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# Synthesis of an Insulin-like Compound Consisting of the A Chain of Insulin and a B Chain Corresponding to the B Domain of Human Insulin-like Growth Factor I†

Satish Joshi, G. Thompson Burke, and Panayotis G. Katsoyannis*
*Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029*
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ABSTRACT: An insulin-like hybrid molecule consisting of the A chain of insulin and a B chain corresponding to the B domain of human insulin-like growth factor I (growth factor I sequence 1–30) has been synthesized essentially by the procedures developed in this laboratory for the synthesis of insulin and analogues. The hybrid competed with $^{125}$I-insulin for insulin receptors in rat liver plasma membranes and was a full agonist in stimulating incorporation of [3-$^{3}$H]glucose into lipids in rat adipocytes. In both assays, the compound displayed ca. 2% of the potency of insulin. The compound was recognized by anti-insulin antibodies but was only ca. 0.25% as potent as insulin in this activity. The hybrid exhibited growth-promoting activity in fibroblasts, displaying 3–8% of the activity of insulin. In contrast, the compound was recognized by insulin-like growth factor carrier proteins, a property not associated with insulin. Two points of nonhomology between the B chain of insulin and the B domain of insulin-like growth factor I are considered in connection with these observations.

The insulin-like growth factors I and II (IGF-I and IGF-II) are polypeptides chemically related to proinsulin (Rinderknecht & Humbel, 1978a,b). The primary sequences of the IGFs contain "B- and A-chain domains", exhibiting ca. 40% homology to the B and A domains of proinsulin and are separated by a short connecting peptide (the "C region"), 12 and 8 amino acid residues long in IGF-I and IGF-II, respectively. Unlike proinsulin, the IGFs contain an extension peptide (the "D region") at the carboxyl terminus, an octapeptide in IGF-I and a hexapeptide in IGF-II. Three-dimensional models for IGF-I and IGF-II constructed by using molecular graphics (Blundell et al., 1978, 1983) suggest that these molecules can assume conformations identical with that of insulin as far as the A- and B-chain domains and the hydrophobic core are concerned. The considerable structural homology of insulin and the IGF's is reflected in the functional behavior of these compounds. Insulin and the IGF's exhibit, qualitatively, a similar spectrum of biological activities. Insulin is more potent than the IGF's in insulin-like effects (i.e., lipogenesis and glucose oxidation) whereas the IGFs are more potent than insulin in growth-promoting effects. A program is under way in our laboratory for the synthesis of hybrid molecules containing structural features of the IGFs and insulin with the goal of identifying the regions of these molecules that contribute to their particular biological activities. We have recently reported the synthesis and biological evaluation of such a hybrid molecule, in which the A chain of insulin has been elongated at the carboxyl terminus with the D region of IGF-II (Ogawa et al., 1984). In this paper we describe the synthesis and biological evaluation of an insulin-like compound consisting of the A chain of insulin and a B chain corresponding to the B-chain domain of IGF-I.

## Experimental Procedures and Results

Details of materials and analytical procedures used in this investigation are given in a previous publication (Schwartz et al., 1981). The homogeneity of all the intermediate peptide derivatives was ascertained by thin-layer chromatography (TLC)[1] on 6060 silica gel (Eastman Chromagram Sheet) in two solvent systems: chloroform–methanol–water (89:10:1 and 45:10:1 v/v). Preparation of the Cellex-D (Bio-Rad Laboratories) column and the washing of the resin were carried out as described previously for the preparation of the comparable Ecteola–cellulose column (Ferderigos et al., 1979). For HPLC, a Laboratory Data Control chromatograph equipped with a gradient maker and a Waters μBondapak $C_{18}$ column (4 × 250 mm) was employed. A gradient of 0–63% acetonitrile in 0.1% aqueous TFA was used to elute the column. The gradient was applied at a flow rate of 2 mL/min over a 30-min period.

*Biological Evaluation.* The potency of the synthetic hybrid relative to bovine insulin was measured in three types of assays: insulin receptor binding in a rat liver plasma membrane fraction, in which relative potency is defined as the ratio of insulin to test compound required to displace 50% of specif-

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[1] Abbreviations: Ac, acetyl; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Bu$^t$, *tert*-butyl; CM, carboxymethyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; $Me_2SO$, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; PMB, *p*-methoxybenzyl; TEA, triethylamine; TFA, trifluoroacetic acid; Tos (tosyl), *p*-toluenesulfonyl; TLC, thin-layer chromatography; Z, benzyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane. Compounds designated by Roman numerals are described fully in the supplementary material (see paragraph at end of paper regarding supplementary material).

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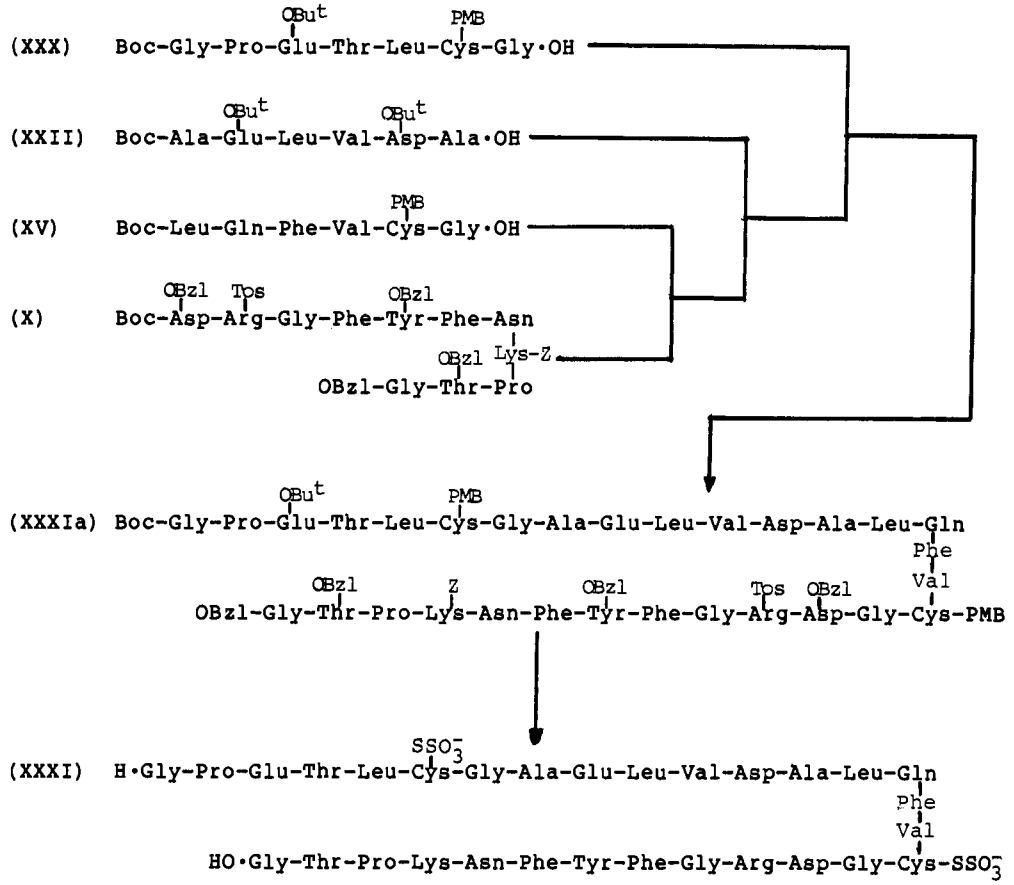
Scheme I




ically bound $^{125}$I-insulin; lipogenesis in rat adipocytes, in which relatively potency is defined as the ratio of insulin to test compound required to achieve 50% of the maximum conversion of [3-$^{3}$H]glucose into organic-extractable material; radioimmunoassay, in which insulin or the test compound was used to compete with $^{125}$I-insulin in binding to guinea pig antibodies raised against insulin. Complete details of all these assays, including sources of reagents and equipment, were described recently (Kitagawa et al., 1984).

*General Aspects of Synthesis of the Insulin-like Compound Consisting of the A Chain of Bovine Insulin and a B Chain Corresponding to the B Domain of Human IGF-I.* The synthesis of this hybrid was carried out by interaction of the S-sulfonated forms of bovine insulin A chain and human IGF-I B-chain domain (IGF-I sequence 1–30) (XXXI) either by the procedures described previously (Katsoyannis et al., 1967b,c) or by the method of Chance et al. (1981).

The S-sulfonated bovine A chain was prepared from bovine insulin as described previously (Katsoyannis et al., 1967a). The procedure for synthesis of the S-sulfonated B-chain domain of human IGF-I was patterned after our synthesis of human insulin B chain (Schwartz & Katsoyannis, 1973). The key intermediate in this synthesis is the protected triacontapeptide XXXIa embodying the entire amino acid sequence of the IGF-I B domain which was constructed by the fragment condensation approach [for a review, see Katsoyannis & Schwartz (1977a,b)] as follows. The C-terminal undecapeptide derivative X (sequence 20–30) was deblocked at the amino end and coupled with the adjacent hexapeptide derivative XV (sequence 14–19) to yield the protected heptadecapeptide (sequence 14–30). Deblocking of the latter peptide derivative at the amino acid and coupling with the adjacent hexapeptide fragment XXII (sequence 8–13) afforded the C-terminal tricosapeptide (sequence 8–30). This product was deblocked at the amino end and coupled with the N-terminal heptapeptide derivative XXX (sequence 1–7) to yield the desired protected triacontapeptide XXXIa. Coupling of the various fragments was carried out by *N,N'*-dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole (Konig & Geiger, 1970) or *N*-hydroxy-5-norbornene-2,3-dicarboximide (Fujino et al., 1974). Deblocking after each synthetic step was performed by exposure to TFA in the presence of anisole. Removal of all blocking groups from the protected triacontapeptide XXXIa was achieved upon exposure to 1 M trifluoromethanesulfonic acid–thioanisole in TFA in the presence of *m*-cresole (Yajima & Fujii, 1981) by using the modification described recently (Ogawa et al., 1984) to ensure complete deprotection. The resulting reduced triacontapeptide was converted to the S-sulfonated form XXXI by oxidative sulfitolysis. The overall synthesis is illustrated in Scheme I.

*Glycyl*-L-*prolyl*-L-*glutamyl*-L-*threonyl*-L-*leucyl-S-sulfo*-L-*cysteinylglycyl*-L-*alanyl*-L-*glutamyl*-L-*leucyl*-L-*valyl*-L-*aspartyl*-L-*alanyl*-L-*leucyl*-L-*glutaminyl*-L-*phenylalanyl*-L-*valyl-S-sulfo*-L-*cysteinylglycyl*-L-*aspartyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*phenylalanyl*-L-*asparaginyl*-L-*lysyl*-L-*prolyl*-L-*threonylglycine* (*Human IGF-I B-Chain Domain S-Sulfonate*) (*XXXI*). The deblocking of the protected triacontapeptide XXXIa was achieved by the procedure of Yajima & Fujii (1981) as modified recently (Ogawa et al., 1984), and the oxidative sulfitolysis of the resulting product was accomplished by the procedure used in the synthesis of human insulin B chain *S*-sulfonate (Schwartz & Katsoyannis, 1973). Briefly, a cooled (0 °C) solution of the protected triacontapeptide (125 mg) in 1 M trifluoromethanesulfonic acid–thioanisole in TFA (4 mL) containing *m*-cresol (1 mL) was stored for 10 min at 0 °C and for 1 h at room temperature.

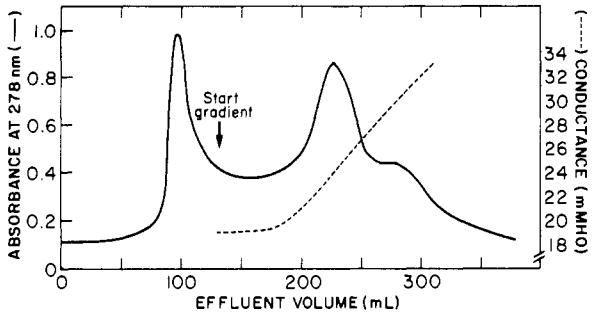



FIGURE 1: Chromatography of crude S-sulfonated human IGF-I B-chain domain on a 1.3 × 50 cm Cellex-D column equilibrated with 0.2 M $NH_4HCO_3$ and eluted with a linear gradient formed between 0.2 and 0.5 M $NH_4HCO_3$. The column was monitored by an ISCO recording spectrophotometer and by a conductivity meter. The S-sulfonated chain was recovered by lyophilization of the effluent (180–250 mL).

Subsequently, to this solution, cooled to −5 °C, was added dropwise a mixture of 8 M guanidine hydrochloride (25 mL) and concentrated $NH_4OH$ (4 mL). During this process, the temperature of the reaction mixture was kept below 5 °C. The resulting mixture (pH ~4.5) was extracted 3 times with ether (30 mL each) and to the aqueous layer, adjusted to pH 8.9 with $NH_4OH$, were added sodium sulfite (0.8 g) and freshly prepared sodium tetrathionate (0.4 g). The reaction mixture was stirred at room temperature for 4 h and then placed in Spectrapor membrane tubing no. 3 and dialyzed against four changes of distilled water (4 L each) at 4 °C for 24 h. Upon lyophilization of the dialysate, the crude IGF-I B-chain domain *S*-sulfonate was obtained as a white powder. For a preliminary purification, this material was dissolved in 0.015 M $NH_4HCO_3$ (5 mL) and chromatographed on a Sephadex G-15 column (4.2 × 50 cm) equilibrated and eluted with 0.015 M $NH_4HCO_3$. The effluent corresponding to the main peak, as monitored by an ISCO recording spectrophotometer (Model UA-5), was lyophilized, and the B-chain domain *S*-sulfonate was obtained as a white fluffy material: weight 95 mg. For further purification, a solution of this material (32 mg) in 0.2 M $NH_4HCO_3$ (2 mL) was adjusted to pH 9 with 1 N $NH_4OH$ and placed on a Cellex-D column (1.3 × 50 cm) equilibrated with 0.2 M $NH_4HCO_3$. Elution of the column was carried out first with 0.2 M $NH_4HCO_3$ and subsequently with a linear gradient formed by adding to 0.2 M $NH_4HCO_3$ (250 mL) and 0.5 M $NH_4HCO_3$ (250 mL). The effluent of the column, as monitored with an ISCO recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 1. The effluent under the major peak (180–250 mL) was collected and lyophilized to give the purified human IGF-I B-chain domain *S*-sulfonate as a white fluffy material: weight 17 mg.

Amino acid analysis of this material after acid hydrolysis gave a composition expressed in molar ratios in excellent agreement with the theoretically expected values (Table I). Digestion of the synthetic material with aminopeptidase M and amino acid analysis of the digest gave the ratios shown in Table I. It is apparent that the synthetic polypeptide chain was completely digested by the enzyme, indicating that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes. On electrophoresis on cellulose acetate membranes in Tris (0.024 M)–barbital (0.006 M)–sodium barbital (0.024 M) buffer, pH 8.8 and 250 V, the synthetic compound exhibited a single band (Pauly positive) (data not shown).

*S-Sulfonated A 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 chains by the procedure we have described previously (Katsoyannis et al., 1967a) with the only difference that the sulfitolysis was carried out for 3 h instead of 24 h.

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

| | acid hydrolysis | | enzymatic hydrolysis | |
|---|---|---|---|---|
| amino acid | theory | found | theory | found |
| Lys | 1 | 1.0 | 1 | 1.1 |
| Arg | 1 | 1.2 | 1 | 0.7 |
| Asp | 3 | 3.0 | 2 | 1.7 |
| Gln | 0 | 0 | 1 | *c* |
| Asn | 0 | 0 | 1 | *c* |
| Thr | 2 | 2.0 | 2 | *c* |
| Glu | 3 | 3.0 | 2 | 1.9 |
| Pro | 2 | 1.9 | 2 | 2.4 |
| Gly | 5 | 5.0 | 5 | 5.0 |
| Ala | 2 | 2.0 | 2 | 2.0 |
| $^1/_2$-Cys | 2 | *b* | 0 | 0 |
| Val | 2 | 2.0 | 2 | 2.0 |
| Leu | 3 | 3.0 | 3 | 3.2 |
| Tyr | 1 | 1.0 | 1 | 1.1 |
| Phe | 3 | 2.9 | 3 | 3.3 |
| *S*-sulfo-Cys | 0 | 0 | 2 | 2.0 |

[a] Number of amino acid residues per molecule. [b] Decomposed during hydrolysis and not determined. [c] Emerge on the same position and not determined.

*Synthesis and Isolation of the Insulin-like Compound Consisting of the A Chain of Insulin and the B-Chain Domain of IGF-I.* The synthesis of this compound by the combination of the insulin A chain and the B chain domain of IGF-I was carried out by two routes. The first route involved the reduction of the S-sulfonated bovine A chain with mercaptoethanol and the interaction of the resulting sulfhydryl form of that chain with the S-sulfonated B-chain domain of IGF-I according to the general procedure used in this laboratory for the synthesis of insulin and analogues (Katsoyannis et al., 1967b,c; Schwartz & Katsoyannis, 1976). The second route involved the interaction of the S-sulfonated derivatives of the insulin A chain and the B-chain domain of IGF-I in the presence of dithiothreitol, according to the procedure of Chance et al. (1981). A typical experiment according to the second route is as follows. To a cold solution of bovine A chain *S*-sulfonate (10 mg) and S-sulfonated B-chain domain of IGF-I (5 mg) in 0.1 M glycine buffer (pH 10.5; 4 mL) was added a cold solution of dithiothreitol (1.7 mg) in 0.1 M glycine buffer (4 mL). The reaction mixture was stirred at 4 °C for 24 h and then processed in exactly the same way as described previously in the synthesis of insulin by combination of the A and B chains (Katsoyannis et al., 1967b,c). Isolation and purification of the insulin-like compound from the reaction mixture was carried out by chromatography on a 0.9 × 23 cm CM-cellulose column with an acetate buffer ($Na^+$, 0.024 M, pH 3.3) and an exponential NaCl gradient according to the procedure we have described previously (Katsoyannis et al., 1967b,c) for the isolation of insulin from the combination mixture of the A and B chains. The elution pattern, as monitored by an ISCO recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 2. As was the case with the combination of natural or synthetic insulin A and B chains (Katsoyannis et al., 1967c), the chromatogram showed the presence of three main peaks. Dialysis and lyophilization of the fractions under the first peak (peak a, 50–75 mL of effluent) yielded a material which, upon amino acid analysis, showed an amino acid composition cor-

Table II: Amino Acid Composition[a] of Acid Hydrolysates of Peaks a–c Obtained from the CM-cellulose Chromatography with an Exponential Sodium Chloride Gradient

| amino acid | bovine A chain theory | peak a found | IGF-I B-chain domain theory | peak b found | insulin-like compound theory | peak c found |
|---|---|---|---|---|---|---|
| Lys | 0 | 0.3 | 1 | 0.9 | 1 | 1.0 |
| Arg | 0 | 0.3 | 1 | 0.9 | 1 | 0.9 |
| Asp | 2 | 2.3 | 3 | 2.9 | 5 | 4.9 |
| Thr | 0 | 0 | 2 | 0.2 | 2 | 1.8 |
| Ser | 2 | 1.9 | 0 | 0 | 2 | 2.4 |
| Glu | 4 | 4.0 | 3 | 3.0 | 7 | 6.8 |
| Pro | 0 | tr | 2 | 2.0 | 2 | 2.1 |
| Gly | 1 | 2.0 | 5 | 4.5 | 6 | 6.4 |
| Ala | 1 | 1.4 | 2 | 2.0 | 3 | 3.0 |
| $^1/_2$-Cys | 4 | *b* | 2 | *b* | 6 | *b* |
| Val | 2 | 1.7[c] | 2 | 1.9 | 4 | 3.1[c] |
| Ile | 1 | 0.5[c] | 0 | 0 | 1 | 0.3[c] |
| Leu | 2 | 2.3 | 3 | 2.8 | 5 | 5.0 |
| Tyr | 2 | 1.8 | 1 | 1.0 | 3 | 2.4 |
| Phe | 0 | 0.6 | 3 | 2.6 | 3 | 2.8 |

[a] Number of amino acid residues per molecule. [b] Not determined. [c] The low recovery of this amino acid residue is due to the well-known resistance of the Ile–Val bond to acid hydrolysis.

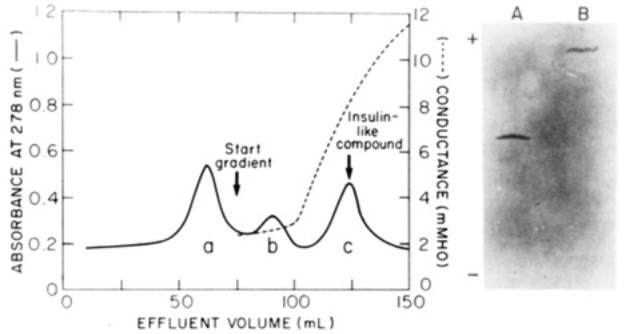



FIGURE 2: (Left) Chromatography of the insulin-like compound obtained by combination of the S-sulfonated bovine insulin A chain and the S-sulfonated human IGF-I B-chain domain (see Experimental Procedures and Results) on a 0.9 × 23 cm CM-cellulose column with an acetate buffer ($Na^+$, 0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by an ISCO recording spectrophotometer and by a conductivity meter. The insulin-like compound (110–140 mL of the effluent) was recovered as the hydrochloride. (Right) Paper print of thin-layer isoelectric focusing of natural bovine insulin (A) and synthetic insulin-like hybrid (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing was at a constant power of 8 W for 4 h.

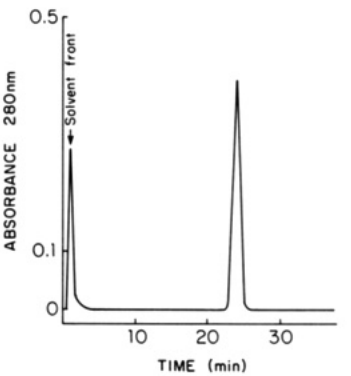



FIGURE 3: Reversed-phase HPLC of the purified insulin-like hybrid on a μBondapak $C_{18}$ column at 2 mL/min with an acetonitrile gradient, 0–63% over 30 min.

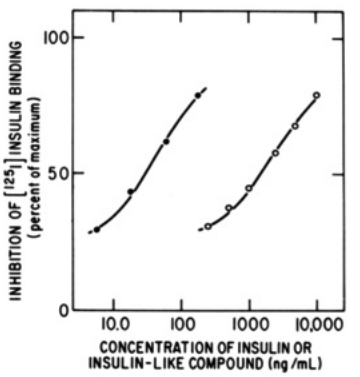



FIGURE 4: Effect of bovine insulin (●) and synthetic insulin-like hybrid (○) on porcine $^{125}$I-insulin binding to rat liver plasma membranes (see Experimental Procedures and Results). The inhibition of $^{125}$I-insulin binding, expressed as percent of maximum, is plotted as a function of the concentration of unlabeled insulin or synthetic compound. The data are taken from a typical experiment which was repeated 3 times. In this experiment the maximum binding represents 8.7% of the input radioactivity.

responding to that of the insulin A chain (Table II). The material present in the effluent under peak b (80–100 mL of effluent) was isolated via picrate as the hydrochloride (Katsoyannis et al., 1967b). Amino acid analysis of an acid hydrolysate of this product showed a composition corresponding to that of the B-chain domain of IGF-I (Table II). Upon chromatography of a combination mixture of synthetic or natural insulin chains (Katsoyannis et al., 1967b,c), insulin was eluted with application of the NaCl gradient and was the slowest moving component. A similar elution pattern was observed with the insulin-like compound in the present studies. This product was isolated from the effluent (peak c, 110–114 mL) via picrate as the hydrochloride (0.9 mg) following the procedure we have employed previously for the recovery of insulin and analogues (Katsoyannis et al., 1967b,c).

Amino acid analysis of the synthetic insulin-like compound after acid hydrolysis gave a composition, expressed in molar ratios, in good agreement with the theoretically expected values (Table II). On isoelectric focusing on thin-layer plates in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, the synthetic compound focused into one band (Figure 2). On reversed-phase HPLC the insulin-like compound behaved as a single component (Figure 3).

*Biological Evaluation of the Insulin-like Compound Consisting of the A Chain of Insulin and the B-Chain Domain of IGF-I.* The displacement of $^{125}$I-insulin from insulin receptors in rat liver plasma membranes by unlabeled insulin and the insulin-like hybrid is shown in Figure 4. Inhibition of $^{125}$I-insulin binding is concentration dependent, producing parallel dose–response curves for the two competitors. The calculated potency for the insulin-like hybrid is 1.9 ± 0.7%

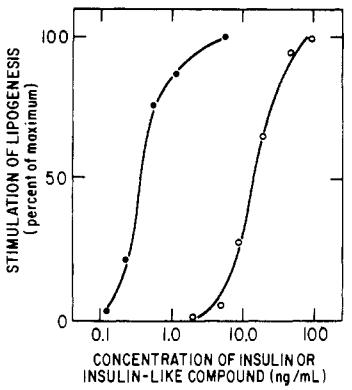



FIGURE 5: Effect of bovine insulin (●) and synthetic insulin-like hybrid (○) on lipogenesis in rat adipocytes. The stimulation of lipogenesis, expressed as percent of maximum, is plotted as a function of the concentration of insulin or synthetic compound. The data are taken from a typical experiment which was repeated three times. In this experiment, 0% stimulation corresponds to 0.08, and 100% corresponds to 1.3 nmol of [3-$^3$H]glucose converted (mg of cells)$^{-1}$ h$^{-1}$.

relative to insulin. Figure 5 depicts the ability of bovine insulin and the insulin-like hybrid to stimulate the conversion of [3-$^3$H]glucose into lipids in rat adipocytes. The synthetic material exhibits the same maximum stimulatory activity as does the natural hormone, and the dose–response curves are again parallel. The calculated potency of the insulin-like compound is 2.3 ± 0.3% relative to insulin in this assay.

The insulin-like hybrid cross-reacts with antibodies raised in guinea pigs against porcine insulin, giving rise to good straight-line plots of $C_0/C_i$ (Hales & Randle, 1963). In this assay, however, the synthetic material displays only 0.25% of the potency of bovine insulin (two experiments; data not shown).

## Discussion

The insulin-like growth factors are polypeptides exhibiting considerable homology with proinsulin (Rinderknecht & Humbel 1978a,b). This homology resides in domains in the IGF's which correspond to the A and B chains of insulin. Further, the amino acid residues in much of the proposed "binding region" of insulin (Pullen et al., 1976) are identical or conservatively varied in the IGF's (Blundell et al., 1978; Blundell & Humbel, 1980). These include amino acid residues (insulin numbering) B$^{12}$ Val, B$^{13}$ Glu (Asp) B$^{23}$ Gly, B$^{24}$ Phe, B$^{25}$ Phe (Tyr), A$^1$ Gly, and A$^{19}$ Tyr. Studies employing computer graphics (Blundell et al., 1978, 1983) suggest that the IGF's can assume a three-dimensional arrangement similar to that of insulin as revealed by X-ray crystallography. In the computer-generated models, the hydrophobic core of all these molecules and the dispositions of the A- and B-chain domains are similar. The IGF's, however, possess an extension peptide at their C-terminal ends (the D region) as well as a connecting peptide (the C region) which joins the C-terminal end of the B domain to the N-terminal end of the A domain. The computer models suggest that the C and D regions may partially cover the conserved "insulin receptor binding region" and account, at least in part, for the diminution of insulin-like activity observed in the IGF's (Blundell et al., 1978, 1983).

A comparison of the B chain of insulin with the B domain of IGF-I (Figure 6) reveals that the central portions of the primary sequences (insulin numbering positions 5–26) are identical or conservatively varied in 17 of 22 positions or 77% homology. We have previously shown that the N-terminal four and the C-terminal five amino acid residues of the B chain are of relatively minor importance for the biological activity of insulin [for a review, see Katsoyannis (1979)]. It was thus of interest to construct a hybrid molecule consisting of the A chain of insulin and a B chain corresponding to the B domain of IGF-I (sequence 1–30). The computer-generated models of insulin and the IGF's suggest that such a compound could adopt an insulin-like conformation. This hybrid would contain all of the amino acid residues contributed by the A chain of insulin to its putative binding region. In addition, the B chain would contribute the residues B$^{12}$ Val, B$^{13}$ Asp, B$^{23}$ Gly, B$^{24}$ Phe, and B$^{25}$Tyr; these residues are identical with or conservatively varied from those residues contributed by the B chain of insulin to this region. The hybrid molecule would lack the peptides corresponding to the C and D domains of the IGF's, which were proposed to be among the structural features responsible for their diminished insulin-like activity.

The synthesis of the hybrid molecule was patterned after the procedures previously employed in this laboratory for the synthesis of insulin and analogues [for a review, see Katsoyannis (1981)].

Combination of the A and B chains was carried out by the interaction of the S-sulfonated forms of the A chain of bovine insulin and a synthetic B chain corresponding to the "B domain" of IGF-I either by the methods described previously (Katsoyannis et al., 1967b,c) or by the method of Chance et al. (1981). Both syntheses involve interaction of the A and B chains at pH 10.5 in the presence of thiols. Under these conditions, the formation of "mispaired" disulfide bridges between the A and B chains of insulin is strongly disfavored (Sieber et al., 1978). We recognize that these observations do not constitute absolute proof of the positions of the disulfide bonds in the synthetic hybrid; such proof could come only from extensive degradative studies. However, the conditions used for combination, together with the close structural similarity of the B chain of insulin and the B domain of IGF-I, and the observed properties of the hybrid, argue strongly for an insulin-like structure. Isolation of the insulin-like compound

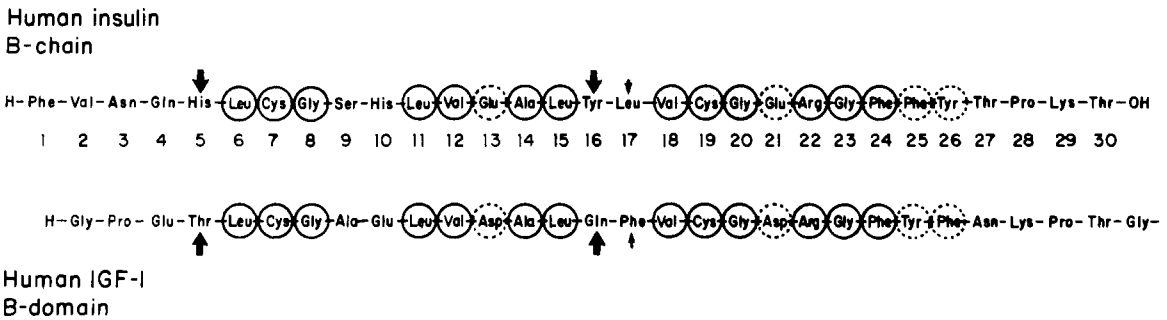



FIGURE 6: Comparison of the sequences of human insulin B chain and human IGF-I B-chain domain (IGF-I sequence 1–30). The numbering for insulin B chain is given between the sequences. Identical residues are enclosed in solid circles while conservatively varied residues are enclosed in dotted circles. The arrows refer to points of nonhomology which are discussed in the text.

from the combination mixture by column chromatography (Figure 2) gave a pattern identical with that seen previously for natural A and B chains of insulin, as well for all insulin analogues we have synthesized (Katsoyannis et al., 1967b,c). As described under Experimental Procedures and Results, the three major components in the chromatogram, a, b, and c, correspond to unreacted A and B chains or polymers thereof and the desired product, respectively. This product displayed the expected amino acid composition (Table II), and was homogeneous upon isoelectric focusing (Figure 2) and upon reversed-phase HPLC (Figure 3).

The hybrid molecule did indeed display insulin-like metabolic properties. It competed with $^{125}$I-insulin in binding to insulin receptors in a rat liver plasma membrane fraction (Figure 4); it was a full agonist in stimulating lipogenesis, and its dose-response curve was parallel to that of insulin (Figure 5). The relative potency of the hybrid (ca. 2%) was, however, unanticipatedly low based on the gross structural predictions mentioned above.

A closer comparison (Figure 6) of the B chain of insulin and the B domain of IGF-I, however, reveals that besides the differences at the N- and C-termini, the two molecules differ at positions $B^5$ (His → Thr), $B^9$ (Ser → Ala), $B^{10}$ (His → Glu), $B^{16}$ (Tyr → Gln), and $B^{17}$ (Leu → Phe) (insulin numbering). We have already shown that modification of the insulin molecule at positions $B^9$ and $B^{10}$ does not greatly affect its biological activity (Schwartz & Katsoyannis 1976, 1977a,b). In contrast, alterations at positions $B^5$ and $B^{16}$ caused substantial decreases in activity. Thus, whereas removal of the N-terminal tetrapeptide Phe-Val-Asn-Gln of the B chain of insulin does not greatly affect the biological activity, elimination of the N-terminal pentapeptide Phe-Val-Asn-Gln-His results in a considerable decrease of the biological activity of insulin (ca. 5% relative to the natural hormone) (Schwartz & Katsoyannis, 1978). We have observed that the substitution of Asn for the $B^5$ His produces an analogue displaying potency ca. 2% relative to insulin and also that replacement of $B^{16}$ Tyr, a member of the putative binding region of insulin (Pullen et al., 1976), with a Gln residue results in an analogue displaying ca. 10% of the activity of insulin (G. Schwartz, G. T. Burke, and P. G. Katsoyannis, unpublished data). The dissimilarity of the B chains in insulin and the hybrid at positions $B^5$ and $B^{16}$ suggests that these residues may play a major role in the expression of the biological profile of the hybrid.

In view of these considerations, the low insulin-like potency of the hybrid is more readily understandable. It would thus appear that, in spite of the considerable homology between the B chain of insulin and that of the hybrid, as well as the presence of the A chain of insulin which results in the conservation of most of the putative receptor binding region of insulin in the hybrid, the substitutions at positions $B^5$ and $B^{16}$ are capable of producing profoundly reduced insulin-like activity. In this connection, the computer graphics studies (Blundell et al., 1978) suggest that the substitution of $B^{16}$ Tyr in insulin by Gln in the IGF's may be of importance for the difference of the biological profiles of these proteins. We cannot at this time exclude any contribution of the substitution at position $B^{17}$; appropriate insulin analogues have not yet been described.

The immunological activity of the hybrid was 10-fold lower than its metabolic activity (ca. 0.25% relative to insulin). Antibodies raised in guinea pigs against porcine insulin have been reported to recognize one of two regions of the molecule (Arquilla et al., 1969; Blundell et al., 1978). One region consists of the N-terminal segment of the B chain together with an adjacent segment of the A chain. The other region includes several A-chain amino acid residues together with the C-terminal end of the B chain. In the present studies, the substitution of the B domain of IGF-I for the B chain of insulin results in a substantial lack of homology in both regions. The low immunological activity is thus not surprising.

The hybrid molecule is endowed with IGF-like activity, stimulating [$^3$H]thymidine incorporation in chick embryo and human fibroblasts with a potency of 3% and 8%, respectively, relative to insulin (M. A. DeVroede, M. M. Rechler, S. P. Nissley, H. Ogawa, S. Joshi, G. T. Burke, and P. G. Katsoyannis, unpublished data). Thus, although the hybrid molecule displayed lower activity than insulin in both metabolic and mitogenic assays, its mitogenic activity was less reduced than its metabolic activity. In contrast, the hybrid molecule was recognized by IGF carrier proteins from both rat and human sources, a property not exhibited by insulin. For example, the hybrid molecule displaced labeled IGF-II from IGF carrier proteins derived from normal adult rat serum, displaying a potency of 16% relative to unlabeled IGF-II (DeVroede et al., 1985). These results indicate that the substitution of the B domain of IGF-I for the B domain of insulin disturbs the structural features required for binding to insulin receptors as well as to IGF receptors. This substitution, however, confers upon the hybrid molecule sufficient similarity to IGF-I to permit its recognition by IGF carrier proteins. Computer graphic studies have, in fact, suggested that amino acid residues $B^{14}$ Ala, $B^{17}$ Phe, $B^{18}$ Val, and $A^{13}$ Leu (insulin numbering) may be involved in binding to IGF carrier proteins (Blundell et al., 1983). The present hybrid conserves all of these residues.

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## SUPPLEMENTARY MATERIAL AVAILABLE

Complete synthetic details of compounds I–XXXIa including references (23 pages). Ordering information is given on any current masthead page.

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# Isolation of Steroid Receptor Binding Protein from Chicken Oviduct and Production of Monoclonal Antibodies†

William P. Sullivan, Benjamin T. Vroman, Vickie J. Bauer, Raj K. Puri, Robert M. Riehl, Gary R. Pearson,‡ and David O. Toft*

*Department of Cell Biology, Mayo Medical School, Rochester, Minnesota 55905*

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ABSTRACT: Previous studies have shown that the molybdate-stabilized progesterone receptor from the chick oviduct contains a nonhormone binding component with a molecular weight of 90 000. This protein has also been shown to be associated with some other molybdate-stabilized steroid receptors of the oviduct. In order to access this larger pool of the receptor binding protein, we have developed an isolation procedure based on the observation that the protein is selectively shed from proteins adsorbed to heparin–agarose when molybdate is removed. The protein obtained by this procedure is shown to be the same as that isolated from affinity-purified progesterone receptor as compared by protease digestion and one-dimensional peptide mapping. Four immunoglobulin G secreting hybridoma cell lines were generated against the 90 000-dalton antigen. All of the antibodies recognize the 90 000-dalton protein obtained by electrophoretic transfer from sodium dodecyl sulfate–polyacrylamide gels. In addition, two of the antibodies complex the molybdate-stabilized progesterone receptor as demonstrated by sedimentation analysis on sucrose gradients. One of these antibodies was used to show the presence of the 90 000-dalton component in molybdate-stabilized glucocorticoid and androgen receptors and also to show its presence in brain, liver, and skeletal muscle, but not in serum.

Although the progesterone receptor from the avian oviduct has been studied extensively, the composition and structure of this protein have not yet been clearly defined. Early studies revealed two monomeric 4S receptor forms that have been termed receptors A and B (Schrader & O'Malley, 1972). These contain similar hormone binding sites but differ somewhat in size; the molecular weight of A is between 75 000 and 80 000, and that of B is about 110 000. More recent studies have shown that the large 8S, molybdate-stabilized, receptor also exists in two forms termed I and II (Dougherty & Toft, 1982). In this case, receptor I contains the A hormone binding component, and receptor II contains the B component (Dougherty et al., 1984). In addition, both 8S receptor forms contain a 90 000-dalton protein (90K protein)[1] that does not bind hormone (Dougherty et al., 1984; Renoir et al., 1984b).

The biological significance of the 90K protein is unknown. There is evidence that it exists not only as a component of the 8S progesterone receptor but also as a component of the ag-

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‡ Present address: Department of Microbiology, Georgetown University Medical Center, Washington, DC 20007.

[1] Abbreviations: 90K protein, receptor binding protein of $M_r$ 90 000; DOC–Sepharose, deoxycorticosterone–Sepharose; Mo, molybdate; $Cl_3C$-COOH, trichloroacetic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum.

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