at residues 18–21 and contributes to its superagonist activity (21). However, binding affinity remains relatively high even when hydrophobic residues are substituted for either Arg^{17} or Arg^{18}. Presumably, enhanced activity may also be attributed to the increased hydrophobicity of the longer aliphatic side chains of lysine and glutamic acid relative to those of the normal arginine and aspartic acid residues.

Thus, the positively charged amino acids Lys^{12}, Arg^{17}, and Arg^{18} of glucagon have large effects but are not absolutely critical for the binding and activation function of the hormone. However, the functional groups of these basic residues bolster both the polar and non-polar aspects of the peptide and protein interactions that occur within the receptor binding site and ensure maximum biological activity. The aliphatic backbone of arginine and lysine residues optimize ligand "fitting" within a hydrophobic pocket in the receptor. Our data demonstrate that at these positions in glucagon, nonspecific hydrophobic interactions are as important a contributing factor to binding affinity as the electrostatic effects. Mutagenesis studies on the receptor have outlined the perimeter of a putative binding site bordered by negatively charged residues. Thus, an overall positively charged glucagon molecule contributes to the stabilization of the hormone-receptor complex and secures the binding conformation that subsequently leads to activation.

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