Insulin Action

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Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors

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Abstract

The physiological roles of insulin and insulin-like growth factors (IGFs) are distinct, with insulin acting to regulate cellular uptake and metabolism of fuels, whereas IGFs promote cell growth, survival and differentiation. The only components of signalling pathways known to be unique to insulin and IGFs are their respective receptors, and even these display substantial structural and functional similarity. Specificity of action *in vivo* must in part reflect relative levels of receptor expression in different tissues. The extent to which the receptors differ in intrinsic signalling capacity remains unclear, but specificity might in principle arise from differences in ligand-binding mechanism or properties of intracellular domains. To identify ligand binding determinants we expressed receptor fragments as soluble proteins. Both N-terminal domains and a C-terminal peptide sequence from the α-subunit are essential for ligand binding with moderate affinity. However, binding of ligand with high affinity and specificity requires higher-order structure. To compare signalling capacities, we constructed chimaeras containing intracellular domains of insulin or IGF receptors fused to the extracellular portion of TrkC. Expression and activation of these chimaeras in cell lines reveals subtle differences in signalling and end-point responses, which may depend on cell background.

Key words: chimaeric receptors, domain structure, glucose transport, hybrid receptors, tyrosine kinase.

Abbreviations used: IR, insulin receptor; IGF, insulin-like growth factor; IGFR, type I IGF receptor; TK, tyrosine kinase; IRR, IR-related receptor; CR, cysteine-rich; JM, juxtamembrane; CT, C-terminal; IRS, IR substrate; SH, Src homology; PI 3-kinase; phosphoinositide 3-kinase; PKB, protein kinase B; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; PH, pleckstrin homology; APS, adaptor protein containing PH and SH2 domains; JAK, Janus kinase; SOCS, suppressors of cytokine signalling; CAP, c-Cbl-associated protein; PTB, phosphotyrosine binding.

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Introduction

The most conspicuous actions of insulin in mammals are on the metabolism of carbohydrate, lipid and protein in muscle, adipose tissue and liver, although longer-term effects on the transcription of regulatory enzymes, especially in liver, are also important. In contrast, the major role of insulin-like growth factors (IGF)s, acting on many different tissues, is in the promotion of cell growth, survival and differentiation. Nevertheless, the insulin receptor (IR) and type I IGF receptor (IGFR) appear to mediate very similar biological effects within a given cell background *in vitro*. It remains an open question whether differences in receptor function, as opposed to receptor distribution, contribute to the undoubted specificity of action of insulin and IGFs *in vivo*. This review will consider briefly the common features of IR/IGFR structure and function, before turning to the evidence for, and mechanistic basis of, signalling specificity.

IR/IGFR structure and intracellular signalling

The IR and IGFR, together with the closely related IRR (insulin-receptor-related receptor), form a subgroup of mammalian receptor tyrosine kinases (TKs) sharing approx. 55% sequence identity that are distinguished by the proteolytic cleavage and disulphide linkage of pro-receptor monomers to generate mature receptors with a

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β-α-α-β subunit structure [1]. Invertebrates (*Drosophila melanogaster* and *Caenorhabditis elegans*) possess only a single receptor for insulin/IGF-like peptides, which co-ordinates growth and development in response to nutritional status [2].

The IR and IGFR are relatively specific for their respective ligands, although at high concentrations insulin cross-reacts with IGFR and IGFs with IR. Both receptors are widely expressed in mammalian tissues, albeit at varying levels. In cells expressing both IR and IGFR, pro-receptors undergo hetero- as well as homo-dimerization, creating IR–IGFR hybrids alongside classical IRs and IGFRs [3–5]. Heterodimers appear to assemble with comparable efficiency to homodimers, and when either receptor is in substantial excess the less abundant receptor is drawn almost entirely into hybrids. Thus in skeletal muscle IGFRs are present largely as hybrids, together with an excess of classical IRs [6], whereas in fibroblasts IRs are present largely as hybrids with an excess of classical IGFRs (B. Navé, K. Dib and K. Siddle, unpublished work). Hybrid receptors bind both insulin and IGF-I, although they have lower affinity for insulin than classical IR does [7]. Binding of either ligand leads to autophosphorylation, and presumably activation, of both TKs within hybrids [8], but outcomes in terms of biological responses have not been studied.

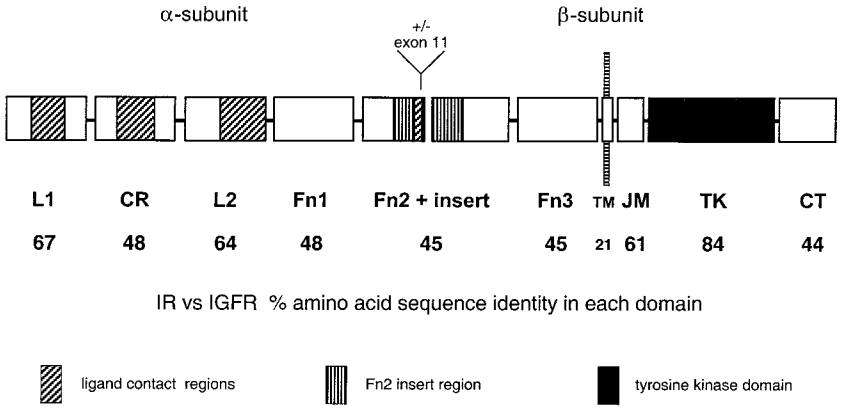
Considerable progress has been made in elucidating the structure of the extracellular portion of IR and IGFR. Sequence analysis and molecular modelling [9] predicts six structural domains of approx. 150 amino acids, designated L1, CR (cysteine-rich), L2, Fn1, Fn2 and Fn3 (Figure 1). The Fn2 domain has a large (135 amino acids) insertion, containing the site of cleavage between α- and β-subunits and, adjacent to this in the IR, the sequence encoded by the alternatively spliced exon 11. A crystal structure has been solved for a 462-amino-acid N-terminal fragment of the IGFR, revealing the L1, CR and L2 domains surrounding a central space with dimensions sufficient to accommodate a ligand molecule [10]. However, this IGFR fragment does not itself bind IGF-I, and it is uncertain whether the orientation of domains in the crystal structure is the same as in native receptor.

Within the intracellular portion of the IR and IGFR, the TK domain proper is flanked by juxtamembrane (JM) and C-terminal (CT) domains (Figure 1). The TK domains are the most highly conserved regions of the receptors, with 84% amino acid identity. Ligand-induced activation occurs via intramolecular *trans*-autophosphorylation between β-subunits, and crystal structures have been solved for the IR TK domain in both basal and activated states [11]. The JM domains contain an autophosphorylation site in an Asn-Pro-Glu-Tyr (NPEY) motif that participates in substrate binding. The function of CT domains remains ill-defined, and signalling is not dramatically affected by either deletion or overexpression of CT sequences [12,13]. However, the

Figure 1

Structural domains of IR and IGFR

The structural domains of the IR and IGFR are indicated as L1, L2 (β-helices), CR (cysteine-rich), Fn1, Fn2, Fn3 (fibronectin type III; seven-stranded β-sandwich), TM (transmembrane), JM (juxtamembrane), TK (tyrosine kinase), and CT (C-terminal). The Fn2 domain additionally has an insert region containing the site of cleavage between α- and β-subunits, the alternatively spliced IR exon 11, and a key ligand contact site. The similarity between IR and IGFR is indicated as the percentage amino acid sequence identity within each domain.



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greater sequence divergence of CT compared with TK and JM domains is consistent with a role in signalling specificity or receptor-specific regulation.

A distinguishing feature of signalling by the IR/IGFR family is the dependence on phosphorylation of intracellular substrates, as opposed to autophosphorylation, for recruitment of downstream signalling components [14–16]. Both IR and IGFR phosphorylate IR substrates (IRS-1–IRS-4) and Shc (Src homology/collagen) proteins (p46/p52/p66), triggering two major signalling pathways. Phosphorylated IRSs recruit and activate class 1a phosphoinositide 3-kinase (PI 3-kinase) via SH2 domains of the adaptor protein p85, leading to synthesis of membrane-associated PtdIns(3,4,5)P_3. This in turn recruits and activates phosphoinositide-dependent kinases (PDKs), which then phosphorylate and activate other protein kinases, including Akt/protein kinase B (PKB), p70rsk and protein kinase Cζ (PKCζ) [17]. A number of other PtdIns(3,4,5)P_3-binding proteins and potential effector systems have been identified within cells, including the small G-protein Rac [18] and dual adaptors for phosphotyrosine and phosphoinositides [19], and the role of these proteins in insulin signalling awaits clarification. There is substantial evidence that PI 3-kinase activity mediates a wide range of insulin/IGF effects, including stimulation of glucose transport, glycogen synthesis, protein synthesis and mitogenesis, inhibition of apoptosis, and regulation of gene transcription [20]. A second signalling pathway involves recruitment to both IRS-1 and Shc of the guanine-nucleotide-exchange factor Sos, via the SH2 domain of the adaptor Grb2. This leads to activation of the small G-protein Ras, which in turn activates the protein serine kinase Raf and the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade. The role of this cascade in insulin/IGF action is limited, although it has been implicated in anti-apoptotic signalling and transcriptional regulation [21]. There is evidence for cross-talk between the two arms of IR/IGFR signalling, especially in relation to interactions between PI 3-kinase and Ras [20]. The signalling contributions of other proteins bound by phosphorylated IRSs, including the phosphotyrosine phosphatase SHP-2, the TK Fyn, and the SH3-containing adaptor Nck, are yet to be clearly defined.

Although the major IR/IGFR signalling pathways involve recruitment of effectors by tyrosine-phosphorylated IRS and/or Shc, there may also be a role for proteins that interact directly with the receptors. In principle, such proteins might initiate distinct signalling pathways acting independently of, or in synergy with, those mediated by IRS and Shc. Alternatively, they might modulate TK activity and signalling via these established substrates, or participate in other aspects of receptor function such as endocytosis and degradation. It is an obvious possibility that differential interaction of proteins with IR and IGFR might contribute to signalling specificity.

The SH2/pleckstrin homology (PH)-domain-containing adaptors SH2-B/PSM and APS (adaptor protein containing pleckstrin homology and SH2 domains) interact with phosphotyrosines in the conserved activation loop of the IR/IGFR TK, and are substrates for tyrosine phosphorylation [22–27] (see also contribution by T. S. Pillay to this colloquium, pp. 529–534). These adaptors also interact with other receptor TKs [28–31], and in terms of cellular distribution and efficiency of phosphorylation APS appears more likely than SH2-B/PSM to play a significant role in IR/IGFR signalling. There is evidence that these adaptors function positively in signalling to mitogenesis and differentiation [32–34]. However, other data suggest that APS plays a role in c-Cbl-dependent ubiquitination and degradation of the IR [35]. The IGFR interacts with and phosphorylates SH2/SH3-containing c-Crk adaptor proteins, mediated predominantly via JM tyrosines [36–38]. Insulin also stimulates phosphorylation of these proteins, but less efficiently than IGF-I does. Tyrosine phosphorylation of Crk may modulate its interaction with guanine-nucleotide-exchange factors, and thus influence the activity of small G-proteins acting on Raf kinase. This pathway thus has the potential to enhance IGFR-dependent mitogenic signalling [39,40]. Phospholipase Cγ has also been reported to associate with IR via undefined sites, and to play a role in both metabolic and mitogenic signalling [41]. A variety of SH2-domain-containing proteins bind to the conserved CT autophosphorylation site of IR and IGFR *in vitro*, including the p85 subunit of PI 3-kinase, GTPase-activating protein (GAP), phosphotyrosine-specific phosphatase SHP2 and CT Src kinase (CSK) [42–46]. However, there is no evidence that these interactions play a significant role *in vivo*.

Several components of cytokine-signalling pathways interact also with IR and/or IGFR. The Janus kinases JAK-1 and JAK-2 bind to and are

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phosphorylated by activated IR and IGFR, and in turn phosphorylate IRS-1 at distinct sites from IR/IGFR [47,48]. The JAK substrates and transcription factors Stat5B and Stat3 also bind to and are phosphorylated by IR [49–51]. SOCS (suppressors of cytokine signalling) proteins, which negatively regulate JAK/Stat pathways, have yielded confusing results. SOCS-1 and -2 were identified as binding partners for IGFR, dependent on receptor activation but not JM Tyr^{950} [52]. SOCS-3 was shown to bind to activated IR, but in this case binding was dependent on the analogous JM Tyr^{960} [53]. Another study reported that SOCS-3 interacted constitutively with both IR and IGFR and, unlike SOCS-2, was phosphorylated upon IGFR activation [54].

Of particular interest is the Grb7/Grb10/Grb14 family of PH/SH2-domain-containing adaptors, of which Grb10 has received most attention. These proteins interact directly with IR and IGFR, via both SH2 and BPS domains of the adaptor and CT and TK domains of the receptor [55–60], and binding is preferential for IR compared with IGFR [61]. Grb10 is not phosphorylated by IR/IGFR, but phosphorylation by TKs of the Src family negatively regulates binding to IR [62]. The role of Grb10 remains obscure, with some studies reporting inhibition of receptor function [55,59,63,64] or of specific signalling pathways [65], but with others suggesting enhancement of mitogenic signalling [56,66]. In either case, the effects of Grb10 were not confined to IR, but were also seen with IGFR, although the potential for differential effects may have been obscured by overexpression. Grb10 may also interact with Ras, Raf and MEK (MAPK/ERK kinase), consistent with a role in signalling [67,68]. Additionally, Grb10 may bind proteins involved in ubiquitination, suggesting a role in targeting receptors for degradation [69].

Various isoforms of 14-3-3 proteins bind to activated IGFR, but not to IR, via Ser^{1272} and/or Ser^{1283}, which are unique to IGFR [70,71]. The consequences of this interaction for IGFR signalling remain to be determined. A human homologue of the yeast protein MAD2, which has been implicated as a cell cycle check-point regulatory protein, was reported to bind to the CT segment of unstimulated IR, but not to IGFR [72]. The interaction was decreased following receptor activation, but the significance for insulin signalling remains obscure.

A novel signalling pathway involving CAP (c-Cbl-associated protein) has recently been proposed to contribute to insulin action on glucose transport and glycogen synthesis, in synergy with PI 3-kinase-dependent pathways [73–75]. CAP interacts constitutively with IR within caveolin-rich lipid rafts, and mediates phosphorylation of c-Cbl, leading to recruitment of Crk and associated guanine-nucleotide-exchange factors [76]. The mechanism of interaction between CAP and IR is unclear because CAP lacks SH2 domains, although it does contain three SH3 domains. It remains to be determined whether CAP interacts similarly with IGFR.

Studies going back over many years have implicated heterotrimeric G-proteins in insulin action [77]. There has recently been renewed interest in this area, with evidence that IR and IGFR may interact with different G-proteins. $G_{\alpha q}$ has been shown to be a substrate for the IR TK, and has been implicated in insulin signalling to glucose transport [78]. In contrast, IGFR, but not IR, appears to be constitutively associated with G_i, and to release $G_{\beta\gamma}$ subunits following receptor activation [79,80]. $G_{\alpha i}$ has been implicated in IGFR-mediated stimulation of MAPK and mitogenesis [80–82], but appears not to be involved in IR stimulation of mitogenesis or in GLUT4 translocation [80].

Evidence for differential responses to IR and IGFR

The original presumption that IR signals primarily acute 'metabolic' responses, whereas the IGFR induces longer-term 'mitogenic' effects, was largely on the basis of the most obvious actions of insulin and IGFs *in vivo*. However, it is now clear that specificity *in vivo* of insulin and IGFs must reflect at least in part the levels of expression of IR and IGFR in target tissues. Hepatocytes, adipocytes and skeletal muscle, the major targets for insulin action, express high levels of IR and low levels of IGFR, whereas the opposite is true for fibroblasts and various types of undifferentiated progenitor cells. However, both IR and IGFR (and therefore also IR/IGFR hybrids) are widely expressed, and a widespread belief persists that specificity also derives substantially from differences in intrinsic signalling capacity of the receptors, notwithstanding the many common elements of signalling [83].

When activities of IR and IGFR are compared *in vitro* by detailed dose–response experiments, selective blockade with specific antibodies or overexpression in transfected cells, it is apparent that the receptors are very similar in

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signalling potential within a given cell background [84]. Indeed, IR and IGFR elicit the same spectrum of 'metabolic' and 'mitogenic' effects in diverse cell types, including fibroblasts, hepatocytes, myoblasts and adipopcytes [85–93]. Moreover, IGFs have been shown to elicit insulin-like metabolic effects *in vivo* in mice and humans lacking functional IR [94,95], and IR has been shown to function in mitogenic signalling *in vivo*, particularly in response to IGF-II [96,97]. There have not been any convincing demonstrations of biological responses that are unique to the IR or IGFR. However, the list of responses that have been studied in any detail is not a long one and the existence of some all-or-nothing differences (for instance, in relation to the expression of specific genes) cannot yet be ruled out.

It has proved surprisingly difficult to establish conclusively whether or not there are more subtle differences in the efficiency with which IR and IGFR mediate biological responses. The range and magnitude of responses available for study in cultured cells is very limited, and typically excludes the most characteristic responses to insulin *in vivo*; for instance, those involving translocation of GLUT4 glucose transporters or inhibition of lipolysis. Studies dependent on endogenous IR and IGFR may be biased by differences in levels of expression and in affinity of the receptors for their respective ligands. More importantly, such studies cannot take account of the contribution of IR/IGFR hybrids, which might obscure differences in signalling by classical IR and IGFR. Overexpression of receptors by cDNA transfection circumvents some of these problems, but may override differences in activity that would be apparent at 'physiological' levels of receptor expression. Moreover, studies of transfected cells are susceptible to problems of random clonal variation unrelated to the expression of receptors, and findings have not always been replicated in different laboratories.

Notwithstanding these problems, there is a substantial body of evidence (reviewed below) that IR and IGFR do differ in the efficiency with which they mediate at least some biological responses. The challenges now are to determine whether such relative specificity is a consequence of differences in extracellular ligand binding or intracellular signalling capacity, and whether it involves differences in efficiency of coupling with a common set of signalling components or the participation of additional receptor-specific signalling pathways.

Extracellular ligand binding

Stimulation of intracellular signalling pathways may be influenced both quantitatively and qualitatively by the kinetics of ligand binding and the persistence of receptor occupancy. For instance, the downstream consequences of ERK/MAPK activation by receptor TKs depend on whether activation is transient and largely restricted to the cytosol, or persistent, allowing more effective nuclear translocation [98–100]. Studies of insulin analogues with different dissociation kinetics demonstrated that, whereas potency for stimulation of glucose metabolism paralleled equilibrium binding affinity, mitogenic potency correlated with dissociation rate, so that slow-dissociating analogues exhibited enhanced mitogenic relative to metabolic responses [101,102]. Slow-dissociating analogues induced more sustained phosphorylation of Shc, but not IRS-1, when compared with normal insulin [102]. Interestingly, phosphorylation of Shc, but not IRS-1, is dependent on receptor internalization [103,104]. The persistence of IR TK activation within the endosomal compartment [105,106] must depend on the rate of ligand dissociation as early endosomes become acidified. Thus the ability of slow-dissociating ligands to induce persistent Shc phosphorylation and an enhanced mitogenic response may reflect the location as well as the extent of receptor activation. It has been proposed that differences in the mitogenic signalling capacities of IR and IGFR may reflect the binding kinetics of the ligands as much as the intrinsic specificity of the receptor TKs [107–109]. Interestingly, it has been reported that IGF-II is not only a high-affinity ligand for the A (exon 11−) isoform of IR, but elicits a more effective mitogenic stimulation than insulin [97]. Direct evidence that IGFs and insulin differ in rates of dissociation from their respective receptors, at either neutral (cell surface) or acidic (endosomal) pH, would add considerable weight to the hypothesis that signalling specificity is imposed by differences in ligand–receptor interaction.

More speculatively, it has been suggested that differences in the mechanism of binding of IGF-I and insulin to their respective receptors may influence the nature as well as the extent of receptor activation [107]. Various lines of evidence suggest that a single molecule of ligand (insulin or IGF) makes asymmetric contacts with both α-subunits within a native receptor heterotetramer [107,110]. It is not known whether cross-linking is

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essential for receptor activation, or whether contacts with a single α-subunit suffice to trigger some conformational change. It is possible that there are distinct components of receptor activation that depend on different aspects of ligand binding, and that differences in the mechanisms of binding of insulin and IGF could influence signalling outcome from the respective receptors.

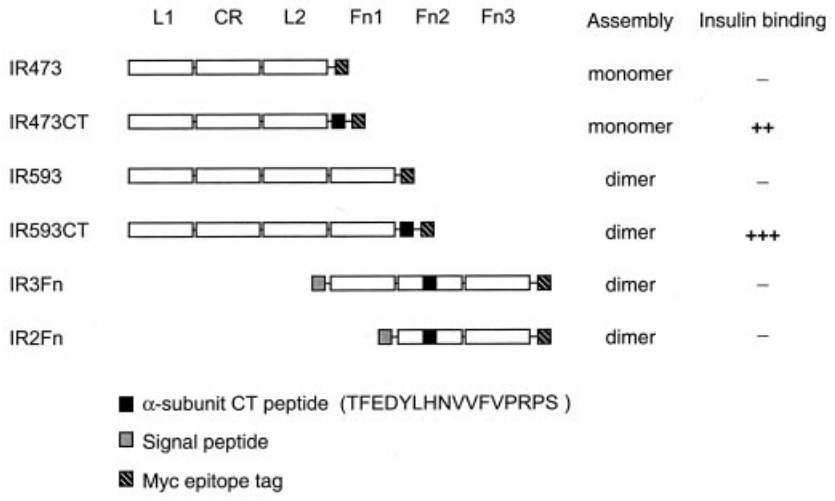
The IR and IGFR display broadly similar affinities (0.1–1 nM at neutral pH), pH dependence and negative co-operativity [107], but differ in other aspects of ligand binding. For instance, IR binds only a single molecule of insulin with high affinity, but the situation is not so clear-cut for IGFR [1,111]. Reduction of α–α disulphides and separation into half-receptors markedly reduces binding affinity of IR for insulin, but not of IGFR for IGF-I, and IR/IGFR hybrids exhibit relatively low affinity for insulin, but retain high affinity for IGF-I [7]. Four regions of primary sequence have been identified as ligand-contact sites by photoaffinity cross-linking, analysis of IR/IGFR chimaeras and alanine-scanning mutagenesis [112–117]. Three of these regions, involving faces of the L1, CR and L2 domains, surround a central space in the structure of an N-terminal fragment of IGFR. Residues in the L1 domain are major determinants of insulin-binding specificity, whereas the CR domain plays a more important role in determining specificity for IGF-I. The fourth contact region, a relatively short sequence at the C-terminus of the α-subunits, contains conserved residues that are important for binding of both ligands [117,118]. Other residues may influence specificity through inhibitory effects on ligand binding. The B (exon 11+) isoform of the IR has markedly reduced affinity for IGFs compared with the A (exon 11−) isoform, although the isoforms differ very little in their affinity for insulin [119]. It seems that the sequence encoded by exon 11, which lies immediately downstream of the α-subunit CT ligand contact site and is absent in IGFR, exerts a selective negative influence on IGF binding. Although the cross-linking model offers an explanation of many phenomena surrounding insulin/IGF binding, it remains to be demonstrated how the different binding regions contribute to provide the high-affinity insulin binding of native IR, and whether the same model holds true for IGFR.

We have studied ligand binding to receptor fragments in an attempt to delineate the minimum requirements for ligand binding. Earlier studies had demonstrated that free α-subunit bound insulin with moderate affinity, whereas smaller fragments were devoid of insulin-binding activity [120]. Subsequently, it was shown that the Fn1 or connecting domain is not essential for insulin

Figure 2

IR constructs expressed as soluble secreted proteins in CHO cells

Fragments of the IR extracellular region containing integral domains (L1, CR, L2, Fn1, Fn2, Fn3; see Figure 1) were expressed in CHO cells [123]. All fragments contained a CT Myc-epitope tag. An N-terminal signal peptide sequence was added to the IR3Fn and IR2Fn constructs; other constructs possessed the normal IR N-terminus with an endogenous signal sequence. All constructs were secreted efficiently either as monomers or as disulphide-linked dimers. Binding of ^{125}I-labelled insulin was assessed relative to wild-type IR (scored as a proportion of '+++').



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binding [121,122]. We have studied a number of receptor fragments expressed in CHO cells (Figure 2). Fragments consisting of three N-terminal domains (L1, CR, L2), designated IR473 and IGFR462, were efficiently secreted as monomeric soluble proteins and recognized by a range of anti-receptor monoclonal antibodies, suggesting that the individual domains adopted a native conformation [123]. A four-domain fragment (L1, CR, L2, Fn1), designated IR593, was secreted as a disulphide-linked dimer, indicating that the Fn1 domain alone is sufficient to mediate α–α dimerization. We confirmed that there was no measurable binding of radioligands to either the three- or four-domain N-terminal fragments or to the complementary CT fragments containing three (Fn1, Fn2, Fn3) or two (Fn2, Fn3) fibronectin domains [123]. These CT fragments were engineered to include an N-terminal signal sequence, and both were secreted as disulphide-linked dimers that had been at least partially cleaved at or near the normal α/β processing site, indicating that dimerization could be mediated by the Fn2 domain alone. It was concluded that ligand binding required both N-terminal and CT contact sites within the α-subunit.

Studies of receptor fragments with variable internal deletions had suggested that the spacing between N-terminal and CT binding determinants was not critical [122]. We therefore investigated the properties of receptor fragments in which the sequence of 16 amino acids from the α-subunit C-terminus was fused directly to the C-terminus of IR473, IGFR462 and IR593 constructs. Surprisingly, addition of the relevant CT peptide generated moderate-to-high affinity ligand binding in all cases. The IR473.CT and IGFR462.CT monomeric proteins bound their respective ligands with 5–10% of wild-type affinity, comparable with full-length half-receptors, and retained appropriate relative specificity for insulin and IGF-I [123,124]. In fact, the CT sequences of IR and IGFR are interchangeable and contribute to the affinity, but not the specificity, of binding of either ligand [124], confirming earlier studies involving alanine-scanning mutagenesis [117,118]. The dimeric IR593.CT construct displayed even higher affinity, in our hands comparable with wild-type IR, but did not exhibit negative co-operativity of ligand binding (K. H. Surinya, L. Molina and K. Siddle, unpublished work). We additionally found that monoclonal anti-receptor antibodies that stimulated or inhibited insulin binding to wild-type solubilized IR did not have the same effects on IR593.CT, even though this construct retained the relevant antibody epitopes. Other workers found that a sequence corresponding to exon 10 was required, in addition to the CT peptide, to confer full wild-type affinity and negative co-operativity on four-domain IR fragments [125]. Certainly it appears that modulation of insulin binding involves elements additional to those required for high-affinity binding *per se*, probably within membrane-proximal fibronectin domains. The dissection of ligand binding determinants has shown the complex interplay of interactions at multiple sites, but has not as yet revealed fundamental differences in the binding mechanisms of insulin and IGFs that might lead to different activation states of their receptors. However, much remains to be done before this possibility can be ruled out.

Intracellular signalling

To compare the signalling potential of IR and IGFR intracellular domains while avoiding problems of interpretation arising from overexpression or the presence of IR/IGFR hybrids, we have utilized chimaeric receptor constructs. This approach builds on the pioneering studies of Ullrich and colleagues [126], who first demonstrated that receptor TKs could be activated via heterologous ligand-binding domains. It was reported that in NIH3T3 fibroblasts DNA synthesis was induced more efficiently (in terms of dose–response) by overexpressed IGFR compared with IR, and that chimaeras containing the intracellular portion of IGFR fused to the extracellular portion of IR behaved more like IGFR than IR [127]. However, IR and IGFR were indistinguishable in their ability to mediate stimulation of glucose uptake (presumably reflecting activity of GLUT1 transporters in these cells). It is notable that other workers did not observe any differences in signalling by the IR and IRR expressed as chimaeras with the CSF-1 (colony-stimulating factor) receptor in 3T3-L1 adipocytes [128]. There is always a possibility that differential responses might in fact reflect simple clonal variability of cell lines unrelated to the expression of specific receptors, and ideally multiple independently derived clones should be studied in an attempt to minimize this risk.

We constructed chimaeras (designated TIR and TIGR) in which the intracellular portions of IR or IGFR respectively were fused to the extracellular portion of the neurotrophin receptor

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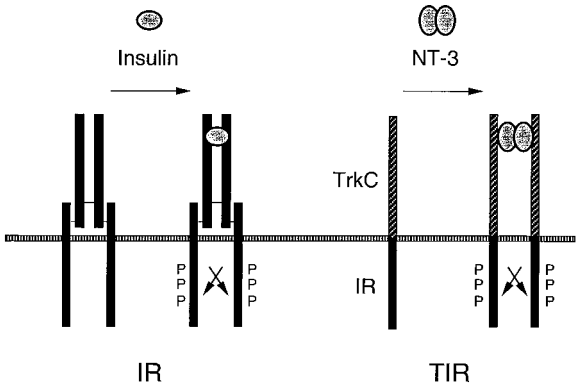
TrkC (Figure 3). We isolated matched clones of murine 3T3-L1 cells stably expressing the chimaeras at levels comparable with endogenous IR and IGFR. Under these conditions there was no detectable constitutive activation, but the chimaeras were efficiently activated by the dimeric TrkC ligand NT-3, which presumably induces chimaera dimerization. The activity of the chimaeras could thus be studied against a null background despite the presence of appreciable levels of endogenous IR and IGFR. In undifferentiated 3T3-L1 fibroblasts, we found no difference between TIR and TIGR chimaeras in their capacity to stimulate DNA synthesis [129] or to protect against apoptosis [130]. However, TIR was markedly more effective than TIGR in mediating stimulation of glycogen synthesis in these cells [129]. It has also been reported that glycogen synthesis is more effectively stimulated by IR than by IGFR in NIH-3T3 cells and in hepatocytes [131,132]. In differentiated 3T3-L1 adipocytes the difference in stimulation of glycogen synthesis by TIR and TIGR was very much less than in fibroblasts [133]. However, we found that TIR induced twice as much stimulation of glucose uptake than did TIGR in adipocytes, and this was paralleled by greater translocation of GLUT4 to the plasma membrane [133], suggesting that IR might signal to a larger pool of intracellular GLUT4 transporters than IGFR. We have recently turned our attention to the possibility that IR and IGFR might differentially regulate the expression of specific genes, through effects on transcription or mRNA stability. Using oligonucleotide and cDNA microarrays representing approx. 12000 genes, we detected some 2000 transcripts whose expression was increased or decreased more than 2-fold by TIR/TIGR stimulation (C. Mulligan, J. Rochford, D. Cope, A. E. Willis, K. Siddle and S. O'Rahilly, unpublished work). Of these, up to 10% appeared to respond differentially in TIR compared with TIGR cells. Although some of these apparent differences may have been artefactual because of problems in quantification of low basal signals, this approach has great potential in scanning for truly specific effects of IR compared with IGFR.

The aspects of receptor structure that confer differential signalling potential remain to be identified, but in principle any or all of the JM, TK and CT domains might be involved. The JM domain is important for recruitment of substrates via its phosphorylated NPEY motif [134,135]. However, not only the NPEY motif itself but also the

Figure 3

Structure and activation of chimaeric receptors expressed in 3T3-L1 cells

Chimaeric receptors contain the extracellular ligand-binding domain of the neurotrophin receptor TrkC fused to the intracellular domain of the IR (TIR chimaera) or IGFR (TIGR chimaera) [129]. Wild-type IRs exist as disulphide-linked dimers, and are activated by intramolecular *trans*-phosphorylation upon binding of monomeric insulin ligand. Chimaeric receptors are monomeric in the basal state, but dimerize and are activated upon binding dimeric NT-3 ligand.



flanking residues that determine affinity for the phosphotyrosine binding (PTB) domains of IRS-1 and Shc are highly conserved between IR and IGFR, so the receptors would not be expected to differ in their recruitment of substrates via PTB domains. IRS-2 has a second interaction domain that has no counterpart in IRS-1 and is thought to bind to the TK domain itself, although there is no evidence for a differential interaction with IR and IGFR [136–138]. The TK domains are the most highly conserved regions of the receptors, and although some differences in the specificity of IR and IGFR TKs towards peptide substrates have been reported [139], these have not been confirmed [140]. The IR and IGFR TKs phosphorylate the same sites on recombinant IRS-1 *in vitro* [141], although this does not exclude the possibility that serine phosphorylation or subcellular compartmentation of IRSs might create differences in susceptibility to phosphorylation by IR and IGFR within intact cells.

The greatest sequence divergence between IR and IGFR is in the CT domains, suggesting that this region may be a source of signalling specificity. Truncation or mutation of the CT domain does not in general dramatically affect IR or IGFR function, although some effects on signalling efficiency have been reported [142]. For instance, studies with chimaeras in which CT

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domains were exchanged between IR and IGFR concluded that the CT domain was responsible for the greater efficiency of IR in stimulating glycogen synthesis [131] and pyruvate dehydrogenase [143], although no clear conclusions could be drawn concerning mitogenic signalling [144]. Attention has focused particularly on IGFR Tyr^{1250} and Tyr^{1251} and Ser^{1280}, Ser^{1281}, Ser^{1282} and Ser^{1283} that have no equivalent in IR, and IR Tyr^{1316} that is absent in IGFR. Direct evidence for ligand-stimulated phosphorylation of IGFR Tyr^{1250} and Tyr^{1251} or IGFR Ser^{1280}/Ser^{1281}/Ser^{1282}/Ser^{1283} is lacking, although IR Tyr^{1316} is a confirmed site of autophosphorylation. Phosphorylation at any of these sites might in principle selectively modulate the activity of the receptor TKs towards endogenous substrates, although mutation of the sites does not affect phosphorylation of IRS-1, Shc or the receptors themselves [145,146]. Alternatively, phosphorylation might create binding sites for direct association of regulatory proteins such as 14-3-3 [70].

Site-directed mutagenesis implicated the IGFR-specific tyrosine residues (Tyr^{1250} and Tyr^{1251}) in transformation and tumorigenesis and, less consistently, in mitogenic effects [145,146]. Mutation of Tyr^{1251} did not affect activation of PI 3-kinase or MAPK [147,148], but led to disruption of the cytoskeleton [149]. The quartet of IGFR-specific serine residues (Ser^{1280}/Ser^{1281}/Ser^{1282}/Ser^{1283}) has been implicated both in transformation [150] and in an alternative IGFR-specific anti-apoptotic pathway that is revealed in IRS-1-deficient 32D cells, in which operation of PI 3-kinase/PKB-dependent pathways is compromised [151,152]. These results illustrate that where there is a redundancy of signalling pathways to a given biological response, the operation of receptor-specific pathways may be obscured by simultaneous activation of pathways common to both IR and IGFR.

Substitution of the CT tyrosines (Tyr^{1316} and Tyr^{1322}) of IR has been reported to enhance activation of MAPK and mitogenic signalling, in part due to decreased expression of MAPK phosphatase-1 [153–155]. The IR-specific residue Tyr^{1316} has also been shown to facilitate the phosphorylation of pp120, a specific IR substrate in hepatocytes [156]. The function of this protein is uncertain, but it has been implicated in down-regulating mitogenic signalling by the IR. These results indicate that signalling specificity may arise through suppression as well as activation of signalling pathways.

At a mechanistic level, there is evidence both for differential effects of IR and IGFR on established signalling pathways and for the existence of novel signalling components that may be receptor-specific. In 3T3-L1 adipocytes, we found that the TIR chimaera mediated greater IRS-1 phosphorylation and association with class Ia (p85/p110) PI 3-kinase compared with TIGR, correlating with the stimulation of glucose uptake [133]. The IR chimaera was also more effective in stimulating class II PI 3-kinase activity [157]. Conversely, the TIGR chimaera mediated greater phosphorylation of p52 Shc, recruitment of Grb2 and stimulation of MAPK activity than did the TIR chimaera [133]. Surprisingly, the two chimaeras elicited similar activation of PI 3-kinase activity assayed in anti-phosphotyrosine immunoprecipitates, and of PKB and p70 S6 kinase, suggesting there may be specific pools of PI 3-kinase activity that are differentially coupled with downstream targets, perhaps reflecting subcellular compartmentation.

Phosphorylation of IRS proteins has also been investigated in hepatocytes, using cell lines derived from wild-type or IR-knockout mice. It was concluded that IRS-2 functions primarily as a substrate for IR and not IGFR, and plays an important role in mediating the metabolic and mitogenic actions of insulin in hepatocytes for which IRS-1 cannot fully substitute [158]. Other studies have also suggested that IRS-1 and IRS-2 are functionally distinct molecules [159,160]. It is not clear which downstream pathways might be unique to IRS-2 in hepatocytes. Both receptors activated Akt/PKB to a similar extent, but there was evidence for an additional IR-specific rapamycin-sensitive pathway for inactivation of glycogen-sensitive kinase 3 (GSK-3) and stimulation of glycogen synthesis that cannot be triggered by IGFR [132]. It is known that multiple mechanisms contribute to the regulation of glycogen synthesis, which may differ in relative significance in different cell types [161].

A protein kinase that is specifically activated by IR and not IGFR in hepatocytes has also been implicated in regulation of the transcription factor FKHR, suggesting a mechanism for differential effects of IR and IGFR on gene expression [162]. In NIH3T3 fibroblasts, IR and IGFR apparently induce increased expression of vascular endothelial growth factor (VEGF) mRNA by distinct mechanisms [163]. Stimulation by IR required PI 3-kinase, but not MAPK, activation, whereas the opposite was the case for IGFR, even though both

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receptors elicited similar stimulations of PI 3-kinase and MAPK. These data suggest that unidentified receptor-specific signalling pathways may synergize with known kinase cascades in mediating transcriptional regulation.

Conclusions

There is as yet no indication that either the IR or IGFR can elicit unique biological responses, although compelling evidence now exists that the two receptors do differ in their effectiveness in mediating some endpoint responses. Differences in both ligand-binding kinetics and coupling to intracellular signalling pathways may contribute to this relative specificity. Differences in signalling may in turn reflect efficiency of activation of common signalling pathways involving phosphorylation of major substrates such as IRS and Shc, or modulation by receptor-specific adaptor proteins. It is tempting to speculate that IR/IGFR hybrids might have unique activity arising from synergy between IR-specific and IGFR-specific pathways.

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Insulin receptor substrate proteins and neuroendocrine function

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Abstract

A family of insulin receptor substrate (IRS) proteins mediates the pleiotropic effects of insulin and insulin-like growth factor 1 (IGF-1) on cellular function by recruiting several intracellular signalling networks. Conventional murine knockout strategies have started to reveal distinct physiological roles for the IRS proteins. Deletion of *Irs1* produces a mild metabolic phenotype with compensated insulin resistance but also causes marked growth retardation. In contrast, mice lacking IRS-2 display nearly normal growth but develop diabetes owing to a combination of peripheral insulin resistance and β-cell failure. As well as the classical metabolic events regulated by insulin signalling pathways, studies in lower organisms have implicated insulin/IGF-1 signalling pathways in the control of food intake and reproductive function. Our analysis of IRS-2 knockout mice shows that female mice are infertile owing to defects in the hypothalamus, pituitary and gonad. IRS-2$^{-/-}$ mice have small, anovulatory ovaries with reduced numbers of follicles. Levels of the pituitary hormones luteinizing hormone and prolactin and gonadal steroids are low in these animals. Pituitaries of IRS-2$^{-/-}$ animals are decreased in size and contain reduced numbers of gonadotrophs. Additionally, IRS-2$^{-/-}$ females display increased food intake and develop obesity, despite elevated leptin levels, suggesting abnormalities in hypothalamic function. Coupled with recent observations that brain-specific deletion of the insulin receptor causes a similar phenotype, these findings implicate IRS signalling pathways in the neuroendocrine regulation of reproduction and energy homeostasis.

Key words: insulin, knockout mouse, metabolism, receptor.
Abbreviations used: IGF, insulin-like growth factor; IRS, insulin receptor substrate; PI-3K, phosphoinositide 3-kinase.
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Introduction

Insulin and insulin-like growth factor 1 (IGF-1) have pleiotropic effects on cellular and organismal function, regulating such processes as metabolism, growth, cell differentiation and survival [1]. Both act via distinct transmembrane receptors with intrinsic tyrosine kinase activity that, once activated, phosphorylate intracellular target molecules [2]. The best defined of these are the insulin receptor substrate (IRS) proteins [2]. These proteins are structurally characterized by C-terminal pleckstrin-homology and phosphotyrosine-binding domains, which are required for high-efficiency coupling to the activated insulin receptor, and an N-terminal region with multiple sites of tyrosine phosphorylation [2]. A family of four mammalian IRS proteins has been identified; homologues of these molecules exist in lower organisms such as *Drosophila*. IRS proteins act as molecular adapters; once phosphorylated on specific tyrosine residues, they recruit a number of SH2 (Src homology 2)-containing effector proteins and thus are able to activate a cascade of intracellular signalling pathways [2]. The best

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