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Minkowski Award, 1981, Amsterdam

Pierre De Meyts studied medicine at the University of Liège in Belgium. In 1973, he obtained a Fogarty International Postdoctoral Fellowship and joined the group of Jesse Roth at the NIH, Bethesda, Md, USA. In 1975, he won the Solomon A. Berson Research and Development Award from the American Diabetes Association. In 1976, he joined the International Institute of Cellular and Molecular Pathology in Brussels. He won the Belgian Alumni Prize in 1978 and the Assubel Prize in 1983, and shared the Diaz Cristobal Prize with Jesse Roth at the IDF Congress in Vienna in 1979. In 1986, he became Director of the Department of Diabetes, Endocrinology and Metabolism and Associate Director of the Department of Molecular Genetics at the City of Hope in Duarte, California, as well as Clinical Professor of Medicine at the University of Southern California. In 1990, Dr. De Meyts was recruited to Denmark to become Director of the Hagedorn Research Institute in Gentofte. He also teaches biochemistry part-time as a Professor in the Department of Chemistry at the Catholic University of Louvain in Belgium. He is also known as a political and scientific cartoonist under the pseudonym Chuck.

The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling

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Summary Insulin and insulin-like growth factor-I exhibit a set of non-classical receptor binding properties suggestive of negative co-operativity or site-site interactions between the two receptor halves: curvilinear Scatchard plots, acceleration of dissociation of bound labelled ligand at high dilution in the presence of unlabelled ligand. The $\alpha_2\beta_2$ receptor dimer binds only one ligand molecule with high affinity. The dose-response curve for the acceleration of ^{125}I-insulin by unlabelled insulin is bell-shaped, with a disappearance of the negative co-operativity at insulin concentrations over 0.1 μmol/l. This phenomenon had been attributed to insulin dimerization, but new data with non-dimerizing analogues and insulins modified at the hexamer-forming surface indicate the presence of a second binding site on the insulin molecule's hexamer face. This site binds to a second domain on the receptor. A new binding model for in-

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Abbreviations: BHK cells, baby hamster kidney cells; IGF-I, Insulin-like growth factor-I; bp, base pair; Gly, glycine; Glu, glutamic acid; Gln, glutamine; Tyr, tyrosine; Asn, asparagine; Val, valine; Phe, phenylalanine; Leu, leucine; Ser, serine; Thr, threonine; Pro, proline; DHPP, desheptapeptide insulin; NMR, nuclear magnetic resonance; PDGF, platelet derived growth factor; CSF, colony stimulating factor; GH, growth hormone; His, histidine; GH, growth hormone; IRS-1, insulin receptor substrate-1; Lys, lysine.

sulin and insulin-like growth factor-I is proposed where the bivalent ligand bridges the two receptor α subunits alternatively at opposite sites in a symmetrical receptor structure. The implications of the model for negative co-operativity, bell-shaped biological curves, and the divergence between mitogenic and metabolic signalling are discussed in the context of the evolution of the properties of insulin and insulin-like growth factor-I. [Diabetologia (1994) 37 [Suppl 2]: S135–S148]

Key words Insulin receptors, IGF-I receptors, negative co-operativity, receptor dimerization, mitogenic signalling, metabolic signalling.

I am not suggesting insulin is aggregating with insulin in cross-linking. I am suggesting that insulin has more than one binding site for the receptor.

Martin Raff [1]

Introduction

It is exactly 20 years ago that my first paper described the unusual kinetic properties of the insulin receptor as a case of negative co-operativity [2]. My early thinking about receptor interactions was much influenced by classical works on allosteric enzymes [3–6]. I clearly did not realize then that it would take two decades more to begin to understand the possible molecular mechanisms underlying insulin's behaviour, and that the notion of negative co-operativity would remain contentious until quite recently. The recognition of our work by the Minkowski prize in 1981 was a major encouragement.

One stumbling block to understanding negative co-operativity was that our sophistication in analysing the kinetic behaviour of insulin and its structure-function relationships through the extensive use of insulin analogues [7] preceded any detailed knowledge of the structure of the receptor itself. A major step forward was the elucidation of the subunit structure of the receptor [8], and another one the cloning and sequence determination of its cDNA [9, 10].

It became apparent that the insulin receptor *is*, after all, a dimeric allosteric enzyme, belonging to the protein tyrosine kinase superfamily [11], and that insulin acts as an effector molecule that turns on its catalytic activity (see review by Van Obberghen in this issue). In this context, it has now become acceptable to propose that such a molecule has co-operative properties or site-site interactions, which is the rule rather than the exception in multimeric enzymes. An important question is whether the negative co-operativity is just an inconsequential side effect of some alteration in the receptor architecture after the first insulin molecule binds with high affinity, or whether it reflects some fundamental mechanism that links regulation of the dissociation rate to transmembrane signalling. This review is an attempt to start answering this question.

Structure of the insulin receptor

The mature insulin receptor is a disulphide-linked dimer, each half of which is made of two distinct disulphide-linked α and β subunits (the often used misnomer "heterotetramer" hides the intrinsic symmetry and functional bivalency of the receptor). The α and β subunits arise from the processing of a single chain preproreceptor precursor. The α subunits, which are entirely extracellular, contain the insulin binding sites and a cysteine-rich domain. They are made of either 719 or 731 amino acids depending on the tissue-specific alternative splicing of the 36 bp exon 11. The presence of the domain encoded by exon 11 decreases insulin binding affinity two to three-fold and reduces the receptor internalization and downregulation [12], but the role of this alternative splicing is otherwise unclear. The β subunits, which cross the cell membrane once, are made of 620 amino acids and contain in their intracellular portion the protein tyrosine kinase.

The gene coding for the receptor (> 130 kbp) is made of 22 exons (11 for each α and β subunit) separated by 21 introns [13]. For a more detailed description of the receptor structure and function, see [11, 14, 15].

Phenomenology of negative co-operativity in insulin receptor binding

The equilibrium and kinetic properties of insulin receptor binding can be summarized as follows:

1. Scatchard plots (that is a plot of bound/free ligand as a function of bound ligand, which is linear in the case of simple reversible reactions) of insulin binding are curvilinear, indicating that the affinity of the reaction (given by the slope of the plot), is not uniform over the saturation range (Fig. 1).

Recent studies with purified receptors have established that the receptor dimer binds only one insulin molecule with high affinity, but at saturation binds at least a second insulin molecule with lower affinity [16–20], a property reminiscent of that called "half-of-the-sites-reactivity" in some negatively co-operative enzymes [21]. Such a model usually gives an ex-

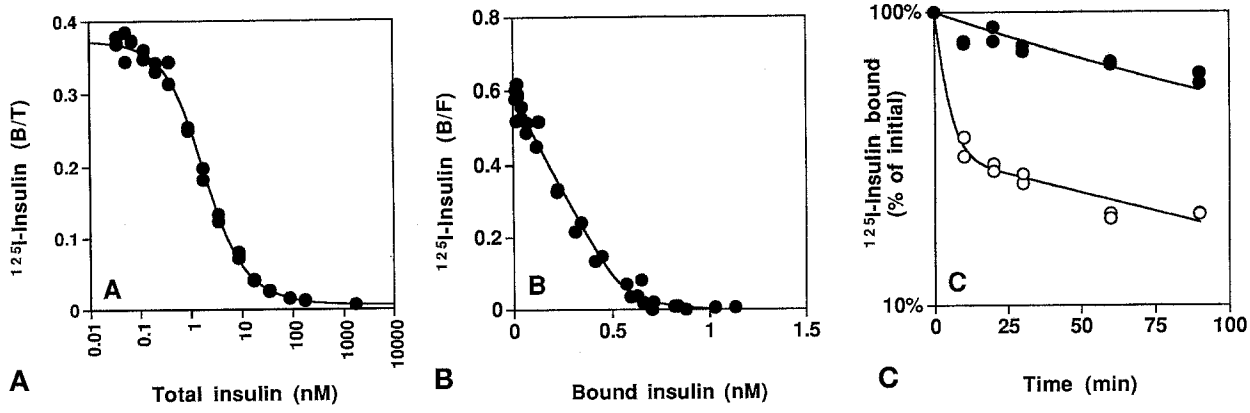

Fig. 1 A–C. Competition curve (**A**) and Scatchard plot (**B**) of insulin binding to insulin receptors on BHK cells overexpressing a cloned insulin receptor cDNA (variant without exon 11). Data from K. Grønskov, computer-fitted by R. Shymko to a model assuming one high affinity and one low affinity site per receptor dimer. (**C**) Dissociation kinetics of ^{125}I-labelled insulin from NIH3T3 cells overexpressing a cloned insulin receptor cDNA (gift from S. Taylor), by dilution in the absence (●) or presence (○) of unlabelled insulin. Data from B. Wallach computer-fitted by R. Shymko to the "K_{super}" model [28]

cellent computer fit of the insulin binding competition data (Fig. 1), as well as of IGF-I binding to its receptor (see below).

When purified under certain conditions, an engineered secreted receptor ectodomain made of the two extracellular α subunits and the two extracellular portions of the β subunits, shows a linear Scatchard plot and binds two insulin molecules with an equal affinity, which is about 20 times lower than the high affinity of the whole receptor [19]. This confirms the potential bivalency of the insulin receptor and demonstrates the structural equivalence of the two halves.

2. The dissociation of a tracer amount of bound ^{125}I-insulin in an "infinite" dilution (in practice, 40 to 100-fold, sufficient to prevent rebinding of dissociated tracer) is markedly accelerated in the presence of unlabelled insulin in the dilution buffer [2, 7, 22] (Fig. 1), a finding not predicted by receptor site heterogeneity, but consistent with the negative co-operativity model: the cold insulin binding to empty sites speeds up the off rate of prebound tracer, the kinetic equivalent of the lower affinity seen in equilibrium experiments (the affinity constant = association rate constant divided by dissociation rate constant). This experimental design was the first to distinguish between site heterogeneity and negative co-operativity, which cannot be discriminated on thermodynamic grounds (equilibrium properties) alone.

The purified ectodomain which shows a linear Scatchard plot does not exhibit accelerated dissociation (Schäffer, L., personal communication). Similarly, a monomeric receptor purified under mild reducing conditions (with an αβ structure instead of $\alpha_2\beta_2$) shows a linear Scatchard plot of low affinity binding, a stoichiometry of one insulin per αβ [16, 17] and a fast dissociation rate with no acceleration by unlabelled insulin [23, 24]. These studies provided the first evidence that high affinity binding requires both αβ moieties [25, 26].

3. The dose-response curve for the dissociation-accelerating effect of unlabelled insulin is bell-shaped: tracer dissociation increases when the cold insulin concentration in the buffer increases from physiological concentrations up to 100 nmol/l, but decreases ("self-antagonism") when the insulin concentration increases between 0.1 and 10 μmol/l [2, 7, 22] (Fig. 2). At high concentrations, insulin self-aggregates into dimers and hexamers. Until recently, the disappearance of negative co-operativity at high insulin concentrations was thought to result from insulin dimerization [7]: the range of insulin concentrations at which it is observed fits the range at which dimerization occurs ($K_d = 7 \times 10^{-6}$ mol/l), and dimerization covers the area of the insulin molecule that we showed to be essential for negative co-operativity (see below). Furthermore, some insulins known not to dimerize, such as hystricomorph insulins, failed to show the disappearance of negative co-operativity at high concentrations, so that the dissociation rate of ^{125}I-insulin remains maximally accelerated in the presence e.g. of 0.1 to 10 μmol/l unlabelled guinea-pig insulin [7]. Recent data obtained with a variety of genetically engineered monomeric insulins have shown, however, that these correlations were fortuitous, and that the bell-shaped negative co-operativity dose-response curve has another explanation – and in fact, is an important clue to the probable molecular mechanism involved (see below).

4. The dissociation rate of ^{125}I-insulin, whether measured by dilution alone or by dilution plus unlabel-

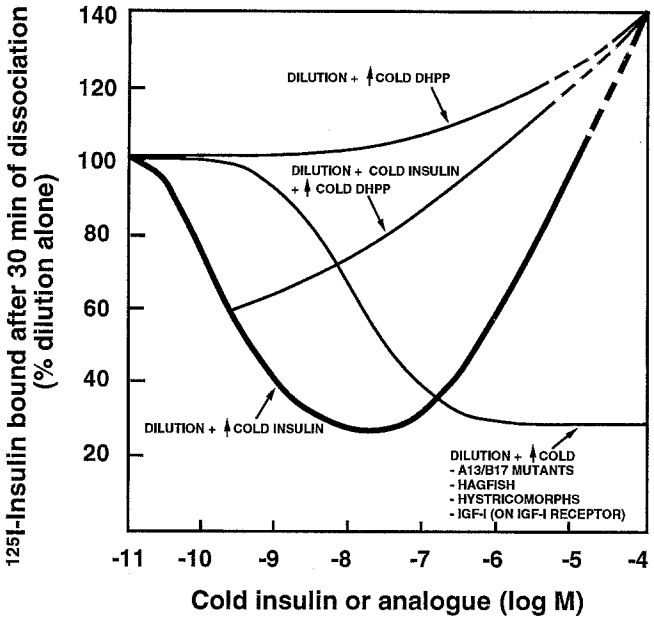

Fig. 2. Schematic representation of the characteristics of the dose-response curve for negative co-operativity in the insulin receptor. The effect of cold insulin on the dissociation rate of the tracer, as a function of the concentration of cold insulin, is bell-shaped. Des-heptapeptide insulin (DHPP) does not accelerate, but in fact slows down, the dissociation of ^{125}I-insulin. DHPP antagonizes the acceleration induced by cold insulin (Gu and De Meyts, unpublished). A number of insulin species or analogues, as well as IGF-I on its own receptor, show a monophasic instead of a bell-shaped curve

led insulin, is not first-order (that is, the dissociation data are not linear when plotted with a logarithmic vertical axis, as a simple reversible reaction would be) (Fig. 1). This is best explained if a fraction of the bound ^{125}I-insulin-receptor complex has isomerized to a tighter binding state (nicknamed the "K_{super} state") before initiation of dissociation [11, 27]. Therefore, a minimum of three states of the insulin-receptor complex is required to account for the complexity of binding kinetics: 1) the initial state to which the first insulin molecule binds with high affinity, or "K_e" state (e stands for "empty"); 2) the low affinity state caused by negative co-operativity and corresponding to the accelerated off rate, or "K_f" state (f stands for "filled"); and 3) the above-mentioned K_{super} state to account for the slower portion of the dissociation curve. This concept has been validated by the fact that monoclonal antibodies against the insulin receptor either induce the negative co-operativity or K_f state like insulin, or alternatively stabilize the K_{super} state [27], suggesting that these kinetic states do indeed represent different conformational states of the complex. A simple mathematical description of this three-state model fits simultaneously the equilibrium and dissociation data [28]. A new molecular interpretation of these three states is presented below.

Structure-function relationships of insulin binding and negative co-operativity: the "classical" receptor binding domain on the insulin molecule

Studies of the receptor binding and biological properties of a vast number of insulins from diverse animal species, chemically modified insulins and more recently genetically engineered insulins, have generally supported the concept that a dozen invariant residues from both A and B chains form a surface that binds to the insulin receptor, including Gly^{A1}, Glu^{A4}, Gln^{A5}, Tyr^{A19}, Asn^{A21}, Val^{B12}, Tyr^{B16}, Gly^{B23}, Phe^{B24}, Phe^{B25} and Tyr^{B26} [7, 29] (Fig. 5). This surface partially overlaps the insulin surface involved in insulin dimerization. Some variable residues are likely to be also involved in receptor binding, in order to explain the fact that the affinities of animal insulins for the receptor vary at least 100-fold, from the hystricomorphs and hagfish 2–5 % to the chicken and turkey 300 %.

A small subset of these residues was shown to be essential for negative co-operativity; substitutions or deletions at B23–26 (e.g. mutation of Phe^{B25} to Leu, as found in a diabetic patient [30], or deletion of Asn^{A21} (substitutions are tolerated) result in a marked decrease or complete loss of the ability of the analogue to accelerate the dissociation of prebound ^{125}I-native insulin, thus defining a "co-operative" subsite within the binding domain [7] (Fig. 5). Phe^{B24} and Phe^{B25} alone provide much of the binding free energy involved in inducing the site-site interactions. Most importantly, all "non-co-operative" analogues generated by alteration of the co-operative site, such as desheptapeptide insulin (DHPP), with a deletion of B23–30, were shown to be active antagonists of the negative co-operativity induced by native insulin, i.e. the dissociation of ^{125}I-insulin is markedly accelerated by 1.7 nmol/l unlabelled insulin, but is slowed down by 1.7 nmol/l insulin + 1.7 μmol/l DHPP [31].

Recent data suggested that, after the initial interaction, insulin undergoes a change in conformation upon receptor binding. The crystal structure of a mini-proinsulin with a peptide bond between A1 and B29 is isomorphic to native insulin, but this analogue has very low activity [32], suggesting that the conformation of insulin in the crystal or in solution (as seen by NMR) is not the conformation assumed in the active state of the hormone-receptor complex. Analogue studies have suggested that in the binding process, the carboxy-terminal residues of the B chain move away from the amino-terminal residues of the A chain, exposing an alternative protein surface (A2-A3) [33]. Receptor binding is therefore not a simple collision process, but rather a multi-step process which may involve successively different residues in the transition towards the active state.

However, even this consensual complexity lacks an essential first step, which involves an interaction

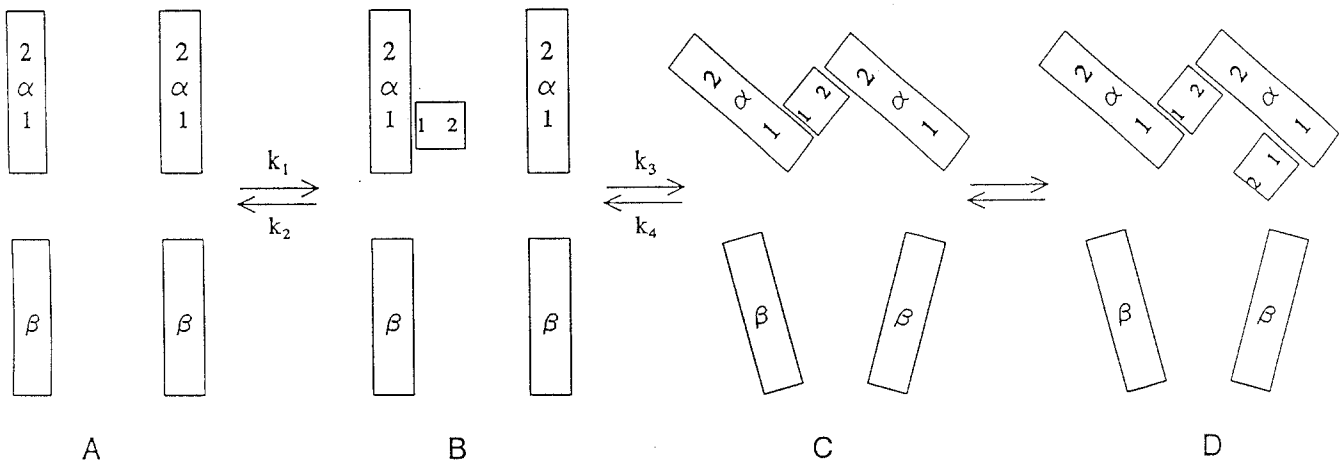

Fig. 3 A–D. Schäffer's cross-linking model for insulin binding to the insulin receptor. (**A**) Empty receptor. (**B**) Initial binding – soluble receptor ectodomain binding mode. (**C**) High affinity holoreceptor binding. (**D**) Low affinity holoreceptor binding. From [26] used with permission

with a completely different area of the insulin molecule and remained undetected until recently.

Clarification of the role of insulin dimerization in the dose-response curve for negative co-operativity: evidence for a second binding site on the insulin molecule

Some authors have argued that insulin dimerization on the receptor may in fact explain the negative co-operativity [34–36], but the concentration dependence did not fit and there was clearly no correlation between the ability of analogues to dimerize and their ability to accelerate the dissociation rate of ^{125}I-insulin [7, 22]. As mentioned above, the evidence available until recently was consistent with the hypothesis that the disappearance of negative co-operativity at insulin concentrations over 0.1 μmol/l was explained by insulin dimerization covering the "co-operative site". One fact, however, was bothering us: like the non-dimerizing hystricomorph insulins, hagfish insulin also failed to show a bell-shaped curve and continued to accelerate ^{125}I-insulin dissociation above 0.1 μmol/l [7], although hagfish insulin dimerizes as well as insulin [37]. Moreover, the hagfish monomer has a structure very similar to that of porcine or human insulin in the crystal [37], except for the position of the two Phe^{B25} residues in the dimer which diverge from each other instead of being stacked as in the insulin dimer [37].

The definite proof that the disappearance of negative co-operativity at high insulin concentrations has nothing to do with insulin dimerization came from studies of genetically engineered insulin analogues with absent or impaired dimerization [38]. The principal interactions in dimer formation involve Gly^{B8}, Ser^{B9}, Val^{B12}, Glu^{B13}, Tyr^{B16}, Gly^{B23}, Phe^{B24}, Phe^{B25}, Tyr^{B26}, Thr^{B27}, and Pro^{B28} [29, 38]. Since some of these are involved in receptor binding, non-overlapping residues were modified in order to prepare monomeric insulins with preserved receptor binding. Non-dimerizing insulins, such as Asp^{B9} insulin, showed the same disappearance of negative co-operativity above 0.1 μmol/l as native insulin [39].

In contrast, insulins mutated at the hexamer-forming surface of insulin, e.g. at residues Leu^{A13} or Leu^{B17}, failed to show a bell-shaped curve, that is, they continued to accelerate the dissociation rate of ^{125}I-insulin at concentrations over 0.1 μmol/l, like hagfish and hystricomorph insulins. It thus became obvious that the common denominator between all the insulins which do not lose the negative co-operativity at high concentrations is the inability to hexamerize, suggesting that an unsuspected interaction of the hexamer-forming surface of insulin stabilizes at high concentrations the tightly bound (K_{super}) state of the insulin-receptor complex and abolishes the negative co-operativity [39].

Either the hexamer-forming surface of unlabelled insulin binds to that of the prebound ^{125}I-insulin so that this bound dimer now binds more tightly to the receptor, or the hexamer-forming surface of the unlabelled insulin binds to another spot on the receptor in a way that stabilizes the K_{super} state. If there was an insulin-insulin interaction, mutations in the hexamer surface should have the same effect whether it is the normal insulin or the mutant insulin which is labelled and bound first to the receptor. This proved not to be true: when labelled A13 or B17-substituted insulins were bound first, the dose-response curve for the acceleration of their dissociation by native unlabelled insulin was bell-shaped, disproving the hypothesis of a direct insulin-insulin interaction (De Meyts, P et al., manuscript in preparation) and supporting earlier similar data with hystricomorph insulins [40].

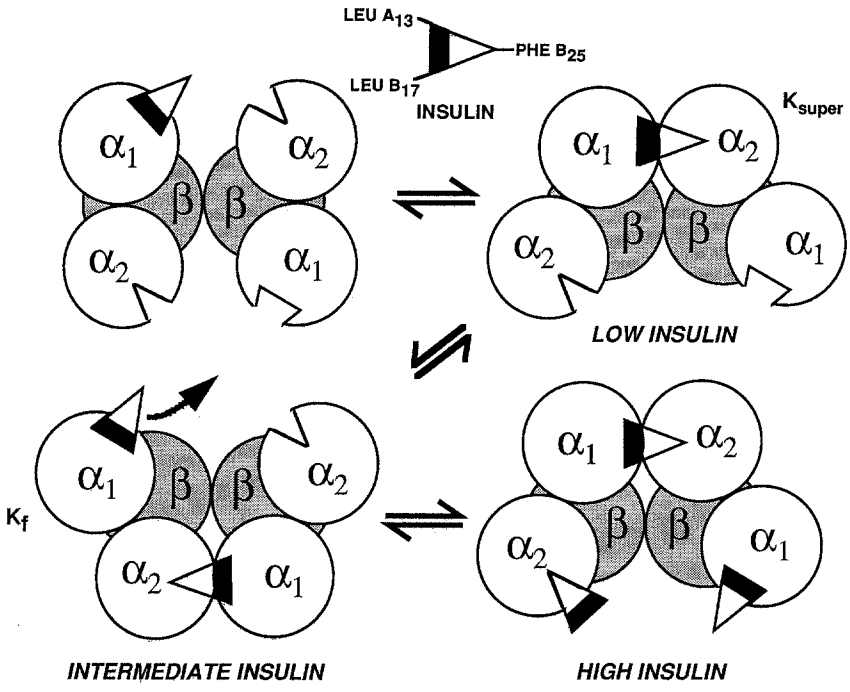

Fig. 4. The symmetrical, alternative cross-linking model for insulin binding to its receptor. The receptor is viewed from the top. Each α subunit is represented as a pseudo-symmetrical dimer, as suggested by Bajaj et al. [55], containing two subsites α1 and α2. The first insulin molecule binds through its hexamer-forming surface to α1, and then cross-links through its dimer-forming surface to α2 on the second α subunit. The resulting tight bivalent binding is referred to as the K_{super} state. If the concentration of insulin is increased, partial dissociation of the first bound insulin allows a second insulin molecule to cross-link the opposite α1-α2 pair, which allows the first molecule to dissociate completely. At very high insulin concentrations, α1 and α2 opposite the first cross-link are both occupied, preventing the second cross-linking and maintaining the first bound insulin molecule in the K_{super} state, explaining the bell-shaped dose-response curve of dissociation kinetics

The self-antagonism for the negative co-operativity may therefore reflect the interaction of a second binding site on the insulin molecule that overlaps with its hexamer-forming surface, with a distinct site on the insulin receptor.

This second insulin binding site on the receptor may be on the same α subunit, which might therefore contain a two-faced binding pocket, one face binding insulin's "classical" binding site which overlaps with the dimer-forming surface, the other face binding insulin's hexamer-forming surface. Alternatively, the second receptor site could be on the opposite α subunit, meaning that insulin binds to one α subunit through its dimer forming-surface and cross-links the other α subunit through its hexamer-forming surface (not necessarily in that order).

The receptor dimerization paradigm

The concept of receptor aggregation by bivalent ligands as a signal-triggering mechanism is not new and has been much discussed in immunology [1]. The idea that monomeric insulin may have two binding sites that cross-link two receptors was first proposed by Raff [1]. Receptor dimerization as a mechanism of trans-membrane activation appears today a rather general paradigm [41]. In some cases, dimerization of the ligand is the driving force bringing the receptors together as with platelet-derived growth factor (PDGF) and colony-stimulating factor 1 (CSF-1) [41]. In other cases, such as for epidermal growth factor (EGF), the monovalent binding of the ligand may create a conformational change which brings together two similarly occupied ligand-receptor complexes; in that case, the binding stoichiometry of the dimeric complex will be 2:2 [41]. A special case is the recent discovery that the class II histocompatibility antigen in the crystal is a dimer of αβ heterodimers [42], suggesting dimerization as a mechanism for initiating the cytoplasmic signalling events in T-cell activation. Dimerization as a mechanism of receptor activation is not reserved to membrane receptors; the receptors of the nuclear hormone receptor family (thyroid homones, retinoids and steroids) form homo- and heterodimers [43], as do other transcription factors such as *fos* and *jun*.

A different mechanism for receptor dimerization that involves a monomeric ligand, with a stoichiometry of 1:2, was proposed for the human growth hormone (GH) receptor [44], and confirmed in the crystallographic structure of the complex [45]. Two distinct binding sites ("site 1" and "site 2") on opposite faces of one GH molecule are involved. GH binds first through site 1 to one receptor molecule, and the complex formed binds sequentially through site 2 to a second receptor molecule at essentially the same receptor site as the one that binds GH site 1. Such a mechanism is probably general in the cytokine receptor family, with some members using a third asymmetric binding component in the complex [46].

Several authors recently proposed that a similar cross-linking mechanism may be operating within the insulin receptor pre-existing dimer, in order to explain the 1:2 stoichiometry [25, 26, 47–49]. Yip [48] speculated that two binding sites on one insulin molecule could bind either to two subsites on the same receptor α subunit (as also proposed by Fabry et al.

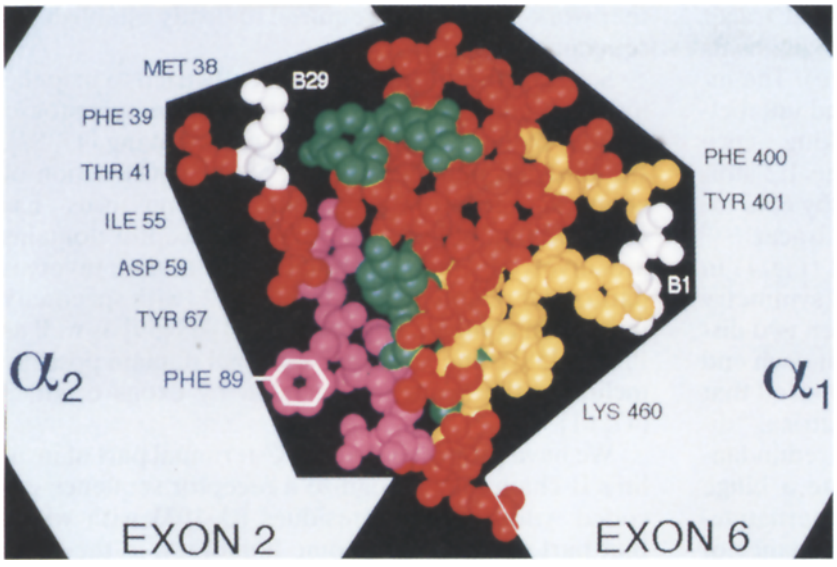

Fig. 5. Revised structure-function relationships for bivalent insulin receptor binding. This figure summarizes the currently available evidence compiled among others from [7, 47, 57, 58, 59, 84]. The classical binding surface, which overlaps the dimer-forming surface, is shown in green and purple (purple: "co-operative site"). The hexamer-forming surface is shown in yellow; the exact residues of this surface that are involved in receptor binding have not yet been mapped, with the exception of Leu^{A13} and Leu^{B17}. Shown in white are the positions where photoaffinity labels have been tagged [47, 57]: the B1 tag was shown to associate with receptor sequence 390–488 (exon 6), and the B29 tag to the first 120 α subunit residues (exon 2). The α subunit Phe 89 has been hypothesized to interact with insulin's Phe^{B25} in a manner similar to the two Phe^{B25} side chains in the insulin dimer [58]. Molecular graphics by R. Shymko, computer artwork by H. Wengholt

[47]), or alternatively cross-link two subsites across the two α subunits [48]; however, two insulins simultaneously cross-linking the α subunits as he proposed does not account for the 1:2 stoichiometry and ignores the negative co-operativity. This model [48] is therefore implausible. Lee et al. [25] have proposed a cross-linking mechanism whereby one insulin molecule has a predominant interaction with one receptor half and a less complete contact with the other half receptor.

Schäffer was the first to propose a cross-linking model [26] (Fig. 3) that accounts for the 1:2 stoichiometry and at the same time accounts for the co-existence of low affinity binding (curvilinear Scatchard plot), as well as for the unusual binding properties of some modified insulins to the holoreceptor and to the soluble ectodomain. In this ingenious model, however, the cross-linking per se does not provide an intrinsic mechanism for the accelerated dissociation of ^{125}I-insulin by unlabelled insulin, or its slowing down above 0.1 μmol/l insulin. Schäffer postulates additional negative site-site interactions between the second insulin binding to the empty second receptor site 1 and the first, cross-linked insulin (Fig. 3), in order to explain the accelerated dissociation. Furthermore, stabilizing interactions between a third insulin binding to the second empty receptor site 2 and the cross-linked insulin (Fig. 3) explain the loss of negative co-operativity at high insulin concentrations. In our studies, it seems possible to explain more simply *all* of the above phenomena through the cross-linking mechanism without having to invoke additional forces, provided that the receptor has an internal symmetry that allows alternative cross-linking at each of the two sets of α subunit subsites [50].

The symmetrical, alternative cross-linking model

An important aspect of the bivalent cross-linking concept is that any biological property that requires the dimeric form of the receptor exhibits a bell-shaped curve, since at high concentration of ligand, all receptor sites would be occupied by a single ligand molecule through its site 1, resulting exclusively in monomeric complexes. In agreement with this concept, GH self-antagonizes itself at high concentrations and produces bell-shaped curves for stimulation of thymidine incorporation in a leukaemic cell line transfected with GH receptors or a GH-granulocyte-macrophage colony stimulating factor chimeric receptor [51], in IM-9 lymphocytes or in the LB murine lymphoma cell line (Ilondo M.M., Ursø, B., De Meyts P., unpublished). This dimerization concept was confirmed by mathematical modelling [52, 53] and by demonstrating bell-shaped curves for GH stimulation of lipogenesis in rat adipocytes [53]. A second implication of the monomeric ligand-receptor dimerization concept is that GH mutations that impair binding of the ligand site 2 to the second receptor should generate antagonists, since at high concentration such mutated analogues would saturate all receptor sites by binding through GH site 1 and promote exclusively monomeric complexes [51]. This was verified experimentally by creating growth hormone antagonists such as G120R-hGH [51, 53].

Returning to insulin, which property has a bell-shaped curve and can be antagonized by mutations in one of the sites of the ligand molecule? The answer is clearly the acceleration of the dissociation of ^{125}I-insulin by unlabelled insulin, which is antagonized by DHPP (Fig. 2). This suggests therefore that the tracer dissociation acceleration is due to the cross-linking of the two α subunits by unlabelled insulin. But since ^{125}I-Tyr^{A14} insulin has the same high affi-

nity as unlabelled insulin [54], the prebound tracer must also have been cross-linking (which explains its slow off rate associated with the K_{super} state). The inescapable conclusion is that both tracer and unlabelled insulin must be alternatively cross-linking (since both cannot do it simultaneously given the 1:2 stoichiometry), and that it is the cross-linking by cold insulin that speeds up the dissociation of the tracer.

This is easily accounted for in a model (Fig. 4) in which the insulin receptor has an internal symmetry that allows alternative cross-linking between two distinct subsites α1 and α2 facing each other at each end of the α subunits. Bajaj et al. [55] have proposed that each α subunit may have a pseudo-symmetrical "dimeric" structure due to internal sequence redundancy. The cysteine-rich region may constitute a hinge around which the two α subunit subsites alternatively see-saw in order to be cross-linked. Occupancy of both α1 and α2 empty sites at super high concentrations of insulin prevents cross-linking at the sites opposite of the tracer-occupied ones, thus stabilizing the first cross-link (K_{super} state). This model may explain all of the known kinetic properties of the insulin-receptor interaction through the cross-linking mechanism without need for additional interactions: 1:2 stoichiometry for high affinity binding, additional low affinity sites (curvilinear Scatchard plot), acceleration of tracer dissociation by unlabelled insulin, self-antagonism for negative co-operativity as well as antagonism by DHPP. It provides structural counterparts to the three previously described kinetic states (Fig. 4).

Location of site 1 and site 2 on the insulin molecule, and α1 and α2 on the receptor α subunit

The insulin binding site overlapping the hexamer-forming surface may bind first, i.e. is "site 1" to use the GH terminology [45]; indeed, insulins mutated in the hexamer-forming surface, such as A13 or B17-mutated analogues, or hystricomorph insulins, have a markedly reduced initial association rate to the receptor [40, 50, 56]. These data suggest that the initial interaction of human insulin with the receptor normally takes place through insulin's hexamer-forming surface, and that the previously described "classical" binding site is the one that subsequently cross-links ("site 2"). The antagonism of negative co-operativity by analogues such as DHPP also suggests, if one assumes a sequential mechanism similar to that observed with growth hormone, that insulin's dimer-forming surface is site 2, i.e. binds last. This binding mode is the opposite to that proposed previously [26, 50]. This implies that much of the structure-activity relationships of insulin binding defined over the past two decades have essentially described the interaction of insulin's "site 2" with the receptor's α2. Further work is obviously required to firmly establish the sequence of binding.

Several laboratories have recently tried to map the insulin-binding domains on the insulin receptor α subunit by ligand photoaffinity cross-linking [47, 57], site-directed mutagenesis [48, 58] or construction of chimeric receptors [59, 60]. A consensus has emerged that at least two separate receptor domains are involved: an N-terminal domain that involves part of the region encoded by exon 2 (with specificity for insulin conferred by sequence 1–68 [59] as well as Phe 89 [58]), and a more C-terminal domain possibly including the sequence encoded by exons 6 and 7 [47, 61].

We have proposed that the C-terminal part of insulin's B-chain may recognize a receptor sequence encoded within exon 2 (residues 83–103) with which that part of insulin has some homology, in the same manner as insulin dimerizes with itself; and that the receptor Phe 89 may interact with insulin's Phe^{B25} [58]. In support of this concept, mutation of the receptor's Phe 89 to Leu markedly decreased insulin binding [58, 60]. Although this notion remains speculative, the hypothesis that the dimer-forming surface of insulin (denoted "site 2" above) binds to the N-terminal part of the receptor was supported by photoaffinity labelling experiments in which an insulin with a tag at Lys^{B29} was shown to attach covalently to a receptor fragment containing approximately the first 120 N-terminal residues [57].

Current evidence supports that insulin's hexamer-forming surface, denoted above "site 1", may bind to the more distal receptor domain probably encoded by exons 6 and 7 [47, 61]. Mutation of Lys 460 to Glu in the exon 6 domain (as found in a leprechaun patient) nearly abolishes the loss of negative co-operativity above 0.1 μmol/l [15, 62]. Mutation of Lys 460 to Arg abolishes the acceleration of ^{125}I-insulin dissociation by unlabelled insulin, suggesting that the first crosslink is essentially irreversible in this mutant. In addition, site-directed mutagenesis has suggested that residues Phe 400 and Tyr 401, also in exon 6, play a role in insulin binding and/or site-site interactions [63, Carbonnelle C. C., et al., submitted for publication]. In agreement with these findings, photoaffinity cross-linking of insulin with a tag at B1, at the hexamer-forming surface, attaches covalently to a receptor fragment starting at residue 390 until approximately 488 [47].

A tentative revised structure-function relationship of insulin-receptor binding in the light of the bivalent cross-linking concept is shown in Figure 5. It needs to be validated (or invalidated) by X-ray crystallography of the complex.

The receptor binding mechanism of IGF-I

The growth-promoting actions of both IGF-I and IGF-II are thought to be essentially mediated by the IGF-I receptor [65].The structure of human IGF-I is very similar to that of human insulin with 45 % homology [66], with the exception that the short 12-residue C-peptide linking the A and B chains is not removed during processing like insulin's, and that the equivalent of the A chain is extended at A21 by an 8-residue long D-region.

Unlike insulin, IGF-I has very weak self-association and does not hexamerize.

The IGF-I's Phe^{B23}-Tyr^{B24}-Phe^{B25} region plays a similar role in IGF-I receptor binding as the equivalent region of the insulin molecule has in receptor binding [66, 67]; in addition, the C-region of IGF-I (but not the D-region) is required for high affinity binding [66]. The additional contribution of the C-region may explain why IGF-I binding has a higher affinity than insulin binding, an affinity which mandates IGF-I tracer dilution to minimal concentrations in binding studies.

The IGF-I receptor cDNA sequence [68] and genomic structure [69] are similar to those of the insulin receptor with ~56 % in overall sequence homology and the same exon/intron organisation except for the absence of an equivalent to the alternatively spliced exon 11 of the insulin receptor. In contrast, an alternative splicing at the 5' end of exon 14 results in a sequence change from Lys-Thr-Gly-Thr to Lys-Arg-Tyr in the β subunit extracellular domain [69].

The kinetics of IGF-I binding have not been studied in detail. Scatchard plots have been variably reported as either linear or curvilinear. On cells expressing the native receptor, the presence of hybrid receptors confuses the picture [49, 65]. In a variety of cells over-expressing an IGF I receptor cDNA, as well as in cells containing the native IGF-I receptor but few insulin receptors (human arterial smooth muscle cells), IGF-I binding exhibits a similar negative co-operativity as the insulin receptor: curvilinear Scatchard plots well fitted assuming a 1:2 stoichiometry, and ligand-accelerated tracer dissociation [70, 71]. The IGF-I receptor $\alpha_2\beta_2$ dimer binds a single IGF-I molecule with high affinity, while each dissociated $\alpha\beta$ half binds an IGF-I molecule with low affinity [72]. These data suggest that the overall mechanism of IGF-I binding is analogous to insulin's and fits a similar cross-linking pattern [70]. The low affinity of insulin and IGF-I for their non-cognate receptor may be due to the fact that they recognize only one of the two receptor subsites and are unable to crosslink.

There was however one major difference in the negative co-operativity of IGF-I: the dose-response curve for IGF-I was not bell-shaped, and resembled the monophasic curve observed with the insulin analogues modified at the hexamer surface [70]. The implications of this difference are discussed later.

As far as the location of the binding sites on the receptor are concerned, interesting differences have been observed between the insulin and IGF I receptors. Using the chimeric receptor approach, it was shown that the residues that confer IGF-I specificity appear to be located differently than in the insulin receptor, in the exon 3-encoded cysteine-rich region [59, 60].

In the second putative binding site, mutations at Arg 450 in the IGF-I receptor (equivalent to Lys 460 in the insulin receptor, a position where mutations markedly affect the pH sensitivity and negative co-operativity of insulin binding [62]) have no effect on IGF-I binding and negative co-operativity (Wallach, B. and De Meyts, P., unpublished). More strikingly, substitution of exons 6, 7 and part of 8 in the IGF-I receptor cDNA by the equivalent segments of the insulin receptor produces a bell-shaped curve for IGF-I negative co-operativity, instead of the monophasic one observed with the wild-type receptor [70]. This suggests that the surface of the IGF-I molecule corresponding to insulin's hexamer-forming surface interface with this part of the receptor but only make actual contact if the side chains present are those of the insulin receptor.

The above data suggest that the binding interfaces for insulin and IGF-I on their respective receptors may be homologous (which would be expected given the homology of both ligand and receptor), but that within this interface the side chains which make actual contact and determine specificity may be quite different between the two ligand-receptor systems. In GH receptor binding, only one quarter of the side chains buried at the interface account for the majority of the binding energy, i.e. the functional binding epitope is much smaller that the structural epitope [73]. Therefore, the homologous interfaces between the insulin and IGF-I ligand receptor complexes may contain differently located functional epitopes [59]. However, if the C-domain of IGF-I is involved in binding, there may be a part of the IGF-I receptor binding domain without counterpart in the insulin receptor binding domain.

Relevance of the proposed binding mechanism to mitogenic and metabolic signalling

It is not clear at this point whether the cross-linking of the two α subunits is required for triggering signal transduction by insulin and IGF-I (e.g. because the structural alteration associated with cross-linking is the conformational change that derepresses the β subunit tyrosine kinase catalytic activity, a mechanism somewhat analogous to an allosteric transition),

Table 1. Properties of insulins altered in dimer-forming surface

- Faster association rate (not tested for all), faster dissociation rate
- Loss of negative co-operativity
- Antagonize negative co-operativity of native insulin
- Retain metabolic bioactivity proportional to affinity with same maximum

Properties of insulins altered in hexamer-forming surface (A13, B17 mutants, hystricomorph insulins, hagfish insulin)

- Very slow association rate, dissociation rate slower than insulin's
- Maintain negative co-operativity at high concentration (dose-response curve not biphasic)
- Decreased intrinsic metabolic bioactivity relative to affinity
- Increased mitogenic potency relative to affinity (not evaluated for all analogues)

or is merely a device that prolongs the duration of the signal by enhancing the half-life of the ligand receptor complex.

While it is clear that both insulin and IGF-I can induce both mitogenic and metabolic effects, insulin is considered as primarily metabolic and IGF-I primarily mitogenic. Whether each ligand can do both via its own receptor, or whether insulin exerts its mitogenic effects through its weak affinity binding to the IGF-I receptor, and IGF-I its metabolic effects through the insulin receptor, remains controversial [74]. Observations that a few cell lines devoid of IGF-I receptors [74, 75] or transfected cell lines over-expressing the insulin receptor show a sensitive mitogenic response to insulin, demonstrate that the insulin receptor can signal to both mitogenic and metabolic pathways.

We have shown recently in the LB lymphoma cell line [75], which is devoid of IGF-I receptors, that the mitogenic potency of insulin analogues is inversely related to their dissociation rate from the insulin receptor: all analogues tested that had a slower off rate than insulin had a higher relative potency in stimulating thymidine incorporation than expected from their relative affinity [76]. These include the Asp^{B10} insulin, previously shown to be supermitogenic [77] and to induce mammary tumours in rats [56]. Moreover, the negative co-operativity of the analogues also decreased as their mitogenic potency increased [76]; the most potent mitogen, His^{A8}, His^{B4}, Glu^{B10}, His^{B27}-insulin (code named H2), bound essentially irreversibly to LB and IM-9 cells (as first shown by Drejer in $HepG_2$ cells [56]); moreover, the off rate was neither accelerated by unlabelled H2 nor by unlabelled insulin, presumably because the first cross-link (Fig. 4) is so strong that the second bridge never gets a chance to form. These data suggest that the mitogenic signal may be strongly dependent on the strength of the first bivalent cross-link, and that the negative co-operativity may act as a device that dampens mitogenic signalling by both insulin and IGF-I.

As discussed for GH previously, one characteristic of a biological response that requires cross-linking of two receptor moieties is its bell-shaped dose-response curve due to self-antagonism at high concentrations. Such responses have been reported for IGF-I stimulation of differentiation in L6 myoblasts (including elevation of myogenin mRNA) [78], and for insulin antilipolytic effects [79]. In contrast, we see a plateau in insulin's stimulation of lipogenesis up to concentrations which are as high as solubility permits, and at which the negative co-operativity has been fully reversed (consistent with the full agonism of insulins modified in the dimer-forming surface), while the stimulation of lipogenesis by GH in the same cells is bell-shaped [53]. The stimulation of thymidine incorporation in the LB cells by insulin, however, shows a bell-shaped curve that closely parallels the dose-response curve for negative co-operativity, suggesting again the importance of the cross-linking mechanism for mitogenic signalling (B. Ursø and P. De Meyts, unpublished).

The selectivity for mitogenic versus metabolic signalling for insulin and IGF-I may naturally result from the selection of different substrates by the β subunit tyrosine kinase, or the involvement of different "docking" molecules (like insulin receptor substrate-1 (IRS-1) or SHC) to which such substrates associate [80]; however, there is currently little evidence that the two receptors utilize different signalling molecules [81, 82].

Table 2. Residues in hexamer-forming surface in various natural insulins and insulin-like peptides

	B1	B2	B4	B13	B14	B17	B18	B19	B20	A13	A14	A14
Human insulin	Phe	Val	Gln	Glu	Ala	Leu	Val	Cys	Gly	Leu	Tyr	Glu
Amphioxus ILP	–	Gln	Glu	Asp	Val	Phe				Tyr	Ser	
Human IGF I	–	Gly	Glu	Asp		Phe					Arg	
Hagfish IGF	–	Leu	Glu	Asp		Phe			Asp		Arg	
Hagfish insulin	Arg	Thr	Gly	Asn		Ile	Ala			Ile		Gln
Guinea Pig insulin			Arg		Thr	Ser			Gln	Arg	His	Gln
Casiragua insulin	Tyr			Asp	Thr	Ser			Lys	Arg	Asn	Leu
Coypu insulin	Tyr			Asp	Thr	Ser			Arg	Arg	Asn	Met
Porcupine insulin												Gln

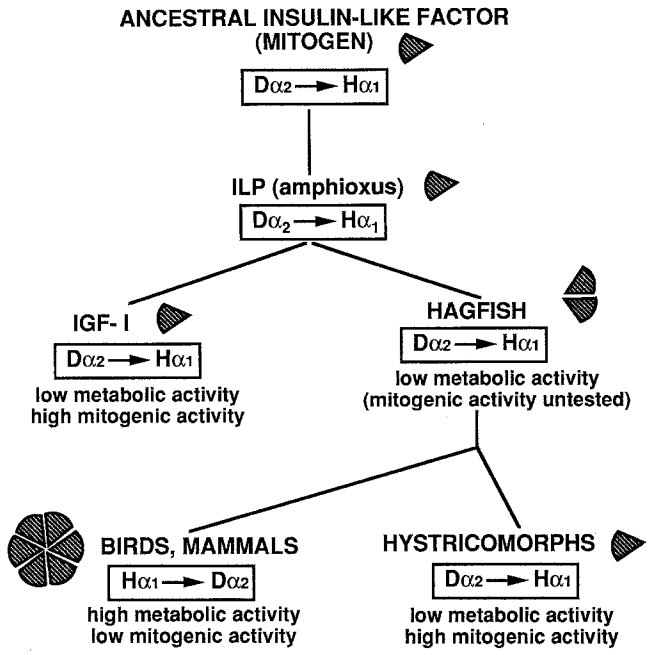

Fig. 6. Proposed evolution of the insulin and IGF-I binding mode, in relation to the mitogenic and metabolic activities of the molecules, and to their self-association properties. ILP (insulin-like peptide): a hybrid insulin/IGF molecule cloned from the amphioxus (*Branchiostoma californiensis*), a primitive cephalochordate that occupies a key position in chordate development as a possible extant relative of the invertebrate progenitor from which the vertebrates emerged [85]. See text for further explanation. Dα2 → Hα1, order of cross-linking; pie symbols, aggregation properties

There is however one aspect of the binding mode that appears to be associated with the selectivity for mitogenic versus metabolic signalling. The lack of self-antagonism for negative co-operativity seen in insulins with alterations in the hexamer-forming surface (already discussed above, Table 1) is associated not only with a set of unusual binding kinetic properties, but also with an unexpectedly high mitogenic potency [83] and unexpectedly low metabolic potency (two to five times lower than relative affinity, as has been long known but never satisfactorily explained for hagfish [36] and hystricomorph insulins (Table 2).

The fact that these insulins have a slow off rate (in fact even slower than insulin's) suggests that the first cross-link does occur. The fact that they accelerate their own as well as native insulin's dissociation (although with a decreased potency) suggests that the second cross-link also occurs. The lack of reversal at high concentration suggests that the binding of a third insulin molecule (Fig. 4) does not occur, or at least not fast enough to prevent the second cross-linking, consistent with the very slow on rate of these analogues.

I would like to propose the following working hypothesis to explain these data, which is speculative but testable:

1. In the model presented above (Fig. 4), the face of the insulin species or analogue which binds first is that with the fastest association rate to its corresponding receptor subsite.

2. In the insulins or analogues with a mutated hexamer surface, the binding of the hexamer surface to α1 (Hα1) does not occur, unless it is "presented" by the analogue being first bound by the dimer-forming surface to α2 (Dα2). The implication is that the very slow on rate of these analogues may reflect the usual slow on rate of Dα2 of wild type insulin, explaining why normally Hα1 occurs first. Once cross-linked, however, the mutated hexamer surface dissociates more slowly than that of native human insulin.

3. The Dα2 initial binding mode enhances mitogenic signalling and decreases metabolic signalling, while the Hα1 does the opposite.

4. IGF-I binding to its receptor, which also shows a monophasic curve for negative co-operativity, and is primarily mitogenic, has a Dα2 binding mode (not slow in this case, however, due to some substitutions in the dimer-forming surface which prevent dimerization but speed up the on rate, and maybe to the contribution of the C region, while substitutions in the hexamer-forming surface and/or in the corresponding receptor domain impair Hα1 like in the other analogues).

These proposals can be tested using appropriately designed analogues, including photoaffinity labelled ones, as well as chimeric receptors.

The way Dα2 may be selective for mitogenesis and Hα1 for metabolic effects may consist in distinct asymmetric autophosphorylation patterns of the receptor β subunit [25] or IRS-1 with the two binding modes, resulting in the ligand-receptor complex making different choices among the signalling molecules available to the two receptors, or in differences in their order of selection or in the kinetics of their activation, or a combination of the above.

This concept has exciting implications regarding the evolution of insulin and IGF-I: the ancestral molecule may have been primarily a mitogenic growth factor (possibly of neuroendocrine origin [64]), which after gene duplication has allowed its surface to evolve in a way that improves aggregation, storage, and at the same time optimizes metabolic signalling while minimizing mitogenic signalling (Fig. 6).

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Note added in proof: Schäffer's model has now been published in full [86].

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