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Understanding Cytokine and Growth Factor Receptor Activation Mechanisms

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Abstract

Our understanding of the detailed mechanism of action of cytokine and growth factor receptors – and particularly our quantitative understanding of the link between structure, mechanism and function – lags significantly behind our knowledge of comparable functional protein classes such as enzymes, G protein-coupled receptors, and ion channels. In particular, it remains controversial whether such receptors are activated by a mechanism of ligand-induced oligomerization, versus a mechanism in which the ligand binds to a pre-associated receptor dimer or oligomer that becomes activated through subsequent conformational rearrangement. A major limitation to progress has been the relative paucity of methods for performing quantitative mechanistic experiments on unmodified receptors expressed at endogenous levels on live cells. In this article we review the current state of knowledge on the activation mechanisms of cytokine and growth factor receptors, critically evaluate the evidence for and against the different proposed mechanisms, and highlight other key questions that remain unanswered. New approaches and techniques have led to rapid recent progress in this area, and the field is poised for major advances in the coming years, which promises to revolutionize our understanding of this large and biologically and medically important class of receptors.

Keywords

Ligand-induced dimerization; ligand-induced oligomerization; receptor pre-association; agonist antibodies; small molecule receptor agonists; bell-shaped dose-response; erythropoietin receptor; receptor-ligand cross-reactivity

1. Introduction

Multicellular life depends on the ability of each cell to sense and respond to its surroundings, so it can coordinate its activities to fulfill the needs of the tissue, organ and organism of which it is a part. To achieve this end a tremendous volume and variety of information must pass through the cell membrane and be integrated and acted upon within the cell. A principal means by which this information transduction occurs is through cytokine and growth factor receptors, which span the plasma membrane and trigger intracellular signals in response to stimulation by soluble cytokine and growth factor proteins in the extracellular milieu (Stroud and Wells 2004; Whitty and Riera 2008; Posner and Laporte 2010). The goal of this article is to summarize the current state of knowledge concerning the molecular mechanisms by which cytokines and growth factors engage their cell surface receptors to bring about an activated receptor complex, to critically evaluate the

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evidence for and against the leading mechanistic hypotheses, and to highlight the major questions that remain unanswered.

Receptors for cytokines and growth factors comprise two or more single-pass transmembrane proteins that span the plasma membrane (Figure 1a). Each receptor component contains an extracellular portion that engages the cytokine or growth factor ligand, a single transmembrane-spanning domain, and a cytoplasmic portion that upon receptor activation engages with intracellular signaling molecules to initiate the biological response (Stroud and Wells 2004; Whitty and Riera 2008; Posner and Laporte 2010). In the simplest case receptors contain two identical receptor chains, as for example in the receptors for epidermal growth factor (EGF) (Ogiso, Ishitani et al. 2002), human growth hormone (hGH) (Cunningham, Ultsch et al. 1991) and erythropoietin (EPO) (Syed, Reid et al. 1998). Other receptors involve more complex combinations of components, such as $\alpha\beta$, α_3 , $\alpha\beta\gamma$, $\alpha_2\beta_2$, or $\alpha_2\beta_2\gamma_2$ (Figure 1b) (Stahl and Yancopoulos 1993; Heldin 1995). The variety of stoichiometric compositions displayed by this large and diverse family of receptors is further complicated by the fact that, in some cases, the binding of two separate molecules of the cytokine or growth factor is required to convert the receptor to an activated state, as illustrated in Figure 1b. So, for example, the activated form of the human growth factor receptor can be represented as $L\alpha_2$, where L represents the bound ligand, whereas for the EGF receptor the activated complex has the composition $L_2\alpha_2$.

Although the historical distinction between cytokines and growth factors was based on the biological roles of the earliest discovered examples, there are also characteristic structural differences in their receptors. Growth factor receptors typically have a kinase domain as part of their cytoplasmic structure (Posner and Laporte 2010), conferring upon them the ability to directly phosphorylate intracellular proteins to initiate signaling (Figure 1a). These receptor kinase domains can be specific for substrate sites in which phosphorylation occurs on a tyrosine residue (receptor tyrosine kinases or RTKs) (Schlessinger and Ullrich 1992; Lemmon and Schlessinger 2010), or alternatively on either a serine or a threonine residue (receptor serine/threonine kinases) (Graham and Peng 2006; Sieber, Kopf et al. 2009; Massague 2012). In contrast, cytokine receptors do not contain a kinase domain as part of their structure. Instead, the cytoplasmic portion of the receptor is noncovalently associated with a cytoplasmic signaling kinase, such as a member of the Janus kinase family that comprises JAK1, JAK2, JAK3 and TYK2 (Figure 1a) (Ghoreschi, Laurence et al. 2009; Harrison 2012; Stark and Darnell 2012).

Receptors for growth factors and cytokines involve at least two polypeptide components of the kind described above – that is components that either possess intrinsic kinase activity in their cytoplasmic domains or are noncovalently associated with cytoplasmic signaling kinases. However, in addition to these kinase-bearing components, other kinds of molecules can be involved in forming the activated signaling complex. In some cases, these additional components are also transmembrane proteins, but with minimal cytoplasmic structure that does not participate in interactions with intracellular proteins. For example, the receptor for the cytokine interleukin-2 (IL-2) comprises three polypeptide components, IL-2R α , IL-2R β and the common gamma chain γ_c (Malek and Castro 2010). Both IL-2R β and γ_c have extensive cytoplasmic structure, and associate with the signaling kinases JAK1 and JAK3. The IL-2R α chain, in contrast, has a minimal cytoplasmic domain containing only 13 amino acids, which does not directly participate in signaling. Thus, only IL-2R β and γ_c are absolutely required for signaling (Malek and Castro 2010). The role of the alpha chain is to further stabilize the complex and so increase the binding affinity for IL-2, thereby conferring greater IL-2 sensitivity on cells expressing all three receptor components compared to cells expressing IL-2R β and γ_c only (Saito and Honjo 1990; Roessler, Grant et al. 1994; Rao, Driver et al. 2004; Malek and Castro 2010). A particularly instructive example in this regard

is the IL-6 receptor, which becomes activated when two IL-6 molecules engage two molecules of IL-6R α and two molecules of gp130 to form a 2:2:2 complex (Boulanger, Chow et al. 2003). Like IL-2R α , IL-6R α has a relatively small cytoplasmic structure (Mihara, Hashizume et al. 2012). Moreover, cells that express gp130 but not IL-6R α can respond to IL-6 if a soluble form of the IL-6R α extracellular domain is also present (Tamura, Udagawa et al. 1993). This result shows that IL-6 signaling is principally driven by the gp130 components of the receptor, and that the role of the IL-6R α chain is to mediate the interaction of IL-6 with gp130 in order to stabilize the activated signaling complex, a function for which IL-6R α need not be directly tethered to the cell membrane.

In other cases, in addition to two or more full-length signaling components a receptor can contain additional polypeptide chains that are not transmembrane proteins, but instead are linked to the outer leaflet of the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. An example is the receptor tyrosine kinase RET, which is activated by members of the GDNF family of growth factors but also requires the participation of one of four additional membrane-associated components, the GPI-linked co-receptors GFR α 1-4 (Airaksinen and Saarma 2002). Each of the four GFR α proteins is more or less specific for one of the four GDNF-family growth factors that can activate RET (Airaksinen, Titievsky et al. 1999). The activated receptor complex comprises two molecules of RET plus one molecule of growth factor plus two molecules of the GFR α that is specific for that growth factor (Jing, Wen et al. 1996). Analogous to the situation with IL-6R α , cells that express RET but not any GFR α can be made responsive to GDNF family growth factors by inclusion of soluble forms of the appropriate GFR α protein (Jing, Wen et al. 1996). Thus, in the RET system the function of the growth factor in combination with the GFR α proteins is principally to bring about the appropriate interaction between RET molecules (Freche, Guillaumot et al. 2005; Schlee, Carmillo et al. 2006).

There are also examples of receptors in which non-protein co-factors are required for full activation to occur. For example, RET requires Ca²⁺ to productively engage its GDNF family growth factor ligands and GFR α co-receptors (Nozaki, Asai et al. 1998). Similarly, Zn²⁺ is required for the high affinity binding of hGH by the human prolactin receptor (Cunningham, Bass et al. 1990). Other growth factors require participation of noncovalently bound carbohydrate molecules to activate their receptor. For example, members of the fibroblast growth factor (FGF) family require heparan sulfate proteoglycan to interact productively with their receptors (Nakamura, Uehara et al. 2011).

The general picture, therefore, is that receptor signaling requires the involvement of at least two full-length transmembrane receptor components that have kinase activity associated with their cytoplasmic domains (Stroud and Wells 2004). But formation of the activation receptor complex can also involve other proteins that do not have intrinsic signaling activities, as well as non-protein co-factors, providing additional ways to regulate activation of the receptor by making it contingent on the presence and concentration of these other molecules. There is some literature to suggest that GPI-linked proteins, despite their lack of cytoplasmic structure, can participate in signaling through clustering in membrane microdomains known as “lipid rafts” (Maeda and Kinoshita 2011), but in general at least two full-length, kinase-associated receptor proteins are required. It should be noted that some receptors require more than two full-length components to achieve signaling. For example, receptors for growth factors from the TGF β family require two alpha and two beta receptor chains for signaling, all four of which are receptor serine/threonine kinases (Massague 2012).

Ligand-Receptor Cross-Reactivity

In the early days of cytokine receptor research it was assumed that each cytokine or growth factor acted through its own specific receptor. However, it was soon discovered that some receptors could be activated by multiple ligands from a given family and, conversely, that some ligands interact with multiple distinct but structurally related receptors. Over the course of the 1990s it became clear that a degree of cross-reactivity between ligands and receptors is in fact the norm rather than the exception (Stahl and Yancopoulos 1993; Heldin 1995). This cross-reactivity takes several forms. In some cases a set of structurally related ligands interact with exactly the same receptor. For example, the Type I interferon receptor, comprising the two chains IFNAR1 and IFNAR2, can be activated by at least 18 different Type I interferon ligands (Pestka, Krause et al. 2004), resulting in complexes that have very similar three-dimensional structures (Thomas, Moraga et al. 2011). In other cases, only one receptor component is common to the different ligands (Stahl and Yancopoulos 1993; Boulanger and Garcia 2004). For example, the cytokines IL-2, -4, -7, -9, -15 and -21 all act via receptors that include the common gamma chain, γ_c , with additional receptor components being cytokine-specific (Alves, Arosa et al. 2007). Other receptor families that use a shared component include the receptors for IL-3, IL-5 and GM-CSF, which use the common beta chain, β_c (Lopez, Hercus et al. 2010), the receptors for the ten cytokines of the IL-6/IL-12 family which use gp130 (Wang, Lupardus et al. 2009), and the four growth factors of the GDNF family that signal through RET (Airaksinen and Saarma 2002), as well as very many cases where a receptor chain is shared among two or three receptors (Stahl and Yancopoulos 1993; Heldin 1995).

One illustrative example of the complexity of ligand-receptor cross-reactivity is provided by the ErbB family of receptors (Figure 2) (Singh and Harris 2005; Ferguson 2008). This family comprises four receptor chains that interact in different combinations to form a set of homo- or heterodimeric receptors that collectively respond to a set of 11 growth factors from the EGF family (Schneider and Wolf 2009). Further complicating matters, ErbB2 is incapable of binding ligands, and can therefore function only by forming heterodimers with ligand-bound forms of the other ErbB family members (Klapper, Glathe et al. 1999; Kochupurakkal, Harari et al. 2005; Baselga and Swain 2009). ErbB3, on the other hand, can bind some ligands, but lacks an active kinase domain and so again can function only through formation of heterodimeric complexes with other ErbBs (Guy, Platko et al. 1994; Baselga and Swain 2009). Thus, within a small family of structurally homologous receptor chains, we see a diversity of homodimeric and heterodimeric activated receptor complexes that allow a limited set of receptor components to mediate the distinct biological functions of a much larger set of ligands. Other examples of receptor families that involve extensive ligand-receptor cross-reactivity include the PDGF/PDGF-R system (Li, Blumenthal et al. 2011), the TGF β receptor superfamily (Piek, Heldin et al. 1999), and the TNF receptor superfamily (Bodmer, Schneider et al. 2002).

The Structure of the Ligand-Receptor Complex

The prototypical cytokine/receptor complex is illustrated by the co-crystal structure of hGH with two copies of the extracellular domain of its receptor, hGH-R (Cunningham, Ultsch et al. 1991). Figure 3a shows that in this complex hGH, which has a four-helix bundle fold, is cupped between the two receptor molecules as if between two opposing hands touching at the wrist. The extracellular structure of hGH-R comprises two fibronectin type III (FNIII)-like domains, and the hormone binds at the inter-domain junction, making contact with both FNIII-like domains of each receptor protein. Two additional important observations were derived from this structure. First, hGH is an asymmetric molecule, and presents two distinct surface sites (called Site 1 and Site 2) to equivalent regions on the two identical receptor molecules. Thus, the receptor is ambiphilic in its binding properties, being able to bind to

either Site 1 or Site 2 on hGH (though with rather different affinities (Cunningham, Ultsch et al. 1991)) despite the lack of structural similarity between these two regions on the ligand. Second, the two receptor molecules make direct contact with each other in the complex, via their membrane-proximal FNIII-like domains. Structures of other cytokine receptors with extracellular domains comprising multiple FNIII-like domains have generally shown a similar topology (e.g. Fig 3b,c), though with other receptor components present in some cases, and with variations in the extent of direct receptor-receptor contact in the complex (Stroud and Wells 2004).

Important variations on the above theme include the following systems for which complex structures have also been obtained. The receptor for IL-2 comprises the IL-2R α , IL-2R β and common gamma (γ_c) receptor chains. Fig 3c shows that, in the activated receptor complex, IL-2, IL-2R β and γ_c combine to form a complex with hGH/hGHR-like topology, with IL-2R α (shown in magenta) making an additional contact to a third, distinct binding site on the ligand (Stauber, Debler et al. 2006). In another variation, the IL-6 receptor comprises two chains, IL-6R α and gp130, that combine with the ligand to form an hGH/hGHR-like complex. However, additional domains of gp130 extend to bind to a separate site on the IL-6 component of a second identical ternary complex, while receiving an equivalent interaction from Domain 1 on the gp130 from the other trimer. The result, shown in Fig 3d, is a hexameric complex containing two copies each of IL-6, IL-6R α and gp130 (Boulanger, Chow et al. 2003). Figure 3e shows a receptor complex from a different structural family, the Transforming Growth Factor- β (TGF β) superfamily of ligands and receptors. TGF β family ligands are covalent homodimers which, by virtue of this two-fold symmetry, present two identical faces that each contain binding sites for both a Type I receptor and a Type II receptor. In the example in Figure 3e, which shows TGF β 1 in complex with two copies each of TGF β R1 and TGF β R2, the two receptors make substantial contact with each other as well as with the ligand. In some other TGF β family complexes the receptors do not make direct contact with each other, such that – at least with respect to extracellular portions of the receptor – complex formation is driven solely by the four receptor molecules interacting separately with the ligand (Radaev, Zou et al. 2010). Considering receptors from the structurally distinct Epidermal Growth Factor (EGF) family illustrates yet another variation on the theme. Figure 3f shows that EGF forms a 2:2 complex with EGFR1, in which a pair of EGF/EGFR complexes associate in a back-to-back orientation such that each EGF makes contact with only a single receptor chain (Ogiso, Ishitani et al. 2002; Ferguson 2008). Another topological variation is illustrated by cytokines of the Tumor Necrosis Factor (TNF) superfamily, which are trimers that contain three (typically identical) receptor binding sites. Figure 3g shows the complex of TNF β (also known as Lymphotoxin- α) with the p55 TNF receptor, showing a 3:1 stoichiometry in which a receptor molecule binds at each of the three subunit interfaces in the ligand to form a complex with three-fold overall symmetry (Banner, D'Arcy et al. 1993). As a final example of the myriad ways in which cytokines and growth factor ligands can interact with their receptors, Figure 3h shows Fibroblast Growth Factor-2 (FGF-2) in complex with its receptor, in which the ligand mediates the interaction between two identical receptor chains, but with the assistance of a heparin sulfate cofactor that makes contact with both ligand and receptor to stabilize the complex (Pellegrini, Burke et al. 2000). As the above examples illustrate, the general picture is of one or two ligand molecules that induce a stabilized complex containing two or more receptor proteins with a defined mutual orientation, but with this end being achieved in a wide variety of different ways (Stroud and Wells 2004). The different stoichiometric compositions used by different receptors presumably have functional consequences for how the concentration of stimulating ligand couples to the level of activated receptor on the cell, and also for how the system will respond to variations in expression levels of the different receptor components (Hendriks, Opresko et al. 2003; Schlee, Carmillo et al. 2006; Levin, Harari et al. 2011). The relationship between receptor composition, the steps involved in the

receptor activation mechanism, and functional properties such as receptor sensitivity, dynamic range, and the slope of the ligand dose-response relationship, remain largely unexplored, however (Schlee, Carmillo et al. 2006).

The above discussion addresses only interactions involving the receptor extracellular domains. However, there is ample evidence that the transmembrane and cytoplasmic portions of receptor proteins can also directly participate in receptor-receptor interactions, and in some cases these contacts appear to be critical to receptor activation. Using NMR methods, direct interactions of the helical TM domains have been observed for ErbB family receptors (Jones, Rigby et al. 1997; Mendrola, Berger et al. 2002; Sharpe, Barber et al. 2002; Sharpe, Barber et al. 2002; Bennasroune, Fickova et al. 2004; Bocharov, Mineev et al. 2008; Mineev, Bocharov et al. 2010). Evidence for TM-TM interactions has also been obtained for the TGF β receptor (Zhu and Sizeland 1999) from functional studies on chimeric receptor constructs transfected into cells, and similar kinds of evidence have demonstrated that TM self-association is critical for EPO-R signaling (Constantinescu, Keren et al. 2001; Kubatzky, Ruan et al. 2001; Ebie and Fleming 2007)(Seubert, Royer et al. 2003; Kubatzky, Liu et al. 2005). Other receptors for which TM-TM interactions have been proposed to occur include FGF-R (Peng, Lin et al. 2009), VEGFR2 (Dosch and Ballmer-Hofer 2010) PDGF-R β (Oates, King et al. 2010) and EphA1 receptor (Artemenko, Egorova et al. 2008). In many other cases it has been proposed that the TM domain interactions are important in ensuring the correct mutual orientation of the receptor components in the activated complex, as discussed in detail below. The extent to which specific TM-TM interactions represent a general feature of the activation of cytokine and growth factor receptors remains unclear, however. To what extent the receptor cytoplasmic structure participates in stabilization of the activated receptor complex is even less studied. An important exception is a study from the Kuriyan group which showed that the EGF-R (ErbB1) kinase domain crystallizes in a form that reveals a direct noncovalent interaction (Zhang, Gureasko et al. 2006). Interestingly, despite the homotypic nature of the interaction, the complex observed crystallographically was asymmetric, with the C-terminal lobe of one EGF-R kinase domain contacting the N-terminal lobe of the other (Figure 4b). This and other evidence led the authors to propose that, in the activated receptor complex on cells, the kinase domain of one EGF-R receptor chain binds to and allosterically activates the kinase domain of the other EGF-R molecule, via an asymmetric interaction closely analogous to how cyclins activate cyclin-dependent kinases.

2. Mechanism of Receptor Activation

Value of Understanding Receptor Activation Mechanisms

It might seem unduly esoteric to worry about exactly how a cytokine or a growth factor activates its receptor. However, there are very practical reasons why it is critical that we understand this phenomenon, not just as an important aspect of our fundamental biological knowledge, but also for direct application to medicine and to drug discovery. Specifically, if we wish to understand how a given level of stimulus (exposure to a given concentration of growth factor for a given time) leads to a particular biological response, a mechanistic understanding of the molecular steps involved, including the affinities and rates of key steps, is essential. The purely empirical characterization of how stimulus inputs correlate with biological outputs, treating the system as a “black box”, is sufficient to define (though not to understand) the behavior of a particular experimental system. But a quantitative, molecular-level understanding is required if we want to be able to extrapolate this knowledge to predict or explain behavior occurring under different conditions, such as in a different cell type or under a particular set of conditions *in vivo* (Whitty, Raskin et al. 1998; Schlee, Carmillo et al. 2006). In addition, it is becoming increasingly recognized that the discovery and development of drugs that target cytokine or growth factor receptors or their ligands can

greatly benefit from a detailed and quantitative knowledge of how the receptor functions and how the pathology in question is coupled to that function (Whitty and Riera 2008). For example, understanding why ligands that induce homotypic receptor complexes - that is complexes that contain two copies of the same receptor component - show a bell-shaped dose-response relationships is important for the rational design of antagonists and “super-agonists” through protein engineering (Fuh, Cunningham et al. 1992; Whitty and Borysenko 1999). Similarly, it has been shown that understanding the activation mechanism of receptors such as IL-4R (Whitty, Raskin et al. 1998) and RET (Schlee, Carmillo et al. 2006) can potentially identify which step in receptor activation is easiest to block with an inhibitor, and which receptor component to target to achieve an inhibitor that is competitive versus noncompetitive with respect to ligand. Our current understanding of the mechanistic details of receptor activation is, in some ways, analogous to the level at which enzymes were understood in the 1960s. The biological functions of the molecules involved are empirically quite well characterized, but in only a few cases do we have detailed insight into the molecular mechanisms involved, and in almost no case do we have a truly quantitative understanding of how function derives from the molecular mechanisms at work (Whitty and Riera 2008). However, as described below, our quantitative and mechanistic understanding of receptor function is advancing rapidly, and promises to bring benefits to biology and medicine comparable to those that resulted from the corresponding advances in enzymology that occurred in previous decades.

Overview of Cytokine/Growth Factor Receptor Activation

If a mutation is introduced into the receptor cytoplasmic domain to inactivate or eliminate the associated kinase function, the result is to ablate the signaling activity of the mutated receptor (Ullrich and Schlessinger 1990). These and other observations, involving a wide variety of different receptor systems, show that the key event in receptor activation is the activation of the kinases associated with the receptor cytoplasmic regions, and the action of these kinases in phosphorylating specific substrate sites on the receptor itself and on cytoplasmic signaling proteins (Lemmon and Schlessinger 2010; Posner and Laporte 2010; Rawlings et al., 2004; Massague 2012). Binding of the cytokine or growth factor to the extracellular portion of the receptor triggers this process by inducing the formation of an activated receptor complex in which the cytoplasmic regions of the signaling chains are brought into the appropriate juxtaposition for this kinase activation to occur (Figure 4a). Generally, the receptor-associated kinases first phosphorylate each other, which converts them to a more active catalytic state (Stroud and Wells 2004; Posner and Laporte 2010). The activated kinases then phosphorylate multiple other sites in the receptor cytoplasmic domains, to create docking sites for the recruitment of cytoplasmic signaling proteins that contain phosphopeptide-specific binding modules such as SH2 and SH3 domains (Pawson and Nash 2003; Brummer, Schmitz-Peiffer et al. 2010).

Some of the signaling proteins that bind to the receptor cytoplasmic region in this phospho-specific manner are themselves substrates for phosphorylation by the receptor-associated kinases. For example, activation of cytokine receptors that engage the JAK/STAT signaling pathway begins with trans co-activation of the JAK kinases associated with the cytoplasmic domains of the main signaling components of the receptor (Rawlings et al., 2004; Stark and Darnell 2012). The activated JAKs then phosphorylate docking sites on the receptor, to which STAT proteins from the cytoplasm can bind. The JAKs phosphorylate the bound STAT proteins, causing them to dimerize upon dissociation from the receptor and migrate as dimers to the nucleus, where they function as transcription factors by binding to promoter sites on DNA to induce the expression of target genes (Rawlings et al., 2004; Stark and Darnell 2012). Other phosphorylation-induced docking sites on receptors serve to recruit additional cytoplasmic kinases to bind to the receptor and thereby expand the range of

downstream signaling molecules that can become activated. For example, Src, Shc1, PKC α and PLC γ are all cytoplasmic kinases that can associate with the intracellular portion of the activated EGF-R through phospho-specific recognition motifs. Other docking sites on receptors can bind adaptor proteins that serve to recruit additional signaling molecules into a multi-protein complex with the receptor, leading to the initiation of other downstream signaling events (Brummer, Schmitz-Peiffer et al. 2010). For example, activation of many RTKs includes phosphorylation of a docking site for the adaptor protein Grb2 (Jang, Zhang et al. 2009). Once bound, Grb2 recruits the guanine nucleotide exchange factor SOS converting it to an active conformation. The activated SOS in turn recruits the GTPase Ras into the complex, and at the same time promotes the dissociation of GDP from Ras and its replacement by GTP from the cytoplasm. The active, GTP-bound form of Ras then binds and activates the signaling kinases Raf and PI3K, to initiate signaling through, respectively, the MAPK and Akt signaling pathways (Rawlings, Rosler et al. 2004). Thus, the phosphorylation of multiple docking sites on the cytoplasmic domain of cytokine and growth factor receptors initiates the assembly of a large multi-protein complex centered on the receptor, triggering a complex cascade of intracellular signaling events (Figure 4a).

Outstanding Questions

Given the detailed knowledge that has been amassed concerning the structure of ligand-receptor complexes, and the phosphorylation, docking and other signaling events that receptor activation brings about on the cytoplasmic side of the membrane, it may come as a surprise to realize how little we know for certain about how the binding of ligand actually brings about receptor activation. In particular, it remains controversial exactly how the binding of the ligand to the extracellular portion of the receptor brings about the necessary juxtaposition between the receptor cytoplasmic domains for signaling to occur. Conflicting evidence exists concerning whether the resting state of the receptor comprises individual, separately diffusing receptor proteins that are brought together into a complex only upon the binding of ligand (Figure 5a), or whether the resting receptor involves the components in a pre-associated complex that is converted to an active state by the binding of ligand (Figure 5b), or indeed whether some receptors function by one of these mechanisms while different receptors employ the other. With few exceptions, some of which are discussed below, it is also unclear whether activation of a common receptor by different ligands merely represent redundant methods to bring the receptor to a common activated state, or alternatively whether different ligands might induce formation of distinct activated states of the receptor with different signaling properties. In addition to these broad uncertainties about fundamental aspects of the activation mechanism, very little is known about quantitative aspects of how ligand binding is coupled to assembly of the activated receptor complex, or how receptor activation is quantitatively coupled to proximal and distal steps in intracellular signaling. Only for a handful of systems have a subset of the key rate and equilibrium constants been estimated and this information used to develop a quantitative picture of how the receptor functions (for selected examples, see (Lauffenburger, Linderman et al. 1987; Whitty, Raskin et al. 1998; DeWitt, Iida et al. 2002; Viswanathan, Benatar et al. 2002; Hendriks, Opresko et al. 2003; Gavutis, Jaks et al. 2006; Jaitin, Roisman et al. 2006; Schlee, Carmillo et al. 2006; Kalie, Jaitin et al. 2008; Kleiman, Maiwald et al. 2011; Thomas, Moraga et al. 2011)).

Ligand-induced oligomerization versus pre-associated receptors

Our evolving understanding of how the binding of a growth factor ligand brings about an activated state of the receptor can largely be traced by considering three well-studied receptors: EGF-R, hGH-R and EPO-R. In 1987 Schlessinger and co-workers observed that purified EGF-R formed reversible, noncovalent dimers when incubated with EGF, and that this dimerization was accompanied by enhanced phosphorylation of the receptor (Yarden

and Schlessinger 1987). This report was followed soon after with evidence that EGF induced EGF-R dimerization on living cells (Cochet, Kashles et al. 1988). These results led the investigators to propose that activation of the receptor occurs when EGF binds to two EGF-R monomers and induces them to dimerize, bringing the receptor cytoplasmic domains together and thereby promoting trans-phosphorylation of the receptor kinase domains to initiate signaling. Shortly thereafter, Wells and co-workers published the co-crystal structure of hGH in complex with the extracellular portion of hGH-R, with supporting biophysical and biochemical data, showing that one molecule of hGH binds two molecules of receptor to form a ternary complex in which the two receptors also make direct contact with each other (Figure 3a) (Cunningham, Ultsch et al. 1991). This study provided the first atomic-level structural picture of the interactions involved in a cytokine/receptor complex. It additionally showed that each receptor molecule uses essentially the same binding site to interact with two quite different surface sites on the hGH ligand, thereby establishing that to stabilize a dimeric receptor involving two identical receptor chains does not necessarily require a symmetrical ligand. Crystal structures of homologous cytokine/receptor complexes, such as EPO/EPO-R (Livnah, Stura et al. 1996; Philo, Aoki et al. 1996), Prolactin/Prl-R (van Agthoven, Zhang et al. 2010), and IL-4/IL-4R α / γ_c (Stauber, Debler et al. 2006) were found to closely resemble that observed for hGH/hGH-R. For the trimeric cytokines of the TNF family, crystal structures of the ligands in complex with soluble forms of their receptor extracellular domains generally show a 3:1 complex (Figure 3g), with a receptor chain bound at each of the three subunit interfaces in the ligand (e.g. (Banner, D'Arcy et al. 1993)), suggesting an analogous mechanism in which binding of a single ligand in this case recruits three receptor molecules into a complex with 3-fold symmetry. Similar structural and biochemical data establishing the formation of ligand-stabilized receptor oligomers have subsequently been reported for multiple other cytokine and growth factor receptors (Stroud and Wells 2004).

Evidence for Ligand-Induced Oligomerization

In addition to the structural evidence discussed above, an accumulation of functional data has been invoked to support the ligand-induced dimerization mechanism illustrated in Figure 5a. Many of these studies used recombinant receptor proteins that were truncated to eliminate the transmembrane and cytoplasmic portions, giving soluble receptor extracellular domains amenable to detailed biophysical and biochemical study. Using soluble receptor proteins of this kind, together with experimental approaches such as size exclusion chromatography, analytical ultracentrifugation and dynamic lightscattering, it has been shown for multiple systems that a stable interaction between soluble receptor extracellular domains is observed only in the presence of the cytokine or growth factor ligand (Cunningham, Ultsch et al. 1991; Ward, Howlett et al. 1994; Horsten, Schmitz-Van de Leur et al. 1995; Wu, Johnson et al. 1995; Philo, Aoki et al.; Treanor, Goodman et al. 1996; Arduini, Strauch et al. 1999; Barton, Hall et al. 2000; McClure, Hercus et al. 2003; Zuniga, Grope et al. 2005).

The validity of the ligand-induced dimerization mechanism was additionally supported by a great number and variety of cell-based measurements. For example, Fuh et al. reported that introducing mutations into hGH to disrupt its "Site 2" binding site (see above) converted the hormone into an antagonist, consistent with the notion that the mutated hormone could bind to hGH-R through the unmodified Site 1, but was unable to recruit a second receptor molecule to form the termolecular activated complex (Figure 6a) (Fuh, Cunningham et al. 1992). These investigators additionally showed that hGH mutants in which Site 2 binding affinity was reduced but not eliminated gave bell-shaped dose-response curves (Figure 6b), indicating that they acted as agonists at low and moderate concentrations but as antagonists when present at high concentrations. This finding was interpreted in terms of the mechanism

shown in Figure 6c, in which a high concentration of ligand forces the receptor into a “dead-end” state in which each hGH-R molecule is bound by a separate hGH molecule through a Site 1 interaction. A bell-shaped dose-response curve has since been demonstrated for wild-type hGH-R (Ilondo, Damholt et al. 1994; Tsunekawa, Wada et al. 1999) as well as for other homotypic receptors that bind a single ligand molecule (Abe, Sasaki et al. 2011; De Meyts, Wallach et al. 1994; Rui, Lebrun et al. 1994; Elliott, Lorenzini et al. 1996; Oberholtzer, Contarini et al. 1996; Schneider, Chaovapong et al. 1997; Tian, Lamb et al. 1998; Schlee, Carmillo et al. 2006), suggesting that this behavior is a general property of such systems (Whitty and Borysenko 1999).

For dimeric receptors involving two dissimilar receptor chains, it has similarly been shown that mutating the region on the ligand that interacts with the second receptor component converts the ligand from an agonist to an antagonist (e.g. IL-4; (Kruse, Tony et al. 1992)); the mutant growth factor can still bind to and occupy receptor chain 1, but cannot recruit receptor chain 2 into a functional complex. Most heterotypic dimeric receptors involve one chain that binds ligand strongly and another that binds ligand alone much more weakly. Therefore, a bell-shaped dose response curve such as was shown for hGH-R, EPO-R, etc. is not expected, unless the ligand is applied at concentrations high enough that even the weaker binding receptor chain becomes independently saturated, which typically does not occur at practically achievable ligand concentrations. However, for the heterotypic receptor for interleukin-4 (IL-4), which comprises the IL-4R α chain and the common γ_c chain, an essentially equivalent observation has been reported. Specifically, it was shown that an antibody that bound to γ_c and blocked its recruitment into the complex acted as a noncompetitive antagonist of IL-4 function on cells, but did not block binding of radiolabeled IL-4 to the cells, whereas an antibody that bound to IL-4R α and blocked the binding of the cytokine was a competitive antagonist of both ligand binding and function (Figure 7a) (Whitty, Raskin et al. 1998). This result is consistent with a mechanism of ligand-induced dimerization, because it shows that recruitment of γ_c into the complex is a separate event from the binding of IL-4 to IL-4R α (Figure 7a), and also shows that in the unbound state γ_c and IL-4R α are far enough apart that binding of a large antibody molecule to the extracellular domain of γ_c does not interfere with the binding of IL-4 to IL-4R α . Such a situation is somewhat difficult (though not impossible) to picture if IL-4R α and γ_c are intimately associated in a pre-formed complex before IL-4 binds, whereas the results are both qualitatively and quantitatively accounted for if a mechanism of ligand-induced dimerization is assumed (Figure 7b) (Whitty, Raskin et al. 1998).

Some of the most compelling evidence for ligand-induced dimerization derived from the great variety of ways in which homotypic receptors can be activated by dimerizing them through artificial means (Ballinger and Wells 1998). The EPO receptor provides the best example of this argument (Watowich 1999). Quite early on it was shown that a number of monoclonal antibodies (mAb) that recognize the extracellular domain of EPO-R can act as agonists in cellular assays (Elliott, Lorenzini et al. 1996; Schneider, Chaovapong et al. 1997). Although only a minority of anti-EPO-R antibodies possessed agonist activity, the fact that a number of them did is consistent with the idea that bringing two receptor molecules together on the membrane is a key event in activation. Moreover, the agonist mAbs showed a bell-shaped dose response indicative of a mechanism in which each EPO-R chain can become bound by a separate antibody molecule at high antibody concentrations to give a non-signaling state, showing that receptor activation requires two EPO-R molecules to be cross-linked by a single mAb. Given the very different structures and sizes of a 150 kDa. antibody versus the 21 kDa. EPO molecule, it would appear highly unlikely that an agonist mAb can bring two EPO-R molecules together in a similar orientation to that observed in the co-crystal structure of EPO-R with EPO (Figure 8a) (Wilson and Jolliffe 1999). Thus, the existence of agonist mAbs suggested that receptor activation involves

bringing two EPO-R molecules together into close proximity, but argues against a requirement for any very specific interaction geometry for the extracellular portions of the receptor. Agonist mAbs have also been reported for many other cytokine and growth factor receptors, establishing this as a general phenomenon (Engelmann, Holtmann et al. 1990; Shalaby, Sundan et al. 1990; Rui, Lebrun et al. 1994; LeSauter, Maliartchouk et al. 1996; Deng, Banu et al. 1998; Prat, Crepaldi et al. 1998; Zhou, Wang et al. 1999; Mori, Thomas et al. 2004; Motegi, Fujimoto et al. 2004; Pietronave, Forte et al. 2010; Kowalczyk, Dunkel et al. 2011).

For EPO-R in particular, it has been shown that the receptor can also be activated in a multitude of other ways, to form complexes that have a range of geometries. These include the following:

- i. As mentioned above, it is possible to convert a cytokine such as EPO, hGH or IL-4 into an antagonist by mutating residues that interact with the second receptor chain (Site 2), while leaving the regions of the ligand that interact with the first receptor chain (Site 1) unchanged. Intriguingly, for EPO and for several other cytokines it has been shown that covalently coupling two such antagonist mutant ligand molecules together results in an agonist (Qiu, Belanger et al. 1998; Langenheim, Tan et al. 2006). This result implies that each of the two antagonist molecules in the engineered ligand dimer can engage a separate receptor chain through their unmodified Site 1 binding sites, and that doing so brings the two receptor chains into a mutual orientation that results in activation (Figure 6d). Importantly, occupancy of EPO-R molecules by separate EPO molecules, each binding through Site 1, does not lead to receptor activation if the EPO molecules are not covalently tethered (Abe, Sasaki et al., 2011). Clearly, the separation and relative orientation of the two receptor extracellular domains must be quite different in the complex with the covalently-linked mutant EPO dimer compared to the activated complex formed upon binding of a single, wild-type cytokine molecule. The crystal structure of EPO bound to EPOR shows that there is not room to bind two covalently-connected EPO molecules, each interacting with the receptor through its Site 1, without substantial reorientation of the EPO-R extracellular domains (Figure 8a). Furthermore, studies with these dimerized EPO variants have shown that their agonistic activity is rather insensitive to the length of the linker tethering the two mutant cytokine molecules together (Qiu, Belanger et al. 1998), implying that a considerable range of relative distances and mutual orientations of the EPO-R extracellular domains are compatible with receptor activation.
- ii. Chimeric receptors can be constructed, comprising the transmembrane and cytoplasmic domains of EPO-R fused to the extracellular domain of a different receptor, that when transfected into cells can be activated by adding the ligand to the different receptor. In the case of EPO-R, active chimeric receptors have been demonstrated using extracellular domains from homologous cytokine receptors such as Prl-R (Dusanter-Fourt, Muller et al. 1994) or the GM-CSF receptor alpha chain (Shikama, Barber et al. 1996; Pless, Norga et al. 1997), or even receptors from a quite different structural class, such as EGF-R or cKit (Ohashi, Maruyama et al. 1994). This result again implies that, in many cases at least, receptor activation is not finely sensitive to the specific mutual orientation of the extracellular domains brought about by the activating ligand.
- iii. Similarly, in a number of systems all or part of the receptor extracellular domain has been replaced by the antigen binding fragment of an antibody. When the receptor/antibody chimera is transfected into cells, receptor activation can be induced by adding a multivalent form of the cognate antigen for the antibody

(Kawahara, Ueda et al. 2002; Kawahara, Ueda et al. 2007; Sogo, Kawahara et al. 2009; Kawahara, Chen et al. 2011; Kaneko, Kawahara et al. 2012).

- iv. Several mutations have been reported, involving introduction of a cysteine residue into the juxtamembrane region of the EPO-R extracellular domain, that result in constitutive activation of the receptor through formation of a disulfide-linked EPO-R dimer (Watowich, Yoshimura et al. 1992; Watowich, Hilton et al. 1994; Kubatzky, Liu et al. 2005; Lu, Gross et al. 2006). Not all of the disulfide-linked receptor dimers reported in these studies resulted in constitutive activation, however, showing that receptor dimerization is necessary but not sufficient for activity in these systems (see below). Constitutive activation by the introduction of transmembrane region cysteine residues has also been reported for other receptors such as fibroblast growth factor receptor-3 (Adar, Monsonego-Ornan et al. 2002).
- v. Seubert et al. showed that a constitutively activated EPO-R could be generated if the receptor extracellular domain was replaced with the leucine zipper coiled-coil motif from the Put3 transcription factor of *S. cerevisiae*, which enforces dimerization (Seubert, Royer et al. 2003). Not all such constructs were active, however, depending on the relative angular orientation of the EPO-R cytoplasmic domains, as discussed more fully below.
- vi. A number of peptides discovered by phage display - some as small as 13 or 14 amino acids - were shown to bind to and activate EPO-R on cells (Wrighton, Farrell et al. 1996; Johnson, Farrell et al. 1998). A co-crystal structure of one such EPO-mimetic peptide (EMP), called EMP1, in complex with the EPO-R extracellular domain (Livnah, Stura et al. 1996) showed a 2:2 complex in which two EMP1 molecules make contact with each other and thereby indirectly mediate contact between the two EPO-R molecules (Figure 8c). A subsequent study showed that covalently dimerizing EMP greatly increased potency (Johnson, Farrell et al. 1997), and dimeric EMPs with a variety of structures and geometries have since been reported (Vadas, Hartley et al. 2008; Sathyanarayana, Houde et al. 2009; Kessler, Greindl et al. 2011). Interestingly, the complex of EMP1 with EPO-R showed a dihedral angle of $\sim 180^\circ$ between the two EPO-R molecules, quite different from the $\sim 120^\circ$ angle seen for EPO-R bound to EPO itself (Figure 8a), indicating once again that more than one mutual orientation between the EPO-R extracellular domains can result in activation of the receptor. Other peptides that stabilize an EPOR dimer are not activating, however, as discussed below. Cytokine mimetic peptides with agonist activity have also been reported for several other receptors including the thrombopoietin receptor TPO-R (Cwirla, Balasubramanian et al. 1997; Krause, Schmoldt et al. 2007), the IL-5 receptor (England et al., 2000), and the neurotrophin receptors TrkA (Pollack and Harper 2002; Zaccaro, Lee et al. 2005; Colangelo, Bianco et al. 2008) and TrkC (Zaccaro, Lee et al. 2005; Chen, Brahimi et al. 2009) (for a review of peptide-based neurotrophin agonists, see (Skaper 2008)).
- vii. A number of reports describe small molecule (i.e. synthetic organic) agonists for cytokine and growth factor receptors (Tarasova, Haylock et al.; Whitty and Borysenko 1999; Boger and Goldberg 2001). An early example was an agonist of the receptor for Granulocyte Colony-Stimulating Factor (G-CSF). In 1998 Tian et al. reported the small molecule G-CSF-R agonist SB247464 (Fig 9a), which was discovered by screening a compound library against a cell-based assay measuring the activation of a reporter gene downstream of the G-CSF-R (Tian, Lamb et al. 1998). This compound, which has C2 symmetry, shows a bell-shaped dose-response curve in the reporter gene assay, consistent with the notion that the two symmetrically-related halves of SB247464 each bind one G-CSF-R molecule to

stabilize a receptor dimer (Whitty and Borysenko 1999). Perhaps not surprisingly given the small size of the compound, SB247464 appears to bind the receptor not at the G-CSF binding site, but at a location in the membrane-proximal region of G-CSF-R (Doyle, Tian et al. 2003) where, by analogy to the homologous receptors hGH-R and EPO-R, it is expected that the receptor chains come close together in the activated complex. For EPO-R itself, it has been shown that appending a weak-binding small molecule ligand to a multivalent scaffold gave a molecule with agonist activity (Qureshi, Kim et al. 1999; Goldberg, Jin et al. 2002), consistent with the idea that ligand-induced clustering of EPO-R brings about activation. Multiple small molecule agonists have additionally been reported for TPO-R (Kimura, Kaburaki et al. 1998; Jang, Okada et al. 2007), including the FDA-approved drug eltrombopag (Cheng, Saleh et al. 2011), as well as compounds that activate the neurotrophin receptors TrkA (Kimura, Kaburaki et al. 1998; Bruno, Clarke et al. 2004; Jang, Okada et al. 2007), TrkB (Jang, Liu et al. 2010; Massa, Yang et al. 2010) and TrkC (Chen, Brahimi et al. 2009) (Figure 9) (for a review of small molecule neurotrophin receptor agonists, see (Skaper 2011)). Small molecule agonists have also been reported for the p75 subunit of the Nerve Growth Factor receptor (Pehar, Cassina et al. 2006).

The diverse ways in which EPO-R and other receptors can be dimerized to bring about receptor activation conclusively establishes that a surprisingly wide range of relative orientations in the extracellular domain of these receptors is compatible with activity. In particular, the observations that monoclonal antibodies and also covalently dimerized forms of antagonistic cytokine variants can bring the receptor into an active state strongly suggests that quite different geometries of the extracellular portions of the receptor can lead to activation of signaling inside the cell.

Importantly, not every means of dimerizing EPO-R results in functional activation of the receptor (Ballinger and Wells 1998). Only four out of a panel of 96 antibodies against the EPO-R extracellular domain were found to have agonistic activity (Schneider, Chaovapong et al. 1997). Presumably all or most of these bivalent mAbs were capable of simultaneously binding two molecules of EPO-R, so this result implies that many antibody-induced EPO-R dimers were inactive. Similarly low percentages of active agonists have also been reported among mAbs raised against the extracellular domains of other cytokine and growth factor receptors (Rowlinson, Behncken et al. 1998; Kai, Motoki et al. 2008). Structural insight into the geometric requirements for activation was provided by the discovery of a phage-derived peptide, EMP33, that binds to and dimerizes EPO-R on cells but does not have agonistic activity (Livnah, Johnson et al. 1998). By occupying the receptor in an inactive state this peptide functioned as an antagonist with respect to EPO or the agonist EMP1. Surprisingly, a crystal structure of EMP33 in complex with the EPO-R extracellular domain (Figure 8b) showed a complex that was grossly similar to that seen for EPO-R with EPO itself or with the agonist peptide EMP1 (Figure 8a, c). As was pointed out at the time (Ballinger and Wells 1998), a puzzling feature of the relationship of structure to activity in these complexes is that the relative orientation of the EPO-R molecules brought about by binding of the antagonist EMP33, with a dihedral angle of $\sim 165^\circ$, is intermediate between the angles seen for the functional complexes of EPO-R with EPO itself (120°) and with the agonist peptide EMP1 (180°) (Figure 8). Assuming these crystal structures accurately reflect the geometries of the complexes that each ligand forms with full-length EPO-R on cells, it is unclear why bringing two EPO-R molecules together into a dimer with the larger and smaller of these angles should both result in activation of the receptor, while formation of a complex with the intermediate angle does not. Similarly, mutation of the transmembrane and juxtamembrane regions of EPO-R show that certain angular displacements between the receptor cytoplasmic domains allow activation while others do not, as discussed in detail below. Thus, although

EPO-R clearly can tolerate a wide range of orientations between the receptor extracellular domains in the activated receptor complex, there are certain quite specific – and as yet not fully understood – geometric requirements that must additionally be met.

A key difficulty in directly comparing results from studies that describe different ways to activate EPO-R or other receptors is that experiments that use different engineered forms of the receptor protein or different cellular contexts can give distinct and sometimes conflicting results. For example, eight out of 14 monoclonal antibodies raised against the extracellular domain of hGH-R were able to activate a chimeric protein containing the extracellular domain of hGH-R fused to the transmembrane and intracellular domains of GCSF-R, but none activated full length hGH-R on FDC-P1 cells (though two of the eight showed weak agonist activity against full-length hGH-R on BaF-B03 cells) (Rowlinson, Behncken et al. 1998). In another instructive example, the four monoclonal antibodies that were reported by Elliott et al., to elicit EPO-R activation showed much lower efficacy than did EPO itself in a functional assay measuring erythroid colony stimulation in primary cells (Elliott, Lorenzini et al. 1996), suggesting that the agonist antibodies induce an activated state of the receptor that is different from that induced by the natural hormone. Furthermore only one of the antibodies promoted cell differentiation, indicating that even among the antibodies different examples activate EPO-R in distinct ways. Similarly, activation of EPOR by the peptide mimetic EMP1 did not recapitulate the full efficacy of EPO, even after covalent dimerization (Wrighton, Farrell et al. 1996; Johnson, Farrell et al. 1997). In the same vein, activation of EPO-R by the Friend virus gp55 protein results in a different JAK/STAT phosphorylation pattern to that seen with EPO (Ballinger and Wells 1998), and the dimerization of EPO-R cytoplasmic domains fused to the Put3 coiled-coil domain supported cell survival and MAPK signaling but not cell proliferation or STAT activation (Seubert, Royer et al. 2003). Drawing conclusions about requirements for receptor activation from data on dimerization of EPO-R or other receptors by different means thus becomes very complicated when the amplitude and the quality of the functional response is considered, given that activity can take so many different forms and the ability of a particular molecule to achieve activity can depend on what read-out is being measured and in what cellular context.

Lodish and co-workers have extensively explored how the geometry of the EPO/EPO-R complex relates to its signaling and functional properties. For example, they measured the activity of mutated forms of EPO-R containing 1, 2, 3 or 4 alanine residues inserted into an alpha-helical segment of the juxtamembrane cytoplasmic domain, resulting in rotation of the receptor cytoplasmic domains relative to one another by about 100° per inserted alanine (Constantinescu, Huang et al. 2001). When cells expressing the different receptor variants were stimulated with EPO, wild type EPO-R and the 3xAla mutant elicited similar levels of cell proliferation, which were much higher than the levels achieved by the 1xAla, 2xAla or 4xAla mutants. Interestingly, the 1xAla mutant was able trigger proliferation when exposed to much higher ligand concentrations, even though its affinity for binding EPO was no different from that of wild type EPO-R and the 3xAla mutant. Moreover, the level of JAK2 phosphorylation upon stimulation with EPO was higher for the less active 1xAla mutant than for the wild type or the 3xAla mutant, but EPO-R phosphorylation was undetectable for the former and almost the same for wild type and 3xAla. In another study they constructed EPO-R mutants in which the extracellular domain was replaced with the leucine zipper coiled coil from the Put3 transcription factor of *S. cerevisiae*, which forced dimerization and was predicted to impose a coiled coil conformation on the downstream TM domain. Further deletion of 1–6 residues from the TM helix rotated the receptors relative to one another to give seven different relative orientations within the dimer. Mutants with three- and six-amino acid deletions were active in the absence of ligand. Interestingly, these mutants were predicted to have similar conformations, rotated only by about 45° relative to each other,

suggesting that achieving approximately the correct rotational orientation is the main requirement for receptor activation in this system (Seubert, Royer et al. 2003). The above results highlight that both the extent and the nature of EPO-R signaling are critically dependent on the mutual orientation of the receptor cytoplasmic domains. Similar results have also been observed for hGH-R. For example, Brown et al. rotated the cytoplasmic domain of hGH-R by inserting up to four alanine residues into the C-terminal α -helical TM sequence. Constitutive activation was achieved only with the four-alanine insertion mutation, which was predicted to rotate the signaling domain about 40° clockwise past the initial position. Ligand-independent activation was also observed upon a single alanine insertion just above the JAK2-binding “Box 1” sequence (Brown, Adams et al. 2005).

Evidence for a Pre-associated Receptor Mechanism

For many years it was generally assumed that essentially all growth factor and cytokine receptors were activated through ligand-induced oligomerization, based on evidence such as that described above, but encompassing also other well-studied receptor systems (Stahl and Yancopoulos 1993; Heldin and Ostman 1996). However, in the late 1990s evidence began to emerge for an alternative activation mechanism, in which receptors exist as preassembled dimers or oligomers even before ligand binds. In this alternative mechanism, the ligand activates the receptor by inducing a conformational reorganization within this pre-associated receptor complex that brings the receptor cytoplasmic domains into the required mutual orientation (Figure 5b). Evidence supporting this mechanism came from both structural and functional studies. The literature on EPO-R and EGF-R again provides a good illustration of the evolution of these ideas.

In 1999 Livnah et al. reported the X-ray crystal structure of the extracellular domain of EPO-R in the absence of ligand (Livnah, Stura et al. 1999). In this structure, the EPO-R molecules are arranged in pairs, with each pair held together by a relatively extensive contact interface encompassing many of the same regions that are involved in binding to EPO itself (Figure 8d). This structure addressed a major gap in our knowledge, in that to gain structural insight into the mechanism of receptor activation it is necessary to consider the structures of both the unligated and bound states of the receptor. However, interpreting X-ray crystal structures of unligated receptors raises the problem of how to distinguish which crystallographically-observed contacts reflect interactions that occur in the native, full-length receptor on cells, and which result from characterizing an artificial receptor construct packed at high density into a crystal. In this particular case the structural report was accompanied by a second paper providing strong functional evidence that EPO-R can pre-associate on cells ((Remy, Wilson et al. 1999); see discussion below), and so the authors felt justified in proposing that the EPO-R homodimer seen in the crystal structure might represent an inactive but pre-associated form of EPO-R that could exist on cells. One notable problem with the interpretation of this structure is that, as the authors point out, the dimer interface seen in the crystal occludes the binding site for the ligand (Figure 8d), making it difficult to conceive of a compelling mechanism for how EPO might bind to a pre-associated receptor dimer with this structure to convert it to an activated state.

Some of the most compelling functional data to support a pre-associated receptor mechanism also involves the EPO receptor. As a companion to the crystallographic paper described above, Remy et al. reported an elegant set of experiments in which complementary fragments of the enzyme dihydrofolate reductase (DHFR) were fused to the cytoplasmic portion of various EPO-R constructs resulting, indirectly, in a fluorescent read-out when two receptor cytoplasmic domains came into close proximity. Only if two complementary DHFR fragments came together to reconstitute the active enzyme would a signal result (Remy, Wilson et al. 1999). The authors showed that EPO-R/DHFR-fragment fusions containing a short linker showed ligand-dependent induction of DHFR

complementation, but that constructs containing a longer linker gave a dimerization signal even in the absence of EPO or the peptide agonist EMP1. The authors concluded that EPO-R exists as a dimer in the absence of EPO, but that in the resting state the receptor cytoplasmic domains are held apart, and that binding of the cytokine induces a reorganization of the pre-associated receptor dimer to bring the cytoplasmic domains into close contact, thereby initiating signaling (Figure 5b).

Many studies have shown that interactions involving receptor TM domains play an important role in regulating receptor activation, and such studies have been interpreted to support a pre-associated receptor mechanism. For example, deletion of a five-residue KWQFP motif in the juxtamembrane region of TPO-R results in a constitutively active receptor (Staerk, Lacout et al. 2006). This result led the authors to propose that the receptor exists as a constitutive dimer in which the KWQFP motif serves to maintain the receptor in an inactive geometry, such that deletion of this motif causes spontaneous, ligand-independent activation. In other receptors, too, the TM or juxtamembrane regions have been shown to be critical in governing the productive orientation of the receptor cytoplasmic domains (Constantinescu, Huang et al. 2001; Kubatzky, Ruan et al. 2001; Seubert, Royer et al. 2003; Kubatzky, Liu et al. 2005; Lu, Gross et al. 2006; Dosch and Ballmer-Hofer 2010), or in mediating receptor self-association (Jones, Rigby et al. 1997; Zhu and Sizeland 1999; Constantinescu, Keren et al. 2001; Kubatzky, Ruan et al. 2001; Mendrola, Berger et al. 2002; Sharpe, Barber et al. 2002; Sharpe, Barber et al. 2002; Bennisroune, Fickova et al. 2004; Ebie and Fleming 2007; Artemenko, Egorova et al. 2008; Bocharov, Mineev et al. 2008; Peng, Lin et al. 2009; Mineev, Bocharov et al. 2010).

The numerous and compelling studies showing that the rotational angle between receptor cytoplasmic domains is important in EPO-R and other Class I cytokine receptors, some of which are described above, led to a more detailed model for how binding of ligand might bring about receptor activation within a preformed receptor dimer (Constantinescu, Huang et al. 2001). In this model, the TM and cytoplasmic domains of the two receptor chains are in close contact in the unligated, inactive state of the receptor, while the extracellular domains are somewhat separated. The cytokine binds initially to one receptor chain, and then by recruiting the extracellular domain of the second receptor chain into a tight complex causes a relative rotation of the two receptor proteins. This rotational motion is propagated to the receptor cytoplasmic domains, bringing the kinase active site associated with one receptor molecule proximal to an activating phosphorylation site on the other. The ensuing trans phosphorylation converts the kinase on the second receptor chain to a high activity state, thereby initiating further phosphorylation events that trigger downstream signaling. This rotational model of receptor activation was further supported by molecular dynamic simulations on the hGH/hGH-R co-crystal structure which suggested that, upon removal of the ligand, the extracellular domains of the receptors rotate approximately 45° counter-clockwise with respect to each other, relaxing into a mutual orientation that was postulated to represent the inactive state (Poger and Mark 2009). In an embellishment of this rotational activation model it has been proposed that, in addition to a mutual rotation, activation also involves a simultaneous scissor-like motion of the two receptors chains (Pang and Zhou). The authors propose that a requirement for both of these motions explains why, in the hPRL-R, mere rotation of the cytoplasmic domain through mutagenesis of the TM domain failed to cause activation (Liu and Brooks 2011). A rotational activation model has also been proposed for the IL-4R, in which the partitioning of certain tryptophan residues on the receptor between associating with the plasma membrane versus with the core of the juxtamembrane cytoplasmic domain was proposed to drive a relative rotation of the TM helices of the IL-4R α and γ_c receptor chains, which is subsequently translated to the cytoplasmic domains to bring the associated JAK1 and JAK3 kinases into the appropriate positions for autophosphorylation (Weidemann, Hofinger et al. 2007).

Multiple reports have appeared in recent years suggesting that other receptors, in addition to EPO-R, hGH-R and PrL-R, also exist on cells as pre-associated dimers or oligomers in the absence of bound ligand (Gadella and Jovin 1995; Siegel, Frederiksen et al. 2000; Krause, Mei et al. 2002; Tenhumberg, Schuster et al. 2006; de Bakker, Bodnar et al. 2008; Jenei, Kormos et al. 2009). For EGF-R in particular, the recent literature contains a multitude of studies addressing the possible existence of preformed dimers or oligomers, of which the following references represent just a sampling (Clayton, Walker et al. 2005; Saffarian, Li et al. 2007; Macdonald and Pike 2008; Tao and Maruyama 2008; Alvarado, Klein et al. 2010; Hofman, Bader et al. 2010). Many of these reports involve imaging studies of one kind or another in which receptors on cells are labeled with fluorescent tags or by other means, and are shown to be clustered even when no ligand is present. However, some studies of pre-associated receptors also involve functional data. An obvious potential weakness of studies that rely on labeled receptors is the possibility that modification of the receptor protein to introduce the label has altered its self-association properties. Moreover, any study that involves recombinant expression of a modified receptor introduces the possibility that artificially high expression levels might induce self-association that would not occur at endogenous levels. Nevertheless, many of the above studies take careful account of these possible artifacts, and control for them very convincingly. For example, a recent study of EGF-R expressed at endogenous levels, using quantum dot-based optical measurements of single molecules, showed that receptor monomers and dimers exist in equilibrium prior to ligand binding (Chung, Akita et al. 2010). Upon stimulation with EGF the receptor molecules appeared to form clusters of three or more monomers. The dimeric state always preceded activation/clustering, but both monomers and dimers bound soluble ligand. Binding of ligand to a kinase-null mutant or a mutant that lacked the dimerizing arm from its extracellular domain did not stabilize the dimer form.

Receptors from the TNF-R superfamily have also been proposed to function by a pre-associated receptor mechanism. These studies exemplify some of the complexities and apparent contradictions that make definitive conclusions on this point so difficult to reach. A crystal structure of the extracellular domain of the p55 TNF receptor has been reported that shows two different types of receptor dimers possessing extensive interaction interfaces (Naismith, Devine et al. 1996). As was the case with EPO-R, described above, these authors proposed that the crystallographically observed dimers might reflect the structure of an inactive state of the receptor that exists under physiological conditions. Furthermore, the notion that TNFRp55 is pre-associated on the cell surface is supported by a compelling set of functional studies conclusively demonstrating that, on H9 lymphoma cells, full-length TNFRp55 predominantly exists as a pre-associated trimer in the absence of its TNF α ligand (Chan, Chun et al. 2000). The trimeric structure is consistent with the 3-fold symmetry of the complex that TNFRp55 forms with the trimeric ligand TNF β (Figure 3g) (Banner, D'Arcy et al. 1993). Experiments using truncated or mutated variants of TNFRp55 showed that self-association is mediated by a so-called Pre Ligand-Binding Assembly Domain (PLAD), which comprises residues 1–56 within the N-terminal cysteine-rich domain (CRD) of the receptor. Similar pre-association behavior was seen for the other TNF-R superfamily members TNFRp75, Fas, CD40 and DR4. However, notwithstanding the compelling nature of the data that establish TNFR receptor pre-association, incorporation of this phenomenon into a plausible mechanism for how ligand binding brings about receptor activation remains problematic. First, it is hard to reconcile the functional data of Chan et al. with the structure observed by Naismith et al. In the former case the data show the receptor to be pre-associated essentially exclusively as a trimer, whereas the crystal structure just as clearly indicates that a dimer is the observed oligomeric state. Even if the X-ray structure were to be dismissed as a crystal artifact, thereby resolving the above discrepancy, the functional data supporting pre-associated receptors are difficult to reconcile with the known properties of TNFRp55/TNF α binding. For example, the crystal structure of the complex of TNF β with

the extracellular domain of TNFRp55 (Banner, D'Arcy et al. 1993), as well as corresponding complex structures for other PLAD-containing TNFR family members, clearly shows that the N-terminal domain of the receptor is not involved in binding to the ligand. And yet deletion of the PLAD domain from TNFRp55 was shown to abolish ligand binding (Chan, Chun et al. 2000). Moreover, if the resting state of the receptor is a trimer connected via the N-terminal PLAD domains, forming a termolecular, cage-like structure on the membrane, then how does the ligand enter this cage to adopt the complex structure observed crystallographically? The dimensions of the TNFRp55 molecule are such that the 55 kDa TNF α trimer appears far too big to squeeze through the small gaps that would exist between the pre-associated receptor chains (Figure 10). Thus, the evidence for receptor pre-association of TNF-Rp55 and these related receptors appears strong, and yet does not easily lend itself to a plausible molecular mechanism for how the binding of ligand to such a pre-associated state could induce formation of the activated receptor complex.

As the astute reader will have observed, the receptor systems that provide the strongest evidence for a pre-associated receptor mechanism are largely the very same ones – including EPO-R, hGH-R, Prl-R, EGF-R and TNFRp55 – that were originally used to formulate and establish the concept of ligand-induced oligomerization. Thus, we are in the uncomfortable position that these well characterized receptor systems appear to provide contradictory information about the mechanism by which they function.

3. Critical analysis of arguments for and against ligand-induced oligomerization versus pre-associated receptors

In the examples cited above, and for cytokine and growth factor receptors in general, the interaction between receptor proteins in the activated complex is strictly noncovalent. Thus, as for any other noncovalent complex, there is no question that any given receptor can in principle exist either as dissociated monomers or as pre-associated oligomers, and which of these is observed will depend on the concentration of receptors on the cell surface relative to the dissociation constant for self-association (Whitty, Raskin et al. 1998; Schlee, Carmillo et al. 2006; Jaks, Gavutis et al. 2007; Kalie, Jaitin et al. 2008; Whitty and Riera 2008; Levin, Harari et al. 2011). Thus, rather than being entirely distinct mechanisms as is typically portrayed, ligand-induced oligomerization and the pre-associated receptor mechanism represent two extremes of a mechanistic continuum (Figure 11). In circumstances where receptor components are expressed at low levels compared to the K_D for their interaction in the absence of ligand (K_{D2}' in Figure 11), the unactivated receptor will exist predominantly as dissociated monomers. But if at least one of the receptor components is expressed at a high level compared to K_{D2}' , in its unactivated state the receptor will predominantly exist as oligomers. It should be noted that when we speak of the expression level or concentration of a receptor protein on the cell membrane, we must think in terms of moles per unit area, not per unit volume; the K_D for interactions between membrane proteins similarly has “two-dimensional” units of moles per unit area (often most conveniently expressed as molecules/ μm^2) (Wu, Vendome et al.; Dustin, Golan et al. 1997; Whitty, Raskin et al. 1998; Schlee, Carmillo et al. 2006; Jaks, Gavutis et al. 2007; Zhu, Dustin et al. 2007; Whitty 2008; Wu, Vendome et al. 2011). These units are appropriate because the separate, membrane-associated molecules are constrained to diffuse in the two dimensions of the cell membrane, and thus it is the dispersion of these species across the available membrane surface and not their distribution in the total volume of the experimental system that governs the change in free energy that occurs when they associate noncovalently into complexes (Whitty 2008). Importantly, it is the local receptor density rather than the average density across the whole cell surface that governs the position of the binding equilibrium (Whitty, Raskin et al. 1998; Schlee, Carmillo et al. 2006). The contextual and dynamic nature of receptor self-association has received growing attention of late, especially for the cases of the EGF receptor (Ozcan,

Klein et al. 2006; Saffarian, Li et al. 2007; Macdonald and Pike 2008; Alvarado, Klein et al. 2010; Chung, Akita et al. 2010; Nagy, Claus et al. 2010), the Type I interferon receptor (Lamken, Lata et al. 2004; Gavutis, Lata et al. 2005; Gavutis, Jaks et al. 2006; Gavutis, Lata et al. 2006; Jaitin, Roisman et al. 2006; Jaks, Gavutis et al. 2007; Kalie, Jaitin et al. 2008; Levin, Harari et al. 2011), and the RET receptor tyrosine kinase (Schlee, Carmillo et al. 2006). Other important advances in this area include the recognition that interactions with other species might be involved in governing the monomer-dimer-oligomer equilibrium for EGF-R (Klein, Mattoon et al. 2004) and that the impact of EGF-R dimer formation on the affinity for binding ligand can be affected by the receptor phosphorylation state (Macdonald-Obermann and Pike 2009). Notwithstanding the increasing sophistication of our thinking concerning the coupling of ligand binding to receptor dimerization, work of this kind is still in its infancy, and great advances in this area can be anticipated over the next decade or so.

The above considerations lead to several conclusions concerning what we can and cannot say, from the available evidence, about whether a given receptor is activated by ligand-induced oligomerization versus a pre-associated receptor mechanism:

Conclusion 1: Much of the mechanistic evidence that has been used to support one or the other of the candidate mechanisms of ligand-induced dimerization versus pre-associated receptors is less conclusive than is commonly recognized.

In the case of the evidence supporting a pre-associated receptor mechanism, the following criticisms can be made:

- The fact that a receptor dimer or oligomer can be observed by x-ray crystallography, or other biophysical or biochemical methods that require high protein concentrations, is not by itself evidence that such complexes will occur to any significant degree on cells. Even if the crystallographically-observed interface looks “real”, in the sense that it shares structural characteristics of known protein-protein interfaces, the structure alone cannot tell us whether the binding affinity between the proteins is sufficient to drive substantial association at the levels of receptor expression that are present on any given cell.
- For the same reason, experiments in which recombinant receptor proteins are heterologously expressed on cells can show spurious self-association if the protein is expressed at levels higher than would be seen physiologically. Studies that measure receptor complex formation using endogenous receptors on untransfected cells are rare, due to the technical difficulties involved. Consequently, most functional experiments have involved recombinant expression of receptor proteins engineered to include a reporter module of some kind, such as the cytoplasmic domain of a different receptor, a fluorescent protein fusion partner, or complementary enzyme fragments. The observation of ligand-independent receptor pre-association by methods such as these must be interpreted with extreme caution, unless careful controls are done to show that the effect is seen at physiologically-relevant receptor expression levels, and also that the reporter module does not itself enhance self-association.
- The observation that a fraction of receptors are observed to exist in pre-associated clusters on cells, as is undoubtedly true in many cases, does not prove that the receptor is activated by a pre-associated receptor mechanism. To draw this conclusion it must additionally be shown that these pre-associated dimers or clusters are on the reaction pathway for receptor activation, and do not instead represent an inactive “depot” state of the receptor. In this regard, the crystallographically-observed ligand-free dimers that have been reported for EPO-

R (Livnah, Stura et al. 1999) and TNFRp55 (Naismith, Brandhuber et al. 1996) are troubling, because in both cases the ligand binding site is occluded by the self-association interface seen in the crystal structure, and so it is not clear how ligand would bind to either complex to bring about its conversion to an active state. In these cases, for the observed complexes to represent the resting state of the receptor it is necessary to propose either (i) that ligand first binds to the receptor through a different and as-yet undiscovered binding site that remains available in the pre-associated receptor complex, and in so doing induces a change in the mutual orientation of the receptors allowing the ligand to subsequently migrate in a separate step to occupy the final binding site observed in the intact ligand-receptor complexes; or alternatively (ii) that for ligand to bind the pre-associated receptors must first spontaneously dissociate to reveal the ligand binding site. In this latter case the ligand is actually binding to monomeric receptor and activating it by ligand-induced oligomerization. This is not merely a semantic distinction; a receptor with a resting state comprising off-pathway dimers or oligomers is anticipated to display many of the functional properties expected for ligand-induced dimerization rather than a pre-associated receptor mechanism. An alternative possibility is that the pre-associated receptor dimers involve additional interactions between the cytoplasmic and/or transmembrane portions of the receptor proteins, such that the extracellular domains can come apart to reveal the ligand binding site without entirely dissociating the receptor dimer. However, complexes observed for receptor extracellular domains are irrelevant to such a mechanistic hypothesis - receptors could pre-associate through their cytoplasmic domains either with or without any interaction between the extracellular domains - and so crystallographically-observed complexes between receptor extracellular domains, such as those reported for EPO-R and TNFRp55, provide no evidence either for or against such a mechanism.

- The fact that deletion of a five-residue motif in the juxtamembrane region of TPO-R leads to constitutive activation of the receptor (Staerk, Lacout et al. 2006) is also not conclusive, as it does not rule out the possibility that self-association of the mutant was caused by the deletion. These and similar results therefore do not exclude the possibility that the resting state of wild-type TPO-R is monomeric, and deleting the five juxtamembrane residues altered the structural or electrostatic properties of the receptor to induce or favor constitutive dimerization that led to ligand-independent activation. Similarly, the many other studies, discussed above, showing that forcing receptor dimerization through TM or juxtamembrane domain mutations, or showing that TM domain interactions regulate the relative orientation of the cytoplasmic domains in the activated receptor, do not directly address the question of whether the wild-type receptor is monomeric or dimeric in its unligated state.

Much of the evidence that has been used to argue for a mechanism of ligand-induced dimerization, when examined closely, can be seen to be similarly inconclusive. Specifically:

- As is widely recognized, the observation of ligand-receptor co-crystal structures in which the ligand appears to bring together two or more receptor proteins, which provided some of the early evidence supporting ligand-induced dimerization, does not rule out a pre-associated receptor mechanism. Such structures do not tell us anything about the oligomeric state of the receptor before ligand binds, which is the key distinction between these two mechanisms.
- Biophysical or biochemical studies showing that a soluble form of the receptor can be induced to dimerize or oligomerize in solution upon adding ligand cannot be taken as proof that the receptor functions by ligand-induced dimerization in a

cellular context. There are several reasons for this. Clearly, if the transmembrane or cytoplasmic domains of the receptor contribute to self-association, then any study that uses engineered receptor constructs that lack these domains will underestimate or entirely miss the tendency of the receptors to self-associate in the cellular context. Moreover, any experiment in which a receptor is studied in solution rather than on the membrane will alter the thermodynamics of binding in ways that are difficult to account for in any quantitative manner (Whitty 2008), so that it is effectively impossible to say whether the degree of self-association that is seen in solution resembles that which would be observed on the membrane. Thus, two receptors that are predominantly self-associated when present on the cell might interact so weakly in solution that this interaction is missed, leading the investigator to incorrectly assume that oligomerization only occurs in the presence of ligand. Similarly, experiments in which receptors are artificially captured on a solid surface or other matrix will not recapitulate the situation that exists on a cell membrane (Day, Cachero et al. 2005), and so cannot be assumed to accurately indicate the degree of self-association that would be observed on cells.

- The observation that a ligand can be mutated to abolish its “Site 2” interaction with the second receptor chain, thereby converting the ligand from an agonist to an antagonist, is consistent with a mechanism of ligand-induced dimerization but does not prove this mechanism. The effect of such mutations can be explained equally well in terms of a pre-associated receptor mechanism, in which the mutated ligand can bind to one receptor chain but cannot make the productive contacts with the additional receptor components that are required to stabilize the activated state (Figure 12a).
- Similarly, the observation of a bell-shaped dose-response when a homodimeric receptor such as hGH-R or EPO-R is activated by its natural ligand or by a monoclonal antibody is again perfectly consistent with ligand-induced dimerization, but does not prove this mechanism. Assuming trivial explanations such as cytotoxicity or rapid down-regulation of the receptor at high ligand concentrations can be ruled out, formally speaking the observation of self-inhibition at high ligand concentrations indicates that binding of one or more additional ligand molecules converts the activated receptor into a “dead-end” (non-signaling) state (Schlee, Carmillo et al. 2006). In a ligand-induced dimerization mechanism this dead-end state would involve each monomeric receptor chain being occupied by a separate ligand molecule, as shown in Figure 6c. However, this result can be explained equally well in the context of a pre-associated receptor mechanism, where the dead-end state might involve each receptor chain being occupied by a separate ligand molecule as shown in Figure 12b. A similar caveat applies to the observation, for a heterotypic receptor, that antagonists that bind to receptor chain two and block its interaction with chain one are noncompetitive with respect to ligand (Whitty, Raskin et al. 1998).

Conclusion 2: Because ligand-induced dimerization and receptor pre-association represent extremes of a mechanistic continuum, there is no reason to suppose that one or the other of these mechanistic alternatives will apply to all cytokine and growth factor receptors, or even to the same receptor under different conditions.

It is quite likely that some receptors act by ligand-induced oligomerization while other receptors use a pre-associated receptor mechanism. Indeed, some receptors might function by either mechanism depending on the cellular context, using ligand-induced dimerization on cell types where the receptor is expressed at low levels compared to K_{D2} (Figure 11), but a pre-associated receptor mechanism on other cell types in which the receptor is

expressed at higher levels. This behavior would lead to the interesting and potentially biologically significant result that two different cells expressing the same receptor might respond quite differently when exposed to the same concentration of ligand. It is even possible that some receptors will be found to use both mechanistic pathways, in parallel, on the same cell. A receptor that is expressed at a level comparable to K_{D2} will have a fraction of molecules present as pre-associated oligomers and the remainder as monomers, such that some receptors might be activated by ligand binding to pre-associated receptor oligomers while activation of the remainder requires ligand binding to induce them to associate. A heterogeneous distribution of receptors between monomeric and pre-associated states has been invoked to account for the observation of two affinity classes for the EGF-R on cells (Boni-Schnetzler and Pilch 1987), though it should be noted that many other phenomena can lead to binding data that give nonlinear Scatchard plots, and so such interpretations must be treated with great caution. Given the tendency of nature to use similar molecules in different ways, it would not be surprising if examples of all of these mechanistic variations were found to exist for different receptors in different cellular contexts. This diversity of mechanistic possibilities for a given receptor might account for some of the conflicting results that have been reported concerning the activation mechanism of particular receptors, though the intrinsic difficulty in performing unambiguous experiments in this area undoubtedly contributes as well.

Conclusion 3. The extent to which a given receptor will self-associate in the absence of ligand is not an intrinsic property of the receptor, but instead is highly contextual, depending as it does on local receptor expression levels.

The contextual nature of the receptor activation mechanism constitutes an important and underappreciated aspect of the biology of these systems. It potentially provides a means for cells to dynamically regulate their responsiveness to a given cytokine or growth factor (Schlee, Carmillo et al. 2006; Levin, Harari et al. 2011)(Jaitin, Roisman et al. 2006), either by up- or down-regulating the expression of one or more receptor components, or by dynamically regulating how uniformly or heterogeneously the receptor is distributed on the cell membrane, for example by elevating local receptor density by induction into membrane microdomains such as lipid rafts (Simons and Sampaio). This contextual plasticity in receptor activation mechanism, if it occurs, could potentially have important consequences for the relationship of stimulus to response in cytokine and growth factor signaling processes. To address this question will require that we abandon the tacit assumption that a given receptor functions by a particular activation mechanism as an intrinsic and unchanging characteristic, and instead move towards a more nuanced picture in which the sequence of steps by which a cytokine or growth factor activates its receptor is governed by the equilibrium and kinetic behavior of molecules in a particular cellular situation.

Ligand-Induced Dimerization versus Pre-associated Receptors

So what can we conclude about whether a given receptor functions by ligand-induced oligomerization versus pre-associated receptors?

- Direct evidence to distinguish these mechanisms most often requires functional studies that use the full-length receptor on cells, preferably present at endogenous levels. Other kinds of experiments, for example involving structurally modified receptors or soluble receptor extracellular domains, are highly relevant and valuable in elucidating structural and biophysical details of the mechanism, but in general cannot definitively establish which of these two reaction pathways is operative in a particular cell type and biological situation.
- To prove a pre-associated receptor mechanism it is not sufficient to show that receptors pre-associate on cells in the absence of ligand. It is additionally necessary

to show that the pre-associated receptor complexes are on the pathway for receptor activation, and do not represent a depot or other off-pathway state.

- Even if it is definitively established that on a particular cell type a given receptor functions by one or the other of these mechanisms, it cannot be assumed that this is an intrinsic characteristic of the receptor that will be true on different cells or in different circumstances, where the local receptor expression levels might be much lower or higher thereby placing the receptor system in a different position in the mechanistic continuum shown in Figure 11.

Despite the enormous amount of work that has been done on this problem, what we can say for sure, at the present time, about which of these two mechanistic possibilities applies to a given cytokine or growth factor receptor is rather limited. We can say that, for a number of receptors at least, pre-associated receptor clusters exist in the absence of ligand. However, the number of receptors involved and the role of these clusters in receptor activation remains unclear, although important recent advances of the sort described in the preceding sections are bringing us closer to a definitive answer. We can also say that receptor activation is a multi-step process, with the first step involving binding of the ligand to one receptor chain, followed by recruitment or re-organization of the remaining receptor chains to form the activated receptor complex. But we generally cannot say whether the later steps involve recruitment of freely diffusing receptor chains or structural reorganization within a pre-formed receptor complex. We can say that in many cases there is considerable plasticity in the mutual orientation of the receptor extracellular domains that can bring about receptor activation, but currently we do not understand in detail which orientations are productive and why. Without developing the experimental methods and theoretical insights required to achieve definitive answers to these questions for a given system of interest – in the same way that, in the arena of enzymology, current methods allow us to achieve a quantitative description of the mechanism of action of essentially any enzyme of interest – our quantitative understanding of signaling and inter-cellular communication will remain severely limited.

4. Other Key Unanswered Questions Concerning Receptor Activation and Signaling

This review has focused primarily on the longstanding controversy over whether various well-studied receptors are activated by a mechanism of ligand-induced oligomerization versus a pre-associated receptor mechanism. Consideration of this question illustrates both the relative paucity of definitive mechanistic knowledge concerning how cytokine and growth factor receptors function, and also the technical challenges associated with trying to perform definitive experimental work in this difficult area. We do not wish the reader to conclude, however, that this is the only important unresolved question in this field. Indeed, there are very many other aspects of the quantitative and mechanistic behavior of cytokine and growth factor receptors about which we know very little. Below, we briefly summarize some other major areas in which our knowledge and understanding of these systems is severely limited, in the hope of stimulating additional research directed towards filling in some of the many blank areas concerning these biologically and medically important systems.

The role of higher-order receptor clustering

When we consider the structure of an activated receptor complex as it exists on cells, we tend to think of the smallest structural unit in which all binding sites on both the ligand and the receptor are satisfied. So, for example, it is commonly assumed that the functional form of the hGH/hGH-R complex on cells involves a termolecular complex in which a single

hGH molecule is bound to two molecules of hGH-R, and similarly that the functional complex for EGF-R is a 2:2 complex in which two EGF-bound EGF-R molecules are associated back-to-back. In this, we are undoubtedly influenced by crystallographic studies of receptor-ligand complexes, in which the unit cell typically contains only one or two such assemblages, and by solution-phase biophysical studies in which formation of small complexes of this type dominates the observable behavior. There is, however, substantial evidence to indicate that formation of these small, prototypical complexes, at least for some receptor systems, is not sufficient to trigger a full biological response. Instead, in these cases the complexes that are formed when growth factor binds to one or two receptor chains is an intermediate state that goes on to aggregate into larger clusters that represent the fully active form of the receptor. The ligand-induced formation of large clusters is well established for immune receptors such as the T Cell Receptor (Germain 1997), the IgE Receptor (Huang, Liu et al. 2009), and also for Eph receptor tyrosine kinases (Himanen, Saha et al. 2007; Himanen, Yermekbayeva et al. 2010). Formation of higher order clusters is not, however, commonly considered as an essential part of the activation mechanism of cytokine and growth factor receptors, partly due to the technical limitations in characterizing receptor interactions on cells that are discussed in detail in previous sections, and partly because such behavior has not often been directly looked for. Nevertheless, evidence exists for several receptors such as EGF-R (Whitson, Beechem et al. 2004; Clayton, Walker et al. 2005), IL-5R (Zaks-Zilberman, Harrington et al. 2008), leptin receptor (Zabeau, Defeau et al. 2004), and several TNF family receptors (Holler, Tardivel et al. 2003) that higher order oligomers form and are required for full receptor activation. Although data substantiating this point has been accumulating in the literature for some years, we have yet to integrate this phenomenon into a complete and coherent mechanistic picture of how ligand binding brings about receptor activation.

Differential activation of a common receptor by different cytokine or growth factor ligands

As we have described in the preceding sections, for many receptors it appears that there is considerable plasticity in the mutual orientation of the receptor extracellular domains that can bring about receptor activation, but currently we do not understand in detail which orientations are productive and why. Moreover, there is considerable uncertainty about the extent to which these structural differences in the orientation of the extracellular domains are transmitted to the cytoplasmic portions of the receptor (Strunk, Gregor et al. 2008), and the consequences for the quality of the signaling response that results. This issue has important implications in the many cases in which a receptor can be activated by two or more different ligands.

Different ligands that activate a common receptor typically have different biological activities *in vivo*, as has been well established for example through genetic knock-out experiments in mice. In general, however, it is unclear to what extent these different *in vivo* activities are due simply to the different times, places and levels at which each ligand is expressed, versus to biochemical differences in how the ligands engage their common receptor. One possibility is that different ligands represent essentially redundant ways to achieve a common activated state of the receptor; that is, the receptor can be thought of as analogous to a light switch, with a single “on” state regardless of how activation is brought about. Alternatively, different ligands might induce activated receptor complexes with quite different conformations, causing geometrically distinct interactions between the receptor cytoplasmic domains. In this latter case, different ligands might induce distinct patterns of intracellular signals leading to different biological responses. Qualitative analysis generally shows that different ligands activate a common set of signaling pathways downstream of a given receptor. However, in relatively few cases have responses of a common receptor to different ligands been compared in detail to assess differences in the levels, timing and

durations of the various downstream signaling events. Moreover, we know very little about the functional consequences that such quantitative differences in the quality of the signal might bring. One instructive exception to this paucity of knowledge is provided by the work of Schreiber, Piehler and their co-workers and collaborators on the activation of the Type I Interferon Receptor by interferon (IFN)- β versus IFN α 2. Both of these homologous cytokines are used as drugs, but they can have quite distinct activities in certain disease states leading, for example, to the use of IFN β as a treatment for multiple sclerosis, a condition for which IFN α 2 is not beneficial. In an elegant series of studies, Schreiber and Piehler showed that the different biological activities of these two ligands results from the different kinetic lifetimes of the activated receptor complexes that each forms with their common receptor, which in turn results from the higher affinity of IFN β versus IFN α 2 for binding to the IFNAR1 receptor component that is recruited into the complex in the second step in receptor activation (Jaitin, Roisman et al. 2006; Jaks, Gavutis et al. 2007; Kalie, Jaitin et al. 2008; Levin, Harari et al. 2011). Notwithstanding this and a small number of other systems for which we have achieved a measure of understanding of this aspect of receptor signaling, as a general rule we are quite ignorant of the mechanistic and biological implications of the very widely observed phenomenon of ligand-receptor promiscuity.

The link between pathway and function

The great variety of stoichiometric compositions that are observed for cytokine and growth factor receptors from different protein families (Figure 1b) implies a variety of different molecular mechanisms for receptor activation. That is, the number and sequence of steps by which the initial binding of the ligand to the first receptor chain leads sequentially to the assembly of the activated receptor complex presumably differs depending on the number of receptor components involved, and the sequence in which these components undergo the requisite association events and/or conformational rearrangements after initial binding of ligand. The more components are involved, the greater the number of possible activation pathways that exist (Figure 13a). One example of a receptor comprising more than two receptor components for which a detailed mechanism has been proposed is the activation of RET by artemin (ART) in conjunction with the GPI-linked co-receptor GFR α 3 (Schlee, Carmillo et al. 2006). The activated RET receptor complex includes two copies of RET, two copies of GFR α 3 and one copy of the covalently-dimeric ligand, ART (Treanor, Goodman et al. 1996; Schlee, Carmillo et al. 2006). In this study the authors measured how the level of RET phosphorylation varied in response to independent manipulation of the ART concentration and the level of available GFR α 3 on the cell. The results were qualitatively and quantitatively consistent with a mechanism in which ART first binds to one molecule of GFR α 3, followed by recruitment of the first RET molecule, followed by binding of the second molecule of GFR α 3, followed finally by recruitment of the second RET molecule to give the activated complex (Figure 13b). Alternative pathways, for example in which GFR α 3 exists as a preformed dimer, or in which the second GFR α 3 is recruited into the complex before the first RET molecule, were incompatible with the experimental data. Importantly, the results were used to develop a quantitative model for the relationship between the level of ART stimulation and the resulting level of activated RET on the cell, elucidating the roles of individual steps in the activation mechanism in governing the functional coupling of input to output in this system (Figure 13c) (Schlee, Carmillo et al. 2006). Remaining unresolved is whether activation of RET by any of its three other activating ligand/co-receptor combinations – GDNF/GFR α 1, neurturin/GFR α 2 and persephin/GFR α 4 – proceeds by the same mechanism, or whether these alternate receptor complexes assemble by a different sequence of steps, and if so what are the consequences for the functional relationship between ligand concentration and activated RET levels on the cell. The general question of how the particular pathway by which receptor complexes assemble on the cell affects the function and regulation of receptor signaling, and therefore

why nature has evolved the different receptor structures and stoichiometries illustrated in Figures 1b and 3, represents an aspect of receptor signaling about which we know very little.

5. Summary

Our understanding of the detailed mechanism of action of cytokine and growth factor receptors – and particularly our quantitative understanding of the link between structure, mechanism and function – lags significantly behind our knowledge of comparable functional protein classes such as enzymes, G protein-coupled receptors, and ion channels. In particular, it remains controversial whether such receptors are activated by a mechanism of ligand-induced oligomerization, versus by a mechanism in which the ligand binds to a pre-associated receptor dimer or oligomer which becomes activated through subsequent conformational rearrangement. A major limitation to progress has been the relative paucity of methods for performing quantitative mechanistic experiments on unmodified receptors expressed at endogenous levels on live cells. New approaches and techniques have led to rapid recent progress in this area, however, and the field is poised for major advances in the coming years, which promises to revolutionize our understanding of this large and biologically and medically important class of receptors.

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Abbreviations

ART	artemin (also known as neublastin)
CRD	cysteine-rich domain of a TNF receptor superfamily member
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor (also known as ErbB1)
EPO	erythropoietin
EPO-R	erythropoietin receptor
FGF	fibroblast growth factor
γ_c	common gamma chain, a component shared by interleukin-2 family cytokine receptors
GDNF	glial cell line-derived neurotrophic factor
GFRα	GDNF family receptor α chain
GPI	glycosylphosphatidylinositol
hGH	human growth hormone
hGH-R	human growth hormone receptor
IFN	interferon
IL	interleukin
LT	lymphotoxin

mAb	monoclonal antibody
Prl	prolactin
Prl-R	prolactin receptor
RTK	receptor tyrosine kinase
TGFβ	transforming growth factor- β
TNF	tumor necrosis factor
TPO	thrombopoietin
TPO-R	thrombopoietin receptor
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor

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(a)

(b)

Figure 1. Cartoon illustrating the structures and compositions of typical growth factor and cytokine receptors

(a) (Left) growth factor receptor, with a kinase domain as an intrinsic part of its cytoplasmic structure. (Right) cytokine receptor, with a signaling kinase noncovalently associated with its cytoplasmic domain. (b) Examples of stoichiometric compositions that have been reported for cytokine and growth factor receptors. Ligands (L) are shown in green, and receptor proteins (α , β or γ) are shown in blue, red or magenta. (A color version of this figure is available in the inline version of this article.)

	ErbB1/ ErbB1	ErbB1/ ErbB2	ErbB2/ ErbB3	ErbB2/ ErbB4	ErbB4/ ErbB4
EGF	X	X			
TGFα	X	X			
Amphiregulin	X	X			
Epigen	X	X			
HB-EGF	X	X		X	X
Betacellulin	X	X		X	X
Epiregulin	X	X		X	X
Neuregulin-1			X	X	X
Neuregulin-2			X	X	X
Neuregulin-3				X	X
Neuregulin-4				X	X

Figure 2. Ligand-receptor cross-reactivity in the ErbB receptor family

Ligands and receptors in this family show a complex pattern of cross-reactivities.

Complicating matters yet further, the receptor protein ErbB2 does not bind directly to any known ligand, but when present on cells is the preferred heterodimerization partner for other ErbB family members. In contrast, ErbB3 can bind ligands, but lacks an active kinase domain and so can signal only as part of a heterodimer with a kinase-active ErbB partner. See text for further details. (A color version of this figure is available in the inline version of this article.)

Figure 3. Experimental x-ray structures showing examples of the variety of ligand-receptor complex geometries observed for cytokine and growth factor receptors

(a) Complex of hGH (green) with two molecules of the extracellular domain of hGH-R (blue) (Cunningham, Ultsch et al. 1991). (b) Complex of IL-4 (green) with the extracellular domains of IL-4R α (blue) and γ_c (red) (LaPorte, Juo et al. 2008). (c) Complex of IL-2 (green) with the extracellular domains of IL-2R α (magenta), IL-2R β (blue) and γ_c (red) (Stauber, Debler et al. 2006). (d) Side view (left) and top view (right) of the hexameric complex comprising two molecules of IL-6 (green) with two molecules each of the extracellular domains of IL-6R α (blue) and gp130 (red) (Boulanger, Chow et al. 2003). (e) Top view of the complex of TGF β 1 (green) with two molecules each of the extracellular

domains of TGF β RI (red) and TGF β RII (blue) (Radaev, Zou et al. 2010). **(f)** Complex comprising two copies of EGF (green) plus two copies of the EGF-R extracellular domain (blue and red) (Ogiso, Ishitani et al. 2002). **(g)** Complex of TNF β (green), also known as LT α , with three copies of the TNFRp55 extracellular domain (blue) (Banner, D'Arcy et al. 1993). **(h)** Side view (left) and top view (right) of the complex containing two copies of FGF2 (green) in complex with two copies of FGF-R extracellular domain (blue) and two molecules of heparin (colored by atom; C = yellow) (Pellegrini, Burke et al. 2000). (A color version of this figure is available in the inline version of this article.)

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Figure 4. Scheme illustrating the generic mechanism for growth factor receptor signaling

(a) Step (i): upon assembly of the activated receptor complex the kinase domains associated with the receptor's cytoplasmic region phosphorylate each other and then additional sites (added phosphate groups are indicated by red stars). Step (ii): Cytoplasmic signaling proteins (orange and purple) are recruited to the newly phosphorylated docking sites on the receptor, and (Step iii) are themselves in turn phosphorylated. Some of these recruited proteins serve as docking sites for additional cytoplasmic signaling proteins (Step (iv)), which mediate additional downstream signaling processes, while others dissociate from the receptor and then form phosphopeptide-mediated complexes with each other or with other

transcription factor proteins (Step (v)), after which they migrate to the nucleus where they interact with specific promoter sites on DNA to modulate the transcription of target genes.

(b) Illustration of the asymmetric interaction between EGF-R kinase domains that results in kinase activation (Zhang, Gureasko et al. 2006). EGF-R is colored blue or pink, while the bound EGF is colored pale green. The extracellular and cytoplasmic domains represent separate experimental crystal structures of these complexes, while the connecting segment is drawn arbitrarily. The N-terminal portion of one EGF-R kinase domain is colored dark blue, while the corresponding portion of the identical kinase domain from the other EGF-R molecule is colored dark red, to highlight the asymmetric nature of the interaction. (A color version of this figure is available in the inline version of this article.)

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Figure 5. Two possible mechanisms accounting for how ligand binding brings about the activation of a dimeric receptor

(a) Ligand-induced receptor dimerization. (b) Allosteric rearrangement of a pre-associated receptor dimer. (A color version of this figure is available in the inline version of this article.)

Figure 6. Potential explanations for key mechanistic findings in terms of ligand-induced dimerization

(a) The observation that a ligand that is mutated to inactivate its Site 2 binding surface can act as an antagonist of homotypic receptors such as hGH-R and EPO-R in cellular assays can be accounted for if each receptor chain binds a separate ligand molecule through its intact Site 1, with no Site 2 interaction to drive recruitment of a second receptor chain into an activated ternary complex. (b) Mutants of hGH with reduced but not eliminated Site 2 binding show a bell-shaped dose response. Reproduced from (Fuh, Cunningham et al. 1992), with permission. ©American Association for the Advancement of Science, 1992. (c) The observation that ligands for homotypic receptors such as hGH-R and EPO-R give bell-

shaped dose-response relationships in cellular assays can be explained if high ligand concentrations drive the receptor to a non-signaling state similar to that described in (a). **(d)** The observation that an antagonistic ligand mutant of the type described in (a) can be converted to an agonist by covalent dimerization can be explained if each ligand monomer binds a separate receptor chain via the unmutated Site 1, thereby bringing two receptor chains together due to the covalent tether. (A color version of this figure is available in the inline version of this article.)

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Figure 7. Competitive versus noncompetitive inhibition reveals mechanistic properties of the heterodimeric receptor for IL-4

(a) (Left panel) antibody that binds to the IL-4R α chain and directly blocks ligand binding acts as a competitive antagonist in assays measuring the IL-4 dependent proliferation of T cells. Antibody concentrations are 0 ($\circ$), 0.41 ($\square$), 1.23 ($\triangle$), 3.7 ($\diamond$), 11.1 (∇) 33.3 ($\boxtimes$) or 100 ($\bullet$) μ g/ml. Inset plot shows that the concentration of IL-4 required to achieve a 50% maximal proliferative response (EC₅₀) increases linearly with the concentration of inhibiting mAb, consistent with simple competitive inhibition. (Right panel) antibody that binds to the γ_c chain and blocks its recruitment into the activated receptor complex acts as a noncompetitive antagonist. Inset: EC₅₀ for IL-4 is independent of inhibitor concentration.

Figure reproduced from (Whitty, Raskin et al. 1998), with permission. ©1998 by The National Academy of Sciences. **(b)** Scheme illustrating how noncompetitive inhibition by anti- γ_c mAb can be explained in terms of a mechanism of ligand-induced dimerization, in which IL-4 and the inhibitor bind to distinct and independent receptor components, and so the antibody cannot be outcompeted by increasing IL-4 concentration (Whitty, Raskin et al. 1998). (A color version of this figure is available in the inline version of this article.)

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Figure 8. X-ray crystal structures of activated and inhibited forms of EPO-R

(a) The native EPO/EPO-R complex showing two molecules of the EPO-R extracellular domain (dark blue and light blue) bound to one molecule of EPO (green), as viewed from the side (left image), or from the top (right image) to show the angle at which the two receptor molecules are juxtaposed (Syed, Reid et al. 1998). (b) The complex of EPO-R with the antagonistic EPO mimetic peptide EMP33 (Livnah, Johnson et al. 1998). (c) The complex of EPO-R with the agonistic EPO mimetic peptide EMP1 (Livnah, Stura et al. 1996). (d) The native EPO/EPO-R complex (left image) and the antiparallel dimer seen for unbound EPO-R (right image) (Livnah, Stura et al. 1999), with the residues that make direct

contact with EPO colored in red in both images, showing that the same regions of the receptor proteins that contact EPO also mediate receptor/receptor contact in the crystal of unbound EPO-R. (A color version of this figure is available in the inline version of this article.)

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Figure 9. Small molecules that have been reported to activate cytokine or growth factor receptors

(a) G-CSF receptor agonist SB247464 (Tian, Lamb et al. 1998). (b) TPO-R agonist TM-41 (Kimura, Kaburaki et al. 1998). (c) The FDA-approved TPO-R agonist drug, eltrombopag (Cheng, Saleh et al. 2011). (d) TrkA agonist gambogic amide (Jang, Okada et al. 2007). (e) TrkB agonist 7,8-dihydroxyflavone (Jang, Liu et al. 2010).

Figure 10. Scheme illustrating the difficulty in reconciling TNF-R pre-association via N-terminal PLAD domains with the known structure of the ligand-receptor complex

The extracellular portion of TNF-Rp55 comprises four cysteine-rich domains (CRDs). The cartoon show three molecules of TNF-Rp55 (blue) pre-associated via their N-terminal PLAD motifs, illustrating the difficulty in accounting how the large TNF ligand (green) gains access to the interior of this three-fold cage. The experimental structure on the right shows the relative sizes of TNF β (green) compared to CRDs 2 and 3 of TNF-Rp55 (blue) (Banner, D'Arcy et al. 1993). (A color version of this figure is available in the inline version of this article.)

Figure 11. The mechanistic continuum that connects the ligand-induced dimerization and pre-associated receptor mechanisms

The scheme reflects the fact that any receptor with even a weak tendency to self-associate will form pre-associated dimers if present at local concentrations on the membrane that exceed K_{D2}' . Conversely, even strongly self-associating receptors will predominantly exist as independently diffusing monomers if the local expression density falls below K_{D2}' . Thus, which mechanism is followed is not an intrinsic property of a particular receptor, but rather is a contextual function of expression level and of other factors that affect the membrane localization or interaction affinity of the receptor components. (A color version of this figure is available in the inline version of this article.)

Figure 12. Potential explanations for key mechanistic findings in terms of pre-associated receptors

(a) The observation that a ligand that is mutated to inactivate its Site 2 binding surface can act as an antagonist of homotypic receptors such as hGH-R and EPO-R in cellular assays (see Figure 6a) can be accounted for if each receptor chain in the pre-associated receptor dimer can bind a separate ligand molecule through its Site 1 binding surface, with no activating structural reorganization. (b) The observation that ligands for homotypic receptors such as hGH-R and EPO-R give bell-shaped dose-response relationships in cellular assays (see Figures 6b, c) can similarly be accounted for by high ligand concentrations driving the

receptor to a non-signaling state similar to that described in (a). (A color version of this figure is available in the inline version of this article.)

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Figure 13. Proposed mechanism for the activation of RET by ART plus GFRα3, and its link to function (Schlee, Carmillo et al. 2006)

(a) "Reaction tesseract" illustrating potential pathways by which initial binding of ART to different possible resting states for the receptor (green squares) could lead to assembly of the activated receptor complex (pink square with bold red outline). The proposed pathway is indicated by the black equilibrium arrows. (b) Proposed mechanism for the activation of RET by ART plus GFRα3. (c) Simulation showing how the activation mechanism shown in (b), together with the experimentally-determined values for K_{D1} – K_{D4} , predicts how the different species on the activation pathway will vary with ART concentration, for NB41A3-GFRα3 cells expressing RET plus GFRα3. The filled circles show experimental

measurements of phosphoRET levels on NB41A3-GFR α 3 cells stimulated with different concentrations of ART, normalized to the same y axis scale. (A color version of this figure is available in the inline version of this article.)

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