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Isolation of Glucagon Antagonists by Random Molecular Mutagenesis and Screening

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SUMMARY

Glucagon has an important role in the regulation of glucose homeostasis, and glucagon antagonists may be effective therapeutic agents in the control of diabetes mellitus. We were able to identify a number of analogs with antagonist activity by creating libraries of mutant glucagon coding sequences, expressing them in a yeast (*Saccharomyces cerevisiae*) secretion system, and screening for clones that produce analogs that inhibit the glucagon stimulation of rat hepatocyte membrane adenylate cyclase. These libraries were constructed by allowing random misincorporation during the synthesis of oligonucleotides that contained the complete coding sequence for mammalian glucagon or for an analog (desHis^1-glucagon) that had partial antagonist activity. We developed and used a simplified screening assay to test culture broths from >3500 individual transformant yeast clones for their ability to inhibit glucagon-dependent adenylate cyclase activity. Ultimately, >20 different analogs with antagonist activity were identified by recovering and sequencing plasmid DNA from yeast strains that were positive in the screening assay. Interestingly, several analogs were identified repeatedly in independent yeast clones and certain amino acid substitutions occurred in more than one analog. This clustering of randomly isolated mutations clearly delineates the regions of the glucagon molecule that are important for designing improved glucagon antagonists. A subset of the antagonists identified in yeast broth were produced by peptide synthesis to confirm their activities as pure compounds.

Glucagon is a polypeptide hormone of 29 amino acid residues that is produced in the pancreas and is important in the control of plasma glucose. Its primary target is the liver, where it stimulates both glycogenolysis and gluconeogenesis through a cAMP-mediated intracellular signaling system.

Elevated levels of plasma glucagon have been associated with hyperglycemia in diabetes mellitus. In this disease insulin deficiency or insensitivity is clearly responsible for abnormal glucose utilization, but excessive glucose production may also contribute significantly to the metabolic imbalance. Thus, inhibiting the action of glucagon may be one way to restore normoglycemia, and glucagon antagonists may prove to be important therapeutic agents in the control of diabetes. Certainly the availability of an effective antagonist would be very useful for experimentally determining the importance of glucagon in diabetes and in glucose metabolism in general.

Most recent efforts to develop glucagon antagonists have been stimulated by improvements in *in vitro* peptide synthesis that enable one to routinely produce polypeptides the size of glucagon. Analogs can be designed using structural models, synthesized, and then tested for biological activity. More than 100 different glucagon analogs have been studied in this way. Several have been reported to have antagonist activity (1–5).

Using these methods, however, the number of peptide variants that can reasonably be produced and tested for biological activity is still relatively small and, in the case of peptides as complex as glucagon, can hardly be considered exhaustive. For example, there has not been a systematic check of at least one amino acid substitution at each position in the glucagon sequence. Recent advances in protein modeling and structure prediction have allowed researchers taking this approach to make educated guesses about what types of variants might be effective, but the basic hit or miss nature of this research remains a prominent feature. We wished to determine whether there were additional amino acid substitutions that could be used to design new, improved, glucagon antagonists.

Molecular mutagenesis can readily be used to create diversity based on the 20 amino acids found in the genetic code. Also, because nucleotide substitution can be performed in a random fashion, a large number of variant coding sequences can be created and pooled into a library that can be processed *en masse*. The ability to routinely perform heterologous gene expression enables one to transform such a library of mutagenized coding sequences into a library of variant polypeptides. Thus, molecular cloning techniques can be used to generate, rapidly and randomly, a large number of protein variants to be tested for biological activity.

The practice of high-throughput screening has been funda-

ABBREVIATION: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

mental to the development of pharmacologically active compounds. In the past it has been applied mostly to natural products and compounds produced by synthetic organic chemistry, but it is clearly an appropriate method to use whenever one is confronted with a large number of compounds to be tested. In this paper we describe a method that applies a biochemically based high-throughput screening procedure to a collection of glucagon variants that were generated by molecular mutagenesis and cloning techniques. We believe that this is a novel integration of methods from different disciplines that will be generally useful in the field of peptide antagonist design.

An initial assumption for this work was that antagonists of peptide hormones such as glucagon can best be generated by making minor changes in the structure of the peptide itself. An analog that competes for receptor binding without initiating signal transduction will block the action of the hormone and act as an antagonist. Presumably there are amino acid substitutions that will disrupt the signal transduction process without interfering with the ability of the peptide to fold into a structure recognized by its receptor.

Basically, our method was to generate a library of randomly mutagenized glucagon coding sequences and to express this library in a yeast (*Saccharomyses cerevisiae*) secretion system. Yeast clones expressing independent members of this library were then used to obtain a collection of culture broths containing substituted glucagon molecules that could be tested individually in a screening assay. This assay measured the effects of the analogs on the glucagon-dependent stimulation of adenylate cyclase activity from rat liver membranes and thus could be used to detect antagonism directly. Plasmid DNA was recovered from yeast clones that were positive in this assay, and potential antagonists were identified by DNA sequence analysis. In this way we were able to test a large number of analogs for antagonist activity and to identify several new sites and substitutions important to the design of glucagon antagonists. The significance of several of these substitutions was emphasized by the fact that they were observed repeatedly among the set of analogs with antagonist activity.

Materials and Methods

Construction of glucagon expression libraries. Secreted expression of glucagon and its analogs was achieved by inserting sets of synthetic oligonucleotides into the autonomously replicating yeast expression vector pBS114 (Fig. 1). These oligonucleotides were designed to contain an 87-base pair sequence encoding mammalian glucagon or an 84-base pair sequence encoding desHis[1]-glucagon, flanked with bridge sequences that provided the correct 3′ overhangs for ligation into the *Hind*III and *Bgl*II sites of pBS114. These bridge sequences also functioned to correctly fuse the glucagon coding sequence to the precursor region of the gene for the yeast mating factor α (8) and to provide a post-translational cleavage site for the product of the yeast *KEX2* gene (9). This construct is similar to one used in a previous report of glucagon secretion from genetically engineered yeast (10).

Oligonucleotides were synthesized on a modified Applied Biosystems 380A automatic DNA synthesizer, using phosphoramidite chemistry on a controlled pore glass support. In the case of mutagenized oligonucleotides, synthesis was interrupted between unmutagenized bridge sequences and mutagenized coding regions, to change reagent bottles. Cross-contamination of the phosphoramidite solutions was carried out as described by Hutchinson *et al.* (11), except that the amounts of solutions transferred were adjusted to achieve slightly different degrees

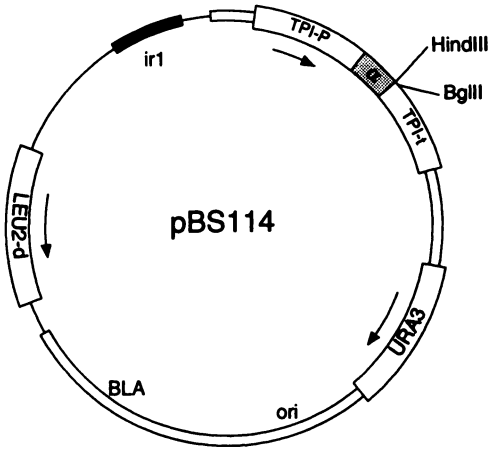

Fig. 1. The plasmid pBS114 is a vector designed for secreted expression of glucagon analogs from *S. cerevisiae* and the recovery of clones for DNA sequencing. It consists of DNA segments derived from three different sources, the bacterial plasmid pBR322, yeast chromosomal DNA, and the yeast extrachromosomal element known as 2-μm circular DNA. Two-micron DNA sequences are indicated by a *narrow line* and *ir1*, which is an inverted repeat element that provides autonomous replication in yeast. pBR322 sequences are indicated by an *intermediate-width line* and include a bacterial origin of replication (*ori*) and the β-lactamase gene (*BLA*) for selection in *E. coli*. Yeast chromosomal sequences are indicated by *boxed segments*. They include the *URA3* gene and a promoter-defective *LEU2* gene (*LEU2-d*) (6) for low and high copy number selection, respectively, the transcriptional promoter and terminator regions from the gene encoding triose phosphate isomerase (*TPI-P* and *TPI-t*, respectively) (7), and a segment of the *MAT* α*2* gene (α), which encodes the precursor region of the yeast mating pheromone α-factor (8). Unique restriction endonuclease sites (*Hind*III and *Bgl*II) facilitate fusion of peptide coding sequences to the α-factor precursor sequence and thus provide for secreted expression of the peptide.

of overall impurity for each synthesis. After synthesis the oligonucleotides were deprotected by standard methods and purified by polyacrylamide gel electrophoresis.

DNA manipulations were carried out using methods described by Sambrook *et al.* (12). *Escherichia coli* strain DH10B (Bethesda Research Laboratories, Inc.) was used as a host to prepare plasmid DNA for sequence analysis and yeast transformation.

Plasmid clones were reserved from each mutagenized library for restriction endonuclease digestion and DNA sequence analysis. Restriction digests indicated that plasmids without inserted glucagon coding regions were rare (none were observed among 50 that were tested) and that misincorporation mutagenesis had yielded approximately the expected number of mutations in each sequence. DNA sequencing indicated a mutation rate of 2.7 misincorporated bases/100 for the full length glucagon library and 2.5/100 for the desHis[1]-glucagon library. These were slightly more than the rates of 2.2% and 2.0%, respectively, predicted from the extent of cross-contamination and the results of mismatch repair. Mutations appeared to be distributed randomly, both among the clones and within each sequence. The remainder of the mutagenized plasmid clones (approximately 10,000 for each library) were pooled and processed *en masse* for yeast transformation.

S. cerevisiae strain ZY100 (*ade2–101, leu2–3,112 ura3–52, suc2-D9, gal2, pep4::TPI1p-CAT*), which is part of the Zymogenetics collection, was used as a host for secreted expression. Yeast culture and transformation were by the methods of Sherman *et al.* (13). Yeast transformants were selected initially for uracil prototrophy and were then transferred to leucine-free medium to select high copy number clones. Plasmid DNA was recovered and sequenced from a number of these clones to show that most produced a single analog. However, it was observed that as many as 20% contained two different plasmids and could be expected to produce a mixture of two different analogs.

Adenylate cyclase assays. Rat liver membranes were prepared

from young (80–100 g) female Sprague-Dawley rats as described by Pohl *et al.* (14) and were stored at −80°. The amount of membrane preparation used for each 100-μl microtiter well reaction was adjusted empirically according to the activity of each batch and was generally equivalent to 50–150 μg of membrane protein. Final concentrations of assay components were 0.1% bovine serum albumin, 1 mg/ml creatine phosphokinase (Sigma C3755), 30 mM phosphocreatine, 20 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM ATP, 1 mM isobutylmethylxanthine, 1 mM EDTA, and 10 μM GTP. For the screening assay, glucagon was added to a final concentration of 5 nM and clarified yeast broth made up 5% of the assay solution. The reaction was started by mixing membrane suspension with the other assay components and was stopped after 12 min at room temperature by adding an equal volume of an ice-cold solution of 100 mM acetic acid and 50 mM EDTA. The microtiter plates were then centrifuged briefly to precipitate the membranes, and an aliquot was removed for cAMP assay. cAMP was assayed using a commercially available scintillation proximity radioimmunoassay kit from Amersham Corporation.

Receptor binding. Receptor binding was measured by competition with ^{125}I-glucagon (Amersham), using the same rat liver membrane preparation used for adenylate cyclase assays. Approximately 50 nCi (100 fmol) of radiolabeled glucagon were mixed in a 1.5-ml microfuge tube with 100 μg of membrane protein and varying concentrations of unlabeled glucagon or an analog in buffer containing 100 mM HEPES, pH 7.1, 300 mM NaCl, 2 mM EDTA, 2% bovine serum albumin, and 20 mg/ml bacitracin, to give a final volume of 250 μl. After a 90-min incubation at 30°, 500 μl of fresh buffer were added and the tubes were centrifuged at high speed for 5 min. Each pellet was washed once by resuspension in 500 μl of buffer and centrifugation for another 5 min. Counts bound were determined by resuspending the pellets in 100 μl of buffer, transferring them to 12- × 75-mm plastic tubes, and counting them in a Packard Cobra Autogamma counter. Samples were assayed in duplicate, and a glucagon standard curve was obtained for each experiment. Counts bound in the absence of competing unlabeled glucagon were generally 25–35% of total counts.

Peptide synthesis. Peptide synthesis was carried out on an Applied Biosystems model 431A peptide synthesizer, using standard protocols and reagents for 9-floromethyloxycarbamyl chemistry. After deprotection, peptides were purified by reverse phase chromatography on a Waters Deltaprep high performance liquid chromatography system. desHis^1[Glu^9]-Glucagon amide was a gift from Dr. Ole Kirk of Novo-Nordisk, Inc.

Results

Secreted expression of mutant glucagon sequences. Two separate libraries of yeast strains producing glucagon analogs were created by allowing random misincorporation of phosphoramidite bases into synthetic DNA. One library was based on a full length glucagon coding sequence and the other on a sequence encoding an analog that lacks the amino-terminal histidine residue (desHis^1-glucagon). This analog has been shown to be a partial glucagon antagonist (15), and a number of other antagonists are known that combine this deletion with substitutions in other parts of the molecule (4, 5, 16). In each case the mutation rate was adjusted to result in an average of approximately 1.4 amino acid substitutions/polypeptide chain.

Secretion levels were measured by radioimmunoassay in a small subset (≈50) of the cultures used for antagonist screening. Clarified broths from approximately 90% of these cultures were found to be positive for glucagon immunoreactivity, with apparent concentrations ranging from 0.5 to 25 mg/liter. This implies that unpurified analogs from the culture broths should be present in approximately 2–80-fold molar excess when diluted 20-fold into adenylate cyclase activation assays containing 5 nM glucagon.

Screening of yeast broths for glucagon antagonist activity. Broths from approximately 2900 colonies from the library based on full length glucagon were screened for the ability to inhibit the glucagon-dependent stimulation of rat hepatocyte andenylate cyclase, by making a single measurement of cAMP produced under standard conditions. The range of cAMP values observed was broadly distributed around a mean that was slightly more than the value obtained when broth from a nonproducing control strain was assayed but less than that seen with a strain producing wild-type glucagon (Fig. 2). This is consistent with the idea that some of the strains produced unmodified glucagon, or analogs that retained partial agonist activity, and were able to stimulate adenylate cyclase beyond the level that resulted from the subsaturating concentration of exogenous glucagon present in the assay. When 192 library members were tested for their effect on adenylate cyclase in the absence of exogenous glucagon, approximately 10% showed significant stimulation above background. Among these positive clones the average level of stimulation was approximately equal to that seen with a strain making wild-type glucagon.

Strains that corresponded to the lowest tenth percentile of cAMP values in the antagonist assay were chosen for secondary screening. These were regrown and assayed in duplicate. Although the cAMP concentrations observed for duplicate samples in this second assay were in reasonable agreement, there was little correspondence between the relative response observed in the two assays (i.e., strains resulting in the least cAMP production in the first assay did not necessarily result in the smallest responses in the second). The normalized mean of values from the second assay was only slightly lower than that from the first, and the overall distributions were quite similar. Nonetheless, strains were chosen for further evaluation based upon the fact that they consistently resulted in cAMP values less than that observed for an isogenic control strain that contained a plasmid lacking a glucagon coding sequence.

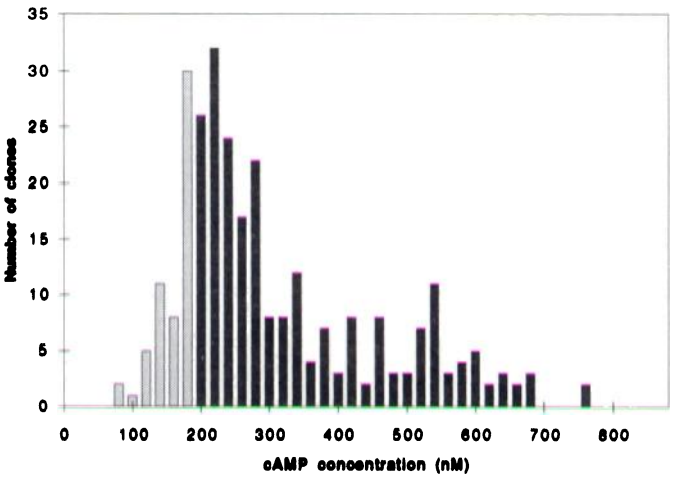

Fig. 2. Distribution of cAMP concentrations observed by screening approximately 280 individual yeast colonies producing glucagon analogs based upon the full length glucagon sequence. In this experiment, the mean observed cAMP concentration for 10 independent assay wells containing broth from a control strain that had been transformed with a plasmid lacking the glucagon coding sequence was 204 nM, with a standard error of 48 nM. Broth from a control strain secreting unmodified glucagon resulted in a mean cAMP concentration of 592 ± 44 nM. The mean value observed with strains making analogs was 300 ± 150 nM. □, Colonies selected for rescreening.

The distribution of cAMP concentrations observed with culture broths from the library of yeast strains producing desHis[1]-glucagon analogs was bimodal (Fig. 3). Roughly one quarter of the 1800 strains screened fell into a group with cAMP values distributed around the value obtained with culture broth from a strain making unmodified desHis[1]-glucagon. The remainder resulted in higher cAMP values distributed around the value obtained with a nonproducing control strain. This is consistent with the hypothesis that a minority of the strains produce analogs that have antagonist activity equal to or greater than that of desHis[1]-glucagon, whereas the remainder either produce analogs that do not have activity or are nonproducers. Strains corresponding to particularly low cAMP values were chosen for secondary screening, and those that consistently resulted in lower cAMP production than that seen with a control strain producing desHis[1]-glucagon were saved for further analysis.

Yeast strains producing glucagon antagonists. Eventually eight strains from the full length glucagon library and 45 from the desHis[1]-glucagon library were chosen as those producing increased antagonist activity, relative to the appropriate control strains. The deduced polypeptide sequence of each glucagon antagonist was determined by sequencing the appropriate DNA coding region from a plasmid clone recovered from each strain.

Seventeen of the 45 plasmids isolated from selected desHis[1]-glucagon library strains were found, in fact, to encode desHis[1]-glucagon itself without any substitutions. The apparent increase in antagonist activity produced by these strains may have been due to increased expression of this analog by some clones or it may be indicative of noise in our assay system. In either case, there is the implication that some of the selected mutant sequences encode antagonists that actually may not be significantly more effective than desHis[1]-glucagon.

The remaining 28 plasmids recovered from the desHis[1]-glucagon library contained mutations that resulted in amino acid substitutions. Table 1 presents a summary of the amino acid substitutions encoded by these plasmids. Several DNA sequences were observed more than once, and in three cases pairs of sequences were found that differed at the nucleotide level but encoded identical peptides. Often, when a sequence encoded several amino acid changes one or more of those changes would be present as a single change in another sequence. In all, 19 different deduced mutant peptide sequences were observed, involving 16 different amino acid substitutions in 11 positions.

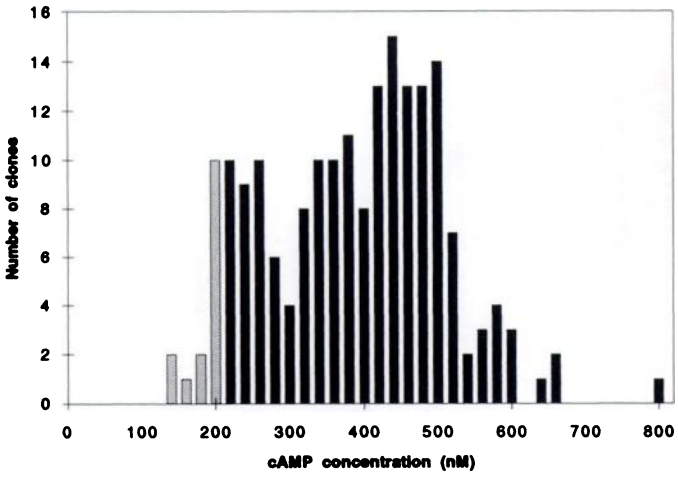

Fig. 3. Distribution of cAMP concentrations observed by screening broths from approximately 190 yeast strains producing desHis[1]-glucagon analogs. In this experiment the mean observed cAMP concentration for a nonproducing control strain was 459 ± 49 nM. The value was 237 ± 39 nM with broths from a strain producing desHis[1]-glucagon. □, Colonies selected for rescreening.

All eight of the plasmids selected and sequenced from the full length glucagon library were found to contain mutations that corresponded to amino acid substitutions in glucagon. These are also included in Table 1. Two contained identical coding sequences, but the remaining six were unique. As with the desHis[1]-glucagon library, some of the amino acid substitutions were present in more than one analog but in conjunction with other changes. Many of the substitutions observed were identical to, or in the same position as, substitutions observed in the desHis[1]-glucagon library.

The antagonist activity of each unique peptide sequence in Table 1 was confirmed by using individual plasmid clones to create a set of retransformed yeast strains. Culture broths from each of these strains were assayed by glucagon radioimmunoassay and were diluted to normalize the concentrations implied by the immunoreactivity of the broths. These normalized broths were then added to adenylate cyclase reactions containing varying amounts of glucagon, to generate an inhibited glucagon response curve for each antagonist. Fig. 4 shows examples of these data that demonstrate that [Ser[4]]-glucagon is a weak glucagon antagonist, whereas desHis[1][Ala[9]]-glucagon, desHis[1][Ala[11]]-glucagon, and desHis[1][Glu[21]]-glucagon are all more effective antagonists than is desHis[1]-glucagon itself.

Synthetic glucagon antagonists. A number of polypeptides corresponding to the mutant glucagon coding sequences observed in our screening experiments have been produced by peptide synthesis. These have been used in experiments designed to prove that the peptide sequences inferred from DNA sequencing are, in fact, effective glucagon antagonists. For comparison, synthetic desHis[1][Glu[9]]-glucagon amide, as described by Unson *et al.* (4), was included in these experiments to represent a state of the art glucagon antagonist.

All of the analogs tested were effective antagonists for glucagon. Fig. 5 shows examples of adenylate cyclase response and inhibition curves generated by stimulating rat liver membranes with various concentrations of a number of analogs, with and without added glucagon. These data have been used to estimate an inhibition coefficient for each analog (Table 2). Table 2 also shows the apparent binding coefficient, calculated by displacement of ^{125}I-glucagon from rat hepatocyte membranes, and the residual adenylate cyclase stimulation for each analog. Although none of our new antagonists were as effective as desHis[1][Glu[9]]-glucagon amide, direct comparison is not necessarily appropriate. Only this analog was synthesized as a carboxyl-terminal amide, and such amidation has been shown to significantly improve receptor binding for a number of glucagon analogs (4, 16). We chose not to make the amidated versions of our antagonists so that they would be exactly analogous to the peptides produced in yeast.

Discussion

Molecular genetics and random screening. We have described a method that effectively identifies glucagon analogs with antagonist activity. The combination of random mutagenesis and a rapid screening method enabled us to produce and

TABLE 1

Glucagon analogs with antagonist activity

	Isolate	Analog	Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Wild-type				His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr
1	J15	desHis^1[Ala^{11}]-Glucagon		des										Ala																		
2	H53	desHis^1[Ala^{11}]-Glucagon		des										Ala																		
3	M14	desHis^1[Glu^9,Ala^{11}]-Glucagon		des								Glu		Ala																		
4	K41	desHis^1[Glu^9,His^{24}]-Glucagon		des								Glu															His					
5	D28	desHis^1[Glu^9,Phe^{15}]-Glucagon		des								Glu				Phe																
6	G60	desHis^1[Asn^9,Phe^{15}]-Glucagon		des								Asn				Phe																
7	O84	desHis^1[Asn^9,Leu^{27}]-Glucagon		des								Asn																		Leu		
8	J36	desHis^1[Asn^9]-Glucagon		des								Asn																				
9	H21	desHis^1[Ala^9]-Glucagon		des								Ala																				
10	E58[a]	desHis^1[Ala^9]-Glucagon		des								Ala																				
11	L2[a]	desHis^1[Ala^9]-Glucagon		des								Ala																				
12	I1	desHis^1[Ile^7]-Glucagon		des						Ile																						
13	BB65	[Asp^1,Ala^2,Ile^7]-Glucagon		Asp	Ala					Ile																						
14	J95	desHis^1[Ala^2]-Glucagon		des	Ala																											
15	F59	desHis^1[Thr^2]-Glucagon		des	Thr																											
16	BB25	[Cys^2]-Glucagon			Cys																											
17	EE89[a]	[Cys^2]-Glucagon			Cys																											
18	FF21	[Pro^2]-Glucagon			Pro																											
19	BB64	[His^3,Ser^4]-Glucagon				His	Ser																									
20	P60	[Ser^4]-Glucagon					Ser																									
21	FF30	[Asp^4,Ser^8]-Glucagon					Asp	Ser																								
22	K93	desHis^1[Ser^8]-Glucagon		des				Ser																								
23	G56	desHis^1[Ser^4]-Glucagon		des			Ser																									
24	E5	desHis^1[Ala^4]-Glucagon		des			Ala																									
25	K9	desHis^1[Ala^4]-Glucagon		des			Ala																									
26	M28	desHis^1[Ser^4,Ala^{29}]-Glucagon		des			Ser																									Ala
27	G68	desHis^1[Pro^3,Ser^{29}]-Glucagon		des		Pro																										Ser
28	T63[a]	desHis^1[Pro^3,Ser^{29}]-Glucagon		des		Pro																										Ser
29	F73	desHis^1[Ser^{29}]-Glucagon		des																												Ser
30	D45	desHis^1[Ser^{29}]-Glucagon		des																												Ser
31	D16	desHis^1[Glu^{21},Ser^{29}]-Glucagon		des																				Glu								Ser
32	H33	desHis^1[Glu^{21}]-Glucagon		des																				Glu								
33	B6[a]	desHis^1[Glu^{21}]-Glucagon		des																				Glu								
34	E34[a]	desHis^1[Glu^{21}]-Glucagon		des																				Glu								
35	R47	desHis^1[Glu^{21}]-Glucagon		des																				Glu								

[a] These isolates may not be independent. They have the same DNA sequence as the first isolate listed encoding the same polypeptide. All other peptide replicates differ at the DNA sequence level.

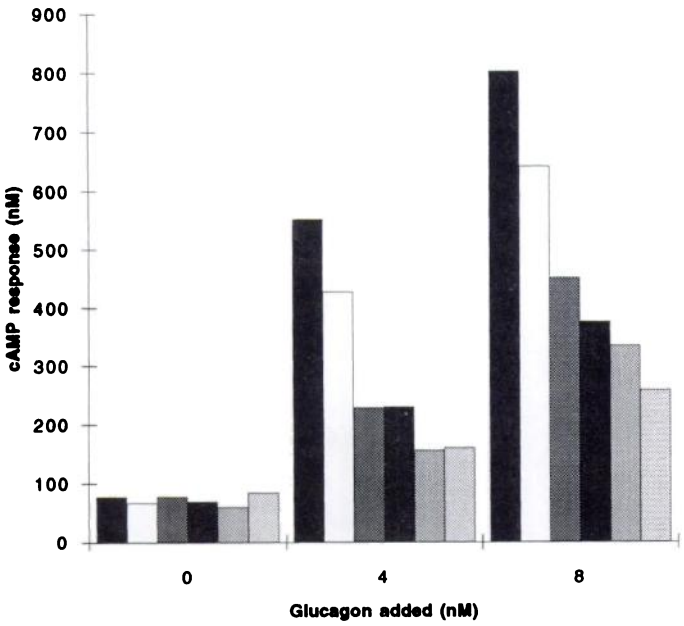

Fig. 4. Diluted culture broths from yeast strains producing various glucagon analogs were added to adenylate cyclase reactions containing 0, 4, or 8 nM glucagon, and the resulting cAMP concentrations were measured. Before dilution the concentration of each analog was measured by a glucagon radioimmunoassay (Linco, Inc.), so that each was present in the assay mixture at an apparent concentration of 100 nM. *No analog*, broth from a nonproducing control strain used at an equivalent dilution. Each value represents the average of three independent assay wells.

test a number of glucagon analogs that is greater than the combined total that has been produced in more than one decade of work by several different peptide synthesis groups. Because of our ability to examine relatively large numbers of analogs, we were able to identify positions within the glucagon molecule and specific amino acid substitutions that were previously not known to contribute to antagonist activity.

We scaled our screening efforts to be virtually certain of testing several different amino acid substitutions for each of the 29 positions in glucagon. As with other approaches that utilize gene expression to produce peptide analogs, we were limited to the 20 naturally occurring amino acids. Our approach was further limited because it was unlikely that more than one base change would occur in any given codon and not all base changes result in unique amino acid substitutions. Thus, only six or seven different substitutions were likely at each position and, because the analogs were screened at random, we cannot be certain that each of these changes was rigorously tested. However, this represents considerably more diversity than that in other approaches, such as alanine-scanning mutagenesis, that are designed to pinpoint functionally important residues.

Antagonist design. Although none of the new synthetic antagonists described were as effective as desHis^1[Glu^9]-glucagon amide, we believe that we have discovered information that can be used to design a new generation of improved antagonists. It is clear that modification of one or more of the first four amino acid residues of glucagon is the most effective way to create an analog with antagonist activity. All the antagonists identified in the full length glucagon library had at least one change in this region, and effective antagonists were quite common in the desHis^1-glucagon library. This fits with the

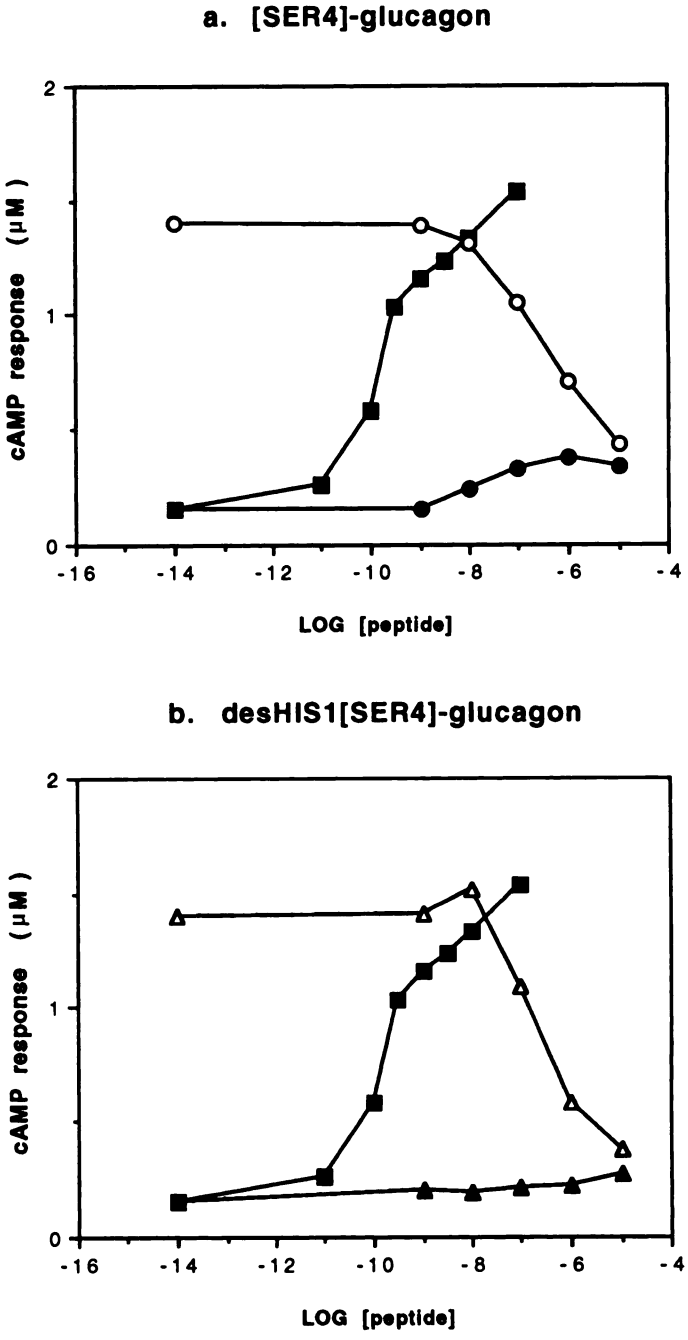

Fig. 5. The response of rat liver membrane adenylate cyclase to various concentrations of glucagon, glucagon analogs, or mixtures of the two was determined by measuring the cAMP concentration after stimulation. a, Dose-response curves for glucagon (■) and [Ser^4]-glucagon (●) as well as an dose-inhibition curve for [Ser^4]-glucagon in the presence of 20 nM glucagon (○). b, Dose-response curve for glucagon (■) and desHis^1[Ser^4]-glucagon (▲) as well as a dose-inhibition curve for desHis^1[Ser^4]-glucagon in the presence of 20 nM glucagon (△). Each *data point* represents the average of three independent assay wells.

generally accepted dogma that the amino-terminal portion of glucagon is responsible for signal transduction, whereas the carboxyl terminus is important for binding. Apparently amino-terminal modifications reduce or eliminate signal transduction and leave receptor binding more or less intact.

In this regard, deletion of the amino-terminal histidine residue appears to be at least as effective as other individual

TABLE 2
Synthetic glucagon antagonists

Analog	Membrane binding, apparent K_d	Adenylate cyclase stimulation		
		Maximum activity	Relative potency	$(I/A)_{50}$[a]
Glucagon	3.1×10^{-8}	1.00	1.00	
desHis[1]-Glucagon	3.4×10^{-7}	0.20	0.001	>100
[Ser[4]]-Glucagon	1.7×10^{-7}	0.16	0.0006	45
desHis[1][Ser[4]]-Glucagon	2.6×10^{-7}	0.08	0.0001	36
desHis[1][Ala[11]]-Glucagon	3.1×10^{-7}	0.00	<0.0001	20
desHis[1][Glu[21]]-Glucagon	1.7×10^{-7}	0.00	<0.0001	25
desHis[1][Ser[29]]-Glucagon	2.1×10^{-7}	0.17	0.0001	33
desHis[2][Glu[9]]-Glucagon amide	9.4×10^{-8}	0.00	<0.0001	10

[a] $(I/A)_{50}$ is the ratio of inhibitor to agonist that causes a 50% reduction in the response seen with the same concentration of agonist alone. It was calculated by dividing the analog concentration at the midpoint of the inibition curve by the concentration of glucagon present in the assay (20 nM).

substitutions, but there appear to be additive or synergistic effects of having more than one change in this region. For example, desHis[1][Ser[4]]-glucagon, desHis[1][Ala[2]]-glucagon, desHis[1][Thr[2]]-glucagon, and desHis[1][Ala[4]]-glucagon were all selected in the desHis[1]-glucagon library screening. The data in Table 2 show that [Ser[4]]-glucagon and desHis[1]-glucagon are both partial agonists with some residual adenylate cyclase-stimulating activity. desHis[1][Ser[4]]-glucagon results in no detectable adenylate cyclase stimulation and is a better antagonist than either of the singly modified analogs.

The antagonist activity of desHis[1]-glucagon or other amino-terminally modified glucagon analogs can be improved in a number of other ways. Substitutions in positions 5, 7, 9, 11, 13, 21, and 29 were each observed more than once among the antagonists we identified. Merrifield and co-workers (4, 5, 16) have shown improved antagonism for a variety of desHis[1] analogs that have substitutions in position 9 and amidated carboxyl termini (i.e., position 29). Our own data (Table 2) also demonstrate improved inhibition indexes $[(I/A)_{50}]$ for desHis[1][Ala[11]]-glucagon, desHis[1][Glu[21]]-glucagon, and desHis[1][Ser[29]]-glucagon, relative to desHis[1]-glucagon itself.

Some of the substitutions identified by our study as contributing to antagonist activity were previously incorporated into synthetic glucagon analogs and tested for their effects on binding and signal transduction. We found that the effect of a given substitution in isolation was not always predictive of its effect in combination with other modifications. One should not be too literal in applying the model of receptor binding and signal transduction as separable carboxyl- and amino-terminal functions.

For example, it is possible that one may have predicted the improved antagonism of desHis[1][Glu[21]]-glucagon from the fact that [Glu[21]]-glucagon was previously described as a superagonist with 3-fold greater receptor affinity and increased andenylate cyclase stimulation (17). If the Glu[21] substitution has a similar independent effect on desHis[1]-glucagon, it could increase the effectiveness of this partial antagonist simply by improving its ability to compete for receptor binding. desHis[1][Glu[21]]-glucagon does, in fact, have increased receptor affinity, relative to desHis[1]-glucagon. However, its improved antagonist activity also appears to result from a further reduction in adenylate cyclase activation (see Table 2).

It is even more difficult to explain the improved activity of desHis[1][Ala[11]]-glucagon. [Ala[11]]-Glucagon has been described as having about 4-fold reduced receptor binding and adenylate cyclase activation, relative to glucagon (18). However, when introduced into desHis[1]-glucagon the Ala[11] substitution provides a moderate increase in receptor binding and a specific reduction in adenylate cyclase stimulation (Table 2). desHis[1][Ala[11]]-Glucagon was the most effective new synthetic antagonist we tested. Clearly there was no way to predict this result from the independent effects of the two modifications present in this analog.

Another unexpected finding of our study was the effect of substituting a serine residue for threonine at position 29. This substitution was observed in at least four independent analogs selected for their improved antagonism. It is surprising that such a conservative replacement in a seemingly unimportant region of the molecule is an effective way to improve the antagonism of desHis[1]-glucagon. Previous studies noted improved activities for analogs that were amidated at this residue, but these improvements were generally ascribed to reduced susceptibility to carboxypeptidase activity.

Finally, our data also support the idea that glucagon function appears to be exceedingly sensitive to amino acid substitution. The distribution of cAMP values in our screening experiments indicates that most of the analogs tested had lost function even though the average number of substitutions per molecule was relatively low. When a subset of the full length library was tested without glucagon in the assay mixture, only 10% showed adenylate cyclase stimulation. With the desHis[1]-glucagon library, approximately three fourths of the analogs had lost the partial antagonism of the starting molecule, presumably because they no longer had receptor-binding activity. All of the analogs we tested, and almost all of those described in the literature, have markedly reduced receptor binding, regardless of where substitutions are made in the molecule. It is also significant that the amino acid sequence of glucagon has been highly conserved in evolution. Very few interspecific changes are known, and all mammalian glucagons are identical. Thus, it appears to be very difficult to modify a specific region of the glucagon molecule without affecting its structure as a whole. This helps to explain why the result of combining several modifications in an single analog is often difficult to predict from the effects of the individual changes.

Conclusions

We believe that our approach is a useful complement to the more traditional methods of antagonist design that are based on modeling and peptide synthesis. It is both reassuring and gratifying that our random approach identified not only those residues, such as His[1] and Asp[9], that were previously known to be important for glucagon function and antagonist design but also others. The preexisting knowledge base for glucagon was very useful in providing validation for our methods. Given this validation, our approach of molecular genetic dissection should be even more useful in analyzing the structure-function relationship of peptides that are more poorly understood than glucagon.

At the same time, random screening of large numbers of variants can also be used to answer specific questions whenever the so-called rational approach is overwhelmed by an unmanageable number of alternatives. It can reveal unexpected interactions between residues and unexpected effects of specific substitutions. The ability of peptide chemists to incorporate an unlimited variety of amino acid analogs into polypeptides may

ultimately be required for production of an effective antagonist, but molecular genetics can be used to discover how best to apply that synthetic ability.

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