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Structural Basis of the Aberrant Receptor Binding Properties of Hagfish and Lamprey Insulins[†]

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ABSTRACT: The insulin from the Atlantic hagfish (*Myxine glutinosa*) has been one of the most studied insulins from both a structural and a biological viewpoint; however, some aspects of its biology remain controversial, and there has been no satisfying structural explanation for its low biological potency. We have re-examined the receptor binding kinetics, as well as the metabolic and mitogenic properties, of this phylogenetically ancient insulin, as well as that from another extant representative of the ancient chordates, the river lamprey (*Lampetra fluviatilis*). Both insulins share unusual binding kinetics and biological properties with insulin analogues that have single mutations at residues that contribute to the hexamerization surface. We propose and demonstrate by reciprocal amino acid substitutions between hagfish and human insulins that the reduced biological activity of hagfish insulin results from unfavorable substitutions, namely, A10 (Ile to Arg), B4 (Glu to Gly), B13 (Glu to Asn), and B21 (Glu to Val). We likewise suggest that the altered biological activity of lamprey insulin may reflect substitutions at A10 (Ile to Lys), B4 (Glu to Thr), and B17 (Leu to Val). The substitution of Asp at residue B10 in hagfish insulin and of His at residue A8 in both hagfish and lamprey insulins may help compensate for unfavorable changes in other regions of the molecules. The data support the concept that the set of unusual properties of insulins bearing certain mutations in the hexamerization surface may reflect those of the insulins evolutionarily closer to the ancestral insulin gene product.

Insulin belongs to a superfamily of structurally related peptide hormones essential for growth, development, reproduction, longevity, and metabolism (reviewed in refs *1* and *2*). In humans, members of this family include insulin, the insulin-like growth factors (IGF-I and IGF-II), relaxins (H1, H2, and H3), INSL3, INSL4, INSL5, and INSL6 (reviewed in ref *3*).

Insulin, IGF-I, and IGF-II bind to receptors that share sequence and structural features. They belong to a superfamily of receptor tyrosine kinases, which in vertebrates includes the insulin receptor, the type I IGF[1] receptor, and the orphan insulin receptor-related receptor (reviewed in ref *4*). In contrast, the relaxin-like members bind to G protein-coupled receptors with seven transmembrane domains (reviewed in ref *5*).

Distinct insulin and IGF-I-like molecules have been cloned in vertebrate species, including extant representatives of ancient chordates, such as the Atlantic hagfish (*Myxine glutinosa*) (*6*−*8*) and lamprey (*Lampetra fluviatilis* and *Petromyzon marinus*) (*9*, *10*). Likewise, separate insulin and IGF-I receptors have been cloned in these animals (*11*).

The study of insulins from various species has shed light not only on the evolution of the molecule but also on the relationship between insulin structure and activity. The finding that a number of amino acids were highly conserved during evolution led to the suggestion that a dozen of these residues located on the surface of the molecule were involved in receptor binding and biological activity. This cluster of residues is now termed the "classical binding surface" or "site 1" (*12*−*15*) (see Table 1). Some of the same residues are involved in the dimerization of the insulin molecule (*14*).

Early studies of the biological properties of hagfish insulin generated somewhat conflicting results. The affinity of hagfish insulin for isolated rat adipocytes was determined to be 23% of that of porcine insulin, while its biological potency in stimulating lipogenesis in the same cells was only 3−5% (*16*−*18*). Others, however, found an affinity for isolated rat hepatocytes or human IM-9 receptors more consistent with the biological potency (*19*, *20*). Indeed, studies of the receptor binding kinetics of ^{125}I-labeled hagfish insulin demonstrated that this insulin had an unusually slow association rate (*21*). Consequently, the earlier studies comparing affinity and biological activity were not performed under equilibrium conditions, and affinity was therefore underestimated. Another anomalous behavior seen with hagfish insulin was a dose−response curve for accelerating the dissociation of ^{125}I-labeled porcine insulin from IM-9 cell receptors [measuring the so-called negative cooperativity (see ref *22*)] that was not bell-shaped (*19*).

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[1]Abbreviation: IGF, insulin-like growth factor.

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Table 1: Sequence Alignment and Comparison of Residues of Human, Hagfish, and Lamprey Insulin in Classical and Novel Binding Surfaces[a]

	Classical Binding Surface (Site 1)												
	Al	A2	A3	A5	A8	A19	A21	B12	B16	B23	B24	B25	B26
human	Gly	Ile	Val	Gln	Thr	Tyr	Asn	Val	Tyr	Gly	Phe	Phe	Tyr
hagfish	–	–	–	–	His	–	–	–	–	–	–	–	–
lamprey	–	–	–	–	His	–	–	–	–	–	–	–	–

	Novel Binding Surface (Site 2)					
	A12	A13	A17	B10	B13	B17
human	Ser	Leu	Glu	His	Glu	Leu
hagfish	–	Ile	–	Asp	Asn	Ile
lamprey	–	Ile	–	–	–	Val

	Other Sites											
	A9	A10	A15	A16	Bl	B2	B3	B4	B21	B29	B30	B31
human	Ser	Ile	Gln	Leu	Phe	Val	Asn	Gln	Glu	Lys	Thr	–
hagfish	Lys	Arg	Asp	–	Arg	Thr	Thr	Gly	Val	Thr	Lys	Met
lamprey	Arg	Lys	Asp	Met	Ala	Gly	Gly	Thr	Asp	Ser	Lys	Thr

[a]Dashes represent identical residues compared to that of human insulin. The sequences are shown with the three-letter code for the amino acids.

These data together with the fact that Ala substitutions in human insulin at A13 and B17 showed the same binding properties as hagfish insulin were used to propose a novel binding surface ("site 2", which coincides more or less with insulin's hexamerization surface) (*22*−*24*). This surface was mapped more recently by alanine mutagenesis of insulin using high-affinity binding to IM9 cells (see Table 1) (*1*, *15*, *25*, *26*), supporting the cross-linking binding model proposed by De Meyts (*22*) and Schäffer (*24*) in 1994. The model proposed by De Meyts (*22*) in which site 1 and site 2 on the insulin molecule alternatively cross-link two receptor α-subunit binding surfaces (site 1 and site 2) with an antiparallel disposition has been supported by the recent crystal structure of the insulin receptor ectodomain (*27*) as well as by alanine scanning mutagenesis of the insulin receptor (*28*, *29*) and mathematical modeling (*30*).

However, to date no rational explanation for the aberrant binding behavior of hagfish insulin has been provided.

In this work, we have re-examined the binding behavior and biological properties of hagfish insulin as well as that of insulin from the river lamprey (*L. fluviatilis*). Structurally, hagfish insulin is 65% identical to human insulin, bearing 19 amino acid substitutions. In comparison, the sequence similarity of lamprey insulin with human insulin is 72%, with 14 amino acid substitutions as well as a pentapeptide extension at the N-terminus (Table 1 of the Supporting Information). Both insulins have a single-residue extension at the C-terminus of the B chain. By analyzing singly substituted human and hagfish insulin analogues, we show that a small number of residues explain the aberrant behavior of hagfish and lamprey insulins.

EXPERIMENTAL PROCEDURES

Materials. Molecular biology procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing, were performed by standard methods. Oligonucleotides were purchased from DNA Technology (Aarhus, Denmark). Restriction enzymes and ligase were from New England BioLabs (Hitchin, U.K.). Recombinant human insulin, ^{125}I-Tyr^{A14} insulin, and *Achromobacter lyticus* protease were from Novo Nordisk A/S (Bagsværd, Denmark). IM9 cells were purchased from ATCC (Middlesex, U.K.). Hagfish insulin was a gift from S. Falkmer (University of Umea, Umea, Sweden). Lamprey insulin was purified to near homogeneity (>98% pure) from the islet organ of the river lamprey *L. fluviatilis* as previously described (*9*). Both hagfish and lamprey insulins were labeled at Tyr ^{A14} at Novo Nordisk's Chemistry and Isotope laboratory using the lactoperoxidase method and high-performance liquid chromatography (HPLC) purification (*31*). Hagfish cDNA production was described previously (*32*).

Vector Construction and Yeast Expression of Insulin Analogues. Substitutions were introduced into the sequence encoding the insulin precursor by QuikChange site-directed mutagenesis (Stratagene). Resultant constructs were subcloned into yeast expression vector pIM45 (kind gift from J. Brandt, Novo Nordisk A/S). The yeast expression procedure has been described previously (*33*). Briefly, an insulin precursor was coupled to a synthetic prepropeptide sequence consisting of a prepropeptide, a KR Kex2 cleavage site, a removable spacer peptide $E(EA)_3EPK$, and insulin precursor (B1-B29/B30)-AAK-(A1-A21). The hagfish insulin cDNA was inserted into pIM45 using standard PCR and restriction enzyme digestion/ligation. To express the hagfish insulin in the system, we substituted Lys at positions A9 and B9 with Ser (like in human insulin) to prevent cleavage at these positions during processing, and we removed residue Met-B31, leading to the sequence Glu-$(Glu-Ala)_3$-Glu-Pro-Lys-(B1-B30)-(A1-A21). The single-chain insulin precursor was expressed in *Saccharomyces cerevisiae* strain MT663. Yeast cells were transformed and selected on yeast nitrogen base/2% glucose plates. Yeast cultures were grown in yeast/peptone medium with 2% glucose and 50 mM $CaCl_2$ for 72 h at 30 °C (*33*, *34*).

Maturation and Purification of the Insulin Precursor. The single-chain insulin precursors were converted to des-B30-insulin using a lysine specific endoprotease (*A. lyticus*) that removes the spacer peptide (*34*). The pH of the cell-free yeast culture medium with the insulin precursor was adjusted to >8.5

with 1 M Tris-HCl (pH 9), and the mixture was applied to a column with *A. lyticus* immobilized on Sepharose. The column was incubated for 30−60 min at room temperature before the cleaved precursor was eluted from the column with 50 mM Tris-HCl. The insulin analogues were analyzed by mass spectroscopy and were shown to have correct masses. Quantitation was performed by liquid chromatography and mass spectroscopy (LC−MS) using human insulin as a standard.

Binding Assays. Whole cell receptor binding assays were performed as previously described (*35*, *36*) using IM9 cells, which strongly express the IR-A isoform (ca. 20000 binding sites/cell). All experiments were repeated three times in duplicate unless otherwise stated.

For association assays, 1×10^7 cells/mL were incubated with 150000 cpm/mL ^{125}I-labeled human, hagfish, or lamprey insulin at 15 °C. At specified time points, duplicate 200 μL aliquots were centrifuged through 200 μL of ice-cold HBB [100 mM Hepes, 100 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, and 1% BSA (w/v) (pH 7.6)]. The bound ^{125}I-labeled ligand was counted in a Wallac WIZARD γ counter (Perkin-Elmer). Two additional aliquots were not centrifuged but counted as the total.

Dissociation kinetics were determined as previously described (*36*). Cells were incubated with ^{125}I-labeled lamprey or human insulin for 6 h at 15 °C. The cells were centrifuged, the supernatants aspirated, and the cell pellets resuspended in an equivalent volume of fresh HBB. The samples were then diluted 40-fold in the absence or presence of 170 nM human insulin. Duplicate tubes were centrifuged at the specified times to determine the cell-bound counts per minute.

For dose−response curves for negative cooperativity (accelerated dissociation) (*36*), 5×10^7 cells/mL were incubated with ^{125}I-labeled hagfish, lamprey, or human insulin (150000 cpm/mL of cells) for 2 or 4 h at 15 °C. Afterward, the cells were centrifuged and the supernatant (unbound ligand) was removed. The cell pellet was resuspended to the same concentration in HBB, and 25 μL aliquots of cells were transferred to tubes containing increasing concentrations of cold analogue in 1 mL of HBB at 15 °C. The dissociation of prebound ligand was stopped after 30 min by centrifugation at 4000 rpm for 5 min. The pellet was counted in the γ counter.

For competition assays, 2.5×10^6 or 1×10^7 cells/mL were incubated with ^{125}I-labeled ligand (20000 cpm) in the presence of increasing concentrations of the analogue in a final volume of 500 μL for 2.5 or 6 h at 15 °C in HBB. Afterward, duplicate 200 μL aliquots were transferred to centrifuge tubes and centrifuged at 14000 rpm for 5 min. The bound ^{125}I-labeled ligand was counted. Binding data were corrected for nonspecific binding and analyzed by computer fitting to a one-site model using an Excel program developed from the model of Wang (*37*) in our laboratory by A. V. Groth and R. M. Shymko, yielding the dissociation constant (K_d).

Metabolic Potency. Insulin-stimulated lipogenesis in freshly isolated rat adipocytes was used to evaluate metabolic potency. Isolated rat adipocytes were prepared by a modification of the method of Gliemann (*38*) from the epididymal fat pads of male Sprague-Dawley rats. Adipose tissue was digested with 1 mg/mL collagenase (Sigma catalog no. C6885) in Krebs Ringers Hepes buffer (KRH) containing 3.5% (w/v) BSA (Sigma catalog no. A4503), 0.2 μM adenosine, and 2 mM glucose for 45 min at 37 °C. The adipocytes were washed three times in washing buffer [KRH containing 1% (w/v) BSA, 0.2 μM adenosine, and 2 mM glucose] and resuspended in incubation buffer (KRH containing 3.5% BSA, 0.56 mM glucose, and 0.2 μM adenosine). Lipocrit was used as a measure of cell number. Hormone-stimulated conversion of glucose into lipids was measured by the method of Moody et al. (*39*) using triplicate determinations for each point. Briefly, 0.5% (v/v) adipocytes were incubated for 2 h at 37 °C in 0.5 mL of incubation buffer containing 0.05 mCi of [3-^{3}H]-D-glucose (TRK239, Amersham) and various concentrations of hagfish, lamprey, or human insulin. Incubation was terminated by the addition of 3 mL of BetaMax scintillator (ICN, Costa Mesa, CA), and total lipids were extracted by allowing the samples to stand for 24 h at 4 °C. Under these conditions, unmetabolized labeled glucose is not extracted into the organic phase and is not counted. We measured the background by preparing samples with a tracer but without hormone and

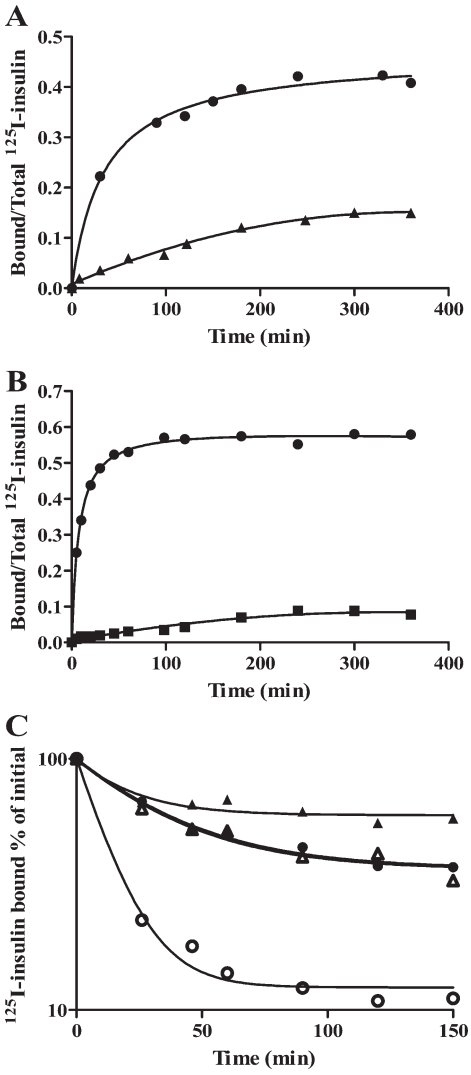

FIGURE 1: Association and dissociation assay. (A and B) Association of ^{125}I-labeled lamprey and hagfish insulin with IM9 cells. The fraction of tracer bound over total is expressed as a function of incubation time. Panel A depicts data from a representative experiment comparing ^{125}I-labeled human insulin (●) and ^{125}I-labeled lamprey insulin (▲), while panel B depicts data from a representative experiment comparing ^{125}I-labeled human insulin (●) and ^{125}I-labeled hagfish insulin (■). (C) Representative curves of dissociation of ^{125}I-labeled human (●) and ^{125}I-labeled lamprey (▲) insulin in dilution alone (filled symbols) or in the presence of 1 μg/mL human insulin (empty symbols). Bound tracer at time t as a fraction (%) of bound tracer at time zero was plotted as a function of time.

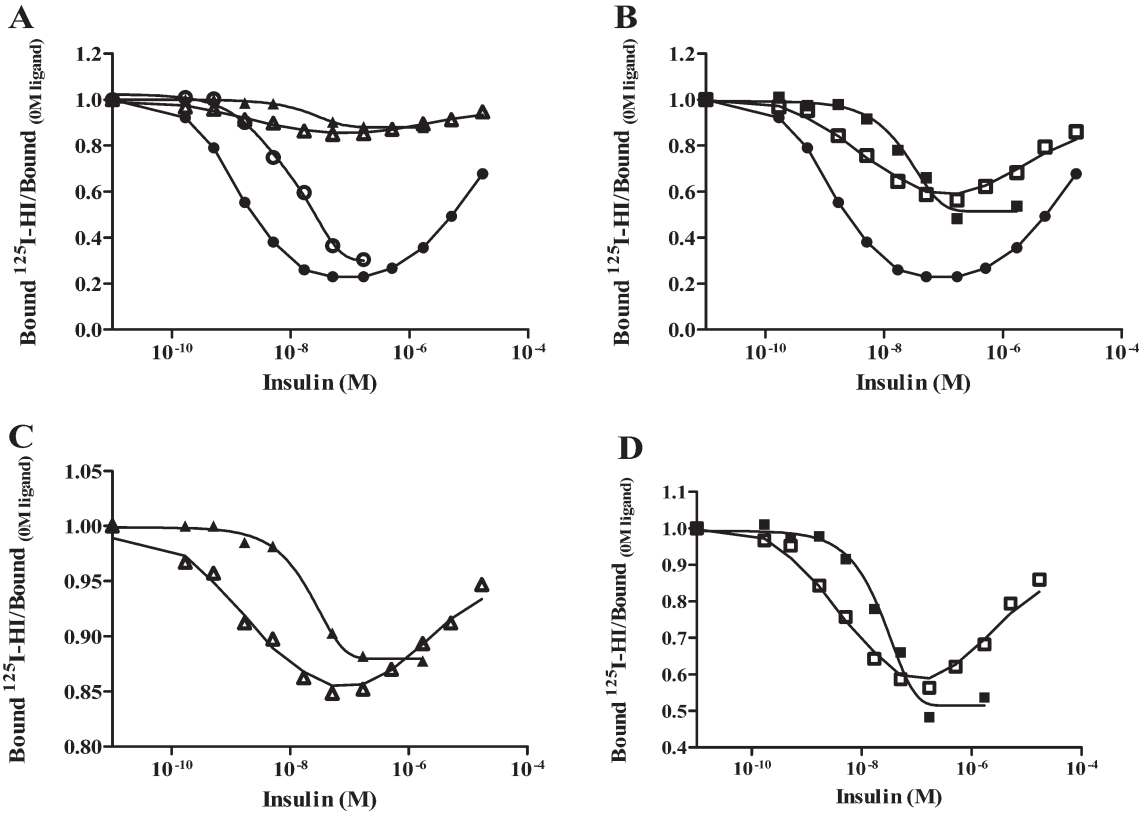

FIGURE 2: Dose−response curves for ligand-induced acceleration of dissociation. Panel A illustrates dose−response curves for the human insulin tracer in the presence of unlabeled insulin (●), the human insulin tracer in the presence of unlabeled lamprey insulin (○), the lamprey tracer in the presence of unlabeled human insulin (△), and the lamprey tracer in the presence of unlabeled lamprey insulin (▲). Panel B illustrates dose−response curves for the human insulin tracer in the presence of unlabeled insulin (●), the hagfish tracer in the presence of unlabeled human insulin (□), and the hagfish tracer in the presence of unlabeled hagfish insulin (■). Panel C is an expansion of the plot of panel A, while panel D is an expansion of the plot of panel B. These expanded scales more clearly demonstrate the presence of reduced negative cooperativity.

immediately adding scintillant. Samples were counted in a β counter (Tricarb 460 C Liquid Scintillation System) for 10 min and the counts corrected for background. Data were fitted to a four-parameter logistics model (*40*, *41*) in Microsoft Excel to determine the concentration that produces 50% of the maximal stimulation (ED_{50}).

Mitogenic potency was measured by thymidine incorporation. NIH 3T3 fibroblasts stably transfected with wild-type human insulin receptor [clone HIR 3.5 (*42*)] were obtained from K. Siddle (Cambridge, U.K.). The cells were cultured with Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and 2 mM glutamine in a humidified atmosphere. HIR 3.5 cells were plated at a density of 10000 cells/well in 96-well microtiter plates and allowed to attach overnight in DMEM containing 10% FBS. Quiescence was achieved after starvation for 2 days in DMEM with 2% FBS at which time hagfish, lamprey, or human insulin was added at increasing concentrations with vehicle alone as a negative control and 10% FBS as a positive control. After incubation for 17 h at 37 °C, the cells were pulse-labeled with 1 μCi of [^{3}H]thymidine (TRK565, Amersham) per well for 3 h. Unincorporated label was removed by washing twice with PBS, and the cells were then solubilized with 0.2 M NaOH. A 200 μL volume of SuperMix Scintillation fluid (Wallac) was added and allowed to mix for 1 h on a plate shaker. Incorporation of [^{3}H]thymidine was determined by β radiation counting (MicroBeta, Wallac). Data were analyzed using a four-parameter logistics model as described for lipogenesis (see above).

RESULTS

Association Kinetics of Hagfish and Lamprey Insulin for the Membrane-Bound Human Insulin Receptor. The kinetics of association of hagfish and lamprey insulin with the human insulin receptor on IM9 cells were determined at 15 °C using ^{125}I-labeled hagfish, lamprey, and human insulin (Figure 1A,B). The association rates for both hagfish and lamprey insulins were much slower than that of human insulin. Via comparison of the initial slopes of the association curves, the initial rate for the lamprey insulin appeared to be 9.4-fold slower than that of human insulin while that of the hagfish insulin appeared to be ~21-fold slower than that of human insulin. An apparent steady state of binding was reached after 6 h at 15 °C with lamprey insulin; 4 h was required for hagfish insulin, while human insulin required incubation for 2.5 h.

Kinetics of Dissociation of ^{125}I-Labeled Lamprey Insulin from the Human Insulin Receptor. Dissociation of ^{125}I-labeled human insulin by dilution alone showed a non-first-order biphasic pattern and was markedly accelerated in the presence of 170 nM unlabeled human insulin as previously described (*12*, *22*, *36*) (Figure 1C). The dissociation of ^{125}I-labeled lamprey insulin by dilution alone was much slower than that of ^{125}I-labeled human insulin, and in the presence of 170 nM human insulin, the dissociation of lamprey insulin was only minimally accelerated to a rate similar to the rate of dissociation of human insulin by dilution alone (Figure 1C). Similar results were shown previously for hagfish insulin (*43*).

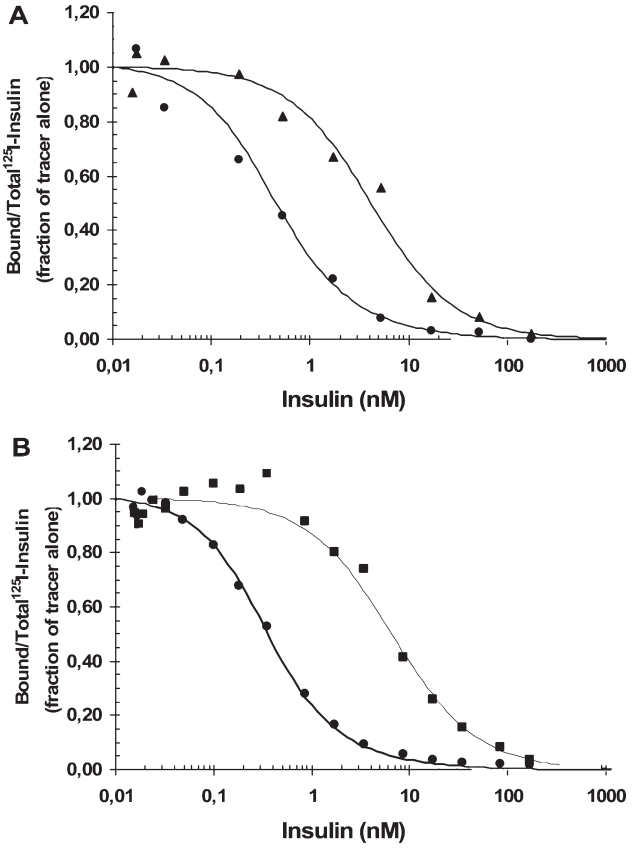

FIGURE 3: Homologous competition for the holo insulin receptor. All curves are plotted as bound:total tracer as a function of the logarithm of the concentration of unlabeled ligand. Competition of ^{125}I-labeled human insulin with increasing concentrations of human insulin (●) is illustrated in the two panels. Competition of ^{125}I-labeled lamprey insulin (▲) is shown in panel A and ^{125}I-labeled hagfish insulin (■) in Panel B. Data points are averages of duplicate values from a representative experiment.

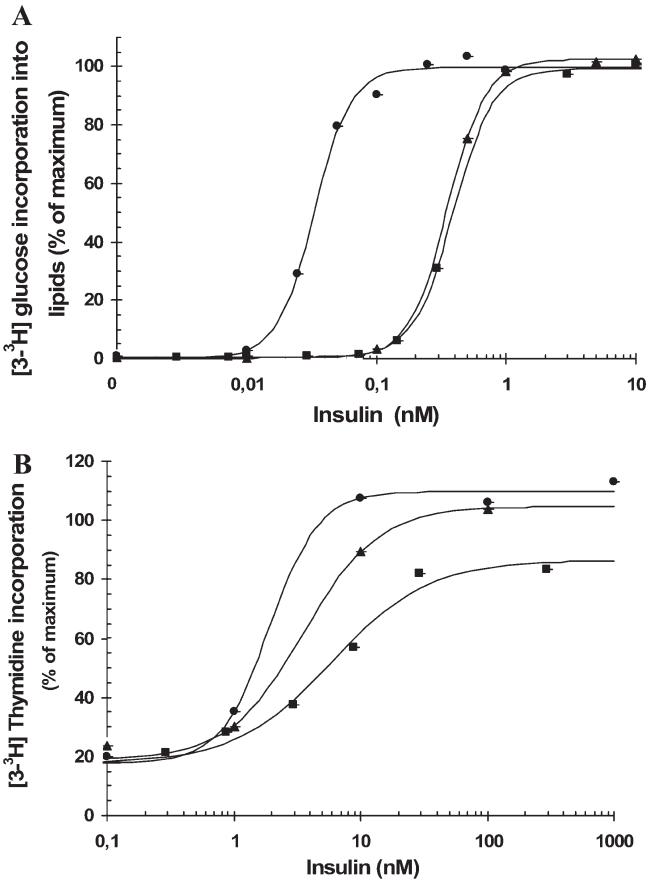

FIGURE 4: Functional studies. (A) Dose−response curves for stimulation of lipogenesis in primary rat adipocytes. Lamprey (▲) and hagfish (■) insulins were compared to human insulin (●) for their ability to stimulate lipogenesis in primary rat adipocytes. Data are averages ± the standard deviation of three experiments performed in triplicate and have been fitted using a four-parameter logistics model. It is plotted as a percentage of the maximum obtained with human insulin as a function of ligand concentration. Panel B illustrates the mitogenic potency by [^{3}H]thymdine incorporation of NIH 3T3 cells overexpressing the insulin receptor. Lamprey (▲) and hagfish (■) insulins were compared to human insulin (●) in their ability to promote DNA synthesis. Data presented are averages of at least three experiments (performed in triplicate) ± the standard deviation and have been fitted to a four-parameter logistics model. Data are plotted as the percentage of the maximum obtained with 10% FBS as a function of ligand concentration.

Dose−Response Curves for Accelerated Ligand Dissociation (*negative cooperativity*). The amount of tracer bound after dissociation for 30 min was measured as a function of the concentration of unlabeled ligand to determine the dose−response curve for negative cooperativity (Figure 2) (*22*, *36*). Dissociation of ^{125}I-labeled human insulin from the human insulin receptor by human insulin produced a bell-shaped curve as previously reported and shown in Figure 2A,B (*22*, *36*). Unlabeled lamprey insulin induced the same maximal acceleration of dissociation of ^{125}I-labeled human insulin as unlabeled human insulin, with a rightward shift in the curve reflecting the reduced affinity of this insulin for the human insulin receptor (Figure 2A). Similar results have previously been shown with hagfish insulin (*19*). Both lamprey and hagfish insulin displayed a decrease in the maximal rate of acceleration of dissociation obtained with cold ligand when these insulins were used as the tracer as seen in Figure 2A,B and on an expanded scale in panels C and D of Figure 2(20 and 56%, respectively, of the insulin tracer). In contrast to unlabeled human insulin, there was no reversal of cooperativity at high concentrations with either lamprey or hagfish insulin when the homologous tracer was used. This was also reported previously with unlabeled hagfish insulin and a porcine tracer (*19*).

Affinities of Lamprey and Hagfish Insulin for the Human Insulin Receptor. The affinities of hagfish and lamprey insulin for the human insulin receptor on IM-9 cells were determined in competition studies using constant tracer concentrations of ^{125}I-labeled human, hagfish, and lamprey insulin and increasing concentrations of the homologous unlabeled ligands. Competition studies were also performed using ^{125}I-labeled human and lamprey insulin with the heterologous insulin as the competitor (data not shown). To allow for the system to reach steady state for the slower associating insulins, the assay was performed at 15 °C for 6 h. As depicted in the competition curves (Figure 3A,B), both hagfish and lamprey insulin have a reduced affinity relative to that of human insulin on the membrane-bound receptor. Lamprey insulin had a relative affinity of 8% relative to that of human insulin, while the relative affinity of hagfish insulin was 4.5%.

Lipogenesis in Primary Rat Adipocytes. Conversion of [^{3}H]glucose into lipids in isolated primary rat adipocytes was used to measure the metabolic potency of the two ancient insulins (Figure 4A). Data from three experiments performed in triplicate were averaged and fitted to a four-parameter logistics model, and

ED_{50} was calculated. In response to insulin, isolated rat adipocytes incorporate labeled glucose into lipids in a dose-dependent manner with an ED_{50} of 33 pM. Both hagfish and lamprey insulin produced the same maximal lipogenesis as human insulin, indicating they are full agonists. However, the potencies were 9.4 and 8.4% of that of human insulin for lamprey (ED_{50} = 358 pM) and hagfish insulin (ED_{50} = 400 pM), respectively.

Thymidine Incorporation. Incorporation of [^{3}H]thymidine into DNA by hagfish, lamprey, and human insulin was measured in NIH 3T3 fibroblasts overexpressing the wild-type human insulin receptor to determine the mitogenic potency of hagfish and lamprey insulin (Figure 4B). The response to 10% FBS was used as an indicator of the maximal response, and data are expressed as the percent of the maximal signal obtained with serum. Potency was calculated by fitting the data from three experiments performed in triplicate to a four-parameter logistics model. Human insulin stimulated DNA synthesis with an ED_{50} of 1.92 nM, while the ED_{50} was 3.55 nM for lamprey insulin and 5.95 nM for hagfish insulin, demonstrating potencies of 54 and 32%, respectively. Lamprey insulin produced the same maximal response as serum or human insulin, while the maximal stimulation by hagfish insulin reached only 75% of that of human insulin (the difference was not statistically significant).

Thus, it appears that hagfish insulin has a relative mitogenic potency that is 3.8 times greater than its relative metabolic potency, and for lamprey insulin, the relative potency is 5.8-fold greater than its relative metabolic potency.

Production, Quantitation, and Binding of the Human Insulin Analogues. Eight human insulin analogues were produced, each containing a single substitution (and one with a double substitution) found in hagfish insulin. The substitutions were selected on the basis of structure−activity relationship considerations of hagfish insulin (see the details in Discussion). All the analogues were expressed successfully in *S. cerevisiae* and had the correct molecular weights as determined by mass spectrometry (data not shown).

Heterologous competition assays were performed using ^{125}I-labeled human insulin to measure changes in the affinity of the analogues compared to human insulin. The average K_d value for each analogue ± the standard deviation (SD) is given in Table 2. Not surprisingly, the two analogues having the conserved Leu to Ile substitution show affinity similar to that of human insulin. Five of the single analogues, IleA10Arg, HisB10Asp, GluB13Asn, GluB21Val, and the double substitution (GlnB4Gly and GluB13Asn), showed a >2-fold change in affinity (Table 2). The 2-fold changes were used as a cutoff on the basis of alanine scanning mutagenesis studies, which have demonstrated that any meaningful change in affinity produced by a single substitution ranges from 2- to 100-fold (*44*, *45*).

The singly substituted analogue that showed the lowest affinity was IleA10Arg. Replacing Ile with Arg had a large impact on receptor binding, leading to an almost 16-fold decrease in affinity. The GluB13Asn analogue exhibited the second largest decrease in affinity, 8-fold. Introducing Val instead of Glu at position B21 resulted in a 4-fold decrease in affinity. The GlnB4Gly analogue exhibited a borderline 1.9-fold decrease in affinity but when combined with the B13 substitution showed a 13-fold decrease, close to an additive effect. The HisB10Asp substitution showed a 5-fold increase in affinity as reported previously (*31*, *46*).

Production, Quantitation, and Binding of the Hagfish Insulin Analogues. Having established that these candidate residues may explain the low affinity of hagfish insulin, we decided to express hagfish insulin with reciprocal substitutions. Heterologous competition assays were again performed on the cell-bound receptors to determine any changes in the affinity of the hagfish analogues with human substitutions compared to human insulin. As shown in Table 3, the hagfish analogue (which contains LysA9Ser and LysB9Ser substitutions, to prevent any undesired cleavage with lysine specific protease during analogue production) shows an affinity that was 5% of that of human insulin similar to the unsubstituted hagfish insulin. The reciprocal substitutions show that they give the opposite effect compared to the substitutions in human insulin. The AspA15Gln, GlyB4Gln, AsnB13Glu, and ValB21Glu analogues show an increase in affinity compared to that of the hagfish analogue, quite similar to the extent of the decrease found with the reciprocal substitutions. In contrast, the HisA8Thr and AspB10His analogues exhibited a decrease in binding affinity as expected.

Dose−Response Curves for Negative Cooperativity. To evaluate the effect of the substitutions on negative cooperativity, we tested whether human and hagfish analogues were able to accelerate dissociation of prebound ^{125}I-labeled human insulin. Both the human and hagfish analogues tested accelerated the dissociation of prebound ^{125}I-labeled human insulin but were less

Table 2: Competitive Binding for the Holo Insulin Receptor with Increasing Concentrations of Singly or Doubly Substituted Human Insulin Analogues and a Fixed Concentration of ^{125}I-Labeled Human Insulin[a]

human insulin analogue	K_d ± SD (nM)	relative affinity[b] (%)
human insulin	0.29 ± 0.02	100
IleA10Arg	5.05 ± 0.84	**6**
LeuA13Ile	0.37 ± 0.04	80
GlnA15Asp	0.48 ± 0.05	63
GlnB4Gly	0.55 ± 0.22	**54**
HisB10Asp	0.05 ± 0.01	**547**
GluB13Asn	2.28 ± 0.47	**13**
LeuB17Ile	0.44 ± 0.04	67
GluB21Val	1.17 ± 0.25	**25**
GluB4Gly/GluB13Asn	3.71 ± 0.66	**8**

[a]The K_d values are averages, and the relative affinity of that of human insulin binding to the insulin receptor [(K_d for human insulin)/(K_d for the insulin analogue) × 100%] is also shown. [b]Bold values show a >2-fold change in affinity relative to that of human insulin.

Table 3: Competitive Binding for the Holo Insulin Receptor with Increasing Concentrations of Singly Substituted Hagfish Insulin Analogues and a Fixed Concentration of ^{125}I-Labeled Human Insulin[a]

hagfish insulin analogue	K_d ± SD (nM)	relative affinity[b] (%) for human insulin	relative affinity[b] (%) for hagfish insulin
human insulin	0.29 ± 0.02	100	2000
hagfish (KA9S, KB9S)	5.49 ± 0.11	5	100
HisA8Thr	7.38 ± 0.75	4	80
AspA15Gln	4.70 ± 0.51	6	120
GlyB4Gln	2.34 ± 0.12	**13**	**260**
AspB10His	14.46 ± 0.41	**2**	**40**
AspB13Glu	1.58 ± 0.13	**19**	**380**
ValB21Glu	1.56 ± 0.29	**19**	**380**

[a]The K_d values are averages ± SD, and the relative affinity of human and hagfish (KA9S, KB9S) insulin binding to the insulin receptor [(K_d for human insulin)/(K_d for the insulin analogue) × 100%] is also shown. The analogues have additional KA9S and KB9S substitutions to prevent cleavage at these positions during processing. [b]Bold values show a more than 2-fold change in affinity relative to that of insulin.

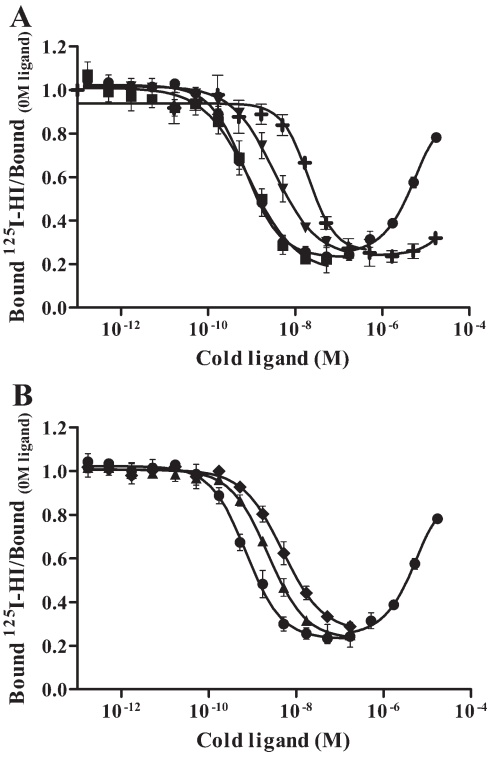

FIGURE 5: Dose−response curves for negative cooperativity (human insulin analogues). Dissociation of prebound ^{125}I-labeled human insulin in the presence of increasing concentrations of human insulin (●) is illustrated in the two curves. Dissociation with increasing concentrations of GlnB4Gly (■), IleA10Arg (+), and the doubly substituted analogue (▼) (A) and GluB13Asn (◆) and GluB21Val (▲) (B). Curves are illustrated as bound to bound at 0 M cold ligand after dissociation for 30 min. The curves are averages of three assays each made in duplicate. Standard deviations are shown for all the data (smaller than symbol when not visible).

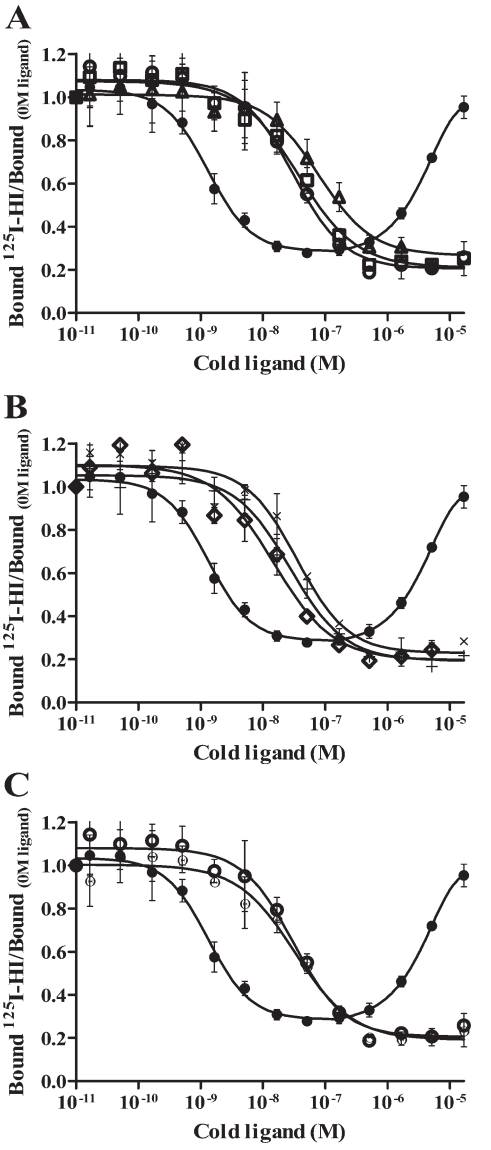

FIGURE 6: Dose−response curves for negative cooperativity (hagfish insulin analogues). Dissociation of prebound ^{125}I-labeled human insulin in the presence of increasing concentrations of human insulin (●) is illustrated in all three curves. (A) Dissociation with increasing concentrations of HisA8Thr (□), hagfish (KA9S, KB9S) (○), and AspB10His (△). (B) GlyB4Gln (×), AsnB13Glu (◇), and ValB21Glu (+). (C) Hagfish (KA9S, KB9S) (○) and AspA15Gln (⊖). Curves are illustrated as bound to bound at 0 M cold ligand after dissociation for 30 min. The curves are an average of three assays each conducted in duplicate. Standard deviations are shown for all the data (smaller than the symbol when not visible).

potent than human insulin as shown by a rightward shift in the dose−response curves (Figures 5 and 6). All single hagfish mutants showed no reversal of cooperativity at high concentrations (Figure 6) within the range tested, as shown previously for wild-type hagfish insulin (*19*), hystricomorph insulins (*47*), and human insulin-like growth factor I (IGF-I) (*48*).

The possible reversal at high concentrations was not possible to assess with the human substituted analogues (Figure 5) due to insufficient material, with the exception of IleA10Arg which showed a clear trend for a bell-shaped curve [the last point of Figure 5A is significantly higher than the nadir of the curve ($p < 0.05$)].

The human insulin GlnB4Gly analogue exhibited a potency almost identical to that of accelerated dissociation of prebound ^{125}I-labeled human insulin as human insulin, while the GluB13Asn and IleA10Arg analogues exhibited the most decreased potency compared to that of human insulin. The B4 and B13 doubly substituted analogue exhibited a higher potency than GluB13Asn, although it had the most reduced affinity of the human insulin analogues. In summary, the analogues ranked as follows in terms of potency for negative cooperativity: human insulin ≈ GlnB4Gly > GluB21Val > the double substitution (B4 and B13) > GluB13Asn > IleA10Arg. All the hagfish insulin analogues exhibited a marked reduction in the potency of accelerated dissociation of prebound ^{125}I-labeled human insulin. The AspB10His analogue had the most reduced potency of all the analogues, in accordance with the relative affinity described in Table 3. Hagfish, HisA8Thr, and AspA15Gln insulin had almost identical potencies, HisA8Thr having the most reduced potency. These results correlate well with the affinities listed in Table 3, although HisA8Thr was expected to give the second largest reduction both in the potency of accelerated dissociation and in the affinity for the receptor, since the reciprocal substitution in human insulin has been reported to increase the affinity for the receptor 3-fold. GlyB4Gln was expected to have a higher potency than hagfish insulin, but it exhibited a marginally reduced potency compared to that of hagfish. AsnB13Glu and ValB21-Glu exhibited higher potencies than hagfish insulin, AsnB13Glu having the highest potency of all the hagfish analogues. To summarize the hagfish analogue potencies, the analogues ranked as follows: human insulin ≫ AsnB13Glu > ValB21Glu > AspA15Gln ≈ hagfish insulin ≈ HisA8Thr > GlyB4Gln ≫ AspB10His.

It should be mentioned that the determination of potencies in this assay is not reliable in the case of analogues with relatively slow kinetics since dissociation acceleration is measured at 30 min while it may take substantially longer for the cold analogues to reach equilibrium during the assay; the curves in this assay move leftward with increasing incubation times (*30*).

DISCUSSION

These results show that hagfish and lamprey insulins share a set of unusual receptor binding and biological properties when compared to most mammalian insulins. Only hystricomorph insulins among mammalian insulins exhibit properties similar to those of hagfish and lamprey insulins (*22*, *49*). Insulin analogues with single substitutions in binding site 2 also were shown to behave in a similar way (*22*, *24*, *50*). This implies that the unusual properties of these insulins may be explained by substitutions in site 2, a hypothesis that has been validated in our laboratory for the hystricomorph insulins (*49*) and is tested in this work for hagfish and lamprey insulins.

Enhanced Mitogenic or Metabolic Potency Ratio of Hagfish and Lamprey Insulins. Both hagfish and lamprey insulins exhibit enhanced mitogenic and metabolic potency ratios, 5.8 for lamprey and 3.8 for hagfish. This is a property that we have seen with other insulins modified in site 2, such as insulins mutated at A13 and B17 (*22*, *24*).

The low biological potency of hagfish and lamprey insulin in rat adipocytes is also in line with insulins like hystricomorph insulins and A13 and B17 mutated insulins, where the biological potency in lipogenesis is proportional to the association rate rather than to the affinity. Holst et al. showed that the potency measured after lipogenesis for 2 h correlated poorly with the amount of analogue bound at the time where lipogenesis is measured ("equilibrium") but correlated strongly with the relative amounts of analogue bound within the first few minutes of incubation (*50*). Thus, insulins and analogues that reach equilibrium quickly will appear to have a potency proportional to their binding affinity, while those with slow kinetics like hagfish and lamprey insulins will have potencies that appear to be proportional to their association rates.

Receptor Binding and Biological Properties of Hagfish Insulin. With regard to hagfish insulin, these results not only confirm and extend previous findings but also reveal several novel features. Although the binding to IM-9 cells was prolonged to 6 h and reached an apparent steady state, the apparent affinity of hagfish insulin was only 4.5% at 15 °C. This agrees with the potency of 5% in competing for ^{125}I-labeled porcine insulin binding to IM-9 cells reported by Muggeo et al. (*19*) in 90 min incubations at 15 °C. Muggeo et al. also found a potency of 5−10% in competing for ^{125}I-labeled porcine insulin binding in rat adipocytes, and Terris and Steiner (*20*) reported a potency of 3% in rat liver cells. These apparent affinities are consistent with the low biological activity of hagfish insulin in stimulating lipogenesis in rat adipocytes: 8.4% in this study, 4.6% by Emdin et al. (*51*), 3.8% by Thomas et al. (*16*), 7% in rat fat pad by Weitzel et al. (*52*) relative to that of bovine insulin, and 5−10% for deoxyglucose transport by Muggeo et al. (*19*).

It is plausible that hagfish insulin's affinity, if it was accurately measured at true equilibrium, would be greater than its metabolic potency (e.g., lipogenesis in rat adipocytes). We do not believe, however, that this implies that hagfish insulin is a partial antagonist but rather that the potency for lipogenesis in rat adipocytes is not determined at equilibrium but early in the binding process, as discussed above. From a physiological viewpoint, it should be noted that the hagfish lives in an environment at ∼5 °C and that the hypoglycemic response of the hagfish to injected insulin takes 2−3 days to develop (*53*).

In contrast, using homologous competition and Scatchard analysis, the relative affinity of lamprey insulin for a purified soluble human receptor ectodomain was found to be 166% on the basis of comparison of IC_{50} and 203% on the basis of comparison of K_d (see Figure 1A of the Supporting Information). This high affinity for the soluble receptor confirms previous findings of 130% based on IC_{50} by Conlon et al. (*9*) for this insulin. The relative affinity of hagfish insulin for the purified soluble human receptor ectodomain was determined to be 22% of that of human insulin (see Figure 1B of the Supporting Information). It is typical for insulin analogues with site 2 substitutions to exhibit much higher relative affinities for the soluble ectodomain than for the cell-bound receptor, since the soluble ectodomain exhibits primarily site 1 binding (*26*; J. Brandt, unpublished data).

Muggeo et al. had shown that hagfish insulin was able to accelerate the dissociation of ^{125}I-labeled porcine insulin with a potency of 5% of unlabeled porcine insulin and showed the same maximal acceleration. A novel finding in this study is that when ^{125}I-labeled hagfish insulin is used as the tracer, the degree of dissociation seen in the presence of an excess of cold hagfish or human insulin is reduced (56% of that obtained with ^{125}I-labeled human insulin), indicating a reduced negative cooperativity, as also seen in rat adipocytes by Emdin et al. (*43*). The dose−response curve for acceleration of ^{125}I-labeled hagfish insulin with unlabeled hagfish insulin was not bell-shaped, while it was with human insulin, as previously reported by Muggeo et al. (*19*).

The mitogenic potency of hagfish insulin in stimulating thymidine incorporation in NIH3T3 cells overexpressing the insulin receptor was 32% relative to that of human insulin. Therefore, hagfish insulin exhibits a mitogenic:metabolic potency ratio of 3.8 in our hands. A previous study reported a ratio of 1.0, but the actual data were not shown (*54*).

Receptor Binding and Biological Properties of Lamprey Insulin. Both association and dissociation rates for lamprey insulin were notably slower than that of human insulin on IM9 cells. While lamprey insulin accelerated the dissociation of ^{125}I-labeled human insulin, albeit with reduced potency, the data suggest a lack of reversal with increasing concentrations of unlabeled ligand. However, a paucity of material did not allow use of very high concentrations of unlabeled lamprey insulin. When ^{125}I-labeled lamprey insulin was used as the tracer, the degree of dissociation seen in the presence of an excess of cold lamprey or human insulin was greatly diminished, indicating reduced negative cooperativity. This was more pronounced with lamprey insulin than with hagfish insulin. Therefore, the previous conclusion about "extraordinary conservation of ... negative cooperativity in the most primitive vertebrate" (*19*), based on the effect of unlabeled hagfish insulin on the dissociation of ^{125}I-labeled porcine insulin, must be tempered by the marked attenuation of this phenomenon when these ancient insulins are used as tracers. The affinity of lamprey insulin for the human insulin receptor on IM9 cells was 8% of that of human insulin. The metabolic potency of lamprey insulin in primary rat adipocytes was similar to that of hagfish insulin, 9 and 8%, respectively, and both insulins gave the same maximal response as human insulin with parallel curves. The mitogenic:metabolic

potency ratio of lamprey insulin (5.8) was greater than that seen with hagfish insulin (3.8).

Structure−Activity Relationships of Hagfish and Lamprey Insulins. The structural reasons for the aberrant binding and biological behavior of hagfish insulin have so far eluded explanation. Hagfish insulin has 33 amino acids in common with human insulin (Table 1 of the Supporting Information) and a very similar three-dimensional structure (*55*). Its ability to dimerize is conserved, but it does not form hexamers because of the HisB10 to Asp substitution (*55*). The "classical binding surface" is conserved (Table 1), and two substitutions are present that confer an increased affinity when introduced into human insulin. The first one is the ThrA8 to His mutation, a mutation that markedly increases the affinity of human insulin to 327% in HepG2 cells (*31*) and 300% in IM-9 lymphocytes (*47*, *56*). Turkey and chicken insulins also have His at A8 and display increased affinity (*57*). The biological activity of HisA8 human insulin is accordingly increased (*58*). The second favorable substitution is the HisB10 to Asp substitution, which enhances 3−5-fold the affinity and activity of human insulin (*31*, *46*). Thus, hagfish insulin would in fact be predicted to have a substantially higher affinity than human insulin if only the classical binding surface was involved. It is clear, therefore, that there must be other contacts between insulin and the receptor outside the classical binding surface, which when substituted in hagfish insulin explain the loss of affinity, a conclusion also reached by Baker et al. (*14*).

Recent investigations of a number of insulin analogues mutated at various positions by site-directed mutagenesis have led us to conclude that there is indeed a second binding surface on the insulin molecule that coincides with insulin's hexamerization surface (*15*, *22*, *26*). It comprises in human insulin residues SerA12, LeuA13, GluA17, HisB10, GluB13, and LeuB17 (Table 1). The central helix of the B chain is at the confluence of both surfaces and is clearly a central motif in binding to the insulin receptor (*59*, *60*).

By excluding substitutions also found in other high-affinity insulins and given the similarity in binding properties between human analogues mutated in site 2 and those of hagfish insulin, we decided to focus on hagfish residues substituted in site 2, namely, LeuA13Ile, HisB10Asp, GluB13Asn, and LeuB17Ile, and to test the effect of reciprocal substitutions at these residues in hagfish and human insulins. We also tested the effect of substitution of HisA8 to Thr in site 1 in hagfish insulin.

We also explored a few residues outside the so far known site 1 and site 2.

We thought that the substitution of GlnB4 with Gly in hagfish insulin and Thr in lamprey insulin could also possibly contribute to the altered binding since B4 is conserved in all mammals but is altered in other low-affinity insulins (Arg in guinea pig, Lys in chinchilla, and Glu in amphioxus).

Baker et al. (*14*) suggested that the GluB21 to Val substitution may be relevant for the low activity of hagfish insulin. However, mutation of B21 to Ala in human insulin increases the affinity to 230% for the low-affinity soluble receptor (*61*) and 226% in IM-9 cells (*26*). In contrast, in a recent study, an ~2-fold decrease in the level of binding to receptors on the human placental membrane was seen, when GluB21 was substituted with Ala (*62*). Studies conducted in our laboratory also indicate that this position is important for receptor binding, since substitutions to Arg, Gly, Pro, Ser, and Thr all show a decrease in affinity of >2-fold (*63*).

Near the completion of this project, we became aware that the IleA10 to Arg substitution in hagfish insulin and IleA10 to Lys substitution in lamprey insulin may also have a deleterious effect on binding. We initially ignored this position because it is not conserved in evolution and is changed to either Thr, Val, or Pro in other insulins (*14*). For unknown reasons, it was also ignored in the previous alanine scanning of insulin (*26*, *61*). However, Gauguin et al. (*64*) recently showed that substitution of IleA10 with Ser as in IGF-I and IGF-II results in a 5-fold loss of affinity and contributes significantly to the low affinity of the IGFs for the insulin receptor. Like A8, A10 lies on the periphery of site 1. Hagfish and lamprey insulins are the only forms in which a charged substitution at A10 is present. It was not possible to test the human insulin with a Lys substitution at A10 like in lamprey insulin since the analogue would be proteolyzed at this position during processing, but the Arg substitutions at this position in human insulin were tested.

Receptor Binding of Human and Hagfish Analogues. On the basis of the structure−activity relationship of hagfish insulin discussed above, nine human insulin and seven hagfish analogues in which residues at different positions were substituted reciprocally were produced (Figure 7A,B), and their binding affinities for the holoreceptor on IM9 cells were determined (Tables 2 and 3). Furthermore, the relative potencies of accelerated dissociation of prebound ^{125}I-labeled human insulin (testing for negative cooperativity) were analyzed for selected analogues (Figures 5 and 6).

The human insulin analogues with hagfish-like substitutions at positions A10, B13, and B21 exhibited decreases in affinity of 16-, 8-, and 4-fold, respectively. While the B4 substitution decreased the affinity to 53% which is borderline, the double mutant GlnB4Gly/GluB13Gly showed a 12.5-fold decreased affinity, greater than that of each single mutation, suggesting that B4 indeed contributes to binding affinity. In contrast, substitution of HisB10 with Asp increased the affinity as previously shown (*31*, *46*). The combined effect of all the substitutions mentioned above would be expected to yield an affinity much lower than that observed for hagfish insulin, suggesting that they are not strictly additive.

Reciprocally, human-like mutations GlyB4Gln, AsnB13Glu, and ValB21Glu in hagfish insulin increased the affinity of hagfish insulin for the human insulin receptor 2.6-, 3.8-, and 3.8-fold, respectively, while HisA8Thr and AspB10His decreased it to 80 and 40%, respectively. This also supported a minor role of B4 in receptor binding.

When these analogues were examined for their ability to induce negative cooperativity via dose−response curves for accelerated dissociation (Figures 5 and 6), the most striking feature was that every single substitution in hagfish insulin produced analogues that induced a sigmoid rather than bell-shaped dose−response curve. This has been observed previously for hagfish insulin (and in this work for lamprey insulin), as well as for insulin analogues mutated at positions A13 and B17 in site 2 (*22*), hystricomorph insulins (*22*, *49*), and IGF-I binding to the IGF-I receptor (*22*, *30*, *47*). The effect of site 2 mutations on the bell-shaped curve has been fully identified in mathematical modeling of the insulin and IGF-I receptors as harmonic oscillators, based on De Meyts' bivalent cross-linking model (*30*). Our initial interpretation for the lack of reversal of negative cooperativity at the IGF-I receptor at high concentrations of IGF-I was that there may not be sufficient space for two IGF-I molecules (due to the presence of the C-peptide) to fit into the

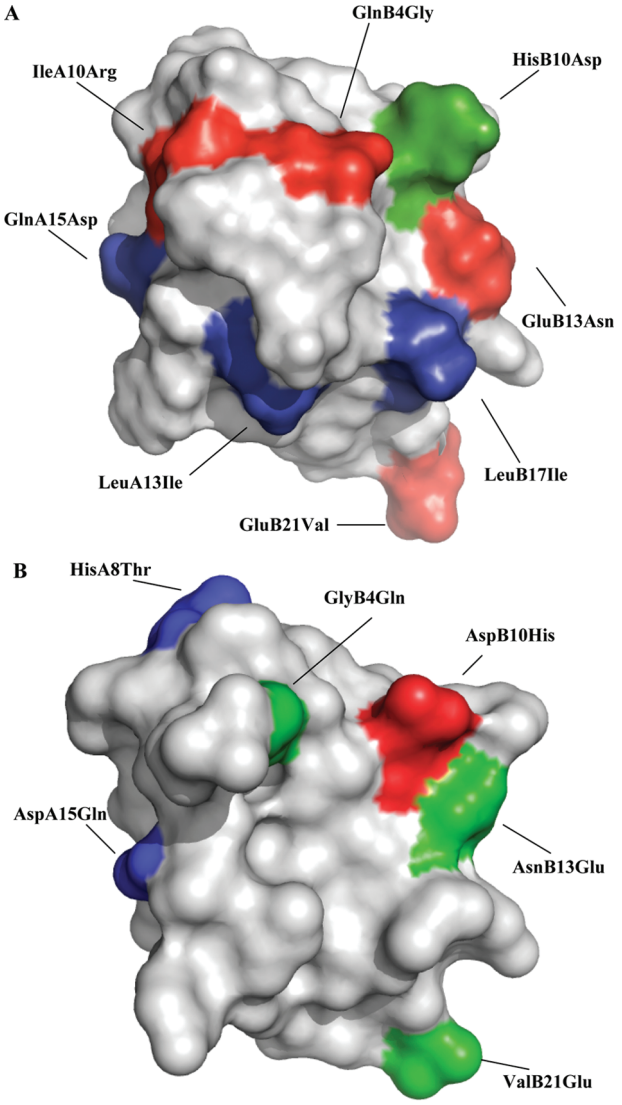

FIGURE 7: Summary of the contribution of each amino acid. Panel A illustrates the effect of each hagfish insulin substitution in human insulin, and panel B illustrates human insulin substitutions in hagfish insulin. The substitutions marked in red led to a significant decrease in affinity, while the substitutions that led to a significant increase in affinity are marked in green. The blue substitutions had no significant change in affinity. The atomic coordinates [(A) human insulin (Protein Data Bank entry 1G7A) and (B) hagfish insulin (a gift from G. G. Dodson)] were used to construct the diagrams. Molecular graphic images were produced using PyMol from DeLano Scientific LLC.

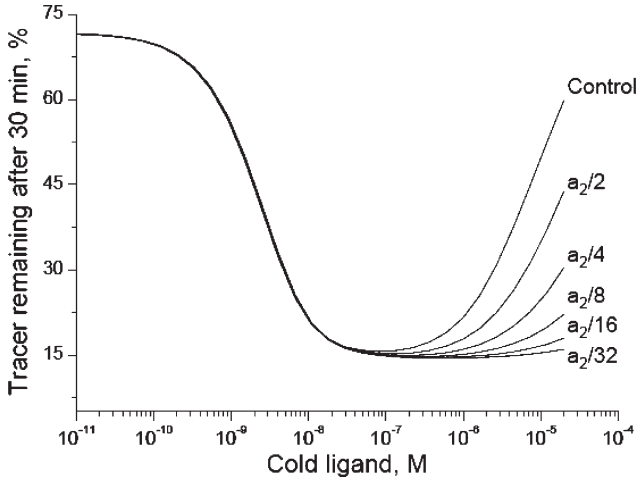

FIGURE 8: Simulated dose−response curves for negative cooperativity. The curves were generated using a harmonic oscillator model as previously described (see ref *30* for a detailed explanation). The model contains five binding parameters: a_1, rate constant for association with site 1; a_2, rate constant for association with site 2; d_1, rate constant for dissociation from site 1; d_2, rate constant for dissociation from site 2; and k_{cr}, cross-linking constant. For the values of the constants, see ref *30*. The simulation shows that for decreasing values of a_2, the dose−response curve goes from bell-shaped to sigmoid within the range of ligand concentrations used in the experiments.

second pair of receptor sites 1′ and 2 for cross-linking (*30*). Obviously, this argument cannot hold for the substituted human analogues studied here. A more plausible argument is that to prevent rebinding of the first cross-link, the rate of formation of the second cross-link must be faster than the rate of reclosing of the first. A slow association rate constant for receptor site 2 may explain the lack of reversal as demonstrated by mathematical simulation in Figure 8. The possibility that a reversal would be observed at much higher concentrations cannot be excluded. This concept probably also applies to the IGF-I receptor since Lawrence et al. have recently shown by small-angle X-ray scattering that the soluble IGF-I receptor ectodomain binds up to three IGF-I molecules (*65*).

The observed potencies for the accelerated dissociation were generally in line with the observed changes in affinity, with the exception of those of GlnB4Gly human insulin which was not different from human insulin and GlyB4Gln hagfish insulin which was slightly less potent than hagfish insulin, showing again a marginal effect of this substitution. It should be stressed, however, that the dose−response curve was done after dissociation for 30 min, and it is known that site 2 mutations can markedly decrease the association rate of the analogue (*22*, *24*). It means that a longer time period may have been needed to determine the exact potency of the analogues in this assay, and to correlate them with the relative affinity observed in competiton assays.

Structural Basis of the Low Affinity and Binding Properties of Lamprey Insulin. On the basis of the findings described above, it is possible to generate a reasonable hypothesis for why lamprey insulin has properties similar to those of hagfish insulin, although they are less pronounced (somewhat higher association rate, higher affinity, and more acceleration of dissociation by unlabeled ligand).

Lamprey insulin shares with hagfish insulin a positively charged substitution at A10 (Lys vs Arg), which is deleterious in hagfish insulin. It is interesting that like hagfish and lamprey insulins, human IleA10Arg was found to have a much higher affinity for a soluble receptor preparation [60% vs 6% as observed in our system (data not shown)]. Such discrepancies have been observed with site 2 insulin substitutions. It is not clear if A10 belongs to site 1 or 2 (it is located between A8 which we have assigned to site 1 and A12 which we have assigned to site 2, on the basis of their respective involvement in dimerization vs hexamerization). The separation between sites 1 and 2 is obviously somewhat speculative in the absence of a structure of the insulin receptor complex, and the two surfaces may in fact be more of a continuum.

Residues A17 and B13 in the hexamer-forming surface are intact, and B21 is conservatively changed from Glu to Asp, in contrast with hagfish insulin. However, lamprey insulin lacks the positive effect of the HisB10 to Asp substitution.

Like hagfish insulin, it also harbors a potentially deleterious B4 mutation, in this case Gln to Thr. However, most interestingly, LeuB17 in the hexamer-forming surface is mutated to Val. In human insulin, as mentioned earlier, single mutations at this position to Ala or Ser are sufficient to reduce the affinity ~3-fold and to induce the peculiar binding behavior seen in hagfish, lamprey, and hystricomorph insulins. Thus, this position is clearly sensitive to the nature of the particular amino acid. It may seem that a Leu to Val substitution is a relatively conservative substitution; however, Leu and Val are not always equivalent, and such substitutions have been found in key proteins in clinical syndromes: first, in insulin itself, where a ValA3 to Leu mutation was found in a diabetic patient (insulin Wakayama of weak potency, 0.2%) (*66*) and second a ValA30 to Leu mutation in transthyretin that results in familial amyloid polyneuropathy (*67*). Systematic mutagenesis at B17 using a solubilized receptor showed that while substitutions at B17 with Met, Phe, and Trp were well tolerated, other substitutions reduced affinity 2−6-fold; substitution to Val reduced the affinity 2.5-fold (*59*). It should be noted that using affinity alone to judge the impact of mutations in site 2 may underestimate the impact of substitutions since reciprocal changes in association and dissociation rates are observed (slow on rate, slow off rate).

In summary, this work confirms and extends previous observations on the receptor binding and biological properties of hagfish insulin and describes very similar properties for lamprey insulin. It provides, at last, a rational explanation for these properties in light of a proposed bivalent receptor binding model and, via comparison of the data obtained with those known for other mutated analogues, reduced the number of candidate substitutions to a small number that was tested by reciprocal site-directed mutagenesis.

Our data also add credibility to our proposal that the set of unusual properties described here may represent the binding mode of the ancestral insulin, which may have been more of a mitogen than a metabolic regulator (*22*) as also proposed by Chan and Steiner (*68*).

It will be of great interest to investigate in detail the properties of the more ancient insulin-like molecules such as those from amphioxus and invertebrates.

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SUPPORTING INFORMATION AVAILABLE

Sequence alignment and comparison of residues of human, hagfish, and lamprey insulins (Table 1) and homologous competition of human, hagfish, and lamprey insulins for soluble insulin receptors (Figure 1), where the curves are plotted as bound to total tracer as a function of the logarithm of the concentration of unlabeled ligand. This material is available free of charge via the Internet at http://pubs.acs.org.

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