Determinants of Growth-Promoting Activity Reside in the A-Domain of Insulin-like Growth Factor I†

Zhen Zhen Chen, Gerald P. Schwartz, Lin Zong, G. Thompson Burke, Jacob D. Chanley, and Panayotis G. Katsoyannis*

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

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ABSTRACT: A two-chain, disulfide linked, insulin-like compound embodying the A-domain of insulin-like growth factor I (IGF-I) and the B-chain of insulin has been synthesized and characterized with respect to insulin-like biological activity and growth-promoting potency. The compound displays a potency of ca. 41% relative to insulin in assays for insulin-like activity (e.g., lipogenesis) but significantly higher activity than insulin, ca. 730% relative to insulin, in growth factor assays (e.g., thymidine incorporation). The compound is, however, a less potent growth factor than IGF-I itself, ca. 26.5% relative to IGF-I, and is not recognized by IGF carrier proteins. We conclude that structural features contained in the A-domain of IGF-I are primarily responsible for the growth-promoting ability displayed by IGF-I, while features in the B-domain are responsible for recognition by IGF carrier proteins.

The insulin-like growth factors IGF-I and IGF-II are polypeptide growth factors in human plasma [for reviews see Humbel (1984), Van Wyk (1984), and Nissley and Rechler (1984)] that show striking homology with proinsulin (Rinderknecht & Humbel, 1978a,b). Like proinsulin, these growth factors are single-chain polypeptides. They contain B- and A-domains which exhibit ca. 40% homology to the B- and A-chains of insulin. The amino-terminal B-domain is connected, via a smaller peptide, the C-domain (11 and 8 amino acid residues long in IGF-I and IGF-II, respectively), to the A-domain. Unlike proinsulin, the IGFs contain an extension peptide, the D-domain at the carboxyl terminus of the A-domain, an octapeptide in IGF-I, and a hexapeptide in IGF-II. The B- and A-domains of IGF-I are ca. 60% identical with the corresponding domains of IGF-II. The primary structure of human IGF-I is shown in Figure 1. The B-domain involves sequences 1–30, the C-domain sequences 31–41, the A-domain sequences 42–62, and the D-domain sequences 63–70. Three-dimensional models for the IGFs have been constructed by using molecular graphics (Blundell et al., 1978, 1980, 1982, 1983); these models suggest that the A- and B-domains and the hydrophobic core of these molecules can assume a conformation identical with that of insulin and proinsulin.

In view of their considerable homology and conformational similarity it is not surprising that the IGFs and insulin exhibit qualitatively similar biological activities. Their relative potencies, however, vary greatly depending on the cell type used for the assay and the particular activity being measured. Insulin is more potent than the IGFs in short-term metabolic effects (e.g., lipogenesis), whereas the IGFs are more potent than insulin in growth-promoting effects (Zapf et al., 1978; King & Kahn, 1981). The IGFs, in contrast to insulin, circulate in plasma bound to specific carrier proteins that appear to modulate their delivery to target tissues [for a review see Nissley and Rechler (1984)].

A program is under way in our laboratory aimed at the identification of the structural features of the IGFs associated with their distinctive biological activities (Katsoyannis et al., 1987). This goal is being approached through the synthesis and biological evaluation of disulfide-linked two-chain insulin-like molecules containing structural features of the IGFs and insulin. We have reported previously the synthesis and biological evaluation of three such hybrid molecules. One compound is an insulin in which the A-chain moiety is extended at the carboxyl terminus with the D-domain of IGF-II (Ogawa et al., 1984; DeVroede et al., 1985). A second compound is an insulin-like molecule in which the A-chain moiety is that of insulin and the B-chain moiety is the sequence corresponding to the B-domain of IGF-I (Figure 1, sequence 1–30) (Joshi et al., 1985a; DeVroede et al., 1985, 1986). The third compound is an insulin-like molecule in which the A-chain moiety is the A-chain of insulin extended at the carboxyl terminus with the D-domain of IGF-II and the B-chain moiety is the sequence corresponding to the B-domain of IGF-I (Joshi et al., 1985b). In the pesent paper we report the synthesis and biological evaluation of an insulin-like molecule in which the A-chain moiety is the sequence corresponding to the A-domain of IGF-I (Figure 1, sequence 42–62) and the B-chain moiety is that of insulin.

Experimental Procedures and Results

Details of materials and analytical procedures used are given in a previous publication (Schwartz et al., 1981). ^{125}I-labeled insulin and [3-^{3}H]glucose were obtained from Du Pont NEN Research Products. A radioimmunoassay kit for insulin was purchased from Amersham. Fatty acid free bovine serum albumin was obtained from Boehringer Mannheim, and crystalline bovine insulin and fraction V bovine serum albumin were from Sigma. Collagenase type II was a product of Worthington, obtained from Cooper Biomedical. The scintillation fluids Filtron-X and Soluscint-O were products of National Diagnostics. Cellulose acetate filters, 0.2-μm pore size, were obtained from Sartorius Membranfilter.

Receptor Binding. A fraction enriched in insulin receptors was prepared from the livers of fasted rats essentially as previously described (Horvat et al., 1975). The ability of a test compound to compete with ^{125}I-labeled insulin in binding to insulin receptors was determined by a filtration technique.

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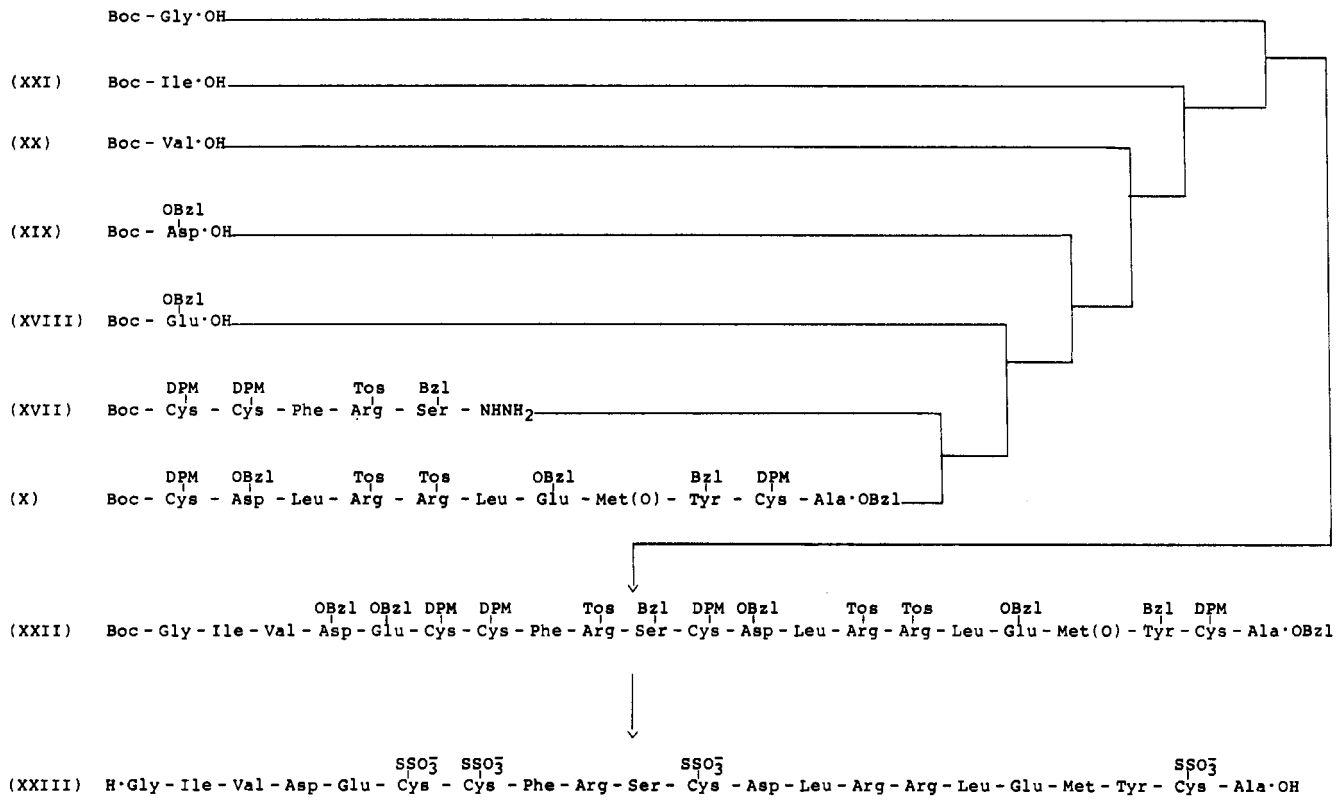
Chart I



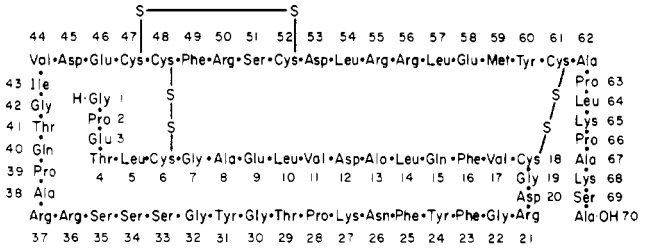


FIGURE 1: Structure of human IGF-I.

Details of the method were described recently (Kitagawa et al., 1984a).

Lipogenesis. Adipocytes were isolated from rat fat pads by incubation with collagenase. The ability of a test compound to stimulate the conversion by adipocytes of [3-^{3}H]glucose into organic-extractable material was then determined. Details of the assay procedure were recently described (Kitagawa et al., 1984).

Radioimmunoassay. A commercial radioimmunoassay kit containing guinea pig antiserum to insulin, prereacted with rabbit antiserum to guinea pig immunoglobulin, was used to assay the ability of a test compound to compete with ^{125}I-labeled insulin for antibodies to insulin. Immune precipitates were collected and washed on cellulose acetate filters, which were dried and dissolved in Filtron-X for liquid scintillation counting. The data were analyzed by the method of Hales and Randle (1963), and potency was calculated from the slopes of the resulting straight-line plots.

Growth factor activity assays are described elsewhere (Tseng et al., 1987).

General Aspects of Synthesis of the Insulin-like Compound Consisting of the B-Chain of Bovine Insulin and an A-Chain Corresponding to the A-Domain of Human IGF-I. This compound was synthesized by the interaction of the S-sulfonated forms of bovine insulin B-chain and human IGF-I A-chain domain (Figure 1, sequence 41–62) at pH 10.5 in the presence of dithiothreitol (Chance et al., 1981). The S-sulfonated bovine B-chain was prepared from bovine insulin as described previously (Katsoyannis et al., 1967). The synthesis of the S-sulfonated IGF-I A-chain domain, patterned after our synthesis of the insulin A-chain [e.g., Katsoyannis et al. (1966)], involved as the key intermediate the construction of the protected heneicosapeptide (XXII) containing the entire amino acid sequence of the IGF-I A-chain domain. The preparation of the latter protected polypeptide was carried out by a combination of the stepwise elongation and fragment condensation approaches [for a review see Katsoyannis and Schwartz (1977)]. Deblocking of the protected heneicosapeptide on exposure to liquid hydrogen fluoride, in the presence of 2-mercaptopyridine and *p*-cresol, and sulfitolysis of the resulting product led to the S-sulfonated IGF-I A-chain domain. The overall synthesis is illustrated in Chart I.

Glycyl-L-*isoleucyl*-L-*valyl*-L-*aspartyl*-L-*glutamyl*-*S*-*sulfo*-L-*cysteinyl*-*S*-*sulfo*-L-*cysteinyl*-L-*phenylalanyl*-L-*arginyl*-L-*seryl*-*S*-*sulfo*-L-*cysteinyl*-L-*aspartyl*-L-*leucyl*-L-*arginyl*-L-*arginyl*-L-*leucyl*-L-*glutamyl*-L-*methionyl*-L-*tyrosyl*-*S*-*sulfo*-L-*cysteinyl*-L-*alanine* (*Human IGF-I A-Chain Domain S-Sulfonate*) (*XXIII*).[1] The deblocking of the protected heneicosapeptide XXII upon treatment with liquid hydrogen fluoride in the presence of *p*-cresol and 2-mercaptopyridine and the oxidative sulfitolysis of the resulting product were accomplished essentially by the procedure used in the synthesis of the human insulin B-chain S-sulfonate (Schwartz & Katsoyannis, 1973). In a typical experiment the protected he-

[1] Abbreviations: Ac, acetyl; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; DPM, diphenylmethyl; EDTA, ethylenediaminetetraacetic acid; EEDQ, 2-ethyl-1-(ethoxycarbonyl)-1,2-dihydroquinoline; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; TEA, triethylamine; TFA, trifluoroacetic acid; Tos (tosyl), *p*-toluenesulfonyl; Tris, tris(hydroxymethyl)aminomethane; troc, β,β,β-trichloroethyloxycarbonyl. Compounds designated by Roman numerals are described fully in the supplementary material.

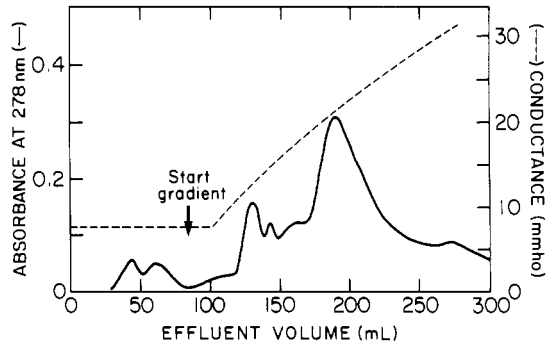


FIGURE 2: Chromatography of crude S-sulfonated human IGF-I A-chain domain on a 1.2 × 25 cm Cellex-E column with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient. The column was monitored with a Gilford recording spectrophotometer and a conductivity meter. The S-sulfonated chain was recovered by dialysis and lyophilization of the effluent (170–250 mL).

neicosapeptide (395 mg) was treated with anhydrous liquid hydrogen fluoride (ca. 15 mL) containing 2-mercaptopyridine (300 mg) and *p*-cresol (1.5 g) at −15 °C for 15 min and at 0 °C for 1 h. The hydrogen fluoride was then removed, and the residue was dried under high vacuum, over KOH, and triturated with AcOEt (4 × 40 mL) and petroleum ether (2 × 40 mL). This material was dissolved in 8 M guanidine hydrochloride (30 mL), and to this solution, adjusted to pH 8.7 by the addition of solid Tris, were added sodium sulfite (1.1 g) and freshly prepared sodium tetrathionate (1.6 g). The mixture was stirred at room temperature for 4 h, placed in Spectrapor membrane tubing No. 3, dialyzed against two changes of distilled water (4 L each) and three changes of 1 N AcOH (2 L each) at 4 °C for 24 h, and lyophilized (weight ca. 250 mg).

For purification the lyophilized material (52 mg) was dissolved in 10 mL of 0.1 M Tris-HCl buffer (pH 7), and this solution, adjusted to pH 7 by the addition of solid Tris, was placed on a Cellex-E column (1.2 × 25 cm) equilibrated with the same buffer. Elution of the column (Ferderigos et al., 1979) was carried out with a linear NaCl gradient formed by adding to the above buffer (200 mL) 0.5 M NaCl in the same buffer (200 mL). The chromatographic pattern, as monitored by a Gilford recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen) is shown in Figure 2. The effluent corresponding to the main peak (170–250 mL) was collected, dialyzed in Spectrapor No. 3 tubing against four changes of distilled water (4 L each) at 4 °C for 24 h, and lyophilized to give the purified human IGF-I A-chain domain S-sulfonate as a fluffy white powder (weight 31 mg).

Amino acid analysis after acid hydrolysis gave a composition, expressed in molar ratios, in good agreement with the theoretically expected values (Table I). Digestion of the synthetic chain with aminopeptidase M and amino acid analysis of the digest gave the molar ratios shown in Table I. It is apparent that the synthetic material was completely digested by the enzyme, indicating that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes.

S-Sulfonated B-Chain of Bovine Insulin. This compound was prepared by oxidative sulfitolysis of bovine insulin followed by CM-cellulose chromatography of the resulting S-sulfonated A- and B-chain by the precedure described previously (Katsoyannis et al., 1967) with the only difference being that the sulfitolysis was carried out for 3 h instead of 24 h.

Synthesis and Isolation of the Insulin-like Compound Consisting of the A-Chain Domain of IGF-I and the B-Chain of Insulin. The synthesis of this hybrid molecule was accomplished by the interaction of the S-sulfonated derivatives of the A-chain domain of IGF-I and the B-chain of insulin in the presence of dithiothreitol at pH 10.5 (Chance et al., 1981). Briefly, human IGF-I A-chain domain S-sulfonate (30 mg) and bovine B-chain S-sulfonate (25 mg) were dissolved in 0.1 M glycine buffer (pH 10.5; 9 mL), and to this solution cooled to 4 °C was added dithiothreitol (7 mg). After 24 h at 4 °C, the mixture was diluted with acetic acid (1 mL) and chromatographed on a Sephadex G-50 column (2.5 × 60 cm), equilibrated and eluted with 1 M acetic acid. The effluent from the peak representing the monomer fraction, which contains the biologically active material (insulin assays), was isolated and lyophilized (weight ca. 15 mg). This product was subjected to reversed-phase HPLC using a Vydac 218 TP column (0.45 × 25 cm) connected to a Laboratory Data Control liquid chromatography system. Batches (ca. 5 mg of protein each) were chromatographed at a flow rate of 0.5 mL/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 60 min. The chromatographic profile is shown in Figure 3. The effluent containing the active material (ca. 36 min) was collected, concentrated to a small volume, and rechromatographed by using the same column and a 20–35% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min over 85 min. The effluent exhibited a single sharp peak (not shown) at ca. 36 min. This is the approximate time of elution of natural insulin under these conditions. From the combination mixture of the A- and B-chains described above, 3 mg of highly purified material was obtained. Amino acid analysis of the purified material after acid hydrolysis gave the following composition, expressed in molar ratios, in agreement with the theoretically expected values (shown in parentheses): $Asp_{2.6(3)}Thr_{0.9(1)}Ser_{1.9(2)}Pro_{0.8(1)}Glu_{5.4(5)}Gly_{4.0(4)}Ala_{2.9(3)}Val_{2.8(4)}Met_{0.6(1)}Ile_{0.6(1)}Leu_{6.0(6)}Tyr_{2.7(3)}Phe_{3.5(4)}Lys_{0.9(1)}His_{2.3(2)}Arg_{3.8(4)}$. Cys was not determined.

Table I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Aminopeptidase M) of the S-Sulfonated Human IGF-I A-Chain Domain

	acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found
Arg	3	2.9	3	3.0
Asp	2	2.0	2	1.6
Ser	1	0.6	1	0.9
Glu	2	2.1	2	1.9
Gly	1	0.9	1	0.9
Ala	1	1.0	1	0.9
$^1/_2$-Cys	4	ND[b]	0	0
Val	1	0.6	1	1.0
Met	1	1.0	1	1.0
Ile	1	0.5	1	1.0
Leu	2	2.0	2	2.0
Tyr	1	0.8	1	1.0
Phe	1	0.9	1	1.0
S-sulfo-Cys	0	0	4	4.5

[a] Number of amino acid residues per molecule. [b] ND, not determined.

Biological Evaluation of the Insulin-like Compound Consisting of the B-Chain of Insulin and the A-Chain Domain of IGF-I. Figure 4 shows the effect of bovine insulin and of the insulin-like hybrid compound upon the binding of ^{125}I-labeled insulin to insulin receptors in rat liver plasma membranes. Inhibition of ^{125}I-labeled insulin binding is concentration dependent, producing nearly parallel dose–response curves for the two competing compounds. The calculated potency of the hybrid compound, on the basis of three separate experiments, is 41.1 ± 2.3% relative to bovine insulin. Figure

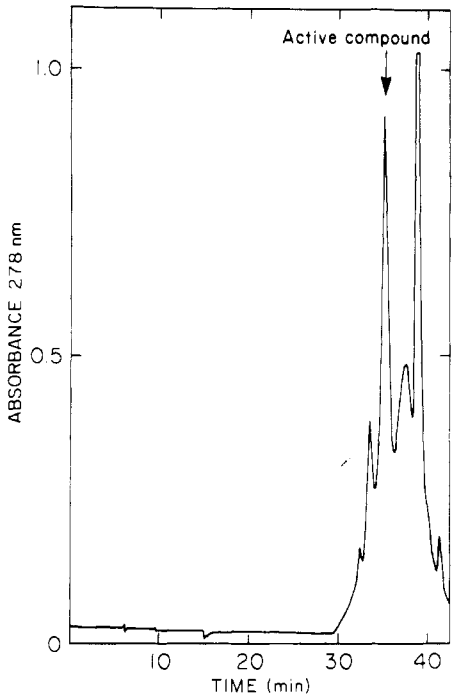


FIGURE 3: Reversed-phase HPLC of the combination mixture of the S-sulfonated human IGF-I A-chain domain and S-sulfonated bovine insulin B-chain on a 0.45 × 25 cm Vydac 218 TP column at 0.5 mL/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 60 min. The active material eluted at ca. 36 min was recovered by concentration of the effluent.

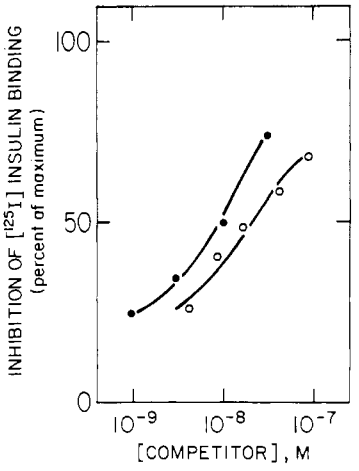


FIGURE 4: Effect of bovine insulin (●) and the insulin-like hybrid compound (○) on the binding of ^{125}I-labeled insulin to rat liver plasma membranes (see Experimental Procedures and Results). The inhibition of ^{125}I-labeled insulin binding, expressed as percent of maximum, is presented as a function of the molar concentration of the competing compound. The data points represent the means of triplicate determinations in a typical experiment that was performed 3 times. In this experiment, the maximum binding represents 4.8% of the input radioactivity.

5 illustrates the effect of bovine insulin and the hybrid compound upon the stimulation of lipogenesis in isolated rat adipocytes. Stimulation is concentration dependent, both agonists produce the same maximum stimulation, and the dose–response curves are essentially parallel. The calculated potency of the hybrid compound, on the basis of five separate experiments, is 41.2 ± 7.1% relative to bovine insulin, in good agreement with the value obtained in receptor-binding assays.

In radioimmunoassays (data not shown) the hybrid molecule was very weak, assaying at 0.17% relative to bovine insulin.

The hybrid was assayed for growth-promoting activity compared with insulin and IGF-I (Tseng et al., 1987). The

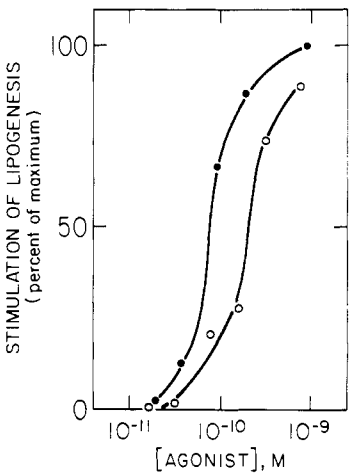


FIGURE 5: Effect of bovine insulin (●) and the insulin-like hybrid compound (○) on lipogenesis in rat adipocytes. The stimulation of lipogenesis, expressed as percent of maximum, is presented as a function of the molar concentration of the stimulating compound. The data points represent the means of triplicate determinations in a typical experiment that was performed 5 times. In this experiment, zero and 100% stimulation refer respectively to 6.5 and 146 nmol of glucose converted into organic-extractable form h^{-1} (mg of cells)$^{-1}$.

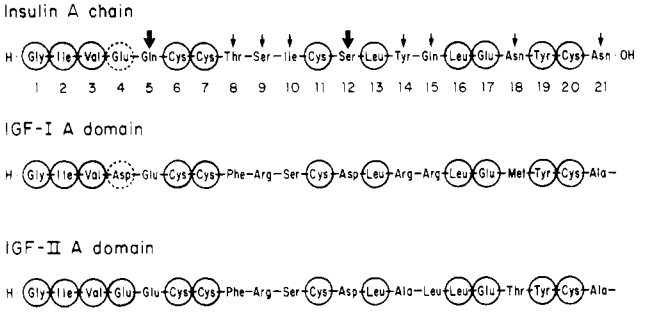


FIGURE 6: Comparison of the primary structure of the A-chain of human insulin with that of the A-domain of human IGF-I and IGF-II. The numbering for insulin is given below that sequence. Amino acid residues common to all three molecules are enclosed in solid circles, while convertive variations are enclosed in broken circles. The arrows refer to points of nonhomology that are discussed in the text.

compound displayed ca. 730% of the potency of insulin and ca. 26.5% of the activity of IGF-I in stimulating the incorporation of [^{3}H]thymidine in chick embryo fibroblasts. The hybrid compound showed no reactivity toward IGF carrier proteins derived from rat serum, a property exhibited by IGF-I but not by insulin.

Discussion

The present hybrid molecule consists of the B-chain of insulin joined by disulfide bonds to a 21 amino acid residue A-chain corresponding to the A-domain of IGF-I (positions 42–62 in IGF-I, Figure 1). Of these 21 amino acid residues, 11 are homologous with insulin (Figure 6); these include the 4 cysteine residues, which are reported to form identical disulfide bridges in IGF-I, IGF-II, and insulin (Blundell & Wood, 1982; Blundell et. al, 1983). It is of particular interest to note that these homologous residues include Gly A^1, Tyr A^{19}, and Ile A^2. Gly A^1 and Tyr A^{19} are members of a putative insulin receptor-binding region (Blundell et al., 1972; Blundell & Wood, 1975; Pullen et al., 1976). Blundell et al. (1978, 1980, 1982, 1983) have reported on the basis of computer graphics studies that the IGFs can assume an insulin-like conformation. We have previously noted that the interaction between the side chains of Ile A^2 and Tyr A^{19} may be among the most critical contributors to the stabilization of the con-

formation of insulin (Kitagawa et al., 1984a,b). These considerations suggest that the above-mentioned hybrid could also adopt an insulin-like conformation and lead to the prediction that the present hybrid molecule might display significant insulin activity.

The synthesis of the hybrid compound was patterned after the procedures employed previously in this laboratory for the synthesis of insulin and insulin analogues [for a review, see Katsoyannis (1981)]. This involves combination of the A- and B-chains by the interaction of their respective S-sulfonated derivatives at pH 10.5 in the presence of thiols. Under these conditions, the formation of "mispaired" disulfide bridges between the A- and B-chains is strongly disfavored; synthetically prepared mispaired insulin analogues are extensively converted into the natural hormone and various polymeric products under these conditions (Sieber et al., 1978). Furthermore, the chromatograpic behavior of the mispaired isomers differs distinctly from that of insulin, and these isomers display greatly reduced biological activity. In contrast, the present compound is the only active species present in the combination mixture of the A- and B-chains, and it elutes upon HPLC as a sharp peak in a position comparable to that of natural insulin. The data indicate that, with regard to the short-term metabolic effects such as binding to the insulin receptor and stimulation of lipogenesis, the hybrid molecule is a potent insulin, although less potent than the natural hormone (ca. 41%). These observations do not, in themselves, provide absolute proof of the positions of the disulfide bridges in the synthetic compound; such proof could come only from extensive structural analysis requiring substantial quantities of material. However, the synthetic methods employed, the close structural similarlity of the A-chain of insulin and the A-domain of IGF-I, and the observed biological and physical properties of the compound argue strongly for an insulin-like structure.

With regard to the very low potency of the hybrid molecule in insulin radioimmunoassay, it should be noted that the A-chain loop (residues A^8, A^9, and A^{10}) is nonhomologous with insulin (Figure 6). This region has been implicated as an important immunological determinant in insulin (Arquilla et al., 1969; Schroer et al., 1983).

In contrast to the modestly reduced potency relative to insulin in short-term metabolic assays, the hybrid compound exhibited greatly increased activity in growth-factor assays, ca. 730% relative to insulin and ca. 26.5% relative to IGF-I in thymidine incorporation. The hybrid compound is not recognized in IGF carrier proteins; such recognition is characteristic of the IGFs but not of insulin. We have previously reported that hybrid molecules containing the B-domain of IGF-I and either the A-chain of insulin or the A-chain of insulin extended at the C terminus with the D-domain of IGF-II have considerably reduced growth-promoting activity, ca. 8% relative to insulin and ca. 0.25% relative to IGF-I, 2 orders of magnitude lower than the present compound (DeVroede et al., 1986; Joshi et al., 1985b). Both these molecules, however, are recognized by IGF carrier proteins (DeVroede et al., 1985).

The properties of the above-mentioned hybrid molecules demonstrate that the growth-promoting activity of IGF-I is associated with the A-domain of the molecule. The B-domain, in contrast, does not contribute directly to growth-factor activity, but contributes features permitting recognition of the growth factor by specific carrier proteins. The D-domain is without obvious effect upon either phenomenon (Joshi et al., 1985b; Tseng et al., 1987).

We have noted that the growth-promoting potency of the present two-chain hybrid compound is lower than that of the single-chain natural IGF-I (ca. 26.5%). In addition, preliminary results (unpublished data) indicate that an insulin-like compound in which both the A- and B-chains correspond to the A- and B-domains of IGF-I, respectively, displays a growth-promoting activity similar to that of the present compound. This suggests that the C-domain, which bridges the B- and A-domains in natural IGF-I but which is absent from the two-chain molecules, may have a role in the expression of growth-promoting activity. The C-domain may affect the disposition of the A- and B-domains in such a way as to enhance the growth-promoting activity of natural IGF-I. A similar role may be attributed to the D domain in view of the fact that this domain does not contain any determinants directly involved in growth-promoting activity (Joshi et al., 1985b; Tseng et. al, 1987). In addition, the presence of a free α-NH_2 group in the A-chain and free α-COOH groups in the A- and B-chains of the hybrid molecule could play a role in reducing the growth-promoting activity of the present compound relative to natural IGF-I. In line with these considerations it is reasonable to assume that the present hybrid compound may exhibit close to the maximum growth factor activity achievable in a two-chain IGF molecule.

We have asserted that the A-domain of IGF-I is primarily responsible for conferring growth-promoting properties upon the molecule. Thus, insulin, embodying its specific A- and B-chains, is a weak growth factor, whereas the present hybrid molecule, embodying the A-domain of IGF-I and the B-chain of insulin, is quite potent as a mitogen. The key to this divergence must reside in the differences between the sequences of the insulin A-chain and the IGF-I A-domain. A comparison of the primary structure of the A-domain of IGF-I with the A-chain of insulin (Figure 6) reveals that 10 of the 21 residues are nonhomologous: $Glu^4Gln^5 \rightarrow$ AspGlu; $Ala^8Ser^9Val^{10} \rightarrow$ PheArgSer; $Ser^{12} \rightarrow$ Asp; $Tyr^{14}Gln^{15} \rightarrow$ ArgArg; $Asn^{18} \rightarrow$ Met; and $Asn^{21} \rightarrow$ Ala. In preliminary studies (unpublished data), we have prepared an analogue of the A-domain of IGF-I in which the residues $Phe^8Arg^9Ser^{10}$ were replaced by the corresponding sequence in sheep insulin, AlaGlyVal. When combined with the B-chain of natural insulin, the hybrid molecule produced displayed growth-promoting properties comparable to those of the present compound. It thus seems reasonable to assume that this sequence may not be importantly involved in the expression of growth factor properties.

The difference between $Tyr^{14}Gln^{15}$ in insulin and ArgArg in IGF-I is profound and would seem to be a likely candidate for conferring a different spectrum of biological activity. In the X-ray model of insulin, Gln A^5 is hydrogen-bonded with Gln A^{15} (Blundell et al., 1971, 1972). An interaction between Glu^5 and Arg^{15} in the A-chain of the present molecule might be more stable and responsible for recognition of the IGF-I receptor. IGF-II, however, is different from both IGF-I and insulin in these positions, having the sequence AlaLeu (Figure 6). Of course, IGF-I and IGF-II react with discrete receptors, and this difference in sequence could reflect that fact; nonetheless, both are growth factors, and they exhibit cross-reactivity with each other's receptors (Nissley & Rechler, 1984). On this basis, we do not consider this area of nonhomology promising as a primary explanation for the different biological activity of IGF-I and insulin.

The substitution of Met for Asn at position 18 also could have an important effect. However, the IGF produced by recombinant DNA technology (AmGen, Thousand Oaks, CA) has threonine at this position (59 in IGF-I numbering) and

is indistinguishable from natural IGF-I. The substitution of Asp for Glu in position A^4 of insulin is a rather conservative variation, and the substitution of the C-terminal Asn residue in insulin with Ala in the present compound is unlikely to have a major influence upon receptor preference (Chu et al., 1987).

The remaining two differences in sequence between the A-chain of insulin and the A-domain of IGF-I are $Gln^5 \rightarrow Glu$ and $Ser^{12} \rightarrow Asp$. Interestingly, these residues are conserved between IGF-I and IGF-II. These differences in sequence involve changes between neutral polar residues in insulin and acidic residues in IGF-I. While one would anticipate that these substitutions would be essentially conservative regarding the conformation of the segment of the molecule within which they reside (Chou & Fasman, 1974; Levitt, 1978), their interaction through intramolecular salt-bridge formation could nonetheless cause disturbances in the overall conformation of the molecules, conferring a preference for different receptors. It is also possible that a direct interaction of these residues with complementary charges in the IGF receptor could contribute to the preference of that receptor for IGF-I versus insulin.

In conclusion, it is striking that a two-chain insulin-like molecule embodying less than one-third of the amino acid sequence of natural IGF-I and displaying considerable homology to insulin is a strong mitogen. Apparently, the compound can assume a conformation similar to that of IGF-I, and the presence of specific amino acid residues associated with the A-domain of IGF-I can, in concert, result in the expression of growth-promoting activity.

Acknowledgments

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Supplementary Material Available

Complete synthetic details of compounds I–XXII, including references (13 pages). Ordering information is given on any current masthead page.

Registry No. I, 115227-96-8; I (deblocked), 115228-12-1; II, 115227-97-9; III, 115227-98-0; III (deblocked), 115244-73-0; IV, 115227-99-1; IV (deblocked), 115228-13-2; V, 115228-00-7; V (deblocked), 115228-14-3; VI, 115228-01-8; VI (deblocked), 115228-15-4; VII, 115228-02-9; VII (deblocked), 115228-16-5; VIII, 115244-72-9; VIII (deblocked), 115228-17-6; IX, 115228-03-0; IX (deblocked), 115244-74-1; X, 115228-04-1; X (deblocked), 115244-76-3; XI, 115228-05-2; XII, 115228-06-3; XII (deblocked), 115228-18-7; XIII, 115228-07-4; XIII (deblocked), 115244-75-2; XIV, 115228-08-5; XIV (deblocked), 115228-19-8; XV, 115228-09-6; XVI, 115228-10-9; XVI (azide), 115228-20-1; XVII, 115269-55-1; XVII (deblocked), 115244-77-4; XVIII, 115269-57-3; XVIII (deblocked), 115269-56-2; XIX, 115269-59-5; XIX (deblocked), 115269-58-4; XX, 115269-61-9; XX (deblocked), 115269-60-8; XXI, 115269-63-1; XXI (deblocked), 115269-62-0; XXII, 115269-64-2; XXIII, 115228-11-0; XXIII (insulin B-chain derivative), 115269-65-3; Ala·OBzl, HCl, 5557-83-5; Boc-Cys(DPM)·OH, 21947-97-7; Boc-Tyr(Bzl)·OH, 2130-96-3; Boc-Met(O)·OH, 34805-21-5; Boc-Glu(OBzl)·ONp, 7536-59-6; Boc-Leu·ONp, 3350-19-4; Boc-Arg(Tos)·OH, 13836-37-8; Boc-Asp(OBzl)·ONp, 26048-69-1; Troc·$NHNH_2$, 31434-96-5; Boc-Ser(Bzl)·OH, 23680-31-1; Boc-Phe·OH, 13734-34-4; Boc-Val·OH, 13734-41-3; Boc-Ile·OH, 13139-16-7; Boc-Gly·OH, 4530-20-5; IGF-1, 67763-96-6; S-sulfonated insulin B-chain, 18175-60-5.

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Activation and Irreversible Binding of Regiospecifically Labeled Catechol Estrogen by Rat Liver Microsomes: Evidence for Differential Cytochrome P-450 Catalyzed Oxidations†

Peter H. Jellinck* and Jack Fishman
The Rockefeller University, New York, New York 10021
Received October 1, 1987; Revised Manuscript Received April 6, 1988

ABSTRACT: Estradiol and 2-hydroxyestradiol labeled with ^3H at different positions in rings A or B were incubated with male rat liver microsomes, and their oxidative transformation was followed by the transfer of ^3H into ^3H_2O. ^{14}C-Labeled estrogen or catechol estrogen was used to determine the fraction that becomes bound covalently to microsomal protein. The further metabolism of 2-hydroxyestradiol involves activation of the steroid at C-4 and, to a much lesser extent at C-1, by a cytochrome P-450 mediated reaction as indicated by the effects of NADPH, spermine, SKF-525A, and CO in the microsomal system. Glutathione promoted the loss of ^3H from C-4 of either estradiol or 2-hydroxyestradiol but had less effect on this reaction at C-1 and inhibited it at C-6,7. It also abolished the irreversible binding of ^{14}C-labeled estradiol and 2-hydroxyestradiol to microsomal protein. NADPH was needed specifically for glutathione to exert its effect both on the transfer of ^3H into ^3H_2O and on the formation of water-soluble products from catechol estrogen by rat liver microsomes. It could not be replaced by NADP, NAD, or NADH. Ascorbic acid inhibited these enzymatic reactions but did not affect significantly the initial 2-hydroxylation of estradiol. Evidence is also provided for the further hydroxylation of 2-hydroxyestradiol at C-6 (or C-7). These results indicate that cytochrome P-450 activates catechol estrogens by an electron abstraction process.

In recent years, the possibility has emerged that some of the actions of estradiol (E_2) may involve significant contributions from its metabolites (Fishman & Martucci, 1978). In particular, the catechol estrogens formed by 2- or 4-hydroxylation of the parent estrogens have been shown to modulate a number of important endocrine functions when administered in pharmacological doses (MacLusky et al., 1981).

Relatively little is known about the further oxidative metabolism of the catechol estrogens although their conversion to water-soluble products (Jellinck & Smith, 1974) and their irreversible binding to liver microsomal protein in the presence of oxygen and NADPH or a superoxide generating system have been reported (Nelson et al., 1976). The synthesis of 2-hydroxyestradiol (2-OHE_2) labeled regiospecifically with ^3H at C-1, C-4, or C-6,7 has made it possible to obtain information about the site of interaction of the steroid with nucleophiles such as glutathione (GSH) (Jellinck et al., 1984) without having to isolate and characterize the products, and more recently, we have reported on the possible mechanism of such adduct formation (Jellinck et al., 1986). In this paper, we assess the contribution of cytochrome P-450 to the further metabolism of the catechol estrogens at specific carbon atoms of the steroid molecule by rat liver microsomes using agents known to increase or inhibit the activity of this enzyme system. We also report on studies of the role of microsomal monooxygenases on the interaction of [4-^{14}C]2-OHE_2 with GSH and show that this reaction is dependent specifically on NADPH. In addition, GSH was found to prevent the irreversible binding of the catechol estrogen to microsomal protein.

Experimental Procedures

Materials. 2-^3H- and 4-^3H-labeled estradiols were prepared and purified as described previously (Jellinck et al., 1984). [4-^{14}C]E_2 (57 mCi/mmol; New England Nuclear Corp., Boston, MA) was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific activity of 2–3 mCi/mmol and kept

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* Address correspondence to this author at the Department of Biochemistry, Queen's University, Kingston, Ontario, K7L 3N6 Canada.

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