

Structure-Activity Studies of Glucagon-like Peptide-1*

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A series of analogs of glucagon-like peptide-1 (GLP-1) was made replacing each amino acid with L-alanine to identify side-chain functional groups required for interaction with the GLP-1 receptor. In the case of L-alanine being the parent amino acid, substitution was made with the amino acid found in the corresponding position in glucagon. Binding assays were performed using the cloned rat GLP-1 receptor, and receptor activation was monitored using RIN 2A18 plasma membranes. The analogs that showed the weakest receptor binding were further compared with native GLP-1 by circular dichroism spectroscopy to investigate possible conformational changes. We conclude that the side chains in positions 7, 10, 12, 13, and 15 are directly involved in the receptor interaction while positions 28 and 29 are important for GLP-1 to adapt the conformation recognized by the receptor.

Glucagon-like peptide-1-(7-36)-amide (GLP-1)¹ is formed by post-translational processing of the proglucagon precursor peptide (1-5). The peptide is secreted from the distal gut into the circulation after the ingestion of, for example, a carbohydrate-rich meal. The peptide has several effects on the endocrine pancreas. It stimulates insulin secretion in the presence of elevated glucose levels; it stimulates proinsulin gene expression and inhibits glucagon secretion (4, 6-8). Accordingly, GLP-1 has a potential impact on glucose metabolism at several levels and has recently shown promising glucose-lowering effects in non-insulin-dependent diabetes mellitus patients when administered in pharmacological doses (9-11).

The sequence of GLP-1 is completely conserved in all mammalian species investigated so far (1, 4, 12-15). The active products of the proglucagon peptide (GLP-1, glucagon-like peptide-2, and glucagon), together with gastric inhibitory peptide, form one branch of the growth hormone-releasing factor (GRF) superfamily of peptides. The members of this superfamily are believed to be derived from a common ancestor (16). The other branch of the GRF superfamily comprises pituitary adenyl cyclase-activating peptide, vasoactive intestinal peptide, peptide histidine isoleucine amide, secretin, and GRF. The high degree of sequence homology among the GRF superfamily is

illustrated in Fig. 1. In spite of the high homology, none of the other peptides tested show any affinity for the cloned GLP-1 receptor (17) except glucagon which, however, has an affinity of at least a factor of 10⁴ less than GLP-1. Surprisingly, Exendin-4, a 39-amino acid peptide from Heloderma masticator venom, which also shares a high degree of homology to GLP-1 (Fig. 1), has recently been shown to be a very potent agonist of GLP-1 while Exendin-4-(9-39)-amide was found to be an antagonist of GLP-1 (18, 19).

So far, very few studies have been performed to investigate the structure-activity relationship of GLP-1. Studies have been performed primarily with N- and C-terminally extended or truncated analogs (20-25). These studies have indicated that the N-terminal histidine is very important for receptor affinity and that N-terminally extended forms of GLP-1 have limited activity only. The C-terminal part of the molecule also seems to be important for the action of GLP-1, although not as critical as the N-terminal histidine. GLP-1-(7-34) and GLP-1-(7-35) exhibit a somewhat reduced receptor affinity while GLP-1-(7-33), GLP-1-(7-22), and GLP-1-(7-20) show no biological activity. The glycine-extended form of the peptide, GLP-1-(7-37), has identical efficacy compared with GLP-1-(7-36)-amide. Furthermore, Exendin-4, having a 9-residue C-terminal extension, is a very potent GLP-1 agonist (19, 24). Some analogs have been made where the specific residues in positions 10, 15, 16, 17, 18, 21, 27, and 31 were exchanged to the residues found in GRF. This study indicated the residues in positions 10, 15, and 17 to be most important for the effect of GLP-1 (23).

As indicated above, the structure-activity studies performed so far have pointed at both the N- and C-terminal part of the GLP-1 molecule being involved in the receptor interaction. However, no study has been performed in order to investigate the function and importance of each amino acid in the peptide. To address this question, we now report on the systematic exchange of each amino acid in the sequence of GLP-1 by L-alanine or, in the case of L-alanine being the parent amino acid, the amino acid found in the corresponding position of glucagon.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The peptides in Table I were synthesized using the ABIMED 422 multiple synthesizer. The nomenclature utilized is based on the sequence of GLP-1 (Fig. 1). 9-Fluorenylmethoxycarbonyl strategy, modified according to Gausepohl *et al.* (26), was used starting from a Rink-resin (NovaBiochem). The side-chain protection was as follows: Asn, Gln, His (trityl), Arg (2,2,5,7,8-pentamethylchroman-6-sulfonyl), Asp, Glu, Tyr, Ser, and Thr (*tert*-butyl), and Trp and Lys (*tert*-butyloxycarbonyl) (NovaBiochem). Each reaction vessel was filled with 25 µmol of resin. The peptides were cleaved from the resin and side chain deprotected in trifluoroacetic acid/triethylsilane/water (92.5:5:2.5) for 120 min and precipitated in *tert*-butylmethyl ether, washed twice with diethyl ether, and lyophilized from 10% acetic acid. The crude peptides were purified by reverse phase HPLC using a gradient of acetonitrile in water (15-40% acetonitrile in 0.01 M HCl; column, Suprapak Pep S C2/C18, 5 µm, 250 × 9.3 mm (Pharmacia LKB Biotechnology Inc.)).

The purified peptides were characterized by analytical HPLC and high performance capillary electrophoresis as previously described (27) and were all of a purity of >95%. Molecular masses were measured by plasma desorption mass spectrometry analysis performed in positive mode on a ²⁵²Cf time of flight mass spectrometer (Bio-Ion) and were in agreement with the calculated mass within ±2 mass units.

Iodination of Tracer—Iodination of GLP-1 was performed by the lactoperoxidase method (28). Purification by HPLC as previously described (29) afforded [¹²⁵I-Tyr¹⁹]GLP-1-(7-36)-amide with a specific activity of 76 kBq/pmol.

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¹ The abbreviations used are: GLP-1, glucagon-like peptide-1-(7-36)-amide; GRF, growth hormone-releasing factor; HPLC, high pressure liquid chromatography; CHL, Chinese hamster lung.

Peptide	7	10	15	20	25	30	35	40	45	50																																	
GLP-1	H	A	E	G	T	F	S	D	V	S	S	L	I	G	Q	A	A	K	E	F	I	A	W	L	V	E	R	amide															
Exendin-4	H	G	E	G	T	F	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	I	E	W	L	K	N	G	P	S	S	G	A	P	P	S	amide							
GLP-2	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	I	Q	T	K	I	T	D	R									
Glucagon	F	S	Q	G	T	F	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T															
GIP	Y	A	E	G	T	F	S	D	Y	S	I	A	M	D	K	I	H	Q	Q	D	F	V	N	W	L	A	Q	K	G	K	K	N	D	W	K	H	N	I	T	Q			
VIP	H	S	D	A	V	F	T	D	N	Y	T	R	L	R	K	Q	M	A	V	K	K	Y	L	N	S	I	L	N	amide														
Secretin	H	S	D	G	T	F	S	E	L	S	R	L	R	D	S	A	R	L	Q	R	L	L	Q	G	L	V	amide																
PACAP-38	H	S	D	G	I	F	T	D	S	Y	S	R	Y	R	K	Q	M	A	V	K	K	Y	L	A	A	V	L	G	K	R	Y	K	Q	R	V	K	N	K	amide				
PHI	H	A	D	G	V	F	T	S	D	F	S	R	L	G	Q	L	S	A	K	K	Y	L	E	S	L	I	amide																
GRF	Y	A	D	A	I	F	T	N	S	Y	R	K	V	L	G	L	S	A	R	K	L	L	Q	D	I	M	S	R	Q	G	E	S	N	Q	E	R	G	A	R	A	R	L	amide

FIG. 1. Comparison of the GLP-1 sequence to the sequence of other peptides in the GRF superfamily and the sequence of Exendin-4. Dark areas represents regions of homology. Position numbers refer to the nomenclature used for GLP-1 (position 7 corresponds to position 1 of the other peptides).

Circular Dichroism Spectroscopy—Peptides were dissolved in 10 mM phosphate buffer, pH 6.5. Concentrations were determined by UV absorbance using a molar absorption coefficient, ϵ_{280} , of $6970 \text{ M}^{-1} \text{ cm}^{-1}$. Far-UV CD spectra were recorded with a Jobin Mark V dichrograph calibrated with (+)-10-camphorsulfonic acid. All spectra were recorded at room temperature using a 0.01-cm cell pathlength and a peptide concentration of about 0.5 mg/ml. All spectra were smoothed by a Fourier transform algorithm before subtraction of the appropriate solvent blanks. The result, $\Delta\epsilon$, is based on the molar concentration of peptide bond.

GLP-1 Receptor Binding Assay—Receptor binding was analyzed using plasma membranes prepared from Chinese hamster lung (CHL) cells expressing the cloned rat pancreatic GLP-1 receptor (17). Cells were cultured in Dulbecco's modified Eagle's F-12 medium containing 17.5 mM D-glucose, 10% fetal calf serum, 1 mM sodium pyruvate, 0.5 g/liter lactalbumin, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 80 µg/ml Geneticin® G-418 (Life Technologies, Inc.). Plasma membranes were prepared by resuspending the cells in cold buffer (10 mM Tris-HCl, pH 7.5, containing 30 mM NaCl, 1 mM dithiothreitol, 5 mg/liter leupeptin, 5 mg/liter pepstatin, 100 mg/liter bacitracin (Sigma), and 15 mg/liter recombinant aprotinin (Novo Nordisk)), homogenization by two 10-s bursts using a Polytron PT 10-35 homogenizer (Kinematica), and centrifugation on a layer of 41% (w/v) sucrose at $95,000 \times g$ for 75 min. The white band located between the two layers was diluted in buffer and centrifuged at $40,000 \times g$ for 45 min. The precipitate containing the plasma membranes was suspended in buffer and stored at -80°C until required.

The receptor binding assay was performed in 96-well 0.65-µm filter microtiter plates (Millipore). The buffer used was 50 mM HEPES (pH 7.4) containing 2.5% human serum albumin grade V (Sigma). The peptide was dissolved in 100 µl of buffer. Tracer (1.13 kBq, 25 µl of buffer) and 5 µg of freshly thawed plasma membrane suspended in 25 µl of buffer were added, and the plates were incubated for 30 min at 30°C . Bound and unbound peptide were separated using a vacuum manifold (Millipore). Filters were washed with 125 µl of buffer and left to dry for 2 h. The filters were then separated using the Millipore Punch System, and the amount of bound tracer was determined.

Adenylate Cyclase Assay—Adenylate cyclase activity was assayed using plasma membranes prepared from the rat insulinoma cell line RIN 2A18. Cells were cultured in media as above with the addition of 5 mg/liter porcine insulin (Novo Nordisk), 1 g/liter dextran T-70 (Pharmacia), 10 mg/liter recombinant aprotinin (Novo Nordisk), and 0.5 g/liter ϵ -aminocaproic acid (Sigma). Plasma membranes were prepared as above.

The incubation was carried out in microtiter plates in a total volume of 100 µl. Peptide and 2 µg of freshly thawed plasma membrane protein were diluted in 50 mM Tris buffer, pH 7.4, containing 0.1% human serum albumin grade V, 1 mM EGTA, 2 mM MgCl_2 , 1 mM ATP, 0.5 mM 3-isobutyl-1-methylxanthine, 15 mM creatine phosphate, 0.5 mg/ml creatine kinase (Sigma). The mixture was incubated for 10 min at room temperature. The reaction was stopped by heating the plates to 80°C for 3 min. The cAMP formed was measured using SPA-RIA (RPA.538, Amersham Corp.).

RESULTS AND DISCUSSION

GLP-1 Analogs—L-Alanine is the smallest optically active amino acid and is not able to fill the same space as any other amino acid except glycine. Furthermore, L-alanine has a high α -helix propensity and an exchange with this amino acid within

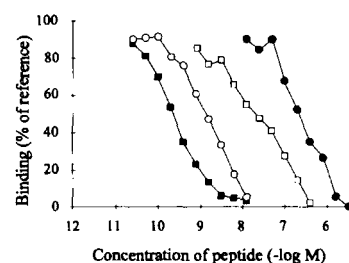


FIG. 2. Displacement of binding of $[^{125}\text{I-Tyr}^{19}]$ GLP-1 to plasma membranes from CHL cells expressing the cloned rat pancreatic GLP-1 receptor with selected GLP-1 analogs. Each point represents the mean of three determinations using plasma membranes from a single preparation, or for native GLP-1 the mean of 92 determinations using plasma membranes from 10 preparations. Assay conditions were as indicated under "Experimental Procedures." ■, GLP-1; □, $[\text{Ala}^{12}]$ GLP-1; ●, $[\text{Ala}^{28}]$ GLP-1.

an α -helix is generally unlikely to disrupt the helix. Accordingly, by substituting each amino acid in GLP-1 successively with L-alanine, positions with side chains important for receptor binding and/or receptor activation may be detected. In GLP-1, L-alanine is found in positions 8, 24, 25, and 30. To test the importance of these positions substitutions were made replacing alanine with the amino acid found in the corresponding position in glucagon. As indicated in Fig. 1, glucagon is the peptide sharing the highest degree of homology to GLP-1 among the related peptides in the superfamily. Even so, glucagon shows almost no affinity for the GLP-1 receptor (17).

Receptor Affinity Studies—Competitive receptor binding was measured for all GLP-1 analogs using plasma membranes derived from a CHL cell line expressing the cloned GLP-1 receptor from rat pancreatic islets (17). IC_{50} for GLP-1 was measured as 0.27 nM in this system (Fig. 2). The binding affinity of the GLP-1 analogs measured is shown in Table I.

As indicated, substitution with L-alanine causes a great loss of receptor affinity when made in positions 7, 10, 12, 13, 15, 28, and 29 ($\text{IC}_{50} > 10 \text{ nM}$). The identification of positions 7, 10, and 15 as important for receptor binding is in agreement with previous observations (23, 24) while positions 12, 13, 28, and 29 have not previously been identified as important. Position 17 was also indicated by Kawai *et al.* (23) as important for receptor binding. However, their conclusion was made based on an analog replacing Ser¹⁷ in GLP-1 with an arginine residue, thereby introducing a charged group in this position. Our data reveal almost no effect of substituting Ser¹⁷ with L-alanine ($\text{IC}_{50} = 0.46 \text{ nM}$), and this position seems, accordingly, not to be involved in receptor binding. The greatest impact on receptor binding was observed by introducing L-alanine in position 28 ($\text{IC}_{50} = 531 \text{ nM}$), thereby removing the hydrophobic Phe²⁸ residue. Interestingly, no effect was observed by introducing residues from glucagon in positions 8, 24, and 30 having alanine as

TABLE I
In vitro activity of various GLP-1 analogs

Activities were measured as indicated under "Experimental Procedures." Binding affinity is expressed as mean $\pm$ S.E. ($n = 3$, or in the case of native GLP-1, $n = 92$) while adenylyl cyclase activity is mean $\pm$ S.E. ($n = 2$, or in the case of native GLP-1, $n = 19$).

Analog	Binding affinity, IC ₅₀	Adenylyl cyclase activity, EC ₅₀
	nM	nM
GLP-1	0.27 $\pm$ 0.09	2.6 $\pm$ 0.4
[Ala ⁷]GLP-1	30 $\pm$ 5	>10 ⁴
[Ser ⁸]GLP-1	2.4 $\pm$ 0.4	2.0 $\pm$ 0.3
[Ala ⁹]GLP-1	8.1 $\pm$ 1.0	2 $\pm$ 1
[Ala ¹⁰]GLP-1	59 $\pm$ 4	>10 ⁴
[Ala ¹¹]GLP-1	3.5 $\pm$ 0.3	5 $\pm$ 2
[Ala ¹²]GLP-1	36 $\pm$ 2	33 $\pm$ 38
[Ala ¹³]GLP-1	36 $\pm$ 6	65 $\pm$ 49
[Ala ¹⁴]GLP-1	0.76 $\pm$ 0.13	5 $\pm$ 4
[Ala ¹⁵]GLP-1	11 $\pm$ 1	>10 ⁴
[Ala ¹⁶]GLP-1	1.7 $\pm$ 0.5	7 $\pm$ 5
[Ala ¹⁷]GLP-1	0.46 $\pm$ 0.10	3 $\pm$ 2
[Ala ¹⁸]GLP-1	0.68 $\pm$ 0.09	2 $\pm$ 1
[Ala ¹⁹]GLP-1	3.5 $\pm$ 0.8	55 $\pm$ 33
[Ala ²⁰]GLP-1	1.7 $\pm$ 0.1	7 $\pm$ 1
[Ala ²¹]GLP-1	4.1 $\pm$ 0.2	65 $\pm$ 49
[Ala ²²]GLP-1	0.57 $\pm$ 0.08	4 $\pm$ 1
[Ala ²³]GLP-1	1.1 $\pm$ 0.2	5 $\pm$ 3
[Arg ²⁴]GLP-1	0.89 $\pm$ 0.20	17 $\pm$ 10
[Ala ²⁶]GLP-1	1.4 $\pm$ 0.3	13 $\pm$ 10
[Ala ²⁷]GLP-1	0.24 $\pm$ 0.04	1 $\pm$ 1
[Ala ²⁸]GLP-1	351 $\pm$ 49	2,600 $\pm$ 780
[Ala ²⁹]GLP-1	25 $\pm$ 3	70 $\pm$ 14
[Gln ³⁰]GLP-1	1.4 $\pm$ 0.1	0.5 $\pm$ 0.2
[Ala ³¹]GLP-1	1.6 $\pm$ 0.2	15 $\pm$ 13
[Ala ³²]GLP-1	4.7 $\pm$ 1.0	4 $\pm$ 3
[Ala ³³]GLP-1	1.4 $\pm$ 0.2	2 $\pm$ 0
[Ala ³⁴]GLP-1	1.7 $\pm$ 0.3	2 $\pm$ 0
[Ala ³⁶]GLP-1	1.3 $\pm$ 0.1	1 $\pm$ 1
[Ala ³⁶]GLP-1	4.6 $\pm$ 0.6	7 $\pm$ 5

the parent amino acid in GLP-1. Even substitution with the charged L-arginine in position 24 had almost no impact on the receptor affinity (IC₅₀ = 0.89 nM).

The accumulation of important residues in the N-terminal region of GLP-1 (positions 7, 10, 12, 13, and 15) suggests this part of the molecule to be most important for receptor binding. Surprisingly, positions 7, 10, 12, and 13 are the most conserved positions among the peptides in the GRF superfamily (Fig. 1). Position 15 appears less conserved, but the differences among the members of the family represent substitutions that are mostly conservative, e.g. aspartic acid to glutamic acid. The N-terminal part of the peptide has also been found to be highly important for receptor binding in other members of the superfamily where detailed structure-activity studies have been performed (30–32). Accordingly, although the N-terminal region of the peptides is highly important for receptor binding, it is unlikely that it is responsible for the selective recognition of the respective specific receptors. More likely, the N-terminal region is carrying a common message important for activation of the receptor. This hypothesis is supported by the observation that Exendin-4-(9–39) is able to completely antagonize GLP-1 (19). Compared with the N-terminal domains, the C-terminal part of the peptides is much more heterogeneous and thus more likely to be involved in specific receptor recognition. Similar ideas have been proposed previously based on the glucagon-glucagon receptor system by Hruby *et al.* (33). Based on our data, positions 28 and 29 in GLP-1 are most important for receptor binding in the C-terminal domain and could, accordingly, be determining the specific interaction between GLP-1 and its receptor.

Interestingly, the only peptide in Fig. 1 that is homologous to GLP-1 in all the important positions identified is Exendin-4, which is a very potent agonist of GLP-1 action (19).

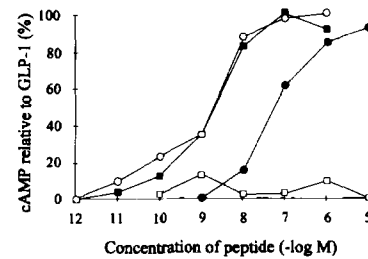


FIG. 3. Adenylyl cyclase activity of selected GLP-1 analogs measured using plasma membranes derived from RIN 2A18 cells. Each point represents the mean of two determinations using plasma membranes from a single preparation, or for native GLP-1 the mean of 19 determinations using plasma membranes from two preparations. Assay conditions were as indicated under "Experimental Procedures." ■, GLP-1; □, [Ala¹⁵]GLP-1; ●, [Ala²⁹]GLP-1; ○, [Ala³⁴]GLP-1.

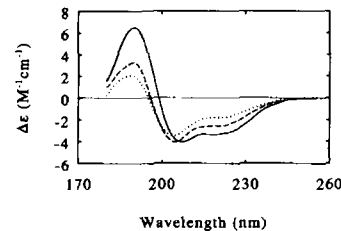


FIG. 4. Far-UV CD spectra of GLP-1 (—) and analogs that cause significant spectral changes, i.e. [Ala²⁸]GLP-1 (····) and [Ala²⁹]GLP-1 (---). Spectra were recorded in 10 mM phosphate buffer, pH 6.5, using a 0.01-cm cell pathlength.

Receptor Activation—Plasma membranes derived from the rat insulinoma cell line RIN 2A18 were used to measure receptor activation as they showed a much higher cAMP response than the plasma membranes from the CHL cell line. Using these membranes, the EC₅₀ for GLP-1 with respect to activation of adenylyl cyclase is 2.6 nM (Fig. 3). The binding affinity for GLP-1 of the membranes (IC₅₀ = 0.44 nM $\pm$ 0.06, $n = 5$) was found to be comparable with the affinity obtained with membranes from the CHL cell line (IC₅₀ = 0.27 nM, Table I).

Overall, analogs with a low receptor affinity were also found to have a higher EC₅₀ with respect to activation of adenylyl cyclase (Table I). In agreement with the above hypothesis, substitutions in the N-terminal positions 7 and 10 were found to have the most dramatic impact on receptor activation as none of these analogs showed any measurable effect on cAMP formation.

The analog with L-alanine substitution in position 15, exhibiting a relative high binding affinity (IC₅₀ = 11 nM) but no ability to activate adenylyl cyclase (EC₅₀ > 10 μ M), was investigated for its ability to antagonize GLP-1 action. Interestingly, the best glucagon antagonists described so far have an amino acid replacement in the same position (34). However, using 10 nM GLP-1, no antagonist effects could, unexpectedly, be observed at concentrations of up to 100 μ M [Ala¹⁵]GLP-1.

Circular Dichroism Measurements—Based on their low binding affinity (IC₅₀ > 10 nM), GLP-1 analogs with L-alanine substitution in positions 7, 10, 12, 13, 15, 28, and 29 were selected for far-UV CD spectroscopy and compared with native GLP-1 to investigate possible conformational changes induced by the substitution.

The far-UV CD spectrum of native GLP-1 was found to be constant in the pH range of 5.5–7.5, indicating a helical content of about 35%. At pH 6.5, CD spectra of the analogs were found to be very similar to that of native GLP-1, except for the ones with L-alanine substitution in positions 28 and 29 (Fig. 4). These analogs gave a significantly smaller CD indicating that

the substitution had disrupted part of the alleged helical structure of the molecule.

Conclusions—The results of our structure-activity studies support the conclusion that the residues His⁷, Gly¹⁰, Phe¹², Thr¹³, and Asp¹⁵ contain side chains important for receptor interaction as substitution with L-alanine in these positions provides analogs with reduced receptor affinity and reduced capacity of activating adenylyl cyclase. Substitution of Phe²⁸ and Ile²⁹ with L-alanine also provides analogs with reduced receptor affinity and activation. However, CD spectroscopy reveals that the latter substitutions introduce a change in the secondary structure of the peptide while none of the former substitutions cause significant change. Rather than being involved in the receptor interaction, Phe²⁸ and Ile²⁹ may, consequently, be more important for the secondary structure of the peptide and consequently for the conformation being recognized by the receptor.

The importance of the residues identified is further highlighted as the only known agonist of GLP-1, Exendin-4, is homologous to GLP-1 in all these positions.

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REFERENCES

- Bell, G. I., Santerre, R. F., and Mullenbach, G. T. (1983) *Nature* **302**, 716–718
- Mosjov, S., Heinrich, G., Wilson, I. B., Ravazzola, M., Orci, L., and Habener, J. F. (1986) *J. Biol. Chem.* **261**, 11880–11889
- Ørskov, C., Holst, J. J., Knuhtsen, S., Baldissera, F. A. G., Poulsen, S. S., and Nielsen, O. V. (1986) *Endocrinology* **119**, 1467–1475
- Holst, J. J., Ørskov, C., Nielsen, O. V., and Schwartz, T. W. (1987) *FEBS Lett.* **211**, 169–174
- Ørskov, C., Bersani, M., Johnsen, A. H., Højrup, P., and Holst, J. J. (1989) *J. Biol. Chem.* **264**, 12826–12829
- Mosjov, S., Weir, C. G., and Habener, J. F. (1987) *J. Clin. Invest.* **79**, 616–619
- Kreymann, B., Ghatei, M. A., Williams, G., and Bloom, S. R. (1987) *Lancet* **II**, 1300–1303
- Drucker, D. J., Philippe, J., Mosjov, S., Chick, W. L., and Habener, J. F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3434–3438
- Nathan, D. M., Schreiber, E., Fogel, H., Mosjov, S., and Habener, J. F. (1992) *Diabetes Care* **15**, 270–276
- Gutniak, M., Ørskov, C., Holst, J. J., Ahrén, N., and Efendic, S. (1992) *N. Engl. J. Med.* **326**, 1316–1322
- Nauck, M. A., Kleine, N., Ørskov, C., Holst, J. J., and Creutzfeldt, W. (1993) *Diabetologia* **36**, 741–744
- Heinrich, G., Gros, P., Lund, P. K., and Habener, J. F. (1984) *J. Biol. Chem.* **259**, 716–718
- Lopez, L. C., Fraizer, M. L., Su, C. J., Kumar, A., and Saunders, G. F. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 5485–5489
- Seino, S., Welsh, M., Bell, G. I., Chan, S. J., and Steiner, D. F. (1986) *FEBS Lett.* **203**, 25–30
- Nishi, M., and Steiner, D. F. (1990) *Mol. Endocrinol.* **4**, 1192–1198
- Campbell, R. M., and Scanes, C. G. (1992) *Growth Regul.* **2**, 175–191
- Thorens, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8641–8645
- Raufman, J.-P., Singh, L., Singh, G., and Eng, J. (1992) *J. Biol. Chem.* **267**, 21432–21437
- Göke, R., Fehman, H. C., Linn, T., Schmidt, H., Krause, M., and Göke, B. (1993) *J. Biol. Chem.* **268**, 19650–19655
- Kawai, K., Suzuki, S., Murayama, Y., Mukai, H., Ohashi, S., and Yamashita, K. (1988) *Biomed. Res.* **9**, 213–217
- Gefel, D., Hendrick, G. K., Mosjov, S., Habener, J. F., and Weir, G. C. (1990) *Endocrinology* **126**, 2164–2168
- Ohneda, A., Ohneda, K., Ohneda, M., Koizumi, F., Ohashi, S., Kawai, K., and Suzuki, S. (1991) *Tohoku J. Exp. Med.* **165**, 209–221
- Kawai, K., Watanabe, Y., Suzuki, S., Ohashi, S., Mukai, H., and Yamashita, K. (1991) *Diabetes* **40**, 71A
- Mosjov, S. (1992) *Int. J. Pept. Protein Res.* **40**, 333–343
- Yanaihara, M., Mochizuki, T., Hoshino, M., Suzuki, M., Nagashima, T., Ishikawa, J., Sato, K., and Takatsuka, N. (1990) in *Gastrointestinal Endocrinology: Receptors and Post-receptor Mechanisms* (Thompson, J. C., eds) pp. 359–371, Academic Press, New York
- Gausepohl, H., Kraft, M., and Frank, R. (1988) in *Peptides 1988* (Jung, G., and Bayer, E., eds) pp. 241–243, Walter de Gruyter & Co., Berlin, Germany
- Vinther, A., Adelhorst, K., and Kirk, O. (1993) *Electrophoresis* **14**, 486–491
- Thorell, J. I., and Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369
- Drejer, K., Kruse, V., Larsen, U. D., Hougaard, P., Bjørn, S., and Gammeltoft, S. (1991) *Diabetes* **40**, 1488–1495
- O'Donnel, M., Garippa, R. J., O'Neil, N. C., Bolin, D. R., and Cottrell, J. M. (1990) *J. Biol. Chem.* **265**, 6389–6392
- Epand, R. M., and Bryszewska, M. (1990) in *Comprehensive Medicinal Chemistry* (Emmet, J. C., ed) Vol. 3, pp. 901–924, Pergamon Press, Oxford
- Kofod, H. (1992) *Acta Endocrinol.* **126**, Suppl. 1, 9–49
- Hruby, V. J., Krstenansky, J., Gysin, B., Peleton, J. T., Trivedi, D., and Mckee, R. L. (1986) *Biopolymers* **25**, 135–155
- Unson, C. G., Macdonald, D., Ray, K., Durrah, T. L., and Merrifield, R. B. (1991) *J. Biol. Chem.* **266**, 2763–2766