Review

Differential regulation of signaling pathways for insulin and insulin-like growth factor I[*]

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The insulin receptor (IR) and the insulin-like growth factor receptor I (IGF-IR) have different functions in cell growth, apoptosis, differentation, and transformation. Although some of these differences may be explained by the relative level of receptor expression and receptor structure (α and β subunits), they may also be attributed to differences in intracellular signals generated by insulin and IGF-I. The presence of hybrid receptors (IR $\alpha\beta$ subunits and IGF-IR $\alpha\beta$ subunits) making up the hetero-tetramers has added a new dimension to our understanding of the functional roles of these receptors. However, to date the results of efforts to understand the differences between these two closely related receptors have indicated mostly similarities. For example, both receptors utilize IRS-1/IRS-2 and Shc as immediate downstream adaptors, leading to activation of the Ras, Raf, ERK kinases and PI-3 kinase pathways. We have used the yeast two hybrid system to identify proteins which bind to the activated IGF-IR but not to the IR. The cytoplasmic domain of the IGF-IR was used to screen a human fetal brain library and two isoforms of the 14-3-3 family were identified. 14-3-3 proteins are a highly conserved family of proteins which have recently been shown to interact with other components of the mitogenic and apoptotic signaling pathways, including Raf, BAD, Bcr/Bcr-Abl, middle-T antigen, Ksr, PKC, PI-3 ki-

nase, ASK1 kinase, and cdc25C phosphatase. We also identified human Grb10, an adaptor protein with SH2 domain associated with the IGF-IR β subunit. Smith's laboratory showed that Grb10 preferentially binds to the IR in intact cells. Using the interaction trap screen (active cytoplasmic domain of the IGF-IR) 55PIK and SOCS-2 proteins were also identified. However, 55PIK and SOCS-2 also interact with the IR in the yeast two hybrid system. These studies raise the possibility that 14-3-3 and Grb10 may play a role in insulin and IGF-I signal transduction and may underlie the observed differences.

The insulin receptor (IR) is essential for glucose metabolism, whereas the type-1 insulin-like growth factor receptor (IGF-IR) is involved in the regulation of normal growth and development as well as the growth of certain cancers [1]. The insulin and IGF-I receptors are separate gene products, having amino acid similarities ranging between 40% and 85% in different domains. The highest degree of homology is found in the tyrosine kinase domain.

The explanation for the differences in the *in vivo* action of insulin and IGFs has to do in part with their different physiology (site of production, control of secretion, etc.), and the relative distribution of the insulin receptor and IGF-I receptor in different tissues and cell types. The interaction of the ligand with the IGF-IR is regulated by the presence of IGF-specific binding proteins, which, however do not bind insulin. Intracellularly, the signaling pathways for the insulin receptor and the IGF-I receptor that have been described to date are very similar (phosphorylation of IRS-1/IRS-2 and Shc, activation of Ras/Raf/Erks and PI 3-kinase signaling pathways) [2, 3].

THE α SUBUNIT STRUCTURE

Given the similarities between the ligands, insulin and IGF-I, and the receptors, the insulin receptor and the IGF-I receptor, it may have been assumed that similar regions of the α subunits of the two receptors would be critical for ligand binding. However, examination of binding of IGF-I and insulin to chimeric in-
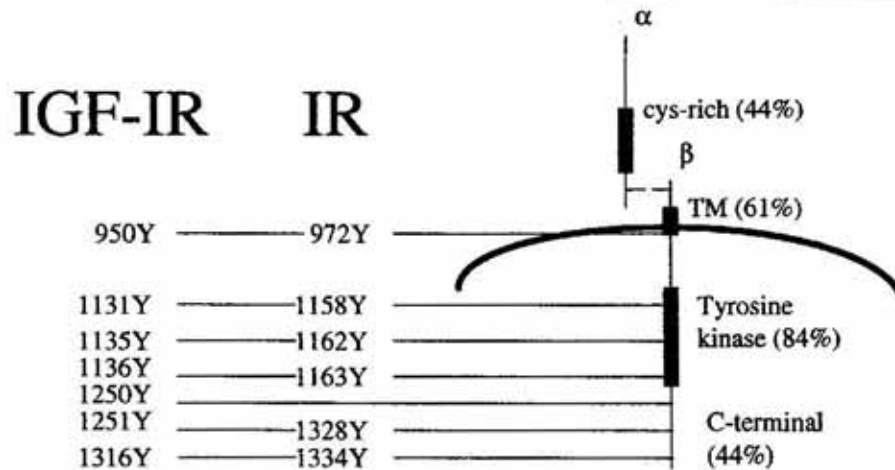
sulin/IGF-I receptors and to insulin receptor α subunit point mutants, showed that the domains of the insulin and IGF-I receptor α subunits which determine high affinity binding of their respective ligands are different [4, 5]. Residues defining IGF-I binding are present predominantly in the cysteine-rich domain of the IGF-I receptor, whereas high affinity insulin binding is largely determined by amino- and carboxy-terminal α subunit regions in the insulin receptor. Moreover, residues 704–715 of IR have been strongly implicated in ligand interaction [6], therefore, it was not surprising that the fragment of α subunit of the IGF-IR for which the structure has recently been determined (residues 1–462) is incapable of binding to its ligand [7, 8]. The fragment is composed of three domains L1 (1–150 aa), L2 (300–460 aa) and cysteine-rich region in the middle. It has been suggested that the large "notch" in the structure with the prominently placed loop (255–265) could be a likely place for ligand to bind [8]. However, studies of chimeric receptors showed that part (1–68 aa) of the IR, which exhibits high homology with IGF-IR, confers insulin binding in the contest of the IGF-IR. Conversely, residues 191–290 of IGF-IR confer IGF-I binding in the context of IR. The "notch" suggested by Garrett *et al.* [8] might contact both these regions in ligand binding. Despite the wide structure of the "notch" (approx. 30 Å between L1 and L2 domains), this fragment of the IGF-IR α subunit does bare the major specificity determinants necessary for the interaction with ligand [9].

RECEPTOR HYBRIDS

Hybrid receptors between an $\alpha\beta$ insulin receptor dimer and an $\alpha\beta$ IGF-I receptor dimer, have been shown to exist using several experimental approaches [10]. Antibodies specific for either the insulin receptor or the IGF-I receptor have been shown to immunoprecipitate a portion of high affinity binding activity for the heterologous ligand and this cross-immunoprecipitating activity was lost following cleavage of class I disulfide bonds which join $\alpha\beta$ dimers to each other. Similarly, a phosphotyrosine antiserum immunoprecipitated both 95 kDa and 100 kDa β subunits from $^{32}P_i$-labeled cells that had been stimulated IGF-I. Insulin receptor antibodies immunoprecipitated both β subunits from the cell extract but only immunoprecipitated the 95 kDa species after reduction of class I disulfide bonds. Phosphopeptide maps of the β subunits showed that the 95 kDa species was very similar to the insulin receptor β subunit, whereas the map for the 105 kDa species was that of the IGF-I receptor β subunit. In addition, it has shown that hybrid receptors can be formed *in vitro* [11]. However, the ligand binding and signaling properties of the hybrid receptors have not been clearly defined although they do not appear to behave as simply the sum of the independent heterodimer halves. For example, Lammers *et al.* [12] reported that the cytoplasmic domain of the IGF-I receptor was 10 times more effective than the insulin receptor cytoplasmic domain in stimulating DNA synthesis. Formation of hybrid receptors may provide another way to regulate signaling by IGF-I and insulin. Since autophosphorylation (activation) of the insulin and IGF-I receptors has been shown to occur by a *trans* mechanism, the formation of hybrid receptors provides one explanation for the dominant negative phenotype observed in syndromes of insulin resistance [13]. A kinase defective $\alpha\beta$ dimer which combines with a wild type $\alpha\beta$ dimer would generate a kinase defective hybrid receptor since the normal receptor half cannot be activated by autophosphorylation. Indeed, when kinase defective insulin receptors have been transfected into cells, the signaling function of the endogenous population of both IGF-I receptor and insulin receptors have been shown to be inhibited [14]. An alternative explanation for the *trans* dominant phenotype is that the mutant receptor competes with wild type receptor for binding of substrates [15]. Early experiments showed that like the insulin receptor, binding of ligand to the IGF-I receptor results in receptor autophosphorylation and activation of the receptor tyrosine kinase activity toward substrates [16]. Although IGF-I binds to $\alpha\beta$ dimers, autophosphorylation does not occur [17, 18]. In the presence of IGF-I, $\alpha\beta$ dimers form heterotetramers and autophosphorylation is possible by a *trans* mechanism between the two $\alpha\beta$ dimers. Direct evidence for a *trans* mechanism was provided by Treadway *et al.* [19]. A hybrid receptor formed between an $\alpha\beta$ kinase defective insulin receptor dimer and an $\alpha\beta$ insulin receptor dimer with a truncated β subunit lacked insulin-stimulated tyrosine kinase activity toward substrates and exhibited autophosphorylation of only the normal sized β subunit in the kinase defective $\alpha\beta$ dimer. In the control experiment, a receptor made up of two $\alpha\beta$ truncated receptor dimers showed normal insulin-stimulated tyrosine kinase activity toward substrates and the truncated β subunits were autophosphorylated. Similarly, a hybrid receptor assembled from the $\alpha\beta$ kinase defective insulin receptor dimer and an $\alpha\beta$ wild type IGF-I receptor, lacked tyrosine kinase activity toward substrate even though receptor autophosphorylation occurred. The autophosphorylation presumably was only on the kinase defective receptor half; the IGF-I receptor half was not autophosphorylated and therefore not activated as a tyrosine kinase toward substrates.

IGF-IR   IR

α

cys-rich (44%)

β

TM (61%)

950Y ———— 972Y

1131Y ————1158Y
1135Y ————1162Y   Tyrosine
1136Y ————1163Y   kinase (84%)
1250Y
1251Y ————1328Y
1316Y ————1334Y   C-terminal
                          (44%)

Figure 1. Domain structure of the IR and the IGF-IR.

The portions of single α and β chains in the heterotetrameric receptors are shown. Receptor domains are described on the right and the percent homology between the two receptors is given. Tyrosine residues known to undergo phosphorylation upon receptor activation are shown. TM: transmembrane domain.

THE β SUBUNIT STRUCTURE AND TYROSINE KINASE ACTIVITY

Despite the high sequence homology in the juxtamembrane and tyrosine kinase domains, the C-terminus region of the two receptors has only limited 44% homology (Fig. 1) [2, 20]. For example, there are two autophosphorylation sites (tyrosines 1328 and 1334) in the C-terminal region of the IR, but only Y1334 is conserved between the IR and IGF-IR. There is evidence that there may be different signaling pathways emanating from the carboxy tail of the two receptors. Baserga [21] summarized amino-acid residues in β subunit of the IGF-I receptor that are required for transformation (as an ability to form colonies in soft agar) and/or protection from apoptosis (as protection of FL5.12 cells from apoptosis induced by IL-3 withdrawal or 3T3 cells from okadaic acid-induced apoptosis) using a series of the IGF-I receptor mutants. These residues include: tyrosine 1250, 1251, and a cluster of serines (1280–1283). Tyrosine 1251 and serine cluster are not represented in the human insulin receptor. However, mutations at tyrosine residues 950 or in the tyrosine cluster (1131, 1135, and 1136) within kinase domain, remained capable of suppressing apoptosis, although such mutations are known to inacti-

vate transforming functions [22]. The tyrosine kinase activity of the insulin receptor has been shown through kinase-inactivating point mutations to be essential for insulin signal transduction (tyrosine residues 1158, 1162, and 1163) [23, 24]. The initial event induced by IGF-I binding is the autophosphorylation of β subunit on tyrosines 1131, 1135, and 1136 in the highly conserved tyrosine kinase region [25]. Although the role of the phosphotyrosine residues in the juxtamembrane region has not been fully elucidated, it is well established that phosphorylation of these three tyrosines enhances the tyrosine kinase activity. The essential role of ligand-stimulated autophosphorylation was demonstrated by substitution of the lysine in the ATP-binding site (glycine rich region of β subunit) with either alanine or arginine, which results that either insulin or IGF-I receptor is unable to induce the tyrosine kinase activity. This also blocks all IGF-I induced actions regarding substrate phosphorylation and activation of signal transducers involved in mitogenic activity and transformation [26].

In 1994, Hubbard and colleagues [27] solved the three dimensional structure of the human insulin-receptor tyrosine kinase domain explaining how receptor tyrosine kinase works. They used the multiwavelength anomalous

diffraction (MAD) phasing method to determine the crystal structure of unphosphorylated (inactive), apo form of the tyrosine kinase domain of the receptor β subunit (306-residue fragment). The insulin receptor tyrosine kinase region revealed those features that are characteristic of members of the protein tyrosine kinase family including IGF-I receptor tyrosine kinase (crystal structure has not been determined). Molecular surface of the tyrosine kinase domain of the human insulin receptor is created by catalytic and activation loops. The activation loop contains three tyrosine residues which are autophosphorylated in response to insulin. In the nonphosphorylated form tyrosine 1162 is bound in the active site of the kinase, acting as an autoinhibitor. The side-chain hydroxyl group of Tyr 1162 is hydrogen-bonded to conserved residues Asp 1132 and Arg 1136 in the catalytic loop. A network of interactions involving residues of catalytic loop hold Tyr 1162 in a position that blocks access to the active site. The insertion of Tyr 1162 into active site not only excludes the entry of exogenous substrates, e.g., IRS-I, but precludes ATP-binding, and as a consequence represses kinase activity [27]. Insulin binding is postulated to move the tyrosine kinase domains closer, and thereby allows transient cross phosphorylation of Tyr 1162 and adjacent tyrosine residues in the activation loop [28]. Upon phosphorylation, phosphorylated activation loop is stabilized in a new conformation displacing Tyr 1162 from the active site. These changes promote the binding of ATP and exogenous substrate to the β subunit of insulin receptor.

RECEPTOR SUBSTRATES

14-3-3 Proteins

Understanding of the mechanism by which the IGFs elicit their mitogenic effect and how this differs from insulin signal transduction requires the identification and characterization of the proteins which interact with the IGF-IR *in vivo*. One year ago, we described recent work from our laboratories and from other investigators that provides evidence for selective interaction of three proteins (14-3-3, Grb10, and MAD2) with either the IGF-IR or the IR [2, 3]. To identify such proteins, we have used a yeast two-hybrid assay, the interaction trap [29, 30] to screen a human fetal brain cDNA library for proteins which bind to the cytoplasmic domain of the IGF-IR. Briefly, yeast are co-transformed with a DNA binding domain plasmid containing the cDNA of the cytoplasmic domain of the IGF-IR and activation domain plasmid containing a cDNA library. If a protein encoded by a cDNA in the library binds to the cytoplasmic domain of the IGF-IR, a functional transcription factor (DNA binding domain plus activation domain) is reconstituted and transcription of two reporter genes, lacZ (β-glactosidase activity) and LEU2 (colony growth on leucine dropout plates) is stimulated. This analysis identified 14-3-3β and ζ proteins when cytoplasmic domain of the IGF-IR was co-expressed. 14-3-3β also binds to the IGF-IR but not the IR *in vitro* and 14-3-3/IGF-IR complexes are present in insect cells over-expressing the IGF-IR cytoplasmic domain. 14-3-3 Proteins are substrates of the IGF-IR in the yeast system and *in vitro*. The interaction of 14-3-3 with the IGF-IR requires receptor kinase activity and maps to the carboxy-terminus of the receptor, but does not depend on tyrosine residues in this or the juxtamembrane regions. Instead, the binding maps to serine residue 1283 and requires phosphorylation of this residue. Also, Craparo *et al.* [31] observed that 14-3-3 ε interacts with phosphoserine based motif in the IGF-IR but does not bind to IR. By binding to phosphoserine motifs (RSXpSXP), 14-3-3 proteins function as effectors of serine phosphorylation [32]. A similar motif (SSSpSLP) was identified as a binding site in the IGF-I receptor [2, 29]. Many proteins are known to bind to 14-3-3

proteins, and the list of proteins continues to expand. In few cases, the function of 14-3-3 binding has been determined. For example, 14-3-3 binding to proapoptotic protein BAD (Bcl-XL/Bcl-2- associated Death Promoter) blocks the interaction of BAD with antiapoptotic protein Bcl-2 [33]. In another case, 14-3-3 binding to Cdc25C phosphatase prevents it from activating Cdc2 kinase [34, 35]. Several other proteins include Bcr, Bcr-Abl, middle-T antigen, Ksr serine kinase, Raf, PKC, PI-3 kinase, A-20, and ASK1 kinase that activates SAPK/JNK and p38 pathways [32, 36–38]. The X-ray crystallography of 14-3-3 ζ and 14-3-3 τ shows a dimer in which bundles of antiparallel helices form an amphipathic groove which is large enough to accept two α helices [39, 40]. This structure suggests that 14-3-3 complexed as homodimers or heterodimers, could function as a bridge to hold signal complexes together. Recently, Avruch's group [41] has shown that the ability of 14-3-3 to support Raf activity is dependent on phosphorylation of serine residues on Raf and on the integrity of the 14-3-3 dimer. A monomeric mutant form of 14-3-3 protein did not activate Raf *in vitro* or restore Raf activity after displacement of 14-3-3 despite capability to bind Raf [41].

We also have shown that 14-3-3β is a substrate for the receptor tyrosine kinase [29]. Perhaps tyrosine-phosphorylated 14-3-3 is released rapidly from the receptor and then performs another function in the cell. In any case, the observation that 14-3-3 proteins interact with the IGF-IR but not with the insulin receptor may lead to an understanding of some of the differences in signaling pathways emanating from these two receptors.

Grb 10

Two other clones that were identified in the screen of human fetal brain library, which we used in the yeast two-hybrid system, encoded the SH2 domain of human Grb10, an adaptor protein which had previously been showed to bind to the EGF receptor and the Ret receptor [30]. Smith's laboratory has provided evidence for preferential binding of Grb10 to the IR in intact cells, suggesting that Grb10 may be selectively involved in signaling pathways emanating from IR (Fig. 2) [42]. Recently, Dong *et al.* [43] observed that unlike other SH2-domain-containing proteins, which bind to either the juxtamembrane domain or the carboxy terminal region of the IR or IGF-IR, human Grb10 binds specifically to the autophosphorylated tyrosine residues in the kinase domain of these receptors. To characterize the structural basis for the interaction between Grb10 and the IR or IGF-IR, they used different mutant receptors containing a segment of deletion in either the juxtamembrane region or in carboxy terminal part of the receptors, or containing Tyr-Phe point mutations in these domains. Because the autophosphorylated tyrosine kinase domain of the re-
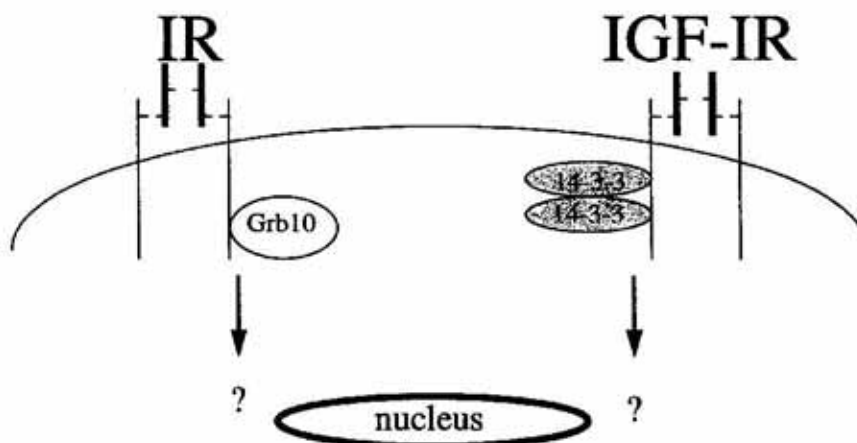

Figure 2. Preferential association of Grb10 with the IR and 14-3-3 with the IGF-IR.

ceptors are critical for receptor kinase activity, the direct binding of human Grb10 at these sites may provide a mechanism for the regulation of insulin receptor signaling [43].

55 PIK and SOCS proteins

Using the interaction trap screen, Furlanetto's and Nissley's groups [44, 45] also identified cDNA inserts encoding all or part of several other proteins. All unique clones were isolated and tested for their interaction with tyrosine kinase active IGF-IR, a kinase-negative mutant in which lysine 1003 in the ATP-binding region was changed to arginine, and wild type IR baits. These clones encoded the SH2 domain of p85 subunit of PI-3 kinase, 55 PIK, and SOCS (suppressor of cytokine signaling)-2. The hp55γ (human homolog of mouse p55PIK) protein interacts strongly with activated IGF-IR but not with the kinase-negative mutant receptor. However, hp55γ also interacts with the IR in the yeast two hybrid system [44]. The SOCS-2 interacted strongly with activated IGF-IR and not with a kinase negative mutant receptor. Mutation of receptor tyrosines 950, 1250, 1251, and 1316 to phenylalanine or deletion of C-terminal 93 amino acids did not result in decreased interaction of the receptor with SOCS-2. hSOCS-1 protein also interacted strongly with IGF-IR in the two-hybrid system. GST-SOCS-2 was associated with activated IGF-IR in lysates of mouse fibroblasts over-expressing IGF-IR and IGF-IR was coimmunoprecipitated with FLAG epitope-tagged SOCS-2 after IGF-I stimulation in human embryonic kidney cells (293) transiently transfected with IGF-IR and FLAG-SOCS-2 [45]. However, SOCS-2 also interacts with the IR (unpublished data). SOCS protein family has been shown to inhibit the JAK/STAT pathway [46, 47]. Recently, two laboratories have identified JAK 2 and STAT5b in yeast two-hybrid library screens using IR as bait [48, 49]. This supports the possibility that SOCS proteins could also function in regulating receptors (such as IR

and IGF-IR) not belonging to the cytokine receptor class.

CONCLUDING REMARKS

Recently DAF-2 receptor which plays an important role in regulating the life span of *Coenorhabditis elegans* has been identified [50, 51]. That insulin/IGF-I receptor homolog is 35% identical to human IR and 34% identical to IGF-IR. Decreasing DAF-2 activity causes fertile adult worms to remain active much longer than normal and to live more than twice as long [52]. This receptor, found in a nematode probably represents the ancestor of IR and IGF-IR, which have diverged during evolution from the function in the regulation of aging so that the modern IR regulates glucose and fuel metabolism and the modern IGF-IR regulates growth.

In summary, 14-3-3 proteins, and Grb 10 appear to interact selectively with either IR or IGF-IR. Therefore, they are good candidates for being signaling molecules on pathways that distinguish IR and IGF-IR. It seems likely that the distal portions of signal transduction pathways for these receptors are shared between these receptors (and with other growth factors), but that some of the more proximal effectors may be more specific for IR or IGF-IR.

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