Insulin-like Compounds Related to the Amphioxus Insulin-like Peptide†

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ABSTRACT: Three insulin-like compounds consisting of two disulfide-linked polypeptide chains have been synthesized. The A-chains of these compounds correspond either to the A- or to the A+D-domain of the putative amphioxus insulin-like peptide (amphioxus ILP), and their B-chains correspond either to the B-chain of insulin or to a slightly modified (i.e., [1-Thr]) B-domain of amphioxus ILP. The biological potency of these compounds was evalulated in mammalian cells or cell fractions containing either human or rat insulin receptors or human or mouse insulin-like growth factor I (IGF-I) receptors, with respect to binding affinity, insulin-like metabolic activity (lipogenesis), and growth factor activity (mitogenesis). Amphioxus ILP A/bovine insulin B and amphioxus ILP A+D/bovine insulin B exhibited potencies ranging from 2.0 to 9.8% relative to natural insulin, and both compounds were full agonists in lipogenesis assays, stimulating lipogenesis to the same maximal extent as seen with natural insulin. Amphioxus ILP A/amphioxus ILP [1-Thr]B stimulated lipogenesis with a potency of 0.01% relative to natural insulin. We consider this compound also likely to be a full agonist. In assays measuring binding to IGF-I receptors and stimulation of mitogenesis, these compounds displayed some activity although the activity was too low for exact quantification. These results suggest that amphioxus ILP has retained an overall structural similarity to mammalian insulin and IGF-I but has also accumulated substantial mutations which markedly reduce its ability to bind and activate their cognate receptors. Furthermore, the binding and biological activities obtained with the hybrid amphioxus A/bovine insulin analogue clearly demonstrate the importance of B-chain determinants in activating the insulin receptor.

In an effort to investigate the evolutionary origin of insulin, a cDNA was cloned and sequenced from *Branchiostoma californiensis* (amphioxus). This DNA sequence coded for a compound that should exhibit some of the structural features of mammalian insulins as well as some of the structural features of the insulin-like growth factors (IGFs) (Chan *et al.*, 1990). This putative insulin-like polypeptide, referred to as amphioxus ILP, is expected to be a disulfide-linked polypeptide molecule with a deduced primary structure embodying B-, C-, A-, and D-domains similar to the IGFs. In contrast to the latter, however, the putative amphioxus ILP has the potential for proteolytic cleavage of the C peptide, which is flanked by a pair of basic residues (Arg-Arg) at the amino terminus and a repeated pair of basic residue (Arg-Arg-Arg-Arg) at the carboxyl end, thus leading to an insulin-like two-chain molecule. The open reading frame of the cDNA does not contain a termination codon between the coding regions for the A- and D-domains, and it allows for an E-domain, which might be subject to proteolytic cleavage since it contains a basic residue at its amino end reminiscent of proIGF.

This paper describes the synthesis and biological evaluation of two-chain, insulin-like molecules consisting of an A-chain corresponding to the A- or A+D-domains of amphioxus ILP and a B-chain corresponding to a slightly modified B-domain of amphioxus ILP or the B-chain of insulin. We have described previously the synthesis of a number of comparable two-chain compounds which are related to insulin and IGF-I and have shown that these compounds displayed variable insulin-like and IGF-like activity (Katsoyannis *et al.*, 1987). It thus seemed reasonable, given the similarity between the putative amphioxus ILP described above and both insulin and IGF-I, that compounds based on the amphioxus ILP mght also display insulin- or IGF-like activity.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials. Commercial reagents were as follows: (butoxycarbonyl)amino acids and derivatives (Bachem and Peninsula Laboratories); 4-methylbenzhydrylamine resin (0.6 mmol of amine/g), used as a solid support for the synthesis of the amphioxus ILP A-domain, and (N^{α}-butoxycarbonyl,N^{G}-*p*-tolylsulfonyl)arginine-PAM resin, (0.4 mmol/g), used as a solid support for the synthesis of the amphioxus ILP B-domain (Vega); (butoxycarbonyl)valine-PAM resin, used for the synthesis of the amphioxus ILP A+D-domain (Dupont). All solvents were high-performance liquid chromatography (HPLC) grade. Details of other materials and methods used are given in previous publications (Kitagawa *et al.*, 1984a). Amino acid analysis of the synthetic chains and insulin-like analogues was done after acid hydrolysis under standard conditions on a Beckman System 6300 high-performance analyzer. For binding studies involving rat insulin receptors, ^{125}I-insulin was purchased from Dupont NEN. For lipogenesis assays, [3-^{3}H]glucose was purchased from Dupont NEN. For binding studies involving human and mouse IGF-I receptors and human insulin receptors, [31-^{125}I-monoiodo-Tyr]IGF-I

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and [A14-^{125}I-monoiodo-Tyr]insulin were prepared by the iodogen method and purified by HPLC (Drejer *et al.*, 1991). Both tracers were gifts from Dr. U. D. Larsen, Novo Nordisk, Bagsvaerd, Denmark. For mitogenesis studies, [*methyl*-1,2-^{3}H]thymidine was purchased from Amersham, United Kingdom. Human recombinant insulin was a gift from Novo Nordisk, Bagsvaerd, Denmark, and human recombinant IGF-I was purchased from Amersham, United Kingdom. Dulbecco's minimal essential medium (DMEM), fetal calf serum (FCS), and newborn calf serum (NCS) were purchased from Biological Industries, Israel. G418 (geneticin) was purchased from Sigma.

Biological Assays. Five types of assays were employed. For binding studies using rat insulin receptors, plasma membranes were purified from rat liver. For lipogenesis assays, adipocytes were prepared from rat fat pads. Details of these assays were described recently (Joshi *et al.*, 1990). For binding studies using mouse and human IGF-I receptors and for mitogenesis assays, cultured cells were employed as described below.

Cell Culture and Transfection. Mouse NIH-3T3 fibroblasts from the American Type Culture Collection were grown in DMEM supplemented with 10% NCS, penicillin, streptomycin, and 2 mM glutamine. Transfection was carried out by calcium phosphate coprecipitation (Graham & Van der Eb, 1973), where 20 μg of human insulin receptor on human IGF-I receptor cDNA was cotransfected with 2 μg of pSV2neo. Neomycin-resistant fibroblasts were selected in medium supplemented with 600 μg/mL G418 and analyzed for ^{125}I-insulin or ^{125}I-IGF-I binding. One native cell line and two transfected cell lines were used, and their characteristics have been described previously: native NIH-3T3 fibroblasts with mouse IGF-I receptors (1.7×10^5/cell), WT 1 cells transfected with human IGF-I receptors cDNA (8.7×10^5 receptors/cell), and C-5 cells transfected with human insulin receptor cDNA (6×10^5 receptors/cell) (Grønborg *et al.*, 1993; B. Ursø, B. S. Wulf, T. Kjeldsen, and S. Gammeltoft, unpublished data).

Insulin and IGF-I Receptor Binding in Cultured Cells. Subconfluent cultures of insulin receptor-transfected or native NIH-3T3 fibroblasts growing in 24-well multidishes (5×10^4 cells/well) were incubated for 20 h at 4 °C with 25 000 cpm/well of ^{125}I-insulin or ^{125}I-IGF-I (approximately 20 pM) and different concentrations of unlabeled human insulin, human IGF-I, or amphioxus insulin-like peptide (ILP)/bovine insulin hybrid compounds in Krebs–Ringer–Hepes buffer (124 mM NaCl, 3.56 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM $KHPO_4$, 25 mM Hepes, pH 7.4) supplemented with 0.1% BSA. The incubation was terminated by washing three times with PBS with 0.1% BSA on ice. The cells were harvested with 0.2 M NaOH and counted in a gamma counter (Nielsen *et al.*, 1991). Receptor-bound ^{125}I-insulin or ^{125}I-IGF-I was determined by the subtraction of non-specific binding determined in the presence of 1.0 μM unlabeled insulin or 0.1 μM unlabeled IGF-I. Data were plotted as bound/free (fraction of maximum) versus the concentration of unlabeled peptide. The concentration giving half-maximal inhibition (IC_{50}) was estimated from the binding curve and the affinity relative to insulin or IGF-I calculated (Gammeltoft, 1990).

DNA Synthesis (Mitogenesis) in Cultured Cells. Subconfluent monolayers of IGF-I receptor-transfected cells in 96-well dishes were cultured in DMEM with 2% NCS for 2 days to achieve quiescence. Insulin, IGF-I, or amphioxus ILP was added in different concentrations, and after 17 h the medium was aspirated and the cells pulse-labeled at 37 °C with 0.2 μCi/well [*methyl*-1,2-^{3}H]thymidine in fresh medium for 3 h. Finally, the cells were solubilized in 0.2 M NaOH, harvested on Whatman glass microfiber filters uing a Betaplate 96-well harvester (Pharmacia), and counted for radioactivity (Nielsen *et al.*, 1991).

General Aspects of the Synthesis of S-Sulfonated A-, A+D-, [1-Thr]B-, and Analogues of [1-Thr]B-Domains of Amphioxus ILP. The synthesis of these compounds was done using procedures routinely used in this laboratory to synthesize S-sulfonated insulin chains. Specifically, the key intermediates for the synthesis of the A- and A+D-chain domains are the protected 21-residue and 33-residue peptide chains, respectively. For the synthesis of the B-chain domain and analogues, the key intermediates are the protected 28-residue peptides, each containing the amino acid sequence of the respective chain domain. The synthesis of the protected 21-, 33-, and 28-residue peptides was done using stepwise solid-phase synthesis (Barany & Merrifield, 1980). For the synthesis of the A-chain domain, the solid support was 4-methylbenzhydrylamine resin, and for the synthesis of the A+D-, [1-Thr]B-, and B-chain analogues, the solid support was PAM resin (Mitchell *et al.*, 1978). The *tert*-butoxcarbonyl group was used for N^{α} protection. Side-chain protecting groups were as follows: *p*-tolylsulfonyl for Arg; 4-methylbenzyl for Cys; cyclohexyl for Glu and Asp; (2-chlorobenzyloxy)carbonyl for Lys; (benzyloxy)methyl for His; benzyl for Ser and Thr; and 2,6-dichlorobenzyl for Tyr. A manual double-coupling protocol (Merrifield *et al.*, 1982) was followed. The protected amino acids were incorporated from preformed 1-hydroxybenzotriazole esters in 3-fold excess. Active esters were prepared with N,N^1-dicyclohexylcarbodiimine and 1-hydroxybenzotriazole in dimethylformamide. Preformed anhydrides (Hagenmaier & Frank, 1972) in dimethylformamide were used for the incorporation of the amino acids following Gln and Glu residues to minimize pyrrolidone carboxylic acid formation (DiMarchi *et al.*, 1982; Orlowska *et al.*, 1987). The completion of the reaction was monitored by the qualitative ninhydrin test (Kaiser *et al.*, 1970), which was negative after each coupling step. The C-terminal Asn residue, in the synthesis of the A-chain domain, was incorporated into the solid support by coupling *tert*-butoxycarbonyl aspartic acid α-benzyl ester with 4-methylbenzhydrylamine resin. After the final deprotection step, the Asp residue was converted to an Asn residue.

After completing the synthesis, the protected peptide resin corresponding to each chain domain was deblocked by treatment with liquid hydrogen fluoride and subjected to oxidative sulfitolysis as previously described (Wang *et al.*, 1991; Chu *et al.*, 1992). The crude S-sulfonated derivatives of the A- and A+D-chain domains were subjected to preliminary purification on a Sephadex G-15 column (4.2×50 cm), using 0.015 M NH_4HCO_3 as the eluting solvent and then purified to homogeneity by chromatography on a Cellex E column (1.2×45 cm) with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient as previously described (Ogawa *et al.*, 1984). A variety of procedures were used to purify the S-sulfonated B-chain domains. Specifically, ion-exchange chromatography on a DEAE Sepharose CL-6B (Pharmacia) column (2×23 cm) with 0.1 M Tris-HCl buffer (pH 7.5) and a linear 0.5 M NaCl gradient (Ogawa *et al.*, 1984) was used to purify the S-sulfonated [1-Thr]B-chain domain. The effluent corresponding to the major peak was collected, dialyzed in Spectrapor membrane tubing no. 3 against four changes of distilled water (4 L each) at 4 °C for 24 h, and lyophilized to yield the purified chain; ion-exchange chromatography on a Cellex E column (1.5×47 cm) equilibrated with 0.1 M NH_4HCO_3 was used to purify the S-sulfonated

[1-Glu,15-Tyr]-, [1-Glu,15-Tyr,16-Leu]-, [1-Glu,9-Asp,15-Tyr]-, and [1-Glu,23-Phe,24-Phe,25-Tyr]B-chain domains. Elution of the column was done first with 0.1 M NH_4HCO_3 (100 mL) and subsequently with a linear gradient formed by adding 0.5 M NH_4HCO_3 (250 mL) to 0.1 M NH_4HCO_3 (250 mL). Lyophilizaiton of the effluent under the major peak afforded the purified chain; ion-exchange chromatography on a CM-cellulose column (2.5 × 45 cm) with a urea acetate buffer, pH 4.0 (Katsoyannis *et al.*, 1967a; Wang *et al.*, 1991) was used to purify [1-Thr,11-Val]B-chain domain S-sulfonate.

The S-sulfonated A- and B-chains of insulin used in this study were prepared by oxidative sulfitolysis of insulin, followed by separating the resulting S-sulfonated A- and B-chains by chromatography as previously described (Katsoyannis *et al.*, 1967a); the only difference was that sulfitolysis was performed for 3.5 h instead of 24 h.

Amphioxus ILP A-Domain S-Sulfonate. A 450-mg sample of peptidyl resin, after deblocking, sulfitolysis, and chromatographic purification, yielded approximately 90 mg of purified amphioxus ILP A-domain S-sulfonate. Amino acid analysis after acid hydrolysis gave the following ratios which were in good agreement with the theoretically expected values (shown in parentheses): $Asp_{2.9(3)}$ $Ser_{1.8(2)}$ $Glu_{4.2(4)}$ $Gly_{1.1(1)}$ $Val_{1.9(2)}$ $Leu_{1.9(2)}$ $Tyr_{2.7(3)}$. Cys was not determined.

Amphioxus ILP A+D-Domain S-Sulfonate. About 146 mg of purified amphioxus ILP A+D-domain S-sulfonate was obtained from 611 mg of peptidyl resin after deblocking, sulfitolysis, and chromatographic purification. Amino acid analysis gave the following ratios: $Asp_{3.0(3)}$ $Thr_{2.6(3)}$ $Ser_{2.7(3)}$ $Pro_{3.4(3)}$ $Glu_{4.2(4)}$ $Gly_{1.1(1)}$ $Ala_{2.8(3)}$ $Val_{2.8(3)}$ $Leu_{1.8(3)}$ $Tyr_{4.0(4)}$. Cys was not determined.

Amphioxus ILP [1-Thr]B-Domain S-Sulfonate. About 40 mg of purified S-sulfonated chain was obtained from 400 mg of peptidyl resin after deblocking, sulfitolysis, and chromatography. After acid hydrolysis, amino acid analysis gave the following ratios: $Asp_{2.8(3)}$ $Thr_{1.7(2)}$ $Ser_{2.4(3)}$ $Pro_{0.7(1)}$ $Glu_{2.1(2)}$ $Gly_{2.9(3)}$ $Ala_{2.0(2)}$ $Val_{2.2(2)}$ $Leu_{3.1(3)}$ $Tyr_{2.0(2)}$ $Phe_{1.1(1)}$ $Arg_{1.7(2)}$. Cys was not determined.

Amphioxus ILP [1-Thr,11-Val]B-Domain S-Sulfonate. About 50 mg of purified S-sulfonated chain was obtained from 400 mg of peptidyl resin after deblocking, sulfitolysis, and chromatography. Amino acid analysis gave the following ratios: $Asp_{3.0(3)}$ $Thr_{1.8(2)}$ $Ser_{2.7(3)}$ $Pro_{1.0(1)}$ $Glu_{2.1(2)}$ $Gly_{3.0(3)}$ $Ala_{1.0(1)}$ $Val_{2.9(3)}$ $Leu_{3.0(3)}$ $Tyr_{1.9(2)}$ $Phe_{1.1(1)}$ $Arg_{1.7(2)}$. Cys was not determined.

Amphioxus ILP [1-Glu,15-Tyr]B-Domain S-Sulfonate. After deblocking, sulfitolysis, and chromatography, about 94 mg of purified S-sulfonated chain was obtained from 610 mg of peptidyl resin. Amino acid analysis gave the following ratios: $Asp_{2.9(3)}$ $Thr_{0.9(1)}$ $Ser_{1.8(2)}$ $Glu_{3.1(3)}$ $Pro_{0.9(1)}$ $Gly_{29.(3)}$ $Ala_{2.1(2)}$ $Val_{2.0(2)}$ $Leu_{3.0(3)}$ $Tyr_{2.8(3)}$ $Phe_{1.0(1)}$ $Arg_{1.9(2)}$. Cys was not determined.

Amphioxus ILP [1-Glu,15-Tyr,16-Leu]B-Domain S-Sulfonate. About 97 mg of purified S-sulfonated chain was obtained from 510 mg of peptidyl resin after processing as previously described. Amino acid analysis gave the following ratios: $Asp_{2.9(3)}$ $Thr_{1.0(1)}$ $Ser_{1.8(2)}$ $Glu_{3.1(3)}$ $Pro_{0.9(1)}$ $Gly_{2.9(3)}$ $Ala_{2.1(2)}$ $Val_{2.0(2)}$ $Leu_{3.9(4)}$ $Tyr_{2.9(3)}$ $Arg_{1.9(2)}$. Cys was not determined.

Amphioxus ILP [1-Glu,9-Asp,15-Tyr]B-Domain S-Sulfonate. After processing as described above, about 109 mg of purified S-sulfonated chain was obtained from 510 mg of peptidyl resin. Amino acid analysis gave the following ratios: $Asp_{3.7(4)}$ $Ser_{1.7(2)}$ $Glu_{3.2(3)}$ $Pro_{1.0(1)}$ $Gly_{3.0(3)}$ $Ala_{2.0(2)}$ $Val_{2.0(2)}$ $Leu_{3.0(3)}$ $Tyr_{2.6(3)}$ $Phe_{1.0(1)}$ $Arg_{2.0(2)}$. Cys was not determined.

Amphioxus ILP [1-Glu,23-Phe,24-Phe,25-Tyr]B-Domain S-Sulfonate. From 500 mg of peptidyl resin, after the usual processing, about 70 mg of purified S-sulfonate was obtained. Amino acid ratios after acid hydrolysis were as follows: $Asp_{2.0(2)}$ $Thr_{0.9(1)}$ $Ser_{1.8(2)}$ $Glu_{3.1(3)}$ $Pro_{1.0(1)}$ $Gly_{2.9(3)}$ $Ala_{2.0(2)}$ $Val_{2.0(2)}$ $Leu_{3.0(3)}$ $Tyr_{1.8(2)}$ $Phe_{2.8(3)}$ $Arg_{2.0(2)}$. Cys was not determined.

General Aspects of the Synthesis of the Insulin-like Compounds Consisting of A-Chains Corresponding to the A- or A+D-Domains of Amphioxus ILP and B-Chains Corresponding to the B-Chain of Insulin or the [1-Thr]B-Domain of Amphioxus ILP. The synthesis of these compounds followed the pattern used in the synthesis of insulin and its analogues as previously described [e.g., Wang *et al.*, (1991) and Chu *et al.* (1992)]. It involves the interaction of the corresponding S-sulfonated A- and B-chains in a ratio of approximately 2:1 by weight, in 0.1 M glycine buffer, pH 10.6, in the presence of dithiothreitol (Chance *et al.*, 1981). The isolation of the insulin-like compounds from the combination mixture of the corresponding S-sulfonated chains was also accomplished by procedures used to isolate insulin or analogues by interaction of the corresponding A- and B-chain S-sulfonates. Specifically, to isolate the insulin-like compounds consisting of the amphioxus ILP A- or A+D-domains and the B-chain of insulin, the combination mixture was acidified with acetic acid and treated with saturated picric acid solution (Katsoyannis *et al.*, 1967b). The precipitated picrate salt was converted to the hydrochloride and was chromatographed on an 0.9 × 25 cm CM-cellulose column with an acetate buffer (Na^+ 0.024 M, pH 3.3) and an exponential NaCl gradient (Katsoyannis *et al.*, 1967b). The insulin-like compound was isolated from the effluent, via picrate, as the hydrochloride. Both compounds exhibited single peaks on reversed-phase HPLC on a Vydac 218 TP column (0.45 × 25 cm) at a flow rate of 0.5 mL/min with 20–80% linear gradient of 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 80 min. To isolate the insulin-like compound, consisting of the A- and the [1-Thr]B-domains of amphioxus ILP, the combination mixture was treated as described above, and the hydrochloride was chromatographed on a Sephadex G-50 column (2.5 × 112 cm), equilibrated, and eluted with 1 N acetic acid (Chen *et al.*, 1988). The effluent representing the monomer fraction, which contains the active material (localized by precalibration of the column with insulin), was collected and lyophilized. This material was subjected to reversed-phase HPLC under the exact conditions described above, and the active material was eluted in a peak with a retention time almost identical to that of insulin (approximately 40 min). Rechromatography of the material (HPLC under the conditions above) exhibited a single sharp peak.

Amphioxus ILP A/Bovine Insulin B. From the interaction of the S-sulfonated derivatives of amphioxus ILP A-domain (22.4 mg) and bovine insulin B-chain (11.7 mg) in 0.1 M glycine buffer (pH 10.6; 5 mL) in the presence of dithiothreitol (3.77 mg), the amphioxus ILP A/bovine insulin B hybrid (3.91 mg) was isolated, after CM-cellulose chromatography, as the hydrochloride via picrate. On reversed-phase HPLC under the conditions described above, this compound exhibited a single peak. Amino acid analysis after acid hydrolysis gave the following ratios: $Asp_{4.0(4)}$ $Thr_{1.0(1)}$ $Ser_{2.6(3)}$ $Pro_{1.0(1)}$ $Glu_{7.3(7)}$ $Gly_{3.9(4)}$ $Ala_{1.9(2)}$ $Val_{4.7(5)}$ $Leu_{5.9(6)}$ $Tyr_{4.7(5)}$ $Phe_{2.9(3)}$ $Lys_{1.3(1)}$ $His_{2.3(2)}$, $Arg_{1.1(1)}$. Cys was not determined.

Amphioxus ILP A+D/Bovine Insulin B. The interaction of the S-sulfonated derivatives of amphioxus ILP A+D-domain (28.83 mg) and bovine insulin B-chain (9.45 mg) in 0.1 M

Table 1: Summary of Assays for Amphioxus ILP-Related Insulin-like Compounds

compound	binding assays: mouse IGF receptor[a]	binding assays: human insulin receptor[b]	binding assays: rat insulin receptor[c]	biological activity assays: mitogenesis[d] (IGF acty)	biological activity assays: lipogenesis[e] (insulin acty)
insulin	0.17	100	100	2.2	100
IGF-1	100	2.3	nd[f]	100	nd
amphioxus ILP A/bovine insulin B	<0.03	5.0	3.9	0.05	9.8
amphioxus ILP A+D/bovine insulin B	<0.03	2.3	2.0	0.2	4.2
amphioxus ILP A/[1-Thr]amphioxus B	<0.03	<0.07	<0.01	<0.02	0.01

[a] Determined with NIH-3T3 fibroblasts expressing mouse IGF-I receptors. [b] Determined with C-5 cells transfected with human insulin receptor cDNA. [c] Determined with rat liver plasma membranes. [d] Determined with WT 1 cells transfected with human IGF-I receptor cDNA. [e] Determined with isolated rat adipocytes. [f] nd, not determined.

glycine buffer (pH 10.6; 5 mL) in the presence of dithiothreitol (3.77 mg) led to the isolation, using the procedure described above, of approximately 4.05 mg of the hydrochloride of this hybrid. Final purification of this product was achieved by reversed-phase HPLC under the conditions previously described. Amino acid analysis of this material gave the following ratio: $Asp_{4.1(4)}$ $Thr_{3.5(4)}$ $Ser_{4.1(4)}$ $Pro_{4.3(4)}$ $Glu_{7.2(7)}$ $Gly_{4.3(4)}$ $Ala_{4.9(5)}$ $Val_{5.7(6)}$ $Tyr_{5.4(6)}$ $Phe_{2.7(3)}$ $Lys_{1.3(1)}$ $His_{2.2(2)}$ $Arg_{1.2(1)}$. Cys was not determined.

Amphioxus ILP A/Amphioxus ILP[1-Thr]B. From the combination mixture of the sulfonated derivatives of amphioxus ILP A-domain (20.23 mg) and amphioxus ILP [1-Thr]B-domain (10.05 mg) in 0.1 M glycine buffer (pH 10.6; 5 mL) containing dithiothreitol (4.55 mg), hydrochlorides of the products were isolated as described in the general aspects section. The combination procedure were repeated once more on the same scale as above, and the combined hydrochlorides were subjected to Sephadex G-50 chromatography and finally to reversed-phase HPLC as described in the general aspects section. The highly purified amphioxus ILP A/amphioxus ILP[1-Thr]B compound was recovered upon lyophilization of the effluent under a peak in the HPLC chromatography that had a retention time of 40.4 min (0.35 mg); the retention time of insulin under the same conditions is about 40 min. Amino acid analysis of the purified material after acid hydrolysis gave the following ratios: $Asp_{5.9(6)}$ $Thr_{1.7(2)}$ $Ser_{4.2(5)}$ $Glu_{6.4(6)}$ $Pro_{1.0(1)}$ $Gly_{4.3(4)}$ $Ala_{2.0(2)}$ $Ala_{2.0(2)}$ $Val_{4.4(4)}$ $Leu_{4.6(5)}$ $Tyr_{4.5(5)}$ $Phe_{0.9(1)}$ $Arg_{1.9(2)}$. Cys was not determined. A Quattro mass spectrometric (VG) analysis gave a molecular weight of 5450 for the synthetic compound; the calculated weight is 5450.95.

Attempted Synthesis of Insulin-like Compounds Consisting of an A-Chain Corresponding to the A-Chain of Insulin and a B-Chain Corresponding to [1-Thr]B-, [1-Glu,15-Tyr]B-, [1-Glu,9-Asp,15-Tyr]B-, [1-Glu,15-Tyr,16-Leu]B-, [1-Glu,23-Phe,24-Phe,25-Phe]B-, and [1-Thr,11-Val]B-Domains of Amphioxus ILP. As mentioned above, an A-chain corresponding to the A- or A+D-domains of amphioxus ILP combines with a B-chain corresponding to the B-chain of insulin or the [1-Thr]B-domain of amphioxus ILP to produce the respective two-chain insulin-like compounds. The situation, however, was quite different when attempts were made to combine the A-chain of insulin with the [1-Thr]B-domain of amphioxus ILP or the five analogues of that domain mentioned above. Using the methods described above, no product exhibiting the appropriate amino acid analysis for an insulin-like analogue was obtained from combination mixtures each containing S-sulfonated bovine insulin A-chain and any of the six variants of amphioxus ILP B-domain S-sulfonates.

BIOLOGIC EVALUATION OF SYNTHETIC COMPOUNDS

Rat Insulin Receptor Binding. ^{125}I-Insulin binding was measured in a preparation of rat liver plasma membranes as described (Joshi *et al.*, 1990). Nonspecific binding, defined as ^{125}I-insulin remaining in the presence of 1×10^{-5} M porcine insulin, was subtracted from all values, and the potency of the test compound was expressed as the ratio of the concentration of the test compound to the concentration of porcine insulin required to inhibit 50% of the specific binding of ^{125}I-insulin. Quantitative analysis of the data showed that the inhibition constant (IC_{50}) of porcine insulin was 5×10^{-9} M and that the potency of the compound embodying an A-chain corresponding to the amphioxus A-domain and the bovine insulin B-chain was 3.9%, relative to porcine insulin. For the amphioxus A+D bovine insulin compound, the corresponding figure was 2.0%. The amphioxus ILP A/amphioxus ILP [1-Thr]B displayed potency too low to estimate in this assay (Table 1).

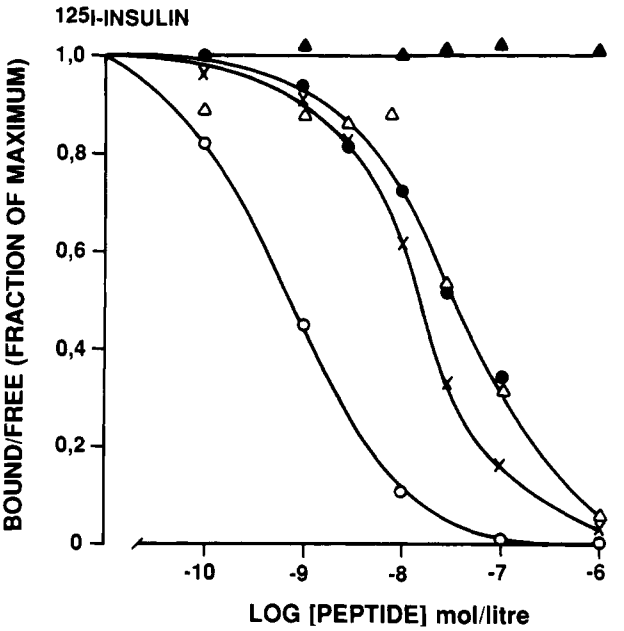

FIGURE 1: ^{125}I-Insulin binding to NIH-3T3 fibroblasts transfected with human insulin receptor. Subconfluent cultures of C-5 cells in 24-well dishes were incubated 20 h at 4 °C with 20 pM ^{125}I-insulin in the absence or presence of human insulin (○), human IGF-I (●), amphioxus ILP A/amphioxus ILP [1-Thr]B (▲), amphioxus ILP A/bovine insulin B (×), or amphioxus ILP A+D/bovine insulin B (△). Receptor-bound ^{125}I-insulin was determined after washing and solubilizing the cells in NaOH and counting radioactivity. Data are mean values of three experiments with a SD of 10–15%.

Human Insulin Receptor Binding. ^{125}I-Insulin binding was measured on the C-5 cell line transfected with human insuiln receptor cDNA (Grønborg *et al.*, 1993; B. Ursø, B. S. Wulf, T. Kjeldsen, S. Gammeltoft, unpublished data). Specific binding of ^{125}I-insulin was completely inhibited by human insulin, human IGF-I, and the two amphioxus ILP/bovine insulin hybrid compounds but not by the amphioxus ILP A/amphioxus ILP [1-Thr]B compound in the concentration range of 10^{-10}–10^{-6} M (Figure 1). The potency of the test

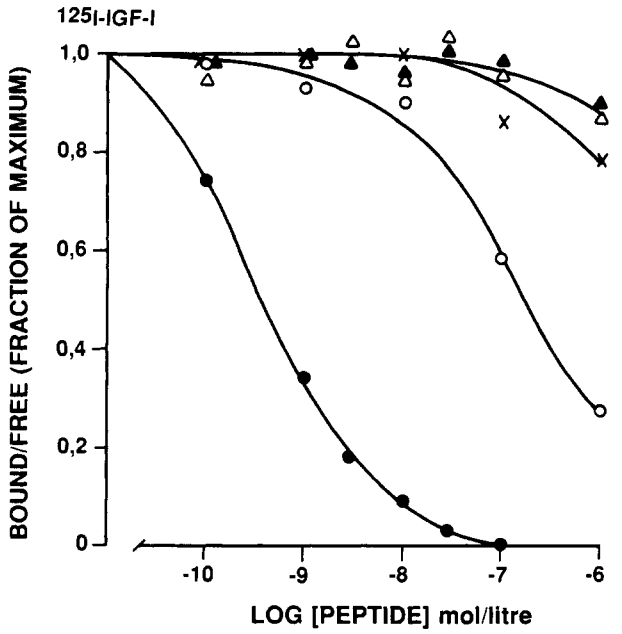

FIGURE 2: ^{125}I-IGF-I binding to mouse IGF receptor on NIH-3T3 fibroblasts. Subconfluent cultures of NIH-3T3 fibroblasts in 24-well dishes were incubated 20 h at 4 °C with 20 pM ^{125}I-IGF-I in the absence or presence of human IGF-I (●), human insuiln (○), amphioxus ILP A/amphioxus ILP [1-Thr]B (▲), amphioxus ILP A/bovine insulin B (×), or amphioxus ILP A+D/bovine insulin B (△). Receptor-bound ^{125}I-IGF was determined after washing and solubilizing the cells in NaOH and counting radioactivity. Data are mean values of three experiments with a SD of 10–15%.

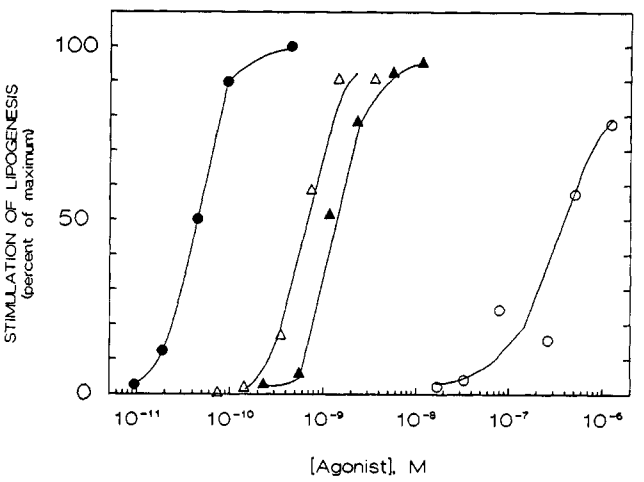

FIGURE 3: Effect of porcine insulin (●), amphioxus ILP A/bovine insulin B (△), amphioxus ILP A+D/bovine insulin B (▲), and amphioxus ILP A/[1-Thr]amphioxus ILP B (○) on lipogenesis in rat adipocytes. The stimulation of lipogenesis, expressed as percent of maximum, is plotted as a function of the concentration of agonists. Data points represent the mean of triplicate determinations in a typical experiment which was performed twice.

compounds ranged from 2.3 to 5% relative to human insulin, whereas the relative potency of the amphioxus ILP A/amphioxus ILP B compound was less than 0.07% (Table 1).

IGF-I Receptor Binding. Human IGF-I completely inhibited the specific binding of ^{125}I-IGF-I in native NIH-3T3 fibroblasts expressing mouse IGF-I receptors. The specific binding of ^{125}I-IGF-I was inhibited 80% by human insulin and 10–20% by the two amphioxus ILP/bovine insulin hybrid compounds as well as by the amphioxus ILP A/amphioxus ILP [1-Thr]B compound, all at the maximum concentration of 10^{-6} M (Figure 2). The relative potency of human insulin was 0.17% relative to human IGF-I, and the relative potency of the amphioxus-related compounds was less than 0.03% (Table 1).

Lipogenesis. The amphioxus-related compounds were compared with porcine insulin for the ability to stimulate the incorporation of [3-^{3}H]glucose into an organic extractable form in isolated rat adipocytes (lipogenesis) (Joshi *et al.*, 1990). The two amphioxus ILP/bovine insulin compounds stimulated lipogenesis to the same maximum extent as porcine insulin, and the amphioxus ILP A/amphioxus ILP [1-Thr]B compound produced about 80% of the maximum activity of porcine insulin. The calculated relative potency was defined as the ratio of the concentration of test compound to the concentration of porcine insulin required to produce 50% of the maximum stimulation produced by porcine insulin (ED_{50}). For porcine insulin, this concentration was 5×10^{-11} M. Amphioxus ILP A/bovine insulin B, displayed a relative potency of 9.8%; amphioxus ILP A+D/bovine insulin B, displayed a relative potency of 4.2%; amphioxus ILP A/amphioxus ILP [1-Thr]B displayed a relative potency of 0.01% (Table 1; Figure 3).

IGF-I-Stimulated DNA Synthesis (Mitogenesis). The stimulation of [^{3}H]thymidine incorporation into DNA by IGF-I was measured in the WT 1 cell line transfected with human IGF-I receptor cDNA (Grønborg *et al.*, 1993; B. Ursø, B. S. Wulf, T. Kjeldsen, and S. Gammeltoft, unpublished data). DNA synthesis was stimulated to the same maximal level by human IGF-I and by the two amphioxus ILP/bovine insulin hybrid compounds, whereas the compound embodying the amphioxus ILP A and the amphioxus ILP [1-Thr]B stimulated DNA synthesis to only ca. 25% of this maximum level (Figure 4). Human IGF-I stimulated DNA synthesis with an ED_{50} of 2.2×10^{-10} M. The potency relative to IGF-I of human insulin was 2.2%; that of amphioxus ILP A/bovine insulin B was 0.05%; that of amphioxus ILP A+D/bovine insulin B-chain was 0.2%; and that of amphioxus ILP A/amphioxus [1-Thr]B was less than 0.02% (Table 1).

DISCUSSION

Cloning of the amphioxus ILP gene revealed that the putative product of that gene shares several structural features with vertebrate insulins and IGFs, indicating a common lineage and suggesting that it is an unusual insulin whose gene may represent a linkage between the genes encoding the IGFs and an ancestral insulin gene (Chan *et al.*, 1990).

Figure 5 illustrates a comparison of the primary structure of the putative amphioxus ILP A-domain with the corresponding regions of human insulin and IGF-I. Twelve of the 21 amino acid residues of the amphioxus ILP A-domain are identical to those found in the human insulin A-chain, a 57% homology; similarly, the human IGF-I A-domain contains identical or conservatively varied residues in 10 of these 21 positions. Interestingly, many conserved residues in the insulin A-chain, e.g., A1, A3, A4, A6, A7, A11, A15, A16, A17, A19, A20, and A21, are also present in the amphioxus ILP A-domain. Some of these residues have been identified as necessary the stability of insulin (Baker *et al.*, 1988), and several of them have been identified as part of the putative receptor-binding region of the hormone (Pullen *et al.*, 1976; Baker *et al.*, 1988). A glaring difference is that in amphioxus ILP, posiiton A2 is occupied by Leu rather than Ile, as in insulin and IGF-I. We have shown that Ile in position A2 is of critical importance to the structure and, hence, to the biological activity of both insulin (Kitagawa *et al.*, 1984a,b) and IGF-I (Zong *et al.*, 1990).

A comparison of the B-chain of insulin and the B-domain of IGF-I (sequence 1–29) with the B-domain of amphioxus ILP (sequence 1–28) indicates that the central portions of the primary structures (positions 5–26 using insulin numbering)

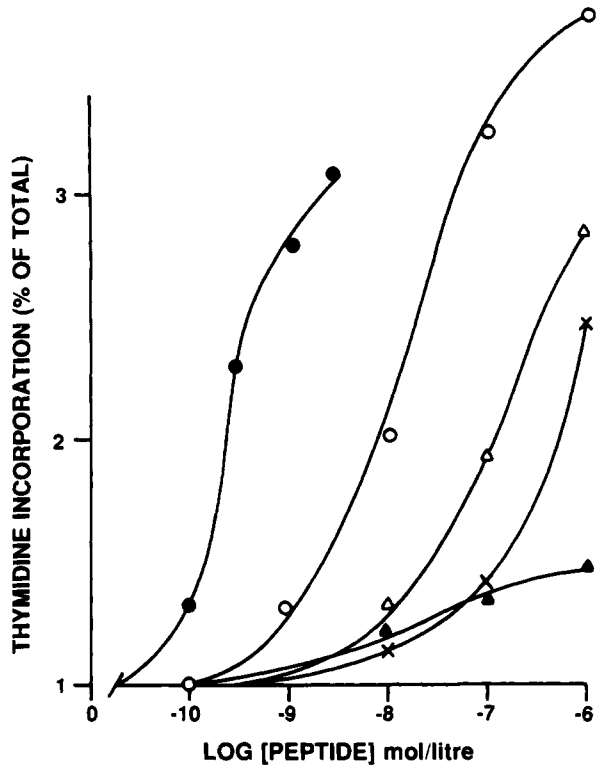

FIGURE 4: IGF-I-stimulated DNA synthesis in NIH-3T3 fibroblasts transfected with human IGF receptor. Subconfluent WT1 cells were cultured for 17 h at 37 °C in the absence or presence of human IGF-I (●), human insulin (○), amphioxus ILP A/amphioxus ILP [1-Thr]B (▲), amphioxus ILP A/bovine insulin B (×), or amphioxus ILP A+D/bovine insulin (△) followed by pulse-labeling for 3 h at 37 °C with [^{3}H]thymidine. Cells were solubilized in 0.2 M NaOH and harvested, and the DNA was collected on filters. After washing, the incorporated radioactivity was counted in a β-counter. The results are expressed as [^{3}H]thymidine incorporation (fold stimulation over basal). Data are mean values of three experiments with a SD of 10–15%.

are identical or conservatively varied in 13 of 22 residues in amphioxus ILP (ca. 59% homology) and in 17 of 22 residues in human IGF-I (ca. 77% homology). We as well as others have previously shown that the N-terminal four and the C-terminal five amino acid residues of the insulin B-chain are of relatively minor importance for biological activity [for a review, see Katsoyannis (1981)]. Despite this high homology, striking variations are apparent between the primary structure of insulin and that of amphioxus ILP, including substitutions for B12 Val, B16 Tyr, B17 Leu, B21 Glu, B24 Phe, B25 Phe, and B26 Tyr (insulin numbering). All of these residues are conserved in most mammalian insulins, and many of them are conserved in IGF-I as well (Figure 5). Several of these residues have been implicated in the stabilization of a structure commensurate with high biological activity in insulin (Baker *et al.*, 1988; Hu *et al.*, 1993). The D-domain of amphioxus ILP, a 12 amino acid extension of the A-domain (Chan *et al.*, 1990), is comparable in length with the D-domain of IGF-I (eight residues) (Rinderknecht & Humbel, 1978), but there is no sequence homology.

We have previously reported the synthesis of a number of two-chain insulin-like compounds consisting of an A-chain corresponding either to the A-chain of insulin or to the A- or the A+D-domain of IGF-I and a B-chain corresponding to the B-chain of insulin or the B-domain of IGF-I [for a review, see Katsoyannis *et al.* (1987)]. Biological evaluation of these compounds showed that they are endowed to a varying degree with insulin- and IGF-like activity (Joshi *et al.*, 1985; Chen *et al.*, 1988; Schwartz *et al.*, 1988). These studies led us to the following tentative conclusions: (1) The A-domain of IGF-I contains the determinants for growth-promoting activity, whereas the B-domain contributes mainly to the recognition by IGF-I of the IGF carrier proteins and of anti-IGF antibodies. (2) The D-domain of the IGFs participates in the suppression of insulin-like activity in these molecules, presumably by partially covering their "insulin receptor binding region." This conclusion is in agreement with computer graphics studies (Blundell *et al.*, 1978, 1983). (3) The B-domain of IGF-I, independent of the D-domain, interferes with the interaction of the molecule with the insulin receptor and may play a role in modulating specificity in receptor recognition.

In view of these considerations, we became interested in constructing analogous two-chain compounds consisting of A-chains corresponding to the A-chain of insulin or to the A- and the A+D-domains of the putative amphioxus ILP and B-chains corresponding to the B-chain of insulin or to the B-domain of the putative amphioxus ILP. We have synthesized the S-sulfonates of amino acid chains with sequences corresponding to the A-, the A+D-, and the B-domains of amphioxus ILP containing 21, 33, and 28 residues, respectively. To eliminate the possibility of pyrrolidone carboxylic acid formation during the final acid deprotection of the B-domain bound to the resin used as a solid support in the solid-phase synthesis of this polypeptide (DiMarchi *et al.*, 1982; Orlowska *et al.*, 1987), the naturally occurring N-terminal Gln residue was substituted by a Thr residue. [1-Thr]B-domain was thus used for the construction of the two-chain molecules under discussion.

As described in the experimental section, the final step in the synthesis of the two-chain insulin-like compounds involves the combination of the appropriate A- and B-chain S-sulfonates, at pH 10.5 in the presence of dithiothreitol. Under these conditions, the formation of "mispaired" disulfide bridges between the A- and B-chains of insulin is strongly disfavored (Sieber *et al.*, 1987; Tang & Tsou, 1990). The marked homology between the amphioxus ILP A- and B-chains and those of insulin and the similarity of the conditions used to combine these chains to generate amphioxus ILP or amphioxus ILP/insulin hybrids, together with the full agonist behavior of the resulting compounds in the lipogenesis assay (see below), argue strongly for an insulin-like structure. The A-chain S-sulfonates corresponding to the A- and to the A+D-domains of amphioxus ILP combined very efficiently with the insulin B-chain S-sulfonate to produce in good yields the two compounds amphioxus ILP A/bovine insulin B and amphioxus ILP A+D/bovine insulin B. This implies that the overall conformation of these A-chains is similar to that of the A-chain of insulin. In contrast, the combination of the A-chain S-sulfonate corresponding to the A-domain of amphioxus ILP with the S-sulfonate corresponding to the amphioxus ILP [1-Thr]B-domain was very inefficient, and the yield of the two-chain insulin-like product, amphioxus ILP A/amphioxus ILP [1-Thr]B was isolated in a poor yield, approximately 28 times lower than the yields of the previous two compounds. Furthermore, the amphioxus [1-Thr]B-domain S-sulfonate failed to combine with the insulin A-chain S-sulfonate, and the anticipated compound, insulin A/amphioxus ILP [1-Thr]B, could not be isolated from the combination mixture. These observations support the idea that there must be large differences in conformation between the insuiln B-chain S-sulfonate and the B-chain S-sulfonate corresponding to the amphioxus ILP [1-Thr]B-domain.

In efforts to overcome these difficulties in combination, we synthesized analogues of the amphioxus ILP B-chain S-sulfonate involving substitutions at positions where variations

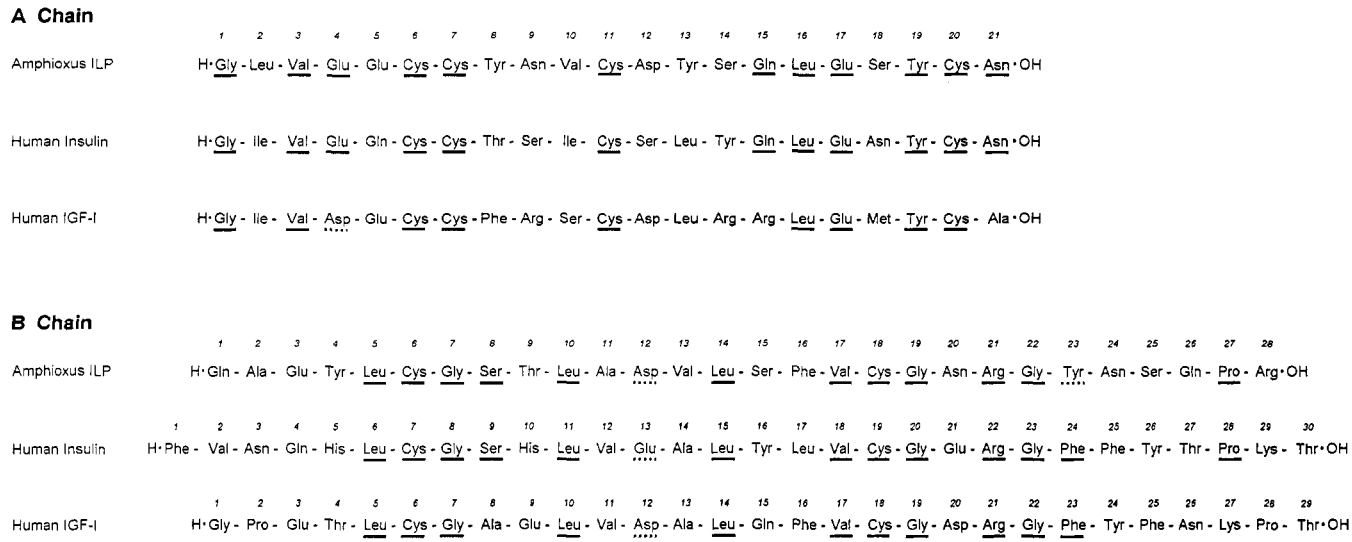

FIGURE 5: Comparison of the primary structure of the A- and B-chains of amphioxus ILP with that of the A- and B-chains of human insulin and IGF-I. Amino acid residues common to all three molecules are underlined with solid lines while conservative variations are underlined with dotted lines.

exist with the insulin B-chain, in the hope that the resulting structure would be more insulin-like and perhaps permit combination with the various A-chain S-sulfonates. We selected residues that are highly conserved in mammalian insulins, specifically B9, B11, B15, B16, B23, B24, and B25 (B10, B12, B16, B17, B24, B25, and B26 in insulin numbering). We have shown that replacement of the naturally occurring B10 residue with an Asp residue in a variety of insulin analogues invariably raises the biological activity of the compound (Burke *et al.*, 1990). Further, we have shown that substitution of the B16 residue of insulin (B15 in amphioxus ILP numbering) decreases the biological activity of the hormone (Schwartz *et al.*, 1985; Hu *et al.*, 1993a). Little information is available concerning the contribution of the B17 residue to the biological activity of insulin. The B24–B26 aromatic triplet in insulin (B23–B25 in amphioxus ILP numbering) has been postulated to influence the structure of the insulin monomer (Mirmira *et al.*, 1991, and references cited therein) and to interact with insulin and IGF receptors (DeMeyts *et al.*, 1990). Finally, we have shown that the Val residue at position B12 is critical to the structure of the B-chain and to the biological activity of insulin (Hu *et al.*, 1993b). Consequently, we synthesized a B-chain corresponding to the amphioxus ILP B-domain in which the B11 Ala residue (B12 in insulin numbering) was replaced with Val. In this compound, the B1 residue was Thr rather than Gln, for the reasons discussed above. In the other B-domain analogues described below, the B1 residue was Glu, which is more isosteric than Thr to the naturally occurring Gln residue in the putative amphioxus ILP. We synthesized B-chain S-sulfonates corresponding to the [1-Glu,15-Tyr]-, [1-Glu,15-Tyr,16-Leu]-, [1-Glu,9-Asp,15-Tyr]-, and [1-Glu,23-Phe,24-Phe,25-Tyr]B-domain of amphioxus ILP. All four of these B-chain S-sulfonates, like the above mentioned [1-Thr]B and [1-Thr,-11-Val]B analogues, failed to combine with insulin A-chain S-sulfonate. Apparently, in spite of the considerably homology between the B-chain sequence of insulin and that of the putative amphioxus ILP, and the increase in homology provided by these substitutions, the conformation of these polypeptides remains different from that of the insulin B-chain. We cannot, at this time, exclude contributions to conformational differences of the substantial differences in the amino acid residues located at the N- and C-terminal ends of these chains or of the differences which still remain between insulin and these analogues at positions B9, B13, and B20 (amphioxus numbering; see Figure 5). We have, however, already noted that the four N-terminal and five C-terminal amino acid residues do not appear to make important contributions to the biological activity of insulin, and in our experience, these residues do not appreciably affect the combination of A- and B-chains in insulin analogues. For positions B13 and B20 (B14 and B21 in insulin numbering), little information is available. For position B10 in insulin (B9 in amphioxus numbering), substitutions have produced profound effects on biological activity in insulin analogues (Hu *et al.*, 1993b, and references cited therein).

In the present study, we have evaluated the potency of the synthetic two-chain, insulin-like compounds in mammalian cells and cell fractions containing either human or mouse IGF-I receptors or human or rat insulin receptors, with respect to binding affinity, insulin-like biological activity, and mitogenic activity. Our data show that amphioxus ILP A/amphioxus ILP [1-Thr]B shows no detectable binding to the human insulin receptor at a concentration as high as 10^{-6} M (Figure 1, Table 1). This compound displays a weak interaction with the mouse IGF-I receptor (Figure 2, Table 1) and low mitogenic activity (Figure 4, Table 1). Further, binding assays using rat insulin receptors showed very low potency (Table 1). This compound did stimulate lipogenesis in rat adipocytes to ca 80% of the level achieved by natural insulin (Figure 3) when present at the highest concentration achievable with the material available, and we consider it likely that it is a full agonist with a calculated potency of 0.01%, relative to porcine insulin (Table 1, Figure 3). In contrast, the hybrid compounds consisting of the B-chain of insulin and either the A-domain or the A+D-domain of amphioxus ILP showed significant binding to both human and rat insulin receptors (Figure 1, Table 1) and also stimulated lipogenesis in rat adipocytes to the same extent as seen with natural insulin (Figure 3). Furthermore, although the hybrid compounds containing the insulin B-chain displayed measurable mitogenic activity, this activity was much lower than their insulin-like metabolic activity, which supports the proposition that the B-chain contributes to the specificity of receptor recognition (Katsoyannis *et al.*, 1987). It is of interest to note that the compound containing the A- and D-domains of amphioxus ILP, i.e., amphioxus ILP A+D/bovine insulin B, is about half as potent as that containing only the A-domain. We have reported an analogous situation with hybrid compounds containing the corresponding domains of the human IGFs (Ogawa *et al.*, 1984; Schwartz *et al.*, 1988). We

conclude that the present two-chain insulin-like molecules embodying the B-chain of insulin possess a region capable of binding to the insulin receptor and that this region can be partially covered by a D-domain, resulting in the suppression of insulin-like metabolic activity. Even the compound containing A- and B-chains corresponding to amphioxus ILP A- and [1-Thr]B-domain stimulates lipogenesis to nearly the level seen with insulin, albeit at much higher concentration (Figure 3). These observations suggest that the putative amphioxus ILP displays a conformation comparable to that of insulin, which itself is similar to the conformation of IGF-I (Blundell *et al.*, 1978, 1983).

In view of the low binding and biological activities obtained with amphioxus ILP A/amphioxus ILP [1-Thr]B, the question arises as to what extent the chemically synthesized compound resembles the native hormone. Very recently, we have successfully sequenced the presumptive amphioxus ILP receptor, and preliminary experiments have shown that amphioxus ILP A/amphioxus ILP [1-Thr] binds to the amphioxus receptor with a 100-fold higher affinity than either human insulin or IGF-I (M. Pashmforoush and S. J. Chan, unpublished data). These results strongly suggest that our synthetic molecule closely resembles the native hormone. Further studies, including utilizing the hybrid analogues described here, should provide important data on the structural determinants recognized by the amphioxus ILP receptor as well as on the evolutionary relationship between the amphioxus ILP, insulin, and IGF-I receptors.

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