The Insulin Receptor and Insulin of the Atlantic Hagfish

Extraordinary Conservation of Binding Specificity and Negative Cooperativity in the Most Primitive Vertebrate

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SUMMARY

The North Atlantic hagfish, a cyclostome that is representative of the most primitive vertebrates still alive, diverged from the other vertebrates about 500 million years ago. Hagfish insulin, which differs from porcine insulin in 18 (38%) of its amino acids, had a potency of 5–10% that of porcine insulin in stimulating glucose oxidation and deoxyglucose transport in rat adipocytes and was 5–10% as potent as porcine insulin in binding to insulin receptors on rat adipocytes and human (IM-9) lymphocytes. Like all other naturally occurring insulins, hagfish insulin accelerated the dissociation of ^{125}I-porcine insulin from insulin receptors and the degree of the acceleration was related to its occupancy of the receptor. The insulin receptor of the hagfish erythrocyte showed the time, temperature, and pH dependence of binding and the negative cooperativity that are characteristic of all other insulin receptors. That the negative cooperativity is fully conserved in such an ancient insulin and receptor suggests that it is an important functional feature of this hormone-receptor system.

The hagfish receptor showed the same absolute affinity and rank order of preference for insulins and insulin analogues (chicken > pork > proinsulin > guinea pig > desoctapeptide) found with other receptors of less primitive vertebrates, which supports the conclusion that the receptor for insulin is functionally better conserved evolutionarily than the hormone. However, uniquely, hagfish insulin was more potent in binding to hagfish receptors than to mammalian receptors; with all other species of insulins studied, the affinity of the hormone for homologous receptor was the same as for receptors of heterologous species.

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Hagfish and lampreys form the class of cyclostomes that are the most primitive vertebrates still alive.[1,2] They occupy a key position in the evolution of the endocrine system in vertebrates, especially with regard to insulin synthesis and insulin action.[3–5] Among the cyclostomes, the North Atlantic hagfish has been the best studied species. In this animal, the islet parenchyma is concentrated in a small whitish organ that is located close to the site of entry of the bile duct into the gut.[3,5–7] In this structure, only insulin and a few somatostatin-producing cells can be found, with neither glucagon-producing cells nor acinar cells. Thus, it is possible to obtain from this organ a relatively rich preparation of insulin by acid-ethanol extraction.[6] Hagfish insulin has been purified and characterized including its physicochemical and biologic properties.[4,8,9] Hagfish insulin contains 52 amino acids (21 in the A-chain, 31 in the B-chain) of which 18 are different from those found in mammalian insulin.[9,10] At low crystallographic resolution, the three dimensional organization of the hagfish insulin monomer is similar to that of the pig insulin monomer.[11] Hagfish insulin dimerizes[11] but does not form hexamers, probably because of structural alterations and the absence of the zinc-coordinating B-10 histidine residue.[8,11] Hagfish insulin had a reduced biologic activity for insulin receptors when tested with mammalian tissues and mammalian insulin receptors.[12]

In previous studies we characterized the interaction of insulin from mammals, birds, and bony fish with insulin receptors of representatives of these and other classes of vertebrates.[13,14] The insulins differ about 100-fold in their biologic potencies in mammals, and this is reflected in their relative affinities for insulin receptors.[15] The insulin receptors of all species reacted the same with all of the insulins, which was independent of the nature of the endogenous insulin in that species.[14]

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The insulin receptor of mammals undergoes characteristic wide changes in affinity in response to changes in temperature, pH and occupancy of receptor by insulin (negative cooperativity). Insulins of all species from bony fish to mammals produced negative cooperativity and receptors of all species showed the negative cooperativity and the other characteristic affinity changes.[14] Thus, the receptor molecule is functionally much more highly conserved than the hormone molecule, which is by evolutionary standards a highly conserved entity. This suggests that the receptor may be more ancient and functionally more important than the hormone.[14]

In the present work, we have extended these studies to the insulin and to the receptor of the hagfish, thereby extending the span of evolution by 100 million years or more.

MATERIALS AND METHODS

Porcine insulin was purchased from Eli Lilly, Indianapolis, Indiana; bovine serum albumin (BSA-Fraction V)* from Miles Laboratories, Elkhart, Indiana; and carrier-free Na^{125}I from Amersham/Searle, Arlington Heights, Illinois. The following insulins and polypeptide hormones were received as gifts: porcine proinsulin from Dr. R. E. Chance, (Eli Lilly); guinea pig insulin from Dr. L. F. Smith, (University of New Mexico, Albuquerque); chicken insulin from J. Simon, (INRA, Novilly, France); and bovine desoctapeptide insulin (DOP) (lacking residues B23-30) from Dr. F. H. Carpenter (University of California, Berkeley). Hagfish insulin was obtained from islet organs of freshly killed hagfish as described by Peterson et al.[9] Porcine ^{125}I-insulin was prepared at specific activities of 150–200 μCi/μg (0.4–0.6 I/molecule) by a modification of the chloramine T method.[16]

Cell preparation and binding assay. The binding of ^{125}I-insulin to IM-9 lymphocytes[17] and isolated rat adipocytes,[18] as well as insulin-stimulation of glucose oxidation[19] and 2-deoxyglucose transport[20] in isolated rat adipocytes were performed by slight modification[21] of the standard methods.

Hagfish erythrocytes. North Atlantic hagfish were obtained from Kristineberg Marine Biology Station, Fiskebäckskil, Sweden. Whole blood was obtained by direct cardiac puncture with heparinized syringes and kept on ice until the start of each experiment. Hagfish erythrocytes are nucleated ovoid cells about 27 μm long and 18 μm broad.[22] All of the experiments on the ^{125}I-insulin binding to hagfish erythrocytes reported here were carried out with aliquots of hagfish blood from the same pool over a 3-wk period. Each aliquot of blood was washed twice with saline solution (900 mosmol/L, pH 7.4) and centrifuged at 2000 rpm for 10 min. Purified erythrocytes were obtained by discarding the supernatant and the top layer of red cells which contained some leukocytes and erythrocyte ghosts. Erythrocytes prepared in this way were homogenous, and no leukocytes were detected by Wright staining. After the wash, the erythrocytes were resuspended in assay buffer (85 mM Tris-HCl, 425 mM NaCl, 10 mM glucose, 10 mg/ml BSA, pH 7.4) at a final concentration of 60–70 $\times$ 10^6 cells/ml. The reaction was initiated by the addition of 400 μl of cell suspension and performed in a total volume of 500 μl in 12 $\times$ 75 mm plastic tubes in the presence of 100 pg of ^{125}I-pork insulin. Unlabeled pork insulin was present over a range of 0–0.7 μM (0–10 μg/ml). After 3 h of incubation at 15°C, duplicate 200 μl aliquots were layered over 100 μl of cold buffer in an 0.4 ml microfuge tube, and the cells were sedimented by centrifugation at 10,000 g for 1 min in a Beckman microfuge. The supernatant was aspirated and discarded, and the radioactivity in the cell pellet was counted in a Nuclear Chicago Autogamma Counter. Nonspecific binding, defined as the radioactivity associated with the cell pellet in the presence of large excess of unlabeled insulin (1.7 μM), has been subtracted from the total ^{125}I-insulin binding to yield specific binding. Nonspecific binding ranged from 0.4 to 1.1% of the total radioactivity. Degradation of labeled insulin was measured by precipitation in 5% trichloroacetic acid (TCA). After 3 h incubation at 15°C, more than 90% of the tracer was precipitable, while about 80% of the tracer was precipitated by TCA after 1 h incubation at 37°C.

RESULTS

HAGFISH INSULIN

Bioactivity of the hagfish insulin. Hagfish insulin stimulated glucose oxidation in isolated rat adipocytes with a potency that was about 5% that of porcine insulin (Figure 1, upper panel) and stimulated 2-deoxyglucose transport with a potency that was 5–10% that of porcine insulin (Figure 1, middle panel). The dose-response curves for hagfish insulin were approximately parallel to those produced by porcine insulin, and, at high concentrations of hormone, the same maximum effects were achieved.

Binding of hagfish insulin to mammalian receptors. Hagfish insulin was 5–10% as potent as pork insulin in competing for insulin binding to insulin receptors on rat adipocytes (Figure 1, lower panel) and was 5% as potent as pork insulin in binding to receptors on IM-9 human lymphocytes (Figure 2). At high concentrations, hagfish insulin produced the same maximal competition as the porcine hormone. As noted previously,[13–15,23] pork insulin was about one-third as potent as chicken insulin in binding to mammalian receptors.

Negative cooperativity. In Figure 3, it can be seen that the hagfish insulin was about 5% as potent as porcine insulin in accelerating the dissociation of ^{125}I-pork insulin from receptors on cultured human lymphocytes and that chicken insulin was about three times more potent than porcine insulin. Thus, hagfish insulin, despite its evolutionary age, has the full capacity to induce negatively cooperative site-site interactions with mammalian insulin receptors that is characteristic of all other naturally occurring insulins studied thus far.[24–26] Thus, hagfish insulin in its interaction with mammalian receptors behaved as a full agonist with an affinity that was 5–10% that of porcine insulin. It generated biologic responses in direct proportion to its ability to bind to the insulin receptor, and induced cooperative site-site interactions.

As can be seen in Figure 3, the negative cooperativity increased with increasing concentrations of chicken and porcine insulins up to some maximum, followed by a decline in the negative cooperativity with further increases

* *Abbreviations used in this paper*: BSA, bovine serum albumin; DOP, desoctapeptide insulin; HEPES, N-2-hydroxyethyl-piperazine N′-ethanesulfonic acid-HCl; TCA, trichloroacetic acid.

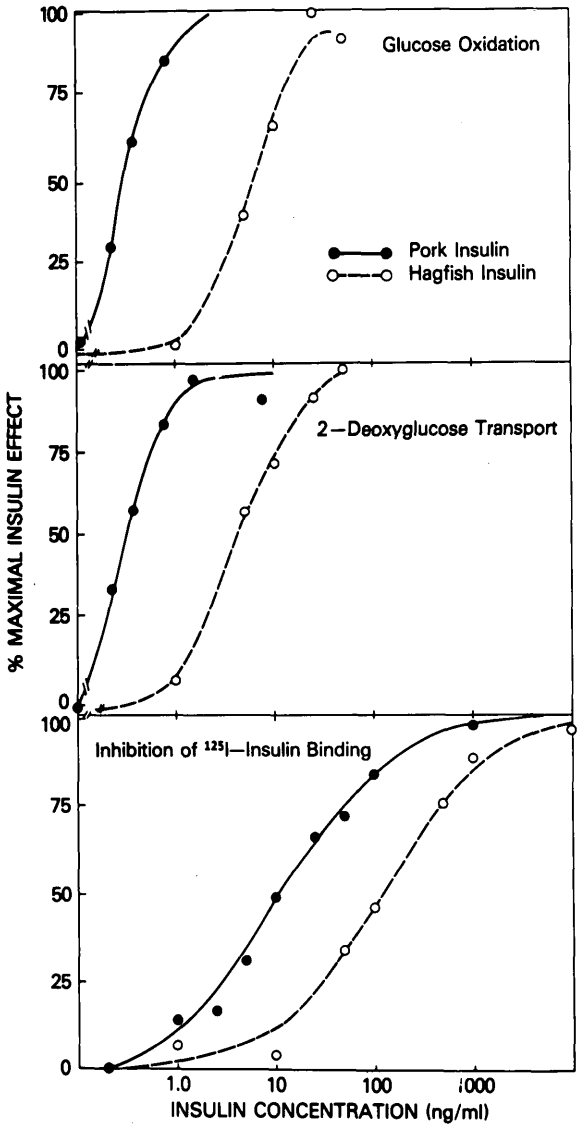


FIGURE 1. Porcine and hagfish insulin in rat adipocytes. Isolated rat adipocytes were incubated with hagfish or porcine insulin. Upper panel shows glucose oxidation measured as conversion of [U-^{14}C]glucose to $^{14}CO_2$ in 2 h.[19] Middle panel shows 2-deoxyglucose transport, measured as [1-^{14}C]-2-deoxy-D-glucose uptake by cells in 2 min.[20] Lower panel shows insulin binding to adipocytes,[18,21] expressed as inhibition of ^{125}I-insulin binding, performed under the same conditions as the measurements of the glucose oxidation.

in the concentration of these insulins. This decrease in negative cooperativity appears to be due to dimerization of the unlabeled insulins.[26] Insulins that do not dimerize, such as guinea pig insulin and tetranitroinsulin, do produce the cooperativity, but do not show the fall-off at high concentrations.[26] Further, the region on the insulin molecule that participates in dimer formation is the same region to which the negative cooperativity has been mapped.[10,26,27] Notice that as the concentration of hagfish insulin was increased, negative cooperativity increased but there was no fall-off in the cooperativity at higher concentrations. Since hagfish insulin does dimerize,[10,11] the finding that cooperativity did not decline in the presence of high concentrations of hagfish insulin raises several possibilities. The dimer of hagfish insulin may be structurally different, e.g. may not cover the cooperativity site as effectively as dimers of insulin of other species.[26] Alternatively, dimers of hagfish insulin may not bind to receptor; that dimers of chicken and porcine insulin show loss of cooperativity requires that these dimers actually bind to receptor.

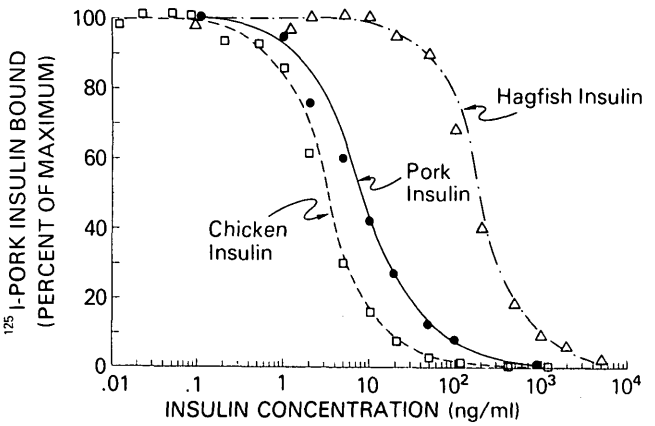
HAGFISH RECEPTORS

Insulin binding to hagfish erythrocytes. Binding of ^{125}I-pork insulin to insulin receptors on the hagfish erythrocyte showed the characteristic time and temperature dependence (Figure 4, left) that had been shown for other insulin receptors in vertebrates.[14] The association rate was directly related to the temperature, whereas the steady state level of binding that was ultimately achieved was inversely related to the temperature. Thus, at 37°C binding was most rapid, reached a peak in about 1 h, and then showed a decline. At 15°C, binding was slower, but reached a higher level and at 4°C, binding was even slower, but achieved even a higher level than that at higher temperatures.

pH Dependence. The binding of ^{125}I-pork insulin to the hagfish receptor showed the same sharp pH dependence as has been described previously for other insulin receptors.[13,14,17,28] Maximal binding was observed at pH 7.4, with a sharp decline on either side of this optimum (Figure 4, right).

Specificity of binding. Insulins from different vertebrates (birds, mammals, and bony fish) vary almost 100-fold in their affinity for insulin receptors. Thus, chicken insulin is two to three times more potent than pork insulin, which is 10 to 30 times more potent than proinsulin, guinea pig insulin, or desoctapeptide derivative of bovine insulin. This preference of insulin receptors for different insulin analogues is characteristic of all vertebrate receptors studied thus far, including the receptor on guinea pig cells.[14] Thus, the affinity of a given species of insulin re-

FIGURE 2. Insulin binding to IM-9 human lymphocytes. Cells were grown at 37°C in Eagle's minimum essential medium. Binding experiments were performed with 4 × 10^6 cells in a total volume of 0.5 ml of 100 mM HEPES buffer, pH 7.8, in plastic tubes with ^{125}I-pork insulin (50 pM) in the absence and presence of unlabeled chicken, pork, or hagfish insulin.[17] After 90 min at 15°C, 200-μl aliquots were layered over 100 μl of cold HEPES buffer in 400-μl microfuge tubes and centrifuged at 10,000 g for 1 min in a Beckman microfuge. The supernatants were aspirated and discarded, and the radioactivity in the cell pellet was counted. Nonspecific binding was considered to be the amount of radioactivity bound to the cells in the presence of 10 μg/ml of insulin and has been subtracted from the total ^{125}I-insulin binding to yield specific binding. The results were expressed as percent of maximum specific binding (i.e. in the absence of unlabeled insulin). Maximum specific binding was 50% and nonspecific binding was 3% of the total radioactivity.



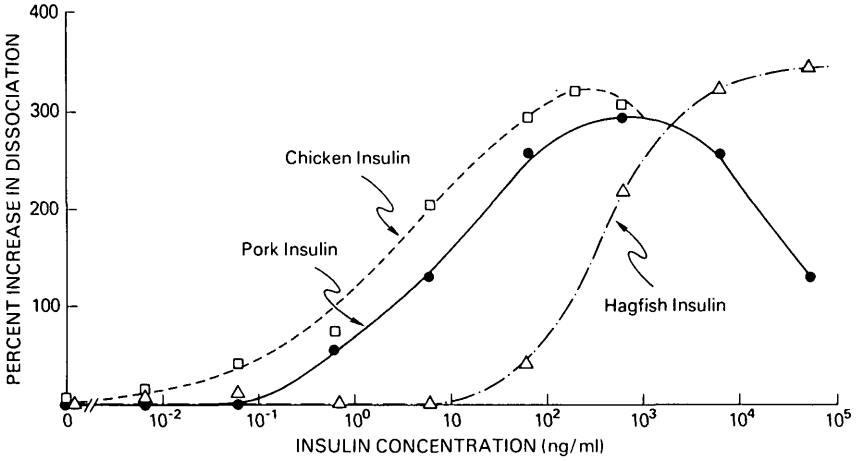


FIGURE 3. Effect of unlabeled insulin on dissociation of ^{125}I-insulin. ^{125}I-porcine insulin (3×10^{-11} M) was incubated with human cultured (IM-9) lymphocytes (2.5×10^{7}/ml) for 30 min at 15°C by which time about half of the labeled insulin was specifically bound to receptors on the cells. Aliquots of the cell suspension were then diluted 100-fold in the absence and presence of unlabeled insulins. The dissociation was allowed to proceed for 30 min at 15°C, after which the cells were sedimented, and radioactivity still bound to the cells was measured. The radioactivity dissociated in 30 min for each experimental point was compared with the radioactivity dissociated during that time period by cells that had been diluted in the absence of unlabeled insulin. This is plotted as function of the insulin concentration after dilution.

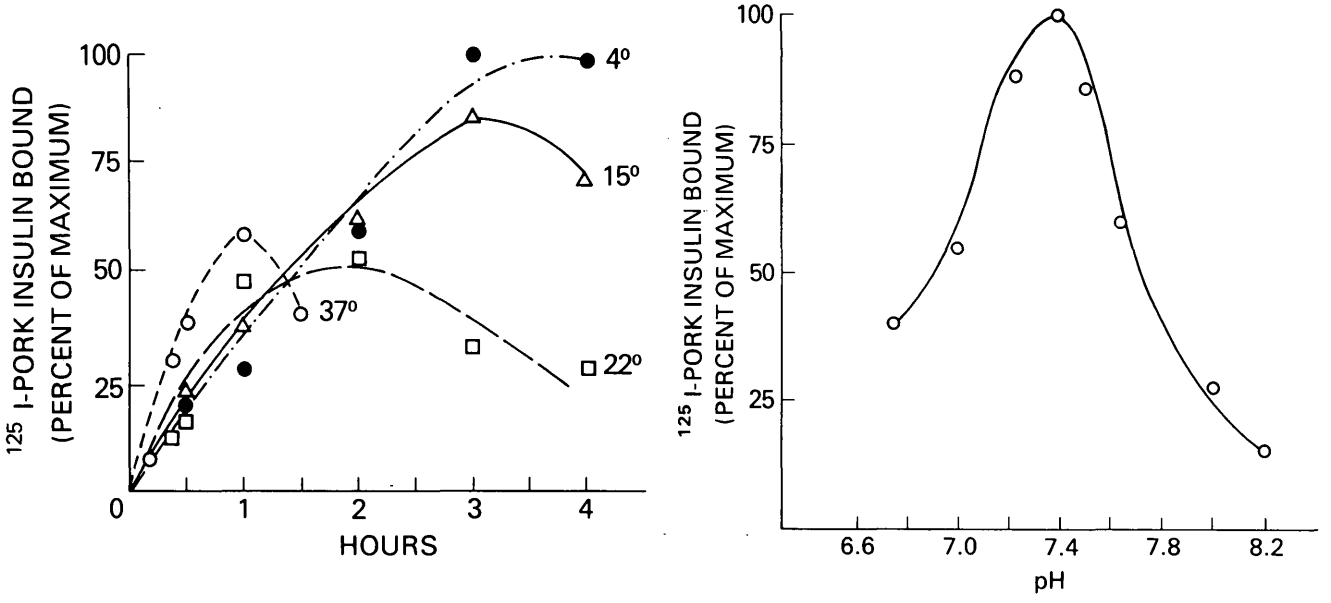
ceptor for insulins is independent of the affinity of the native insulin of that species. In the present study, we found that the binding of ^{125}I-pork insulin to the hagfish receptor showed the same general trend with chicken insulin three times more potent than pork insulin, which was many times more potent than pork proinsulin, guinea pig insulin, and desoctapeptide bovine insulin (Figure 5). Interestingly, hagfish insulin bound better to the hagfish receptor than it did to mammalian receptors. Thus, hagfish insulin was about 25% as potent as porcine insulin in binding to the hagfish receptor, whereas it was only 5–10% as potent as pork insulin in binding to receptors on rat adipocytes or IM-9 human lymphocytes. This finding has been consistent in all experiments (Table 1). It should also be noted that the conditions of the experiments were set to optimize the binding of pork insulin to the hagfish receptor. Thus, the ability of hagfish insulin to compete more effectively in this system is a highly significant observation.

Concentration and affinity of receptors. If we assume that the binding of insulin was uniformly distributed on all of the erythrocytes, we calculate an average of 4000 sites per cell.† Considering the size of the hagfish erythrocyte and its surface area, the concentration of insulin receptors per unit of cell surface was approximately 3 sites/μm^2, which is about comparable to frog and trout erythrocytes (6 and 7 sites/μm^2, respectively).[14,30]

The binding of labeled and unlabeled pork insulin to the hagfish receptor (Figure 6) showed the same shape curve that has been previously described for other insulin receptors on vertebrate cells.[14] Likewise, the affinity of pork insulin for the hagfish receptor was the same and showed the typical decrease in affinity with increasing occupancy of the insulin receptors (Figure 6, inset). The sharp curvilinearity of the Scatchard plot[31] suggested that the hagfish receptor undergoes negatively cooperative interactions (Figure 6). Figure 7 shows that unlabeled insulin

† The number of insulin receptors per hagfish erythrocyte previously reported is incorrect;[29] 4000 sites per cell, obtained from the average of five different experiments, seems to be a better estimate.

FIGURE 4. Binding of ^{125}I-porcine insulin to hagfish erythrocytes. *Left*: The temperature and duration of the incubation are noted in the graph. In this experiment the highest specific binding of ^{125}I-insulin was 2.9% of the total radioactivity and was designated as 100% of the maximum. *Right*: In this experiment, to allow a broader range of pH, the 85 mM Tris buffer and 425 mM NaCl in the assay buffer were replaced by 35 mM Tris buffer, 68 mM HEPES, and 400 mM NaCl. The highest specific binding was 2.6% of the total radioactivity and was designated as 100%. The pH plotted is that measured at the end of the 3 h incubation; pH at the end of the experiment did not differ from that at the beginning.



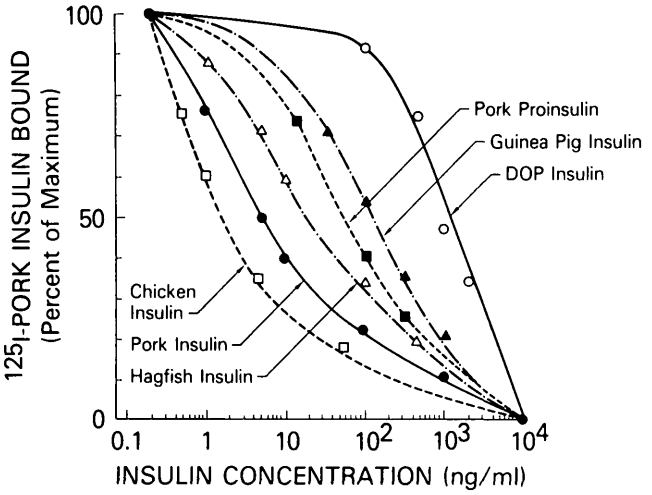


FIGURE 5. Binding of insulin and insulin analogs to hagfish erythrocytes. ^{125}I-pork insulin was incubated with hagfish erythrocytes in the absence and presence of unlabeled hormones for 3 h at 15°C. In this experiment, the maximum specific binding (in the absence of unlabeled hormone) was 2.6% of the total radioactivity. The amount of radioactivity specifically bound to the receptor in the presence of each of the polypeptide hormones was expressed as a percent of maximum specific binding. In Table 1, the results of this experiment are compared with five similar studies.

markedly accelerated the dissociation of labeled insulin from the hagfish receptor which is characteristic of all other insulin receptors. Thus, the sites of the hagfish receptor which are necessary for negative cooperativity are retained in this very primitive insulin receptor as well. That hagfish insulin was able to induce negative cooperativity in mammalian receptors, and hagfish receptor was capable of undergoing negatively cooperative interactions, suggests that negative cooperativity is an important feature in the function of the insulin receptor which is retained intact throughout vertebrate evolution.

DISCUSSION

The insulin from hagfish differs from mammalian insulin in 18 amino acids (38% of the residues). Only one of these amino acid changes is located in the so-called "invariant region" of the insulin molecule which includes both the cooperativity site and the region thought to be the bioactive site of the molecule.[9,10,26] Hagfish insulin has one extra amino acid at the carboxy-terminus of the B-chain (B-31).[9] Also, it lacks the B-10 histidine that is required for the binding of zinc and that appears to be necessary for hexamer formation during the crystallization of insulin.[5,10] In examining the amino acid substitutions, Peterson and his colleagues concluded that hagfish insulin is equally distant from mammalian insulins as it is from fish insulins.[9] Most startling, mammalian insulins and fish insulins are closer to one another than is hagfish insulin to either one, suggesting that hagfish insulin diverged from the mainstem of the vertebrate evolution long before fish and mammalian evolution diverged.[9]

Weitzel found that hagfish was 8% as potent as mammalian insulins in epididymal fat pads of rats.[32] Emdin reported that hagfish insulin is 5% as potent as mammalian insulin in stimulating lipogenesis, 3-0-methyl glucose exchange, and glucose oxidation in isolated adipocytes.[12] These reports are similar to our studies here in isolated rat adipocytes which showed that hagfish insulin was 5–10% as potent as mammalian insulin.

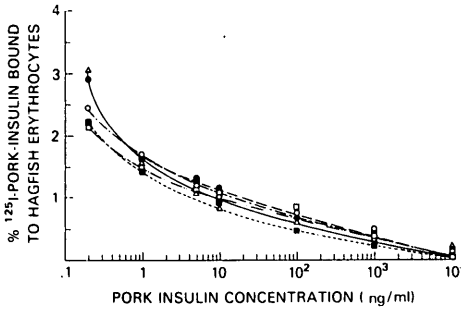
Terris reported that hagfish insulin is 3% as potent as mammalian insulin in inhibiting the binding of labeled mammalian insulin to isolated rat hepatocytes.[33] In our studies of insulin binding to isolated rat adipocytes as well

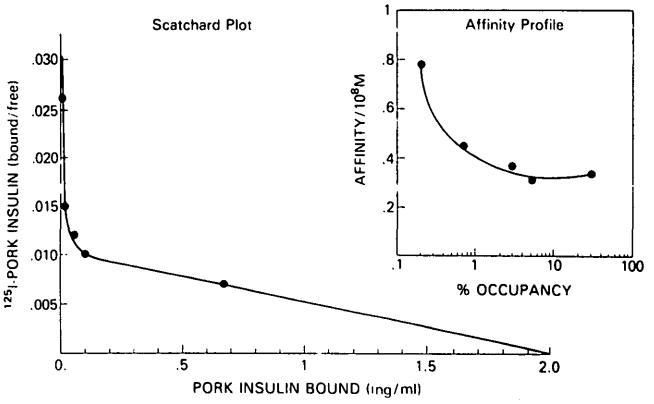
TABLE 1
Affinity of insulins and analogs for hagfish receptors

Insulin	Experiments					
	10/29	10/31	11/3	11/4	11/6	11/9
Chicken	3.0	4.5	4.0	—	—	—
Pork	1.0	1.0	1.0	1.0	1.0	1.0
Hagfish	0.29	0.15	0.33	0.33	0.25	0.13
Proinsulin	0.10	0.05	0.08	—	—	—
Desoctapeptide	0.004	0.004	0.01	—	—	—
Guinea pig	—	—	0.03	0.04	0.06	0.01

Six experiments with ^{125}I-porcine insulin and unlabeled insulins were performed such as the one shown in Figure 5. The affinity of each unlabeled insulin was compared with unlabeled porcine insulin. The relative affinity was expressed as the concentration of porcine insulin that decreased specific binding of the ^{125}I-porcine insulin by 50% divided by the concentration of analog needed to decrease specific binding by 50%.

FIGURE 6. Binding of pork insulin to hagfish erythrocytes. The binding of ^{125}I-porcine insulin to hagfish erythrocytes was studied as described in METHODS. *Upper*: Specific ^{125}I-insulin binding is plotted as a function of the total insulin concentration. Five separate studies are plotted. *Lower*: The mean of the values in the upper panel were used to derive this Scatchard plot where the bound/free (B/F) ratio of labeled insulin is plotted as a function of concentration of bound hormone (B). The intercept of this curve with the horizontal axis yielded the total binding capacity or receptor concentration (R_0). From these data, we calculate that there are 4000 binding sites per hagfish erythrocyte corresponding to about three binding sites per square micrometer of cell surface. In the *inset*, the same data were replotted as an affinity profile to show the average affinity, $\bar{K}$, as a function of occupancy, where $\bar{K} = (B/F)/(R_0 - B)$ and occupancy $= (B/R_0) \times 100$.





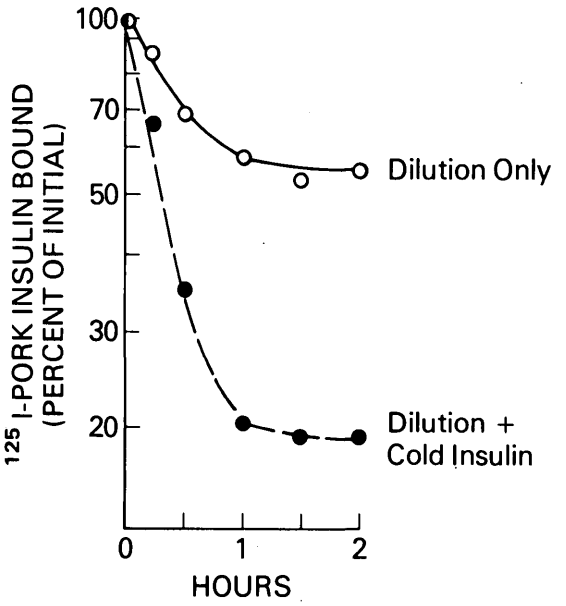


FIGURE 7. Kinetic demonstration of negative cooperativity with hagfish receptors. ^{125}I-pork insulin (100 pg/ml) was incubated with hagfish erythrocytes (50 × 10^6/ml) both in the absence and presence of 10 μg/ml of unlabeled insulin. After 3 h at 15°C, the specific binding was 2.5% of the total radioactivity, and this value was considered as 100%. After incubation, 100-μl aliquots were transferred to a series of tubes, which contained 10 ml of buffer (15°C) with (●– – –●) and without (○——○) 10 μg/ml of unlabeled porcine insulin. At indicated intervals, one tube of each of the two sets was centrifuged. The supernatant was aspirated and discarded, and the radioactivity in the cell pellet was counted. Specific binding at each time interval is compared with the specific binding at the commencement of the dilution.

as human IM-9 lymphocytes, we found hagfish insulin to be 5–10% as potent as mammalian insulin. These studies are in partial disagreement with the earlier finding of Emdin et al.,[12] that hagfish insulin was 23% as potent as mammalian insulin in inhibiting the binding of labeled insulin to isolated rat adipocytes. The reason for this discrepancy is unclear to us at this moment and will require further experiments. Hagfish insulin is less degraded than mammalian insulin. Therefore, any situation in which degradation becomes a major factor, such as prolongation of incubations or cell systems with high degradation, might artificially raise the apparent binding potency or biopotency of hagfish insulin relative to that of pork insulin. The difference noted by Emdin et al. probably cannot be accounted for by an enhanced degradation of porcine insulin, since there was very little degradation of hormone in their experiments.[12] Since binding to receptor was substantially better than the bioactivity of the hormone, hagfish insulin could be defined as a partial antagonist for the fat cell receptor. Because of a large number of spare receptors on the fat cell, they found no antagonism either at steady-state or in studies of dissociation of the hormone from the receptor during the bioactivity studies.

On the basis of our present study of hagfish insulin, we conclude that this insulin, despite its extraordinary evolutionary distance, has retained full intrinsic reactivity for mammalian receptors. It also has full capacity to generate negatively cooperative site-site interactions with mammalian receptors. The biopotency of hagfish insulin is only 5–10% that of mammalian insulins, and this is associated with an affinity for the mammalian receptor that is only 5–10% that of the majority of mammalian insulins.

A more impressive finding in the present study, is that the hagfish receptor is even more highly conserved than is hagfish insulin. Thus, the affinity of ^{125}I-porcine insulin for the hagfish receptor is the same for mammalian receptors. The hagfish receptor for insulin recognizes all of the bioactive insulins that we tested and retains strikingly the same order of biologic potencies that has been seen with all other receptor studies thus far; namely, chicken insulin is more potent than pork insulin, which is more potent than proinsulin which is more potent than guinea pig insulin and desoctapeptide bovine insulin. Not only is the overall preference maintained, but the absolute affinities are quite similar to those reported for other vertebrate receptors.[14,15]

In our previous study we found that the insulin receptors in vertebrates (mammals through bony fish) do not vary at all in their ability to bind different species of insulin, although the insulins themselves have varied over 100-fold range in biopotencies and affinities for receptors. Thus, the affinity of a given receptor for a given insulin is independent of the kind of insulin that is endogenous in that organism. However, in the present study, we find that hagfish insulin is more potent on the hagfish receptor than it is on the mammalian receptors. Thus, in six different experiments, hagfish insulin was on the average about 25% as potent as pork insulin in competing for the binding of ^{125}I-pork insulin (Table 1). In six experiments, the potency ranged from 13 to 33% (in a single experiment, not reported here, the hagfish insulin had a potency nearly that of porcine insulin). Thus, in our studies, hagfish insulin, which was only 5–10% as potent as porcine insulin in producing biologic effects and in binding to mammalian receptors, was clearly more potent in its reactivity with the hagfish receptor.

Another striking feature of the insulin receptor is its preprogrammed series of changes in affinity that it undergoes in response to changes in temperature, pH, and insulin binding (i.e. negative cooperativity); the interaction of porcine insulin with hagfish receptors showed the characteristic sharp temperature dependence of association as well as steady state level of binding, the extremely sharp pH dependence with an optimum pH between 7 and 8, and the accelerated dissociation of labeled insulin in response to the binding of unlabeled insulin. Thus, the hagfish receptor is capable of undergoing the negative cooperative interactions when it binds insulin.

Before finally concluding that hagfish insulin has a greater affinity (relative to porcine insulin) for hagfish receptor than for mammalian receptor, one possible caveat should be raised. For each particular receptor, the conditions of the assay, especially the components of the buffer, are selected to optimize binding of ^{125}I-porcine insulin. It is possible that hagfish insulin truly has an affinity that is greater than or equal to 25% that of porcine insulin with all species of insulin receptors, but that the conditions of assay with the mammalian receptor are especially disadvantageous for the hagfish insulin. The same caveat holds for the bioassay of insulin in vitro with rat adipocytes. This possibility cannot be excluded except by a very extensive reexamination of the buffer conditions for these assays.

In summary, the hagfish receptor shows extraordinary similarity in function to mammalian receptors with regard to its absolute affinities, relative affinities for various species

of insulin, and its characteristic programmed changes in affinity including the negative cooperativity. All these data are consistent with the idea that the insulin receptor is more highly conserved functionally than is insulin itself and that negative cooperativity is an extraordinarily primitive feature of both insulin and the insulin receptor.

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REFERENCES

[1] Adam, H., and Strahan, R.: Systematic and geographical distribution of Myxinoids. *In* The Biology of Myxine. Brodal, A., and Fänge, R., Eds. Oslo, Universitetsforlaget, 1963, pp. 1–8.

[2] Heintz, A.: Phylogenetic aspects of Myxinoids. *In* The Biology of Myxine. Brodal, A., and Fänge, R., Eds. Oslo, Universitetsforlaget, 1963, pp. 9–21.

[3] Falkmer, S., Thomas, N. W., and Boquist, L.: Endocrinology of the cyclostomata. *In* Chemical Zoology, Vol. 8. Florkin, M., and Scheer, B. T., Eds. New York, Academic Press, 1974, pp. 195–257.

[4] Emdin, S. O., and Falkmer, S.: Phylogeny of insulin. Some evolutionary aspects of insulin production with particular regard to the biosynthesis of insulin in *Myxine glutinosa*. Acta Paediatr. Scand. Suppl. *270*:15–23, 1977.

[5] Falkmer, S., Cutfield, J. F., Cutfield, S. M., Dodson, G. G., Gliemann, J., Gammeltoft, S., Marques, M., Peterson, J. D., Steiner, D. F., Sundby, F., Emdin, S. O., Havu, N., Östberg, Y., and Winbladh, L.: Comparative endocrinology of insulin and glucagon production. Am. Zool. *15* (Suppl. I): 255–70, 1975.

[6] Falkmer, S., Elde, R. P., Hellerström, C., Petersson, B., Efendić, S., Fohlman, J., and Siljevall, J. B.: Some phylogenetical aspects on the occurrence of somatostatin in the gastro-entero-pancreatic endocrine system. A histological and immunocytochemical study, combined with quantitative radioimmunological assays of tissue extracts. Arch. Histol. Japan *40* (Suppl.):99–117, 1977.

[7] Falkmer, S., and Ostberg, Y.: Comparative morphology of pancreatic islets in animals. *In* The Diabetic Pancreas. Volk, B. W., and Wellman, K. F., Eds. New York, Plenum Publishing Co., 1977, pp. 15–59.

[8] Peterson, J. D., Coulter, C. L., Steiner, D. F., Emdin, S. O., and Falkmer, S.: Structural and crystallographic observations on hagfish insulin. Nature (London) *251*:239–40, 1974.

[9] Peterson, J. D., Steiner, D. F., Emdin, S. O., and Falkmer, S.: The amino acid sequence of the insulin from a primitive vertebrate, the Atlantic hagfish (Myxine glutinosa). J. Biol. Chem. *250*:5183–91, 1975.

[10] Blundell, T. L., and Wood, S. P.: Is the evolution of insulin Darwinian or due to selective neutral mutation? Nature (London) *257*: 197–203, 1975.

[11] Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Sabesan, M. N.: The low resolution crystal structure of hagfish insulin. J. Mol. Biol. *87*:23–30, 1974.

[12] Emdin, S. O., Gammeltoft, S., and Gliemann, J.: The degradation, binding affinity, and potency of insulin from Atlantic hagfish determined on isolated rat fat cells. J. Biol. Chem. *252*:602–8, 1977.

[13] Ginsberg, B. H., Kahn, C. R., and Roth, J.: The insulin receptor of the turkey erythrocyte: similarity to mammalian receptors. Endocrinology *100*:82–90, 1977.

[14] Muggeo, M., Ginsberg, B. H., Roth, J., Kahn, C. R., De Meyts, P., and Neville, D. M., Jr.: The insulin receptor in vertebrates is functionally more conserved during evolution than insulin itself. Endocrinology. In press, 1979.

[15] Freychet, P.: Insulin receptors. *In* Methods in Receptor Research, Part II. Blecher, M., Ed. New York, M. Dekker, 1976, pp. 385–428.

[16] Roth, J.: Methods for assaying immunologic and biologic properties of iodinated peptide hormones. Methods in Enzymology: Hormones and Cyclic Nucleotides. Colowick, S. P., and Kaplan, N. O., Eds. *In* Peptide Hormones, Vol. 37. O'Malley, B. W., and Hardman, J. G., Eds. New York, Academic Press, 1975, p. 223–33.

[17] Gavin, J. R., III, Gorden, P., Roth, J., Archer, J., and Buell, D. N.: Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem. *248*:2202–7, 1973.

[18] Gammeltoft, S., and Gliemann, J.: Binding and degradation of ^{125}I-labelled insulin by isolated rat fat cells. Biochim. Biophys. Acta *320*:16–32, 1973.

[19] Rodbell, M.: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J. Biol. Chem. *239*:375–80, 1964.

[20] Olefsky, J. M.: Effect of dexamethasone on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. J. Clin. Invest. *56*:1499–1508, 1975.

[21] Kahn, C. R., Baird, K., Flier, J. S., and Jarrett, D. B.: Effect of autoantibodies to the insulin receptor on isolated adipocytes. Studies of insulin binding and insulin action. J. Clin. Invest. *60*:1094–106, 1977.

[22] Wintrobe, M. M.: Variations in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. Folia Haematologica *51*:32–9, 1934.

[23] Simon, J., Freychet, P., and Rosselin, G.: Chicken insulin: radioimmunological characterization and enhanced activity in rat fat cells and liver plasma membranes. Endocrinology *95*:1439–49, 1974.

[24] De Meyts, P., Roth, J., Neville, D. M., Jr., Gavin, J. R., III, and Lesniak, M. A.: Insulin interactions with its receptors: experimental evidence for negative cooperativity. Biochem. Biophys. Res. Commun. *55*:154–61, 1973.

[25] De Meyts, P., Bianco, A. R., and Roth, J.: Site-site interactions among insulin receptors. Characterization of the negative cooperativity. *J. Biol. Chem. 251*:1877–88, 1976.

[26] De Meyts, P., Van Obberghen, E., Roth, J., Wollmer, A., and Brandenburg, D.: Mapping of the residues responsible for the negative cooperativity of the receptor-binding region of insulin. Nature (London) *273*: 504–9, 1978.

[27] Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., and Gammeltoft, S.: Receptor binding region of insulin. Nature (London) *259*:369–73, 1976.

[28] Kahn, C. R., Soll, A. H., Neville, D. M., Jr., Goldfine, I. D., Archer, J. A., Gorden, P., and Roth, J.: The insulin receptor in obesity and other states of altered insulin sensitivity. *In* Obesity in Perspective. Fogarty International Center for Advanced Study in the Health Sciences, Vol. 2, part 2. Bray, G. A., Ed. Washington, D.C., U.S. Government Printing Office, 1975, pp. 301–11.

[29] Muggeo, M., Ginsberg, B. H., Roth, J., De Meyts, P., and Falkmer, S.: The insulin receptor and insulin of the Atlantic hagfish: extraordinary conservation of binding specificity and negative cooperativity in a very primitive vertebrate. Diabetes *26* (Suppl. 1):353, 1977.

[30] Ginsberg, B. H.: The insulin receptor: properties and regulation. *In* Biochemical Actions of Hormones, Vol. 4. Litwack, G., Ed. New York, Academic Press, 1977, pp. 313–49.

[31] De Meyts, P., and Roth, J.: Cooperativity in ligand binding: a new graphic analysis. Biochem. Biophys. Res. Commun. *66*:1118–26, 1975.

[32] Weitzel, G., Stratling, W. H., Hahn, J., and Martini, O.: Insulin vom Schleimfisch (Myxine glutinosa:cyclostomata). Hoppe-Seyler's Z. Physiol. Chem. *348*:525–32, 1967.

[33] Terris, S., and Steiner, D. F.: Binding and degradation of ^{125}I-insulin by rat hepatocytes. J. Biol. Chem. *250*:8389–98, 1975.