Role of the Time Factor in Signaling Specificity: Application to Mitogenic and Metabolic Signaling by the Insulin and Insulin-Like Growth Factor-I Receptor Tyrosine Kinases

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The signal transduction pathways activated by hormones, growth factors, and cytokines show an extraordinary degree of cross-talk and redundancy. This review addresses the question of how the specificity conferred at the binding step is maintained through the signaling network despite the convergence of multiple signals on common efferent pathways such as mitogen-activated protein (MAP) kinase. The mechanism of receptor activation by ligand-induced dimerization provides a signaling device with both a switch and a timer. The role of the time factor, ie, of signaling kinetics, as a determinant of selectivity is discussed with emphasis on the receptor tyrosine kinases and cytokine receptors, and especially mitogenic versus metabolic signaling by insulin and insulin-like growth factor-I (IGF-I).

HORMONES and growth factors exert their intracellular effects through the activation of a complex array of signaling pathways that are closely interconnected (see review by Seedorf in this issue). Although the starting point in the signaling cascades, the cell membrane-bound receptor, is highly specific for a given ligand (and sometimes a small number of closely related molecules), this apparent molecular selectivity is rapidly lost in the next steps of the signaling network given the convergence of multiple receptor-initiated signals on common pathways such as the *ras/raf*/MAPK/ERK kinase (MEK)/mitogen-activated protein (MAP) kinase cascade or the phosphatidylinositol-3 kinase (for review, see De Meyts et al,[1] and Seedorf, this issue). Moreover, cross-talk between the pathways triggered by cytokine receptors, receptor tyrosine kinases, and seven-transmembrane–domain receptors further complicates the understanding of how a specific starting signal travels through the network to generate a specific end-point pattern of cellular responses. Although a number of elements are involved in the selectivity of signals (see below), this brief review will focus on the importance of the kinetic aspects (eg, transient *v* sustained) of the activation of signaling molecules, including the receptors themselves, in deciding which of multiple possible bifurcating pathways will actually be followed.

CELLULAR PROTEIN KINASES

The activity of a large number of cellular proteins is regulated through their reversible phosphorylation by enzymes called protein kinases,[2] which transfer the third (g) phosphate of the cellular energy carrier adenosine triphosphate to an amino acid side-chain containing a hydroxyl (OH) group. Most protein kinases, of which there may be more than 1,000,[3] phosphorylate proteins on serine or threonine. In contrast, the enzymes encoded by oncogenes such as the prototypical *src*,[4] as well as a number of growth factor receptors, instead phosphorylate tyrosines. Although tyrosine phosphorylation may represent less than 0.1% of total cellular phosphorylation events, it plays a crucial role in the control of normal cell growth (both early and late mitogenic events) and cell transformation, as well as in a number of metabolic signaling pathways.

The reverse reaction, protein dephosphorylation, is equally important in cell regulation, and involves a vast array of phosphoprotein phosphatases with either serine/threonine or tyrosine specificity that will not be discussed here (for review, see Hunter,[5] Cohen,[6] and Tonks et al[7]).

Members of the protein tyrosine kinase family show considerable structural diversity: the conserved catalytic domain is combined with a wide variety of extracatalytic, regulatory domains (some of which are receptor-binding domains for extracellular ligands).[8] There are indeed two groups of protein tyrosine kinases: the nonreceptor, cytosolic tyrosine kinases, and the transmembrane, receptor tyrosine kinases.[9]

Several families of cytosolic kinases have been identified and many members molecularly cloned that are not receptors, such as the *src*, *fes*, *abl*, and JAK families.[8] Some modules of *src* molecular anatomy, the so-called *src* homology (SH) domains SH2 and SH3, are found in other unrelated signaling molecules, where they play an important role in their binding to motifs containing phosphorylated tyrosines (SH2) or proline-rich motifs (SH3) on activated protein tyrosine kinases or intermediary docking molecules. Many of the cytosolic tyrosine kinases are activated by growth factors and other cellular activators.[10] Upon activation, some of these kinases are recruited to the plasma membrane and associate with a non–tyrosine kinase receptor to become part of its signaling complex, eg, JAK2 with the growth hormone receptor.[11]

OVERVIEW OF THE SUPERFAMILY OF RECEPTOR PROTEIN TYROSINE KINASES

The members of this family receive environmental information from regulatory domains expressed on the cell surface.[12] These receptors are allosteric molecules in which high-affinity ligand binding outside the cell induces conformational changes that activate the intracellular kinase

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domain. The first substrate of the activated kinase is often the receptor intracellular kinase domain itself, which autophosphorylates at well-defined tyrosine residues. This phosphorylation results in the creation of phosphotyrosine-containing sequence motifs that are recognition sites for other signaling molecules containing the previously described SH2 domains. An exception is the insulin/insulin-like growth factor-I (IGF-I) receptor family, in which no such motifs appear to be created, but in which the autophosphorylation enhances catalytic activity of the kinase (although some controversial evidence suggests that phosphatidylinositol-3 kinase may bind directly to the insulin receptor). Such signaling molecules group themselves around the activated receptor, become tyrosine-phosphorylated by the receptor kinase or otherwise allosterically activated, or interact with one another in specific ways within what is now known as the signal transfer particle[13] (and Seedorf, this issue). Information diffuses from this signal transfer particle along specific signaling pathways within the cytoplasm, eg, through cascades of phosphorylations, with end points as diverse as the activation of specific enzymes, the activation of nuclear transcription factors that turn on specific genes, or the translocation to the cell membrane of vesicles containing specific carriers such as glucose transporters. (We will not review here in detail the intracellular signaling network; for review, see De Meyts et al,[1] and Seedorf, this issue).

Receptor tyrosine kinases contain three major structural domains. The extracellular domain binds the ligand. This is the part of the receptor that confers the most diversity to this family. More than 50 members of the family have so far been cloned, which can be grouped in a number of different subclasses according to molecular architecture of the extracellular domain (Fig 1 of Seedorf, this issue). As pointed out by Wilks,[8] it appears that catalytic activity of the protein tyrosine kinase domain, having been selected as an evolutionary successful theme in the earliest phase of metazoan evolution, has been elaborated on by random variation and by recombination with other evolutionarily successful elements, an extraordinary diversity that testifies to the enzyme's metabolic utility and the evolutionary processes that have produced it.

A single hydrophobic membrane-spanning domain consisting of 22 to 26 amino acids and predicted to be α helical shows no primary structure conservation even between closely related members of the receptor tyrosine kinase family.[14] The transmembrane domain probably plays only a passive role in signal transduction.[13]

The tyrosine kinase domain is the most highly conserved portion of all receptor tyrosine kinases (and is also homologous to other kinases). A consensus sequence, Gly X Gly XX Gly X (15 to 20 amino acids) Lys, is part of the adenosine triphosphate binding site. Mutation of the conserved Lys at this site abolishes kinase activity of several receptor tyrosine kinases,[13] as well as their signal transduction ability.[15] A number of variable tyrosine residues have been identified as autophosphorylation sites in various receptor kinases. Their site-directed mutagenesis to eg, Phe, has been generally shown to abolish some or all of the receptor's signal transduction ability.[15] The current consensus is that all receptor tyrosine kinase signaling activities depend on a functional tyrosine kinase, and that these processes are mediated by tyrosine phosphorylation of cellular substrates.[13]

The x-ray crystal structure of the insulin receptor tyrosine kinase domain has recently been solved at 2.1 Å resolution.[16] The structure has provided interesting clues regarding the determinants of substrate preference for tyrosine rather than serine or threonine, and has revealed a novel autoinhibition mechanism whereby the autophosphorylated Tyr 1162 is bound in the active site.

In addition to the above-mentioned three main domains, a number of other domains have been shown to have a potential regulatory role on kinase function. A juxtamembrane domain of 41 to 50 amino acids lies between the cytoplasmic surface of the plasma membrane and the tyrosine kinase domain; its structure has diverged between the receptor tyrosine kinase subclasses, but is remarkably conserved between members of a given subclass.[14] It may be involved in the modulation of receptor function by heterologous stimuli (receptor cross-talk), in substrate binding (as in the case of insulin receptor insulin substrate-1 [IRS-1][17]), and in receptor endocytosis (as with the insulin receptor[15]).

The kinase domain of some subclasses of receptor tyrosine kinases is divided in two halves by insertion of up to 100 mainly hydrophilic amino acid residues.[13] This kinase insert appears to play a role in the interaction of the receptor with some cellular substrates and effector proteins.

Among the receptor tyrosine kinases, the distal, hydrophilic, carboxy-terminal tail of the intracellular domain is the most heterogeneous in both length (70 to 200 amino acids) and sequence. This heterogeneity is seen even between the closely related insulin and IGF-I receptors.[14] The C-terminal tail had been thought to interact with and perhaps exert a negative control on the activity of the tyrosine kinase domain by folding over it. It contains a number of potential or demonstrated tyrosine autophosphorylation sites that may compete with cellular substrates for the tyrosine kinase active site. Thus, autophosphorylation of these sites would make them unable to compete and would therefore suppress inhibition of the receptor kinase.[13] However, the recent x-ray structure has shown, as mentioned earlier, that it is Tyr 1162 that plays this role rather than the C-terminal tail. There are still discussions as to whether the differing C-terminal tails of insulin and IGF-I receptors may confer specific signaling properties.

ACTIVATION OF RECEPTOR TYROSINE KINASES AND CYTOKINE RECEPTORS BY LIGAND-INDUCED RECEPTOR DIMERIZATION

Allosteric activation of receptor tyrosine kinases (as well as cytokine receptors) is mediated by ligand-induced oligomerization, which allows the interactions between adjacent cytoplasmic domains that activate the kinase to take place,[13,18,19] eg, by putting autophosphorylation sites of one receptor moiety in contact with the active site of the kinase domain of the other moiety (transphosphorylation).[16] Li-

gand binding may dimerize the receptors in several different ways (for review, see De Meyts et al[1]).

1. A monomeric ligand may induce a conformational change that enhances the affinity of one receptor molecule for a second receptor molecule.
2. A dimeric ligand pulls together two receptor molecules.
3. The receptor is constitutively a covalent, disulfide-linked dimer within which the binding of a monomeric ligand induces site-site interactions. This appears to be the case for the insulin/IGF-I receptor subgroup[1,20] (see below).
4. The receptor is made of two asymmetric parts, both with low ligand-binding affinity. One part is a ligand-binding tyrosine kinase, and the other a ligand-binding domain with no tyrosine kinase domain. Eg, nerve growth factor (NGF), a dimer, binds to each receptor (*trk*) component with low affinity, but brings together a heterodimer with high affinity.
5. In some cases, such as the c-kit–encoded stem-cell factor receptor, dimer formation appears to be independent of the bivalency of the ligand and involves stabilization of the dimeric form of the receptor after monovalent binding through an intrinsic dimerization site on the receptor distinct from the ligand-binding pocket.[21]

A variation on the dimerization theme is found in the cytokine receptor family (eg, the growth hormone receptor), in which a monomeric but bivalent ligand brings together two or three components that do not contain a cytoplasmic tyrosine kinase, resulting in recruitment of a cytoplasmic kinase such as JAK2.[22-24]

THE INSULIN/IGF-I RECEPTOR TYROSINE KINASE SUBFAMILY

Insulin belongs to a family of related peptides that includes IGF-I and -II, the relaxins, the invertebrate bombyxins, and the molluscan insulin-like peptides.[25]

Three structurally related receptors for members of this peptide family have been identified: the insulin receptor, the type I IGF receptor (which for simplicity I will call the IGF-I receptor), and an orphan receptor known as the insulin receptor-related receptor. These glycoprotein receptors form a subgroup of the protein tyrosine kinase family with distinct features, most notably their covalent $\alpha_2\beta_2$ dimeric structure. Several detailed reviews have been published on their structure and function.[1,15,20,26-34]

The insulin receptor binds insulin with high affinity, but also IGF-II (with a 10-fold lower affinity) and IGF-I (with a 50- to 100-fold lower affinity).[35] The conventional view is that the insulin receptor is primarily involved in metabolic signaling but may also play a role in mitogenic signaling in some cell types in culture, as well as in some phase(s) of fetal development. Cross-reactivity of IGF-I and -II with the insulin receptor explains in part their insulin-like metabolic effects at high concentrations.

The IGF-I receptor binds IGF-I with high affinity and IGF-II with a similar or slightly lower affinity depending on cell type; it binds insulin with a 500- to 1,000-fold lower affinity, explaining in part the action of insulin as a growth factor in cell culture at high concentrations. The IGF-I receptor is thought of as primarily involved in mitogenic signaling and cell differentiation and in inhibition of apoptosis.[36]

Besides the IGF-I receptor, IGF-II binds in mammals but not in other species to the mannose-6-phosphate receptor, also called for that reason the IGF-II receptor, although it is not clear that this receptor plays any role in IGF-II signaling.[37]

IGF-I and IGF-II also bind to a family of six structurally related circulating binding proteins (unrelated to the receptors) that modulate the IGF's endocrine, paracrine, and autocrine functions[38] and limit the in vivo hypoglycemic effects of the IGFs (see review by Baxter, this issue).

The cDNAs encoding the insulin and IGF-I receptor precursors have been cloned,[39-41] giving valuable clues about the receptor structure and organization and generally confirming structural concepts previously derived from biochemical approaches.[42] The receptors are synthesized as a single-chain precursor, which is split at a tetrabasic sequence into separate α- and β-subunits. The extracellular α-subunit contains the ligand-binding domain. The β-subunit contains a single transmembrane domain, so that a third is extracellular and two thirds (containing the tyrosine kinase domain) are intracellular. The receptor is organized as a functional dimer with an $\alpha_2\beta_2$ structure, with a small number of α-α and α-β disulfide bridges. Various subdomains are recognizable within the sequence of the receptors, which have been probed by mutational analysis, often with conflicting results.[15]

MAPPING OF LIGAND-BINDING DOMAINS IN THE α-SUBUNIT

The α-subunit contains a single cysteine-rich domain flanked by two regions, termed L1 and L2, that have a weak degree of internal redundancy[43] and have been predicted to form a pseudosymmetrical dimer within each α-subunit. Two fibronectin type III repeats are also present in the insulin receptor.[15]

Several groups, including ours, have used mutational approaches (including the construction of chimeric constructs) in an attempt to map the ligand-binding site(s) on the α-subunit of the insulin and IGF-I receptors.[15] More than one segment appears to be involved. For insulin binding to its receptor, an *N*-terminal region located within the first 68 *N*-terminal amino acids (encoded by exon 2) appears to be important for insulin specificity, whereas in the IGF-I receptor a sequence encoded by exon 3 (residues 191 to 290) contained in the cysteine-rich domain defines IGF-I specificity.[44-46] We have suggested the involvement of the receptor region 83 to 103 in insulin binding, especially Phe 89, based on a possible analogy between the insulin-receptor interface and the insulin-dimer interface.[47] By the use of alanine-scanning mutagenesis, Williams et al[48] have identified residues Asp 12, Ile 13, Arg 14, Asn 15, Gln 34, Leu 36, Met 38, Phe 39, Glu 44, Phe 64, Tyr 67, Phe 89, Asn 90, and Tyr 91 as contributing to insulin binding. Photoaffinity cross-linking experiments have suggested that the first 120 residues of the α-subunit are important for insulin binding.[49]

Other data suggest that a second domain in the carboxy-terminal region of the α-subunit is also involved in insulin

binding, perhaps the one encoded by exons 6 and 7 and recognized by the patient's autoimmune antibodies.[50] A mutation at position 460 in this domain found in a leprechaun patient had a complex influence on insulin binding.[51] Schumacher et al[52] have shown that insertion of residues 325 to 524 of the insulin receptor in the IGF-I receptor markedly enhances insulin affinity for the IGF-I receptor. We have shown that inserting the exon 6–encoded domain of the IGF-I receptor (residues 368 to 469) in the insulin receptor enhances affinity of IGF-I for the insulin receptor (in preparation), whereas insertion of the insulin receptor sequence encoded by exons 6, 7, and part of 8 into the IGF-I receptor changes the shape of the dose-response curve for negative cooperativity of IGF-I.[53] These data suggest that the region including and surrounding exon 6 may also contribute specific interactions to both insulin and IGF-I binding to their receptors. A more refined scanning of this region of both receptors is warranted.

Furthermore, it has been shown recently that a novel photoreactive insulin derivative (despentapeptide- (B26-B30) [B25 *p*-azidophenylalanine-α-carboxamide]) cross-links to a carboxy-terminal region of the α-subunit (residues 704 to 718) just upstream of the region encoding for the alternatively spliced exon 11, suggesting that aromatic residues in this region may also be involved in binding or in conformational changes required for the formation of the high-affinity binding state.[54]

Altogether, the mutational data suggest that the area responsible for ligand binding consists of at least two or three separate domains in the insulin and IGF-I receptors, and that the sequences of actual side-chain contact with the ligand are located at different positions within a common pocket in the two receptors.[44-46]

SITE-SITE INTERACTIONS AND NEGATIVE COOPERATIVITY IN THE INSULIN AND IGF-I RECEPTORS

As mentioned earlier, receptor tyrosine kinases, as well as cytokine receptors, become activated enzymes by dimerization.

The mechanistic advantages of the dimer structure are several. It allows lateral transmission of binding energy from outside the cell to inside the cell.[55] It provides a structural framework for making one cytoplasmic domain a substrate for transphosphorylation by the other.[16,56] It also permits regulation of ligand-binding affinity by receptor occupancy through site-site interactions, or cooperativity.

Insulin binding to its receptor indeed exhibits negative cooperativity, ie, the binding of one insulin molecule decreases the affinity of further binding.[20,57-60] The insulin and IGF-I receptor dimers bind only one ligand molecule with high affinity and a second ligand molecule with lower affinity, generating curvilinear Scatchard plots (Fig 1A and C). The binding of one ligand molecule markedly accelerates the dissociation rate of the second ligand molecule (Fig 1B and D). The classic interpretation of such a phenomenon is that ligand binding to one receptor subunit induces a conformational change in the second subunit that reduces its affinity for the ligand. New data from our laboratory suggest that the actual mechanism is in fact analogous to the monomeric-bivalent binding mechanism found in the cytokine receptor family to induce receptor dimerization.[20] Like growth hormone, insulin and IGF-I molecules appear to have two binding domains on two opposite faces of the monomer.[20,53,61] These two binding surfaces probably cross-link the two α-subunits in the preexisting receptor dimer (Fig 2), thereby creating high-affinity binding.[20,61] Since, as discussed earlier, each receptor α-subunit has two binding domains (in contrast to the growth hormone receptor, which has only one), this allows a second cross-linking to take place when a second ligand molecule binds, resulting in release of the first ligand molecule (Fig 2), thereby causing accelerated dissociation.

Scatchard plots of IGF-I binding have been variably reported as either linear or curvilinear. On cells expressing the native receptor, the presence of hybrid receptors made of one half insulin receptor and one half IGF-I receptor confuses the picture.[33,62] We have recently shown that in a variety of cells overexpressing 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 negative cooperativity similar to that of the insulin receptor: curvilinear Scatchard plots well-fitted, assuming a 1:2 stoichiometry, and ligand-accelerated tracer dissociation[1,53] (Fig 1C and D), as also shown by Zhong et al.[63] Like the insulin receptor, the IGF-I receptor $\alpha_2\beta_2$ dimer has been reported to bind a single IGF-I molecule with high affinity, whereas each dissociated αβ half binds an IGF-I molecule with low affinity,[64] although one dissenting report claims that αβ IGF-I receptor halves conserve high affinity.[32] These data suggest that the overall mechanism of IGF-I binding is analogous to insulin's and follows a similar cross-linking pattern.[53] The low affinity of insulin and IGF-I for their noncognate receptor may be due to the fact that they recognize only one of the two receptor subsites and are unable to cross-link.

Interestingly, unlike insulin,[57] the dose-response curve for the negative cooperativity of IGF-I was not bell-shaped (Fig 3), a property that IGF-I shares with insulin analogs modified at the hexamer surface.[53] These analogs, like IGF-I, have enhanced mitogenic properties relative to their metabolic potency.[20]

ROLE OF NEGATIVE COOPERATIVITY AS A MODULATOR OF SUSTAINED SIGNALING

In systems in which the receptor undergoes dimerization, the dimer-bound ligand has a much higher affinity than the monomer-bound one, due to the fact that the dissociation rate of the ligand from the dimeric receptor–bound ligand complex is much slower (corresponding to what we called the K_{super} state of the receptor[65]) than that from the monomeric receptor–bound ligand complex. The importance of receptor dimerization in slowing the rate of ligand dissociation has also recently been recognized in the stem-cell factor receptor.[21]

Thus, the bivalent cross-linking model provides a mechanism by which high-affinity binding and long-lasting association (and therefore more sustained signal transduction) is created from an otherwise weak molecular interaction. We

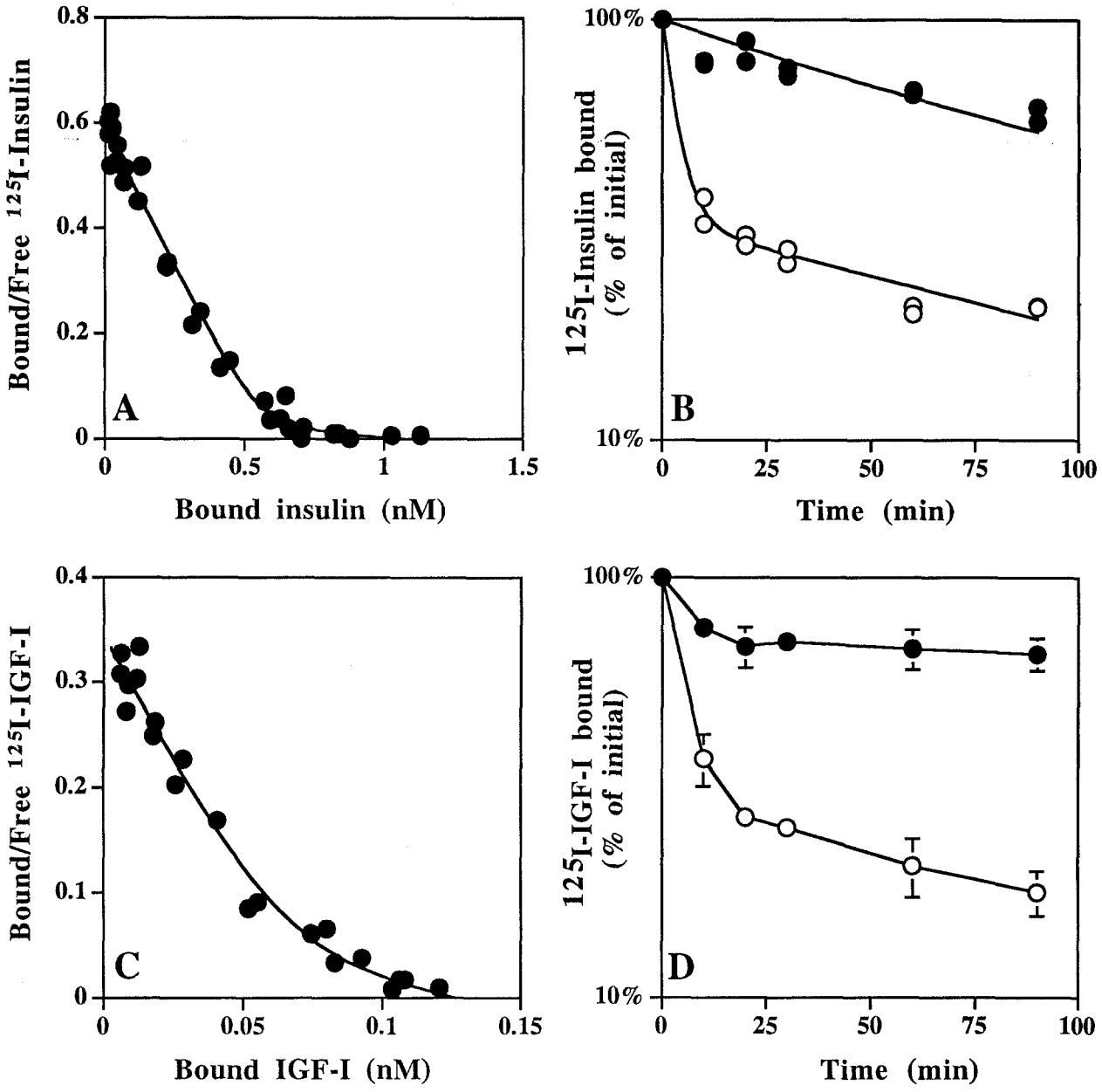

Fig 1. (A) Scatchard plot of insulin binding to insulin receptors on BHK cells overexpressing a cloned insulin receptor cDNA (variant without exon 11). Data computer-fitted to a model assuming one high-affinity and one low-affinity site per receptor dimer. Reprinted with permission.[20] (B) Dissociation kinetics of ^{125}I-insulin from NIH3T3 cells overexpressing a cloned insulin receptor cDNA (gift from S. Taylor), by dilution in the absence (●) or presence (○) of 170 nmol/L unlabeled insulin. Reprinted with permission.[20] (C) Scatchard plot of IGF-I binding to 293 cells overexpressing a cloned IGF-I receptor cDNA. Data computer-fitted to same model as A. Reprinted with permission.[53] (D) Dissociation kinetics of ^{125}I-IGF-I from 293 cells overexpressing a cloned IGF-I receptor cDNA, by dilution in the absence (●) or presence (○) of 100 nmol/L unlabeled IGF-I. Reprinted with permission from *Endocrinology* 135:472-475, 1994.[53] © The Endocrine Society.

will discuss further the importance of this time factor in signaling efficiency, especially regarding mitogenic signaling.

What negative cooperativity adds to the above-mentioned properties is to make the affinity of the complex (and its dissociation rate) dependent on the ambient ligand concentration.

Therefore, negative cooperativity may act as a buffering device that allays the potency of mitogenic signaling and controls the transforming potential of the ubiquitous insulin and IGF-I receptors, which as a result are rather weak mitogens. Impairing this natural restraint by engineering tightly binding analogs has been shown recently to have undesirable consequences, such as an increased carcinogenic or mitogenic potential[66] (see below).

DETERMINANTS OF SIGNALING SPECIFICITY

Since a variety of diverse signals converge on common signaling pathways such as the *raf*-1 to MAP kinase cascade

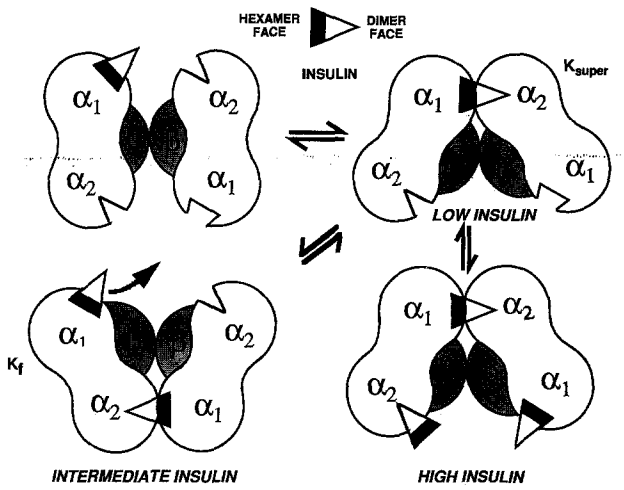

Fig 2. Symmetrical, alternative bivalent cross-linking model for insulin binding to its receptor viewed from the top. The first insulin molecule binds through site 1 to the receptor's α_1 subsite, and then cross-links through its site 2 to the second receptor subunit's α_2 subsite. The resulting tight bivalent binding has been referred to as the K_{super} state.[1,20,53,65] At higher insulin concentration, partial dissociation of the first bound insulin allows a second molecule to cross-link the opposite $\alpha_1\alpha_2$ pair, which allows the first molecule to dissociate completely. This explains the observed accelerated dissociation (negative cooperativity). At high insulin concentrations, α_1 and α_2 opposite the first cross-link are both occupied, preventing the second cross-linking and maintaining the first insulin molecule bound in the K_{super} state. This explains the bell-shaped dose-response curve of dissociation kinetics (see Fig 3). In the case of IGF-I (as well as certain insulin analogs with mutations in site 1), we speculate that the binding of site 1 to α_1 occurs more slowly than that of site 2 to α_2, which therefore occurs first, and then site 1 cross-links to the other subunit's α_1. This binding mode may enhance mitogenic signaling.[20] For the same reason, at high IGF-I concentrations, the third molecule of IGF-I does not have time to bind to the empty α_1 before the second cross-link occurs, and the negative cooperativity curve is therefore not bell-shaped.[53] (see Fig 3).

(see review by Seedorf, this issue, for description of growth factor signaling pathways), one may wonder how the diversity of ligand-specific responses is generated. Response specificity may be generated by the particular pattern of receptors expressed in a given cell, as well as by divergences in the most distal downstream signaling elements (eg, transcription factors) displayed in a given cell. In other words, there may be specificity elements both upstream and downstream of the common pathway. Other factors possibly involved in signaling specificity are shown in Table 1 (see also review by Seedorf, this issue). However, it appears that these answers do not completely resolve the specificity problem, as shown by two examples.

The rat pheochromocytoma PC12 cell line possesses receptors for both NGF and epidermal growth factor (EGF). The effects of the two growth factors on this cell line differ markedly. EGF induces cell proliferation, whereas NGF induces cell differentiation into a sympathetic neuronal phenotype, despite the fact that the two growth factors share the same signaling elements: tyrosine phosphorylation, membrane ruffling, identical immediate early genes, *ras*, extracellular factor-regulated kinase (ERK)/MAP kinases, ribosomal S6 kinase (RSK)/S6 kinases, tyrosine hydroxylase, ornithine decarboxylase, 2-deoxyglucose uptake, Na^+/K^+ pump, and sodium channels.[67] One cannot exclude the possibility that as yet undiscovered, more distal genetic elements provide areas of signaling divergence. However, a concept is emerging that the nature of signaling elements involved is not the only determinant of response specificity, eg, in choosing to pursue a differentiating versus a proliferative program, but that kinetic aspects of their activation play a determinant role in signal specificity (see below).

A second example is the divergent effects of the two closely related ligands (and receptors of) insulin and IGF-I. The conventional wisdom is that insulin is primarily a

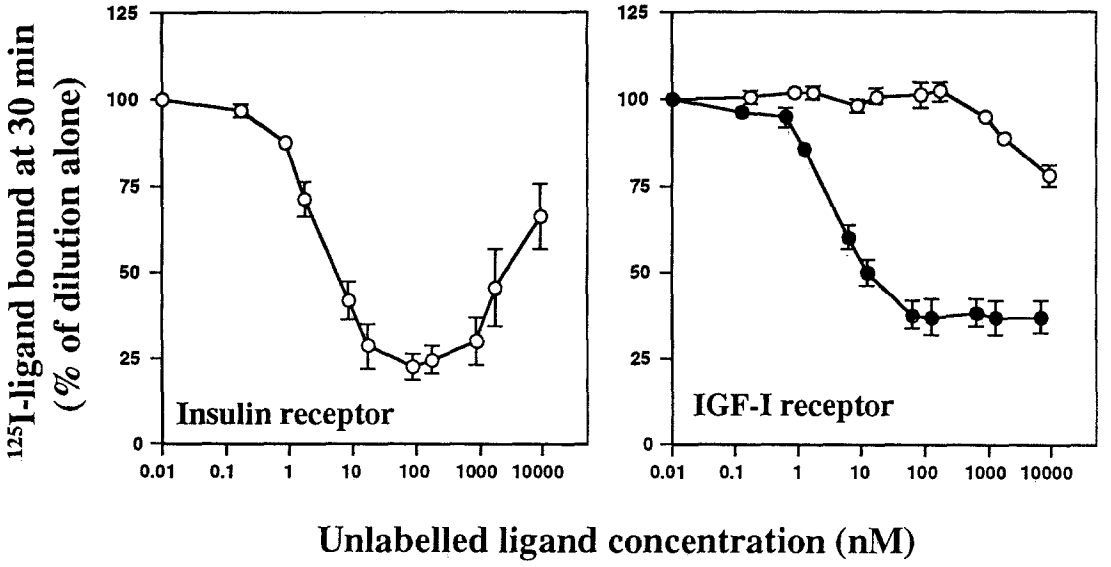

Fig 3. Dose-response curve for dissociation-accelerating effect of unlabeled ligand (negative cooperativity) from 293 cells transfected with cDNA for insulin receptor and IGF-I receptor. Tracer used was ^{125}I-insulin for insulin receptor and ^{125}I-IGF-I for IGF-I receptor. Unlabeled ligand: insulin (○), IGF-I (●). Reprinted with permission from *Endocrinology* 135:472-475, 1994.[53] © The Endocrine Society.

Table 1. Determinants of Signaling Specificity

Types of receptors expressed in a given cell
Stoichiometry of receptors/substrates
Types of substrates/signaling molecules expressed in a given cell
Relative affinity of receptors for substrates/signaling molecules; mass action (competition for same substrates depending on level of receptor expression)
Hybrid receptors
Feedback loops (phosphatases and serine/threonine kinases)
Localization of signaling molecule within the cell
Activation kinetics of signaling molecules (transient *v* sustained, and time delays)

metabolic hormone and IGF-I is primarily a growth factor, and that they share each other's effects at high concentration due to the weak cross-reactivity of each ligand for the other's receptor.[68]

Although the notion that insulin is a physiological growth factor is controversial, several lines of evidence suggest that the insulin receptor can produce a mitogenic signal and support cell growth and in some circumstances may even be involved in cell transformation (for review, see Moses[68] and De Meyts et al[1]).

The fact that many, if not most, cells contain both receptors usually complicates the interpretation. In some of these cell types, such as NIH3T3 cells, stimulation of thymidine incorporation by insulin produces a poorly sensitive curve, suggesting that the response is mediated through the IGF-I receptor.

Few cell lines have been shown to lack IGF-I receptors and to show insulin-dependent growth and sensitive curves for insulin-stimulated thymidine incorporation, such as the H35 or KRC7 hepatoma cell lines.[69-71] Our recent study with a T-cell lymphoma line (denoted LB) devoid of IGF-I receptors, in which insulin acts as a potent mitogen at low concentrations, clearly demonstrates that insulin is capable of mitogenic signaling through its own receptor[72] (Ish-Shalom D, Tzimon G, Christoffersen CT, et al, submitted).

Therefore, although it is clear that insulin can be both metabolic and mitogenic, it is not clear if and where the two pathways diverge, especially since both MAP kinase and phosphatidylinositol-3 kinase have been implicated as being essential for both. It has been suggested that IRS-1 may mediate metabolic responses and that *shc* is important for mitogenesis,[73] but this remains to be proven.

Moreover, there is little evidence that insulin and IGF-I have different signaling modes: both phosphorylate IRS-1 and activate phosphatidylinositol-3 kinase and MAP kinases and c-*fos*.[74-77]

In our studies of the kinetic and biological properties of insulin analogs, we have determined certain elements that may provide some selectivity in metabolic potency versus mitogenic potency at the level of the binding step itself, within the framework of our proposed bivalent binding model. We believe that the kinetics of a signaling event may be as important in selecting response patterns as the nature of the molecules involved.

THE BIVALENT CROSS-LINKING MECHANISM AS A SWITCH AND A TIMER

A signaling device like a receptor must feature as distinct elements a conformational switch and a timer.[78] The switch transforms the input signal from the ligand into a conformational change in the signal-generator domain, thereby activating it. The timer determines how long the signal-generator domain will remain "on" in response to reception of a signal by the detector domain. For example, in the case of G proteins such as *ras* or G_α, binding of guanosine triphosphate turns the switch "on" while the intrinsic guanosine triphosphatase serves as the timer.[78]

In the receptor dimerization model, bivalent cross-linking of two receptor subunits provides both the switch and the timer. Immunologists have long recognized that bivalent ligand binding increases the likelihood that two mobile receptors will remain approximated long enough to transduce a signal[79,80] (Fig 4).

Two elements of the bivalent cross-linking by insulin, insulin analogs, or IGFs may provide selectivity for metabolic versus mitogenic signaling, the first one related to the switch and the second to the timer.

First, the order of cross-linking by the two faces of the ligand may generate asymmetric signaling depending on which face binds first (ie, the dimer-forming surface or the hexamer-forming surface of insulin). In other words, the bivalent switch may be anisotropic. We[20] have recently proposed that insulin and IGF-I may cross-link their receptor α-subunits in opposite order, thereby enhancing, respectively, metabolic or mitogenic signaling.

Second, the tightness of the bivalent cross-link may enhance mitogenic effects by inducing a sustained signal. Analogs of insulin with enhanced mitogenic potency rela-

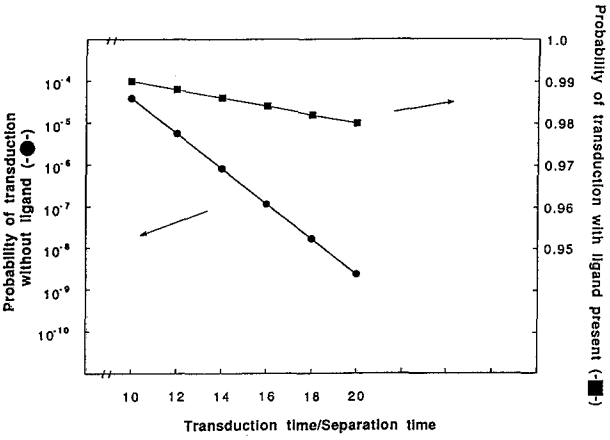

Fig 4. Effect of bivalent binding on the likelihood that two mobile receptors will remain approximated sufficiently long to transmit a signal. Graph shows that if the time required to initiate a signal is appreciably longer than the time the two molecules remain approximated through spontaneous translational diffusion, the probability of signal transduction decreases to virtually 0 (left ordinate). In the presence of even a weakly bound ligand (assumed $K_A = 10^4$ mol/L^{-1}), the probability of signal transduction remains high (right ordinate). Reprinted with permission.[79] Adapted from reference 80. Copyright 1992. The American Association of Immunologists.

tive to binding affinity indeed had a slower rate of dissociation from the insulin receptor of the above-mentioned LB cells, as well as a loss of negative cooperativity. This implies that the first insulin molecule remained bivalently bound even in the presence of an excess of cold insulin. The mitogenic potency of a series of insulin analogs was inversely correlated with the dissociation rate rather than with the affinity.[81] Cross-linking may be required for a sustained activation of the receptor tyrosine kinase in order to induce mitogenic signaling.

The ligand-binding step is not the only one in which the kinetics of signaling play a role in defining specificity. A more downstream example is provided by the role of MAP kinase in the effects of NGF and EGF on cell proliferation and differentiation in PC12 cells discussed earlier. Several groups found that NGF, which induces differentiation, induces a sustained activation of MAP kinase, while EGF, which induces cell proliferation, stimulates MAP kinase activity only transiently[82-85] (see also review by Seedorf, this issue). Many of the nodes in the signaling network may comprise a similarly regulated timer.

As discussed elsewhere,[1] the concept of signal transduction has evolved from that of a collection of unidimensional parallel signaling cascades to that of a two-dimensional network with lateral connectivities between pathways. We believe that time is the third—and not the least important—dimension of the signal transduction network.

To fully understand how the time factor may affect bifurcation decisions at a given node in the signaling network, a quantitative description of integrated signaling pathways is warranted. We are currently working on a comprehensive theory of signal transduction based on approaches devised for the description of regulatory networks such as genetic control circuits, based on Boolean logics.[86-88] Such an approach is needed to go beyond descriptive molecular anatomy and grasp the functional physiology of the interrelationships between signaling elements.

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