High-Potency Hybrid Compounds Related to Insulin and Amphioxus Insulin-like Peptide†

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ABSTRACT: We describe the synthesis and biological evaluation of five two-chain, insulin-like compounds structurally related both to insulin and to a putative insulin like peptide (ILP) whose sequence was deduced from a cDNA cloned from *Branchiostoma californiensis* (amphioxus), a primitive vertebrate [Chan, S. J., Cao, Q.-P., & Steiner, D. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9319–9323]. The present compounds feature an A-chain corresponding to the A-domain of the putative amphioxus ILP A-domain, except that amino acid substitutions have been made at positions A2, A3, A5, and/or A8, linked via disulfide bonds to the B-chain of bovine insulin. Amphioxus ILP [2 Ile] A/insulin B, amphioxus ILP [2 Ile, 8 His] A/insulin B, amphioxus ILP [2 Ile, 5 Gln, 8 His] A/insulin B, and amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His] A/insulin B all display insulin-like metabolic activity and growth-promoting activity (mitogenesis) equal to or greater than that of natural insulin. Amphioxus ILP [8 His] A/insulin B shows activity in these assays greater than that of its parent compound, but not as high as compounds featuring Ile rather than Leu at position A2. In contrast, the parent compound of the present analogues, i.e., amphioxus ILP A/insulin B, displays potencies ranging from 4.0 to 9.8% relative to insulin in insulin receptor binding and lipogenesis assays, respectively. This parent compound displayed activity in growth factor assays too low for exact quantitation [Chu, Y.-C., Hu, S. Q., Zong, L., Burke, G. T., Gammeltoft, S., Chan, S. J., Steiner, D. F., & Katsoyannis, P. G. (1994) *Biochemistry* (in press)]. The results are discussed in connection with the amino acid sequence requirements for metabolic and mitogenic activity in insulin.

A cDNA was cloned and sequenced from *Branchiostoma californiensis* (amphioxus) which could code for a polypeptide that should display structural characteristics of both mammalian insulins and insulin-like growth factors (IGFs) (Chan et al., 1990). This putative polypeptide, referred to as amphioxus ILP, has a deduced primary structure containing B-, C-, A-, and D-domains similar to the IGFs, and its A- and B-domains bear considerable sequence homology to the A- and B-chains of insulin as well as to the A- and B-domains of the IGFs. It was indeed suggested that amphioxus ILP may represent a common ancestor of insulin and the IGFs (Chan et al., 1990).

We have recently completed the synthesis of three insulin-like compounds consisting of two disulfide-linked polypeptide chains, in which the A-chain corresponds either to the A- or to the A+D-domain of the putative amphioxus ILP, whereas the B-chain corresponds to the B-chain of bovine insulin or to a slightly modified B-domain of amphioxus ILP (Chu et al., 1994).

The three synthetic polypeptides (amphioxus ILP A/insulin B, amphioxus ILP A+D/insulin B, and amphioxus ILP A/amphioxus ILP [1 Thr]B) exhibit both insulin-like and IGF-like activities. Specifically, amphioxus ILP A/insulin B and amphioxus ILP A+D/insulin B exhibit insulin-like potencies ranging from 2.0 to 9.8% relative to natural insulin, whereas amphioxus ILP A/amphioxus ILP [1 Thr] B exhibits a potency of 0.01% in lipogenesis assays. In growth-factor assays, these compounds display some activity, but it is too low for exact quantitation. We concluded that the low potencies exhibited by the three synthetic compounds relative to insulin may be attributed to the difference in amino acid sequence in certain positions of these molecules in comparison with the sequence of insulin. In the present paper, we describe the synthesis of five analogues of the hybrid amphioxus ILP A/bovine insulin B whose biological evaluation supports our earlier hypotheses.

EXPERIMENTAL PROCEDURES

Materials. Commercial reagents were as follows: (butoxycarbonyl)amino acids and derivatives (Bachem and Peninsula Laboratories); 4-methylbenzhydrylamine resin (0.6 mmol of amine per gram), used as a solid support (Vega). All solvents were high-performance liquid chromatography (HPLC) grade. Details of other materials and methods used are given in previous publications (Kitagawa et al., 1984; Chu et al., 1992). 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 and [A14 [^{125}I]monoiodo-Tyr]insulin

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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 new-born calf serum (NCS) were purchased from Biological Industries, Israel. G418 (geneticin) was purchased from Sigma, St. Louis, MO.

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 and Van der Eb, 1973), where 20 μg of human insulin receptor or human IGF-I receptor cDNA were 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 receptors/cell), WT 1 cells transfected with human IGF-I receptor cDNA (8.7×10^5 receptors/cell), and C5 cells transfected with human insulin receptor cDNA (6×10^5 receptors/cell) (Grønborg et al., 1993; B. Urso, B. S. Wulff, 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)/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 γ counter (Nielsen et al., 1991). Receptor-bound [^{125}I]insulin or [^{125}I]IGF-I was determined by subtraction of nonspecific 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 one of the amphioxus ILP hybrid compounds 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 using a Betaplate 96-well harvester (Pharmacia) and counted for radioactivity (Nielsen et al., 1991).

General Aspects of the Synthesis of the Insulin-like Compounds Consisting of A-Chains Corresponding to the Amphioxus ILP [2 Ile]-, [8 His]-, [2 Ile, 8 His]-, [2 Ile, 5 Gln, 8 His]-, and [2 Ile, 3 Ile, 5 Gln, 8 His]A-Domains and a B-Chain Corresponding to the B-Chain of Insulin. All five hybrid compounds were prepared by the interaction of S-sulfonated bovine insulin B-chain with the corresponding S-sulfonated derivatives of the amphioxus ILP A-domain analogues. The synthesis of the latter compounds was accomplished by the same procedure employed in the synthesis of S-sulfonated derivatives of the A-chain of insulin and analogues. The first step is the construction of the protected heneicosapeptides, each containing the amino acid sequence of the respective A-domain analogue. The synthesis of the protected heneicosapeptides was carried out by stepwise solid-phase synthesis (Barany & Merrifield, 1980), using 4-methylbenzhydrylamine resin as the solid support (ca. 0.6 mmol of amine per g; ca. 1.0 g). The *tert*-butoxycarbonyl group was used for N^{α} protection. Side-chain protecting groups were 4-methylbenzyl for Cys, cyclohexyl for Glu, benzyl for Ser, and 2,6-dichlorobenzyl for Tyr. A manual coupling protocol was followed (Merrifield et al., 1982). The protected amino acids were incorporated from preformed 1-hydroxybenzotriazole esters in 3-fold excess. Active esters were prepared with *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in dimethylformamide. Preformed symmetric anhydrides (Hagenmaier & Frank, 1972) in dimethylformamide were used for the incorporation of the amino acid residues 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) and was negative after each coupling step. The C-terminal Asn residue was incorporated into the solid support by coupling *tert*-butoxycarbonylaspartic acid α-benzyl ester with 4-methylbenzhydrylamine resin. After the final deprotection step, the Asp residue was converted to an Asn residue.

The protected heneicosapeptides were cleaved from the resin and deprotected from their blocking groups by treatment with liquid hydrogen fluoride and subjected to oxidative sulfitolysis as previously described (Wang et al., 1991; Chu et al., 1992). After a preliminary purification on a Sephadex G-15 column (4.2×50 cm) using 0.015 M NH_4HCO_3 as the eluting solvent, the S-sulfonated amphioxus ILP A-domain analogues were 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 (Chu et al., 1992; Ogawa et al., 1984).

The synthesis of the five amphioxus–insulin hybrids was carried out by the interaction of the respective S-sulfonated amphioxus ILP A-domain analogue and the S-sulfonated bovine insulin B-chain in a ratio of ca. 2:1 by weight in 0.1 M glycine buffer, pH 10.6, in the presence of dithiothreitol as described previously (Wang et al., 1991: Chu et al., 1992). Isolation of the insulin-like analogue from the combination mixture of the corresponding A- and B-chains was carried out by chromatography on a 0.9×24 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 synthetic compound was isolated from the effluent, via picrate,

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

name of 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 activity)	Biological Activity Assays: lipogenesis[e] (insulin activity)
insulin	0.17	100	100	2.2	100
IGF-I	100	2.3	nd	100	nd
amphioxus ILP A/bovine insulin B	<0.03	5.0	3.9	0.05	9.8
amphioxus ILP [2 Ile]A/bovine insulin B	0.3	100	180	2.2	150
amphioxus ILP [8 His]A/bovine insulin B	0.017	25	32	0.2	41
amphioxus ILP [2 Ile, 8 His] A/bovine insulin B	0.5	150	124	1.6	198
amphioxus ILP [2Ile, 5 Gln, 8 His]A/bovine insulin B	0.3	175	126	10	nd[f]
amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His] A/bovine insulin B	0.1	150	nd	1.6	nd

[a] Determined with NIH-3T3 fibroblasts expressing mouse IGF-I receptors. [b] Determined with C5 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] Further substitutions beyond A2 Leu → Ile did not produce increases in insulin-like activity. For some of these compounds, not all types of assay were performed.

as the hydrochloride. Final purification was achieved by reversed-phase HPLC on a Vydac 218 TP column (0.45 × 25 cm) at a flow rate of 0.5 mL/min with a 20–80% linear gradient of 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 80 min. All five analogues, on HPLC rechromatography, under the conditions described above, exhibited single sharp peaks.

RESULTS

The S-sulfonated A- and B-chains of insulin used in this investigation were prepared by oxidative sulfitolysis of bovine insulin followed by chromatographic separation of the resulting S-sulfonated A- and B-chains 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 [2 Ile]A-Domain S-Sulfonate. Peptidyl resin (530 mg), after deblocking, sulfitolysis, and chromatographic purification, yielded ca. 111 mg of purified amphioxus [2 Ile]A-domain S-sulfonate. Amino acid analysis after acid hydrolysis gave the following molar ratios, which were in good agreement with the theoretically expected values (shown in parentheses): $Asp_{2.8\ (3)}$ $Ser_{1.8\ (2)}$ $Glu_{4.0\ (4)}$ $Gly_{1.1\ (1)}$ $Val_{1.7\ (2)}$ $Ile_{0.8\ (1)}$ $Leu_{1.1\ (1)}$ $Tyr_{3.0\ (3)}$. Cys was not determined.

Amphioxus ILP [8 His]A-Domain S-Sulfonate. About 70 mg of purified S-sulfonated chain was obtained from 518 mg of peptidyl resin, after deblocking, sulfitolysis, and chromatographic purification. Amino acid analysis gave the following molar ratios: $Asp_{2.8\ (3)}$ $Ser_{1.6\ (2)}$ $Glu_{4.1\ (4)}$ $Gly_{1.1\ (1)}$ $Val_{1.8\ (2)}$ $Leu_{2.0\ (2)}$ $Tyr_{2.0\ (2)}$ $His_{1.3\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile, 8 His]A-Domain S-Sulfonate. After deblocking, sulfitolysis, and chromatographic purification, 562 mg of peptidyl resin yielded ca. 108 mg of purified S-sulfonated amphioxus [2 Ile, 8 His]A-domain analogue. Amino acid analysis after acid hydrolysis gave the following molar ratios: $Asp_{2.8\ (3)}$ $Ser_{1.7\ (2)}$ $Glu_{4.0\ (4)}$ $Gly_{1.1\ (1)}$ $Val_{1.8\ (2)}$ $Ile_{0.9\ (1)}$ $Leu_{1.2\ (1)}$ $Tyr_{2.3\ (2)}$ $His_{1.1\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile, 5 Gln, 8 His]A-Domain S-Sulfonate. About 98 mg of purified S-sulfonated chain was obtained from 572 mg of peptidyl resin, after deblocking, sulfitolysis, and chromatographic purification. Amino acid analysis gave the following molar ratios: $Asp_{2.7\ (3)}$ $Ser_{1.6\ (2)}$ $Glu_{4.1\ (4)}$ $Gly_{1.1\ (1)}$ $Val_{1.7\ (2)}$ $Ile_{0.8\ (1)}$ $Leu_{1.1\ (1)}$ $Tyr_{2.1\ (2)}$ $His_{0.9\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His]A-Domain S-Sulfonate. From 572 mg of peptidyl resin, after deblocking, sulfitolysis, and chromatographic purification, ca. 100 mg of purified S-sulfonated chain was obtained. Amino acid analysis after acid hydrolysis gave the following molar ratios: $Asp_{2.8\ (3)}$ $Ser_{1.6\ (2)}$ $Glu_{4.1\ (4)}$ $Gly_{1.1\ (1)}$ $Val_{1.0\ (1)}$ $Ile_{1.4\ (2)}$ $Leu_{1.0\ (1)}$ $Tyr_{1.8\ (2)}$ $His_{0.9\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile]A/Bovine Insulin B. From the interaction of the S-sulfonated derivatives of amphioxus ILP [2 Ile]A-domain (23.3 mg) and bovine insulin B-chain (12.0 mg) in 0.1 M glycine buffer (pH 10.6, 5 mL) containing dithiothreitol (6.07 mg), the amphioxus ILP [2 Ile]A/bovine insulin B hybrid (3.07 mg) was isolated after CM-cellulose chromatography, via picrate as the hydrochloride. This product was obtained in a highly purified form upon reversed-phase HPLC under the conditions described above. Amino acid analysis of this product gave the following molar ratios: $Asp_{3.8\ (4)}$ $Thr_{1.0\ (1)}$ $Ser_{2.5\ (3)}$ $Pro_{1.3\ (1)}$ $Glu_{6.9\ (7)}$ $Gly_{3.9\ (4)}$ $Ala_{2.1\ (2)}$ $Val_{4.6\ (5)}$ $Ile_{0.8\ (1)}$ $Leu_{4.7\ (5)}$ $Tyr_{5.0\ (5)}$ $Phe_{3.0\ (3)}$ $Lys_{1.0\ (1)}$ $His_{2.4\ (2)}$ $Arg_{1.0\ (1)}$. Cys was not determined.

Amphioxus ILP [8 His]A/Bovine Insulin B. The hydrochloride of this hybrid (2.15 mg) was obtained from the interaction of the S-sulfonated derivatives of amphioxus [8 His]A-domain (19.46 mg) and bovine insulin B-chain (9.84 mg) in 0.1 M glycine buffer (pH 10.6, 5 mL) containing dithiothreitol (4.82 mg) after CM-cellulose chromatography. This product was obtained in a highly purified form upon reversed-phase HPLC. Amino acid analysis gave the following ratios: $Asp_{3.9\ (4)}$ $Thr_{0.9\ (1)}$ $Ser_{2.5\ (3)}$ $Pro_{0.9\ (1)}$ $Glu_{7.5\ (7)}$ $Gly_{3.9\ (4)}$ $Ala_{2.0\ (2)}$ $Val_{4.6\ (5)}$ $Leu_{5.8\ (6)}$ $Tyr_{3.8\ (4)}$ $Phe_{2.9\ (3)}$ $Lys_{1.1\ (1)}$ $His_{3.5\ (3)}$ $Arg_{0.9\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile, 8 His]A/Bovine Insulin B. The interaction of the S-sulfonated derivatives of amphioxus ILP [2 Ile, 8 His]A-domain (24.75 mg) and bovine insulin B-chain (11.82 mg) in 0.1 M glycine buffer (pH 10.6, 5 mL) in the presence of dithiothreitol (5.12 mg), using the same procedure described above, led to the isolation of 3.7 mg of the hydrochloride of this hybrid. Final purification was carried out by reversed-phase HPLC. Amino acid analysis of this product gave the following molar ratios: $Asp_{3.8\ (4)}$ $Thr_{1.0\ (1)}$ $Ser_{2.2\ (3)}$ $Pro_{1.1\ (1)}$ $Glu_{7.1\ (7)}$ $Gly_{4.1\ (4)}$ $Ala_{2.2\ (2)}$ $Val_{4.6\ (5)}$ $Ile_{0.9\ (1)}$ $Leu_{4.8\ (5)}$ $Tyr_{3.6\ (4)}$ $Phe_{2.9\ (3)}$ $Lys_{1.2\ (1)}$ $His_{3.2\ (3)}$ $Arg_{1.1\ (1)}$. Cys was not determined.

Amphioxus ILP [2 Ile, 5 Gln, 8 His]A/Bovine Insulin B. The hydrochloride of this hybrid (6.35 mg) was obtained by the interaction of the S-sulfonated derivatives of amphioxus ILP [2 Ile, 5 Gln, 8 His]A-domain (26.46 mg) and bovine insulin B-chain (13.71 mg) in 0.1 M glycine buffer (pH 10.6, 5 mL) in the presence of dithiothreitol (6.0 mg) using the same procedure described above. Final purification was achieved by reversed-phase HPLC. Amino acid analysis gave the following ratios: $Asp_{3.9\ (4)}$ $Thr_{0.9\ (1)}$ $Ser_{2.5\ (3)}$ $Pro_{1.0\ (1)}$ $Glu_{7.2\ (7)}$ $Gly_{4.0\ (4)}$ $Ala_{2.0\ (2)}$ $Val_{4.6\ (5)}$ $Ile_{0.8\ (1)}$ $Leu_{5.0\ (5)}$ $Tyr_{3.2\ (4)}$ $Phe_{2.8\ (3)}$ $Lys_{1.0\ (1)}$ $His_{3.0\ (3)}$ $Arg_{1.1\ (1)}$. Cys was not determined.

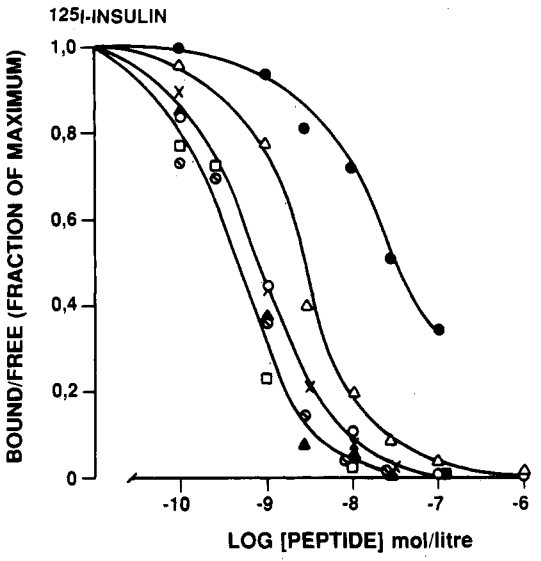

FIGURE 1: [^{125}I]Insulin binding to NIH-3T3 fibroblast transfected with human insulin receptor. Subconfluent cultures of C5 cells in 24-well dishes were incubated 20 h at 4 °C with 20 pM ^{125}I-insulin in the absence or presence of human insulin (○), human IGF-I (●), amphioxus ILP [2 Ile] A/bovine insulin B (×), amphioxus ILP [8 His] A/bovine insulin B (△), amphioxus ILP [2 Ile, 8 His]A/bovine insulin B (▲), amphioxus ILP [2 Ile, 5 Gln, 8 His] A/bovine insulin B (□) or amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His] A/bovine insulin B (⊖). Receptor bound [^{125}I]insulin was determined after washing and solubilizing the cells in NaOH and counting the radioactivity. Data are mean of three experiments with SD of 10–15%.

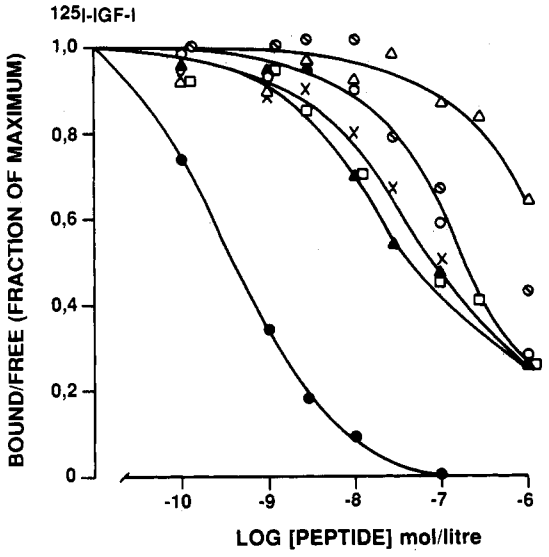

FIGURE 2: [^{125}I]IGF-I binding to mouse IGF receptor on NIH-3T3 fibroblasts. Subconfluent cultures of NIH-3T3 fibroblasts in 24-well dishes were incubated 20 h at 4 °C with 20 pM [^{125}I]IGF-I in the absence or presence of human IGF-I (●), human insulin (○), amphioxus ILP [2 Ile] A/bovine insulin B (×), amphioxus ILP [8 His] A/bovine insulin B (△), amphioxus ILP [2 Ile, 8 His] A/bovine insulin B (▲), amphioxus ILP [2 Ile, 5 Gln, 8 His] A/bovine insulin B (□) or amphioxus ILP [1 Ile, 3 Ile, 5 Gln, 8 His] A/bovine insulin B (⊖). Receptor-bound [^{125}I]IGF was determined after washing and solubilizing the cells in NaOH and counting radioactivity. Data are mean of three experiments with SD of 10–15%.

Amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His]A/Bovine Insulin B. The interaction of the S-sulfonated derivatives of amphioxus [2 Ile, 3 Ile, 5 Gln, 8 His]A-domain (22.47 mg) and bovine insulin B-chain (11.34 mg) in 0.1 M glycine buffer (pH 10.6, 5 mL) in the presence of dithiothreitol (5.35 mg) using the procedure described above led to the isolation of 5.39 mg of the hydrochloride of this hybrid. Final purification

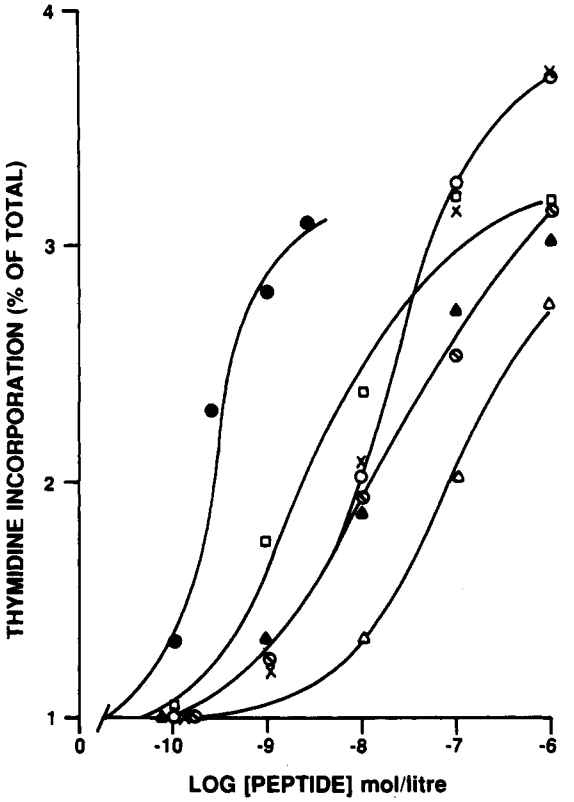

FIGURE 3: IGF-I-stimulated DNA synthesis in NIH fibroblasts transfected with human IGF receptors. Subconfluent WT1 cells were cultured for 17 h at 27 °C in the absence or presence of human IGF-I (●), human insulin (○), amphioxus ILP [2 Ile] A/bovine insulin B (×), amphioxus ILP [8 His]A/bovine insulin B (△), amphioxus ILP [2 Ile, 8 His] A/bovine insulin B (▲), amphioxus ILP [2 Ile, 5 Gln, 8 His] A/bovine insulin B (□), or amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His] A/bovine insulin B (⊖) 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 in 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 SD of 10–15%.

was achieved by reversed-phase HPLC. Amino acid analysis gave the following ratios: $Asp_{3.9}$ (4) $Thr_{0.9}$ (1) $Ser_{2.2}$ (3) $Pro_{1.2}$ (1) $Glu_{7.3}$ (7) $Gly_{4.2}$ (4) $Ala_{2.0}$ (2) $Val_{3.8}$ (4) $Ile_{1.6}$ (2) $Leu_{4.6}$ (5) $Tyr_{3.7}$ (4) $Phe_{2.7}$ (3) $Lys_{1.0}$ (1) $His_{3.3}$ (3) $Arg_{1.1}$ (1). Cys was not determined.

Biological Evaluation of Amphioxus ILP-Related Insulin Analogues. The potency of the present compounds was compared to that of natural porcine insulin or human recombinant insulin, as well as to that of human IGF-I in five types of assays (Table 1and Figures 1–3). The most striking result was an increase in both growth-factor activity and insulin-like metabolic activity of at least 10-fold observed when the Leu residue in position 2 of the A-chain corresponding to A-domain of amphioxus ILP was replaced with Ile. This single change resulted in a compound essentially indistinguishable from insulin in all assays, despite the presence of the remaining 20 residues corresponding to the sequence of amphioxus ILP. Substitution of His for Tyr in position 8 of the same chain led to a smaller but still impressive increase in insulin-like activity. [A8 His]Insulin has been reported to display higher activity than natural mammalian insulin (Marki et al., 1979). We made compounds featuring further substitution of residues corresponding to or conservatively varied from the sequence of the A-chain of insulin for those of the A-domain of amphioxus ILP. Table 1 shows that the replacement of A5 Glu with Gln and replacement of A3 Val with Ile in compounds

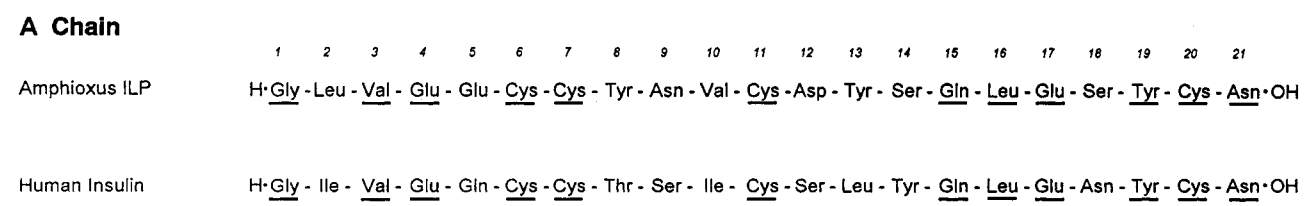

FIGURE 4: Comparison of the primary structure of the A-chain of amphioxus ILP with that of the A-chain of human insulin. Amino acid residues common to both molecules are underlined.

already containing the A2 Leu → Ile modification had no consistent effect upon their potency in insulin assays.

The binding of the present compounds to the human insulin receptor is depicted in Figure 1. A striking increase in potency attends the replacement of A2 Leu with Ile; a lesser increase is produced by the replacement of A8 Tyr with His, and there is little further change upon further modification beyond A2 Leu → Ile, in good agreement with the results obtained using rat insulin receptors.

Figure 2 shows the behavior of the hybrid compounds in binding to the human IGF-I receptor expressed in transfected cultured cells. The general observation is that the A2 Leu → Ile modification produces a compound indistinguishable from insulin, while the replacement of A8 Tyr with His produces a compound with activity higher than the parent compound, but less than those featuring A2 Leu → Ile. Again, further substitution beyond A2 Leu → Ile was without consistent effect.

The activity of the present hybrid compounds in stimulating mitogenesis in cultured cells expressing the human IGF-I receptor is shown in Figure 3. The pattern of activity in this assay is similar to that seen in the IGF-I receptor-binding studies. We note that the potency of [A2 Ile, A5 Gln, A8 His]amphioxus ILP A/ bovine insulin B is higher than that of the other compounds featuring the A2 Leu → Ile modification, but still much lower than IGF-I itself, and we are not sure of the physiological significance of the difference in potency between this compound and the others.

DISCUSSION

Amphioxus ILP A/bovine insulin B, the insulin-like compound which consists of an A-chain corresponding to the putative amphioxus ILP A-domain and a B-chain corresponding to that of bovine insulin, displays potencies of ca. 4% and 9.8% relative to insulin in insulin receptor-binding and lipogenesis assays, respectively. This hybrid compound exhibits potencies of <0.03% and 0.05% relative to human IGF-I in IGF-I receptor-binding and mitogenesis assays, respectively. Natural insulin displays activities of 0.2% and 2.2% relative to IGF-I in these assays (Chu et al., 1994).

A comparison of their primary structures reveals that 12 of the 21 amino acid residues in the amphioxus ILP A-domain are identical to those found in human insulin (Figure 4). Consequently, it appears that the low potency exhibited by amphioxus ILP A/bovine insulin B relative to insulin is attributable to the variation that still exists in the primary structure of these two compounds. We speculate specifically that the key difference is A2 Leu in the amphioxus ILP versus A2 Ile in insulin and IGF-I. We have previously shown that Ile in position A2 is of critical importance for the biological activity both of insulin (Okada et al., 1981; Kitagawa et al., 1984a,b) and of IGF-I (Zong et al., 1990). It should be noted that, in the X-ray model of crystalline hexameric insulin, the side chain of A2 Ile is in van der Waals contact with the phenyl ring of A19 Tyr (Blundell et al., 1972), and this appears to be critical for the maintenance of a conformation commensurate with high receptor-binding affinity and hence high biological activity in insulin.

Our speculation that the low potency of the hybrid amphioxus ILP A/insulin B is attributable to the presence of Leu instead of Ile at position A2 is fully substantiated in the present study. Substitution of Ile for A2 Leu in amphioxus ILP A/insulin B leads to amphioxus ILP [2 Ile] A/insulin B, which is essentially indistinguishable from natural insulin in both insulin-like metabolic assays and growth-factor assays. Implicit in this finding is that the overall structure of amphioxus ILP A/insulin B is comparable to that of insulin, and that it becomes identical upon the substitution of Ile for A2 Leu.

Further evidence for the similarity in overall structure between insulin and amphioxus ILP A/insulin B is provided by the synthesis of other analogues as well. Substitution of A8 Thr in human insulin by His results in [A8 His]insulin, which displays ca. 2–3-fold higher potency than that of the natural hormone (Marki et al., 1979). It was suggested that the His residue at position A8 may be involved in interactions with the insulin receptor which result in higher affinity of the receptor for insulin. Alternatively, the presence of His at position A8 may modify the disposition of neighboring residues involved in receptor binding, thus increasing the affinity of the molecule for the receptor (Pullen et al., 1976; Derewenda et al., 1991). Substitution of His for the Tyr residue at position A8 in amphioxus ILP A/insulin B led to the analogue amphioxus ILP [8 His] A/insulin B, which displayed 4–8-fold higher potency than the parent compound, analogous to the situation with [A8 His]insulin.

Further modification of the A8 His analogue involving the substitution of A2 Leu with Ile yielded the amphioxus ILP [2 Ile, 8 His] A/insulin B hybrid compound, which displayed a potency equal to or higher than that of insulin, in all types of assays.

The last two analogues in this series involve modifications of residues corresponding to or conservatively varied from the human insulin A-chain sequence and the sequence of the amphioxus ILP A-domain. Both analogues contain, in addition, substitutions of Ile for A2 Leu and His for A8 Tyr. Amphioxus ILP [2 Ile, 5 Gln, 8 His] A/insulin B features Gln, the A5 residue of insulin, rather than Glu at position A5. This analogue displays similar potency to that of the parent compound, amphioxus ILP [2 Ile, 8 His] A/insulin B, but substantially higher growth-factor activity. We are at present unable to delineate the significance of the difference in growth-promoting potency between these two compounds. More intriguing is the case with the last analogue described here, amphioxus ILP [2 Ile, 3 Ile, 5 Gln, 8 His] A/insulin B. This hybrid compound differs from the previous three in that Val, present at position A3 in most insulins and in amphioxus ILP, has been replaced by Ile. It displays, in both insulin-like metabolic assays and growth-factor assays, potency comparable to the aforementioned highly active A2 Ile-substituted analogues. This observation is in contrast to the biological behavior of an abnormal insulin identified in some diabetic patients (Nanjio et al., 1986), in which the Val residue at

position A3 has been substituted by Leu (insulin Wakayama). [A3 Leu]Insulin displays a potency ca. 1% relative to the natural hormone (Kobayashi et al., 1986). It has been suggested (Tager, 1990) that the A3 Val → Leu substitution disturbs the A2–A8 distorted α-helix, identified in the X-ray structure of crystalline insulin (Blundell et al., 1972). This disturbance affects the A2 Ile–A19 Tyr van der Waals contact resulting in an insulin with an altered conformation and defective receptor-binding characteristics. Apparently the side-chain packing of Ile at position A3 is sufficiently similar to that of Val at this position to avoid conformational changes to the molecule deleterious to receptor-binding affinity.

In summary, hybrid compounds featuring a single amino acid substitution in the sequence of the A-domain of amphioxus ILP, A2 Leu → Ile, together with the B-chain of insulin, display high insulin-like potency in metabolic and mitogenic assays. This indicates that amphioxus ILP A/insulin B may have an overall conformation comparable to that of insulin and furthermore that the conformation may become identical upon a single amino acid substitution. Given that insulin and IGF-I are structurally related (Blundell et al., 1978, 1983), this observation provides additional evidence for the concept (Chan et al., 1990) that amphioxus ILP may represent a common ancestor of insulin and IGF-I.

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