Position 9 Replacement Analogs of Glucagon Uncouple Biological Activity and Receptor Binding*

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Recent studies on the glucagon antagonist des-His1-[Glu9]glucagon amide have resulted in pure inhibitors of the hormone, suggesting that the inhibitory properties may be centered around position 9. The present study was designed to investigate the chemical characteristics of substitutions in position 9 of glucagon that determine binding affinity and biological activity. Twenty replacement analogs of position 9 of glucagon were synthesized and assessed for their ability to bind to the glucagon receptor in rat hepatocyte membranes and to activate adenylate cyclase. Any substitution of aspartic acid 9 was accompanied by a severely diminished capacity to transmit the biological signal, while retaining receptor binding affinity. These results are an indication of an uncoupling of receptor binding and biological activity at this locus and define a central role of aspartic acid 9 in glucagon activity. Single replacement or deletion of either His1 or Asp9 in glucagon caused a 20- to 50-fold decrease in cyclase activity, whereas these same changes made in tandem caused virtually complete loss of activity, with decreases of 104- to 105-fold. These observations have led us to speculate that, at the molecular level, the region of glucagon required for transduction of the biological response may be distinct from the binding region and is mediated by a coupled interaction between His1 and Asp9 of the hormone and a complementary functional site of the glucagon receptor.

Glucagon is a 29-residue peptide hormone that is secreted by the α cells of pancreatic islets. Together with insulin, functioning within a complex network of counterregulatory mechanisms, it is primarily responsible for the maintenance of the plasma glucose concentration that is critical to survival in man and animals. Most of the effects of glucagon are mediated by its interaction with specific receptors in the liver plasma membrane, followed by activation of adenylate cyclase to increase the intracellular cyclic AMP level. The result is an increase in glycogenolysis and gluconeogenesis with a consequent rise in hepatic glucose production within minutes.

Glucagon is a well-studied member of a peptide family that includes vasoactive intestinal peptide, gastrin-releasing factor, and secretin. These peptide hormones may have evolved from a common precursor, but in spite of startling similarities in sequence each one couples to a specific receptor protein that will trigger distinct physiological events. The glucagon receptor also belongs to a class of receptors linked to their effector enzymes by GTP-binding proteins (G proteins) and probably shares sequence similarity and a conserved structural motif described as membrane-spanning α-helices. This suggests that topologically equivalent domains of receptor protein interact with ligand, and particular variations in the amino acid sequences at the binding site allow receptors to discriminate among the subset of peptide hormones. The structural basis for recognition as well as the subsequent generation of the transmembrane signal is not understood. Structure activity studies have been directed towards sorting out the functional groups and conformational features of the hormone that are responsible for recognition and binding from those that transduce the biological response. The ability to define these properties will allow us to design glucagon antagonists that may be clinically relevant to the management of diabetes mellitus.

Our search for antagonists of the hormone glucagon led us to explore the design of synthetic secretin-glucagon chimeric analogs (1). Although their sequences are highly conserved at the amino terminal region, indicative of a common evolutionary precursor, variant amino acid residues at positions 3, 9, 12 should have functional significance and indeed may confer specificity to each hormone. Subsequent structure-function studies of secretin-glucagon hybrids incorporating single as well as multiple substitutions of residues 3, 9, and 12 of glucagon with those of secretin (2, 3), resulted in the potent antagonist, des-His1-[Glu9]glucagon amide (4) and the presumption that the position 9 hybrid was responsible for the observed inhibition of adenylate cyclase activation by the natural hormone. The in vitro data (5, 6) were in agreement with further in vivo studies (6) that showed that the antagonist was capable of suppressing glycogenolysis in normal rabbits and lowering hyperglycemia in diabetic rat models. The conservative replacement of Asp9 residue with Glu9, which caused the complete loss in adenylate cyclase activity while retaining 40% receptor binding affinity, led us to speculate that the glutamic acid residue bestowed some of the features necessary for antagonism. Efforts to maximize the inhibitory potency of des-His1-[Glu9]glucagon amide by accentuating the putative α-helix at the carboxyl-terminal region (7) or by increasing the hydrophobicity of the peptides (8) provided a series of new antagonists, some with improved inhibition. These findings raised the possibility that the strategic topology around position 9 rather than the glutamic acid residue itself was determining the inhibitory effect, and warranted a study of the consequence of varying the amino acid residue in position 9, in ways that will help define the structural features that contribute to glucagon binding and transduction.

Evidence is presented here showing that position 9 of the hormone determines glucagon biological activity independently of glucagon receptor binding, that substitution at

* This work was supported by U. S. Public Health Service Grant DK 24039. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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this position uncouples binding from transduction. In addition, it is demonstrated that an aspartic acid residue at this position is critical for the biological response and acts in concert with histidine at position 1.

MATERIALS AND METHODS

Synthesis and Purification—Position 9 replacement analogs were synthesized by the solid phase method with 4-methylbenzhydramine resin on an Applied Biosystems 430A peptide synthesizer. After t-butoxycarbonyl deprotection, the peptide resins were treated with 50% piperidine in N,N-dimethylformamide to remove the formyl group on tryptophan prior to high HF cleavage from the resin support. The crude peptides were extracted with 10% glacial acetic acid and purified as described previously (4). The integrity of the peptide derivatives was verified by analytical high performance liquid chromatography, amino acid analysis, and mass spectral analysis which determined the (M + H)+ peak to be within ±0.3 Da of theory.

Receptor Binding and Adenylate Cyclase Activity—Rat liver membranes were prepared from male Sprague-Dawley rats (Charles River Laboratories) by the Neville procedure as described by Pohl (9), resuspended in NaHCO3, and stored as aliquots under liquid nitrogen until use. Protein was determined by a modified Lowry method (10). The receptor binding assay was performed according to the procedure of Wright and Rodbell (11) in 1% bovine serum albumin, 1 mM dithiothreitol, 50 mM Tris-HCl buffer, pH 7.2. The amount of radioiodinated glucagon displaced from receptor sites by increasing concentrations of antagonist was measured. Binding affinity was expressed as the ratio of the concentration of natural glucagon to that of the antagonist required to displace 50% of receptor-bound labeled glucagon. Activation of adenylate cyclase in rat liver membranes was measured by a procedure described by Salomon et al. (12) in an assay medium containing 1% bovine serum albumin, 25 mM MgCl2, 2 mM dithiothreitol, 25 mM GTP, 5 mM ATP, 0.3 mM theophylline, 17.2 mM creatine phosphate, and (1 mg/ml) creatine phosphokinase. The cAMP released was determined with a commercial kit from Amer sham Corp. in which unlabeled cAMP was allowed to compete with [8-3H]cAMP for a high-affinity CAMP-binding protein. Inhibition of adenylate cyclase using the same procedure above, except that increasing concentrations of antagonist were allowed to compete with a constant concentration of natural glucagon. The ratio of the concentration of antagonist to agonist concentration when the response is reduced to 50% of the response to glucagon in the absence of antagonist, is the inhibition index, I/Aso. The pA2 value, calculated also from the dose-response curve (13), is the negative logarithm of the concentration of antagonist that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

RESULTS AND DISCUSSION

Substitution or Removal of Asp9—Twenty new glucagon analogs were synthesized and tested for receptor binding and adenylate cyclase activity. These included the deletion of position 9, or the replacement of Asp9 by D-Asp, Asn, Glu, D-Glu, Gln, Glu-OMe (where OMe is methoxy), Gly, Nle, or Lys. Each change was made both in the presence or absence of histidine at position 1. The data in Table I demonstrate that any of these substitutions of the aspartic acid 9 residue resulted in a marked loss in the ability to produce cAMP. However, all the replacement analogs maintained appreciable binding affinity for the glucagon receptor. Binding is thus necessary but not sufficient for biological activity.

Removal of the side chain functional group in position 9 in the analog [Gly9]glucagon amide (compound 8) produced an analog with 32% receptor binding affinity relative to glucagon yet caused a 600-fold loss in adenylate cyclase specific activity. Deletion of the Asp9 residue altogether in [des-Asp9]glucagon amide (compound 2) was accompanied by 100-fold loss in activity (Fig. 1) but only a 55% reduction in binding affinity (Fig. 2). Single replacements of aspartic acid 9 with glutamic acid (compound 4), Glu-OMe (compound 6), Asn (compound 7), Lys (compound 9), or Nle ((compound 10) were enough to drastically reduce the ability to transduce the signal while forfeiting only approximately half of its binding affinity. In contrast, inversion of the configuration at position 9 by the substitution of D-amino acids in the analogs [D-Asp9] (compound 3) and [D-Glu9] (compound 5) had adverse effects on ligand-receptor fit and brought about a loss of at least 90% binding affinity. Thus the receptor protein is able to tolerate the absence of a side chain or the presence of hydrophobic, neutral, or basic as well as acidic groups at position 9 and does not require a charge for its binding function. It seems clear that the side chain of the position 9 residue is not specifically involved in binding contacts but provides a major functional requirement for agonist activity. Aspartic acid is critical at this position and cannot be replaced even by the closely related dibasic glutamic acid. These results demonstrate an uncoupling of glucagon binding from adenylate cyclase stimulation.

Substitution or Removal of His1—Although our observations have led to the conclusion that Asp9 is crucial for the biological activity of glucagon, but not for receptor binding, it is obvious that His1 also contributes some contact points to secure the putative binding configuration that will trigger the cascade of events leading to cAMP production. It was already known from our work2 that and that of others (14) before us that very few changes in histidine 1 can be made with retention of cyclase activation. Thus, deletion of His1 or replacement of His1 by other amino acid residues in every case reduced activity to <0.2% (15) and acylation of the α-amino nitrogen by 2,4-difluorobenzoyl (8) or acetyl (14) was also incompatible with activity. These and other changes that affect binding can be allosteric and cause their effect at a distance by allowing a change in conformation at a remote site. Thus, des-(1-2)glucagon amide and des-(1-4)glucagon amide remove the specific transducing residue (His1) but did not seriously affect the 19-29 binding region, whereas, removal of 2 more residues (Thr2 and Phe6) in des-(1-6)glucagon amide allowed a large change in conformation at the 19-29 end of the molecule and abolished binding (7).

Changes at Both Positions 1 and 9—It is also evident that His1 can provide a functional group partially capable of transducing the hormonal signal. Thus, compounds 2-10 show that in the absence of the side chain carboxyl of Asp9, these His1-containing analogs retain low agonist activity, whereas when His1 is removed (compounds 13-20) the activity is essentially zero (Table I).

When His1 was removed from glucagon amide, the additional absence of a functional side chain at position 9 as in des-His1-[Gly9]glucagon amide (compound 19) still allowed 100% binding affinity (Fig. 2). Substitution with neutral Glu9-OCH3 (compound 16), Asn9 (compound 17), or Gln9 (compound 18) or with positively charged Lys9 (compound 20) and His9 (compound 21) afforded peptide derivatives with 30-70% binding affinities. Des-His1-[Nle9]glucagon amide (compound 22), containing a long hydrophobic side chain, bound even slightly better than natural glucagon (125%). An aromatic side chain in the analog des-His1-[Phe9]glucagon amide (compound 23) was an exception and may have perturbed the binding conformation significantly, since it lost 89% of the binding potency. All of these derivatives were essentially inactive in the cyclase assay even though they bound well to the receptor. Finally, elimination of residues at both position 1 and position 9 in the analog des-[His1,Asp9]glucagon amide (compound 24) caused a complete loss of cyclase activity, while retaining only 7% binding (Fig. 2). Clearly, an amino acid residue at position 9 contributes towards stabilization of

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1. D. Andreu, personal communication.

2. C. G. Unson, manuscript in preparation.
The binding conformation and emphasizes the perception that virtually the entire polypeptide backbone is essential for the proper fit into a binding site in the receptor protein.

The effects on binding are probably due largely to steric and conformational properties that influence the efficiency of the contact of the hormone with the receptor. They may also involve electrostatic binding interactions. The binding region would be situated either in the extracellular domains or within any one of the transmembrane helices of the glucagon receptor (Fig. 3). At this binding site, histidine 1 and aspartic acid 9 may interact not only with each other but also with a specific residue in the receptor protein, causing the glucagon-receptor complex to adopt the conformation that leads to adenylate cyclase activation. The effects on transduction of the hormonal signal leading to activation of adenylate cyclase may be a consequence of functional groups in the hormone that are involved in proton transfer reactions with functional groups on the G protein, or on the receptor protein itself, which is thereby modified to react with the G protein, promoting the exchange of GDP for GTP, and the subsequent dissociation of the $\beta_\gamma$ subunits, which ultimately leads to cyclase activation (15, 16). Evidence that strategic placement with regard to the receptor, which is immobilized in the cell membrane, is essential is provided by the observation that reversal of the positions of His$^1$ and Asp$^9$ in the ligand led to the derivative [Asp$^9$,His$^9$]glucagon amide (compound 11) which lost 75% binding potency and had no cyclase activity.

In the case of the $\beta$-adrenergic receptor it is specifically the Asp$^9$ residue in the third transmembrane helix of the protein that interacts with the ligand amine, epinephrine (17, 18), because the mutants Asn$^9$ and Glu$^9$ receptor had $K_0$, values 1000-fold higher than the wild type for agonist stimulation of adenylate cyclase (18). Our data suggest that an analogous situation for glucagon and its receptor may exist. Competitive labeling experiments in secretin have shown (19) that amino-terminal histidine interacts with Asp$^9$ and raises the measured $pK_a$ value of the His$^1$ amino group, and correspondingly its...
imidazole function, one unit higher than the model compound histidylglycine. There is also a remarkable increase in the histidine reactivity, which was interpreted to be evidence for the existence of an ordered conformational state in aqueous dilute solution, in which amino-terminal histidine interacts with another region of the molecule, causing a measurable perturbation of its chemical properties. The phenomenon in glucagon, while less startling, also exists (19, 20). Unlike secretin and vasoactive intestinal peptide, which both have an aspartic acid in position 3, glucagon has an aspartic acid at position 9 and its electrostatic effect with positively charged histidine 1 could still be attained by proper folding. Note that the derivative Lysg glucagon amide (compound 9) showed no adenylate cyclase activity in contrast to the other position 9 replacement analogs which were weak agonists. This may be attributed to the positively charged functional group that interacts unfavorably with His1. Thus, an electrostatic interaction between His1 and an aspartic acid in close proximity may play a role in the biologically active conformation of these peptide hormones.

These replacement analogs did not promote cAMP production but competitively inhibited the ability of natural glucagon to produce cAMP and thus were pure antagonists. The values obtained from the competitive binding with radiolabeled hormone and inhibition of adenylate cyclase were generally in agreement for each analog. However, in the case of des-His1-[Nle6]glucagon amide (compound 22), a high binding affinity of 125% only gave an inhibition index of 19 and a pA2 value of 7.6, while des-His1-[Gln6]glucagon amide (compound 18) which only bound 33% as well as natural glucagon was able to inhibit glucagon activity appreciably better with an inhibition index of 8.3 and a pA2 value of 8.2. We have attributed the anomaly in part to the different conditions utilized in the binding and adenylate cyclase assays. However, it is also known that Mg2+ ion and GTP, both of which are required for the activation of the G protein in the adenylate cyclase assay medium, are known to decrease ligand receptor affinity (21). It has also been demonstrated that the hepatocyte contains a heterogeneous population of saturable glucagon binding sites and that there is a nonlinear relationship between binding and adenylate cyclase activation (22-24).

Discovery of a site in the peptide molecule in which binding is uncoupled from transduction should now allow us to speculate on the interactions of glucagon with its membrane-bound receptors, dissect the binding and transduction phenomena, and study them independently of one another. Whether this phenomenon of strategically positioned residues that control transduction can be extended to other members of the glucagon family of hormones also remains to be tested. A generalization would be of significance and might lead to receptor- and therefore effector-specific antagonists.

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