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Review

Structure and function of the type 1 insulin-like growth factor receptor

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Abstract. The type 1 insulin-like growth factor receptor (IGF-1R), a transmembrane tyrosine kinase, is widely expressed across many cell types in foetal and postnatal tissues. Activation of the receptor following binding of the secreted growth factor ligands IGF-1 and IGF-2 elicits a repertoire of cellular responses including proliferation, and the protection of cells from programmed cell death or apoptosis. As a result, signalling through the IGF-1R is the principal pathway responsible for somatic growth in foetal mammals, whereas somatic growth in postnatal animals is achieved through the synergistic interaction of growth hormone and the IGFs. Forced overexpression of the IGF-1R results in the malignant transformation of cultured cells; conversely, downregulation of IGF-1R levels can reverse the transformed phenotype of tumour cells, and may render them sensitive to apoptosis in vivo. Elevated levels of IGF-1R are observed in a variety of human tumour types, whereas epidemiological studies implicate the IGF-1 axis as a predisposing factor in the pathogenesis of human breast and prostate cancer. The IGF-1R has thus emerged as a therapeutic target for the development of antitumour agents. Recent progress towards the elucidation of the three-dimensional structure of the extracellular domain of the IGF-1R represents an opportunity for the rational assembly of small molecule antagonists of receptor function for clinical use.

Key words. Insulin-like growth factor; insulin; receptors; 3D structure; signalling pathways; apoptosis; cancer therapy.

Introduction

The insulin-like growth factors (IGFs) are essential for normal foetal and postnatal growth and development. Targeted ablation of the *Igf-1* gene results in embryonic growth deficiency, manifested as dwarfism at birth, and impaired postnatal growth and infertility in mice [1, 2]. Functional inactivation of the *Igf-2* gene also compromises embryonic growth but has little effect on relative growth rates following birth [3]. Two members of the same family of cell-surface receptors mediate the diverse cellular effects elicited by IGF-1 and IGF-2. The type 1 IGF receptor (IGF-1R), a member of a family of transmembrane tyrosine kinases that includes the insulin receptor (IR) and the orphan insulin receptor-related receptor (IRR), binds IGF-1 with high affinity and initiates the physiological response to this ligand in vivo [4]. The IGF-1R also binds IGF-2, albeit with lower affinity, and is in part responsible for the mitogenic effects of this polypeptide during foetal development [2]. Mice lacking functional IGF-1Rs are born weighing less than half the normal weight, and die soon

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thereafter [1]. An alternately spliced form of the IR that lacks exon 11 and is expressed in many foetal tissues has recently been identified as binding IGF-2 with high affinity [5], confirming an earlier genetic study implicating the IR in the growth-promoting effects of IGF-2 [6]. A third receptor, the mannose-6-phosphate receptor, also binds IGF-2 and regulates its bioavailability by internalizing and targeting bound ligand for lysosomal degradation [7]. Finally, there exist at least six soluble IGF-binding proteins (IGFBPs), to which the majority of circulating IGFs are bound, that act to modulate the biological activity of both growth factors [8].

Endocrinologically, signalling through the IGF-1R has traditionally been viewed in the context of its impact on somatic growth, in particular the synergistic relationship with growth hormone (GH) that is essential for normal postnatal growth [9]. However, a number of experimental observations made earlier this decade have served to broaden the cellular roles played by this axis, under both normal and pathophysiological conditions. First, it was found that IGF-1 was effective in rescuing neuronal [10], haematopoietic [11] and fibroblast [12] cell types from programmed cell death (apoptosis), thereby establishing IGF-1 as a 'cell survival factor'. Second, ablation of IGF-1R expression significantly impaired the progression of fibroblasts through the cell cycle in serum-rich conditions, and prevented entry into S phase in the presence of mitogenic growth factors [13]. These in vitro studies reflected the in vivo effects of the germline deletion of both *Igf-1r* alleles on embryonic growth, in that the severe growth retardation observed during the second half of gestation resulted principally from a deficit of cellular proliferation events. Finally, and perhaps most provocatively, fibroblast cell lines established from *Igf-1r* knockout mice were resistant to oncogenic transformation by a variety of viral and cellular oncogenes [13]. Conversely, overexpression of IGF-1Rs promoted the neoplastic transformation of cell lines in a ligand-dependent manner [14]. These observations have provided a foundation that has resulted in a wealth of knowledge about the role played by the IGF axis not only in normal cellular development, but also in malignant transformation [15]. As a result, the IGF-1R has emerged as a candidate therapeutic target for the treatment of human cancer.

IGF-1 receptor: discovery and sequence

The first evidence for the presence of an IGF receptor distinct from IR came in 1974 when ^{125}I-labelled insulin and ^{125}I-labelled NSILAs (soluble fraction of nonsuppressible insulin-like activity) were used to label distinct proteins in purified rat liver plasma membranes [16, 17]. The IGF-1R could be solubilized with nonionic detergents [18] and was subsequently shown by SDS gel electrophoresis to resemble IR in being a homodimer composed of two α and two β chains held together by disulfide bonds [19–21]. The solubilized IGF-1R and IR proteins could be selectively immunoprecipitated by the three monoclonal antibodies αIR-1, αIR-2 and αIR-3 [22]. When expressed in the presence of monensin, an inhibitor of posttranslational processing, the IGF-1R was shown to be synthesized as a 180-kDa precursor [23] which is glycosylated, dimerized and proteolytically processed to yield the mature $\alpha_2\beta_2$ receptor. The next key discovery was the demonstration that IGF-1R, like IR, is a tyrosine kinase which is activated and autophosphorylated following IGF-1 binding [24, 25].

The complementary DNA (cDNA) for human (h) IGF-1R was cloned and sequenced in 1986 [26]. It consists of 4989 nucleotides and codes for a 1367-amino acid precursor (fig. 1). The preproreceptor includes a 30-residue signal peptide (residues -30 to -1) and an Arg.Lys.Arg.Arg furin protease cleavage site at residues 708–711 which results in the production of an α chain (residues 1–707) and a β chain (residues 712–1337). The α chain and 195 residues of the β chain comprise the extracellular portion of the IGF-1R and contain 11 and 5 potential N-linked glycosylation sites, respectively [26]. There is a single transmembrane sequence (residues 906–929) and a 408-residue cytoplasmic domain containing the tyrosine kinase. The cDNA for the hIR and the third member of the IR family, hIRR, have been cloned and sequenced and are similarly organized [27–29].

The human IGF-1R gene is greater than 100 kb in size and contains 21 exons, 10 in the α chain and 11 in the β chain [30]. No evidence for an exon equivalent to the alternatively spliced exon 11 of hIR was found. An alternate human IGF-1R messenger RNA (mRNA) transcript has been reported, in which a 3-bp (CAG) deletion results in the substitution of Arg for Thr^{898}Gly^{899} (fig. 1), eight residues upstream from the start of the transmembrane region of hIGF-1R [31]. The CAG$-$ isoform shows reduced internalization and enhanced signalling properties compared with the CAG$+$ isoform [32].

The ligands (insulin, IGF-1 and IGF-2) share a common three-dimensional (3D) architecture [33] and can bind to each other's receptor in a competitive manner. The hIRR ligand is unknown. The major feature which separates the IGF-1R and other members of the IR family from most other receptor families is that they exist on the cell surface as disulphide-linked dimers and require domain rearrangements rather than receptor oligomerization for cell signalling.

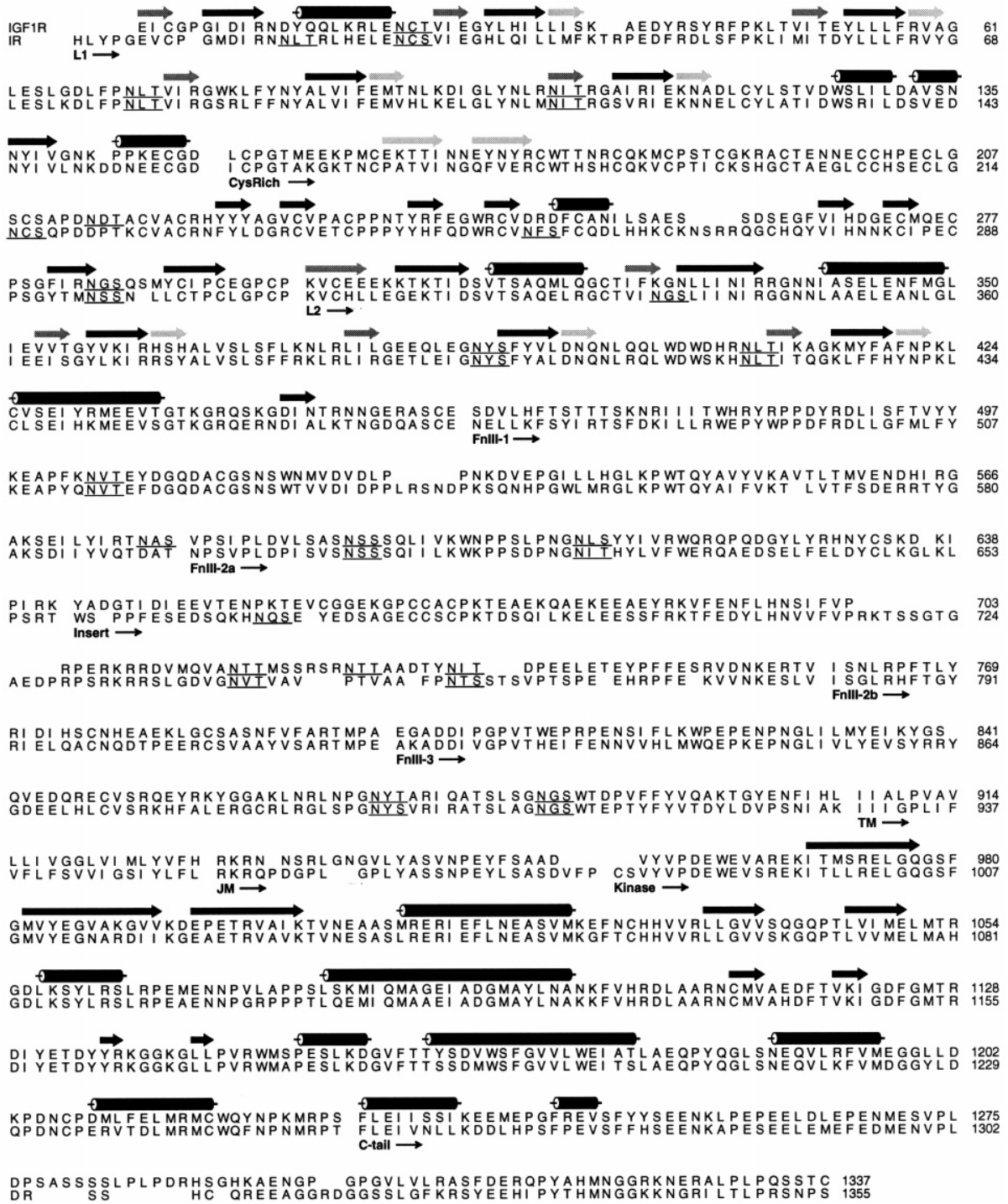

Figure 1. Amino acid sequences of hIGF-1R and hIR. The secondary structural assignments for L1/cys-rich/L2 domains and the tyrosine kinase domain are depicted above the sequences as cylinders for α helices and arrows for β strands. The demarcation boundaries for the various domains and modules are shown underneath the sequences. Based on [26–28, 62, 86].

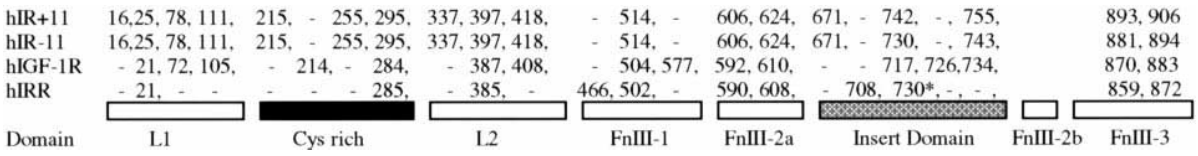

Figure 2. Distribution of potential N-linked glycosylation sites in hIR, hIGF-1R and hIRR ectodomains. Residue numbers for the potential N-linked glycosylation sites are shown above the schematic cartoon of the structural modules that make up the ectodomains. There are two isoforms of the hIR that differ in the presence or absence of 12 amino acids coded for by exon 11. From [50]. (Reproduced with permission from Elleman et al. (2000) Biochemical Journal 347, 771–779.)

Receptor biosynthesis

In mammalian cells the correct folding and oligomerization of complex, multidomain proteins is controlled by N-glycosylation [34]. Reversible trimming of newly synthesized N-glycosylated polypeptides by glucosidase and glucosyltransferase enzymes controls their transient association with the two lectin chaperones calnexin and calreticulin [34]. Binding to calnexin and calreticulin, together with BiP, the homologue of heat-shock protein 70, slows down the rate of folding, and prevents the occurrence of premature associations before all protein domains are synthesized. This reversible process of glucosylation/lectin binding and deglucosylation/lectin release continues until the glycoprotein is correctly folded and assembled [34, 35].

Studies aimed at elucidating the steps involved in the expression, folding and assembly of the mature hIR homodimer have been reviewed recently [36] and serve as a good model for IGF-1R biosynthesis. Glycosylation is critical for IR biosynthesis. Inhibition of N-linked glycosylation by tunicamycin leads to intracellular accumulation of aglyco-proreceptors which fail to form correctly assembled tertiary and quaternary structures [37]. Calnexin, and to a greater extent calreticulin, have been shown to associate with both the early monomeric and late monomeric proreceptor species of IR but not with the IR dimers [38]. This suggests that once the IR monomers are correctly folded, these lectin chaperones no longer bind, enabling receptor dimerization to occur, followed by export from the endoplasmic reticulum (ER). When calnexin and calreticulin binding is blocked by castanospermine, IR dimerization is accelerated, but the receptor protein is retained in the ER through interactions with BiP until it is correctly folded and transported to the trans-Golgi [38]. The ability to form functional receptors in the absence of ER lectin binding but not in the absence of glycosylation indicates that N-linked carbohydrate also functions to enhance the solubility of IR and to counteract aggregate formation [39].

The hIR is heavily glycosylated and is estimated to contain 58–64 kDa of carbohydrate [40]. Oligosaccharides of both the high mannose and complex type are present, the latter containing additional fucose, N-acetylglucosamine, galactose and sialic acid residues [41–44]. The hIR has 18 potential sites for N-linked glycosylation, 14 on the α chain and four on the β chain, of which 16 have been confirmed as glycosylated [L. G. Sparrow, personal communication]. O-linked glycosylation has been demonstrated only in the β subunit [41, 45, 46]. Other members of the insulin receptor family have fewer potential N-linked glycosylation sites (fig. 2), suggesting that not all sites are required for correct folding, assembly and function. The hIGF-1R has 16 potential N-linked glycosylation sites [26], whereas the hIRR has 11 sites [29].

Studies of the effects of removing N-linked glycosylation sites indicate that there are many redundancies in hIR glycosylation. Every site, with one exception, can be mutated individually without detriment to cell-surface expression, receptor processing and ligand binding [45–52]. When combinations of sites are examined, it appears that the major domains of the receptor, particularly those closer to the N-terminus (i.e. L1, cys-rich, L2), require at least one intact glycosylation site to ensure correct folding and processing [50]. Removal of the four glycosylation sites in the N-terminal L1 domain (at residues 16, 25, 78 and 111) resulted in complete retention of proreceptor in the ER and no proreceptor processing [46, 49, 51]. The mutant receptor was a dimer and could bind insulin, but failed to show insulin-induced kinase activity in vitro [49]. No single site was critical, as cells expressing single-site mutants at either residue 16 or 25 showed negligible defects compared with the significant reduction in cell-surface receptor levels when both residues 16 and 25 or 25, 78 and 111 were mutated [51]. The double mutant 78 and 111 and the triple mutant 16, 78 and 111 showed some reduction in processing and cell-surface expression [51], whereas the double mutants 16 and 78, and 16 and 111 were normal [50]. None of the three sites (residues 215, 255 and 295) in the cys-rich region appear to be critical individually. However, the triple mutant 16, 215 and 255 was poorly transported to the cell surface, and little processed receptor was produced [50].

There are three N-linked sites in the L2 domain at 337, 397 and 418 (fig. 2). Simultaneous removal of two of these sites, residues 397 and 418, has been shown to severely affect receptor biosynthesis, whereas individual mutation of either of these sites [52] or mutation at both 337 and 418 [50] were without detrimental effect. The site at 514 has been removed with 297 and 337, or 337 and 418, without significant effect [50]. The four N-linked glycans of the β chain (at residues 742, 755, 893 and 906 in the exon 11+ isoform) can be removed without affecting proreceptor processing, surface expression or insulin binding [48]. This quadruple mutant, however, was defective in autophosphorylation and kinase activity and was unable to transduce the signals for glycogen or DNA synthesis [47, 48]. Analysis of a series of single-site and double-site mutations established that this signalling defect was predominantly caused by the 906 mutation, although removal of the site at 893 also depressed kinase activity by 40% [47, 48]. The quadruple mutants $606+730+743+881$ and $671+730+743+881$ in the exon 11− isoform appeared normal by all criteria examined, whereas the quadruple mutant $624+730+743+881$ showed normal processing and ligand binding but exhibited a constitutively active tyrosine kinase [50]. Finally, three higher-order mutants with six or seven N-linked sites removed have been investigated. Mutants $\Delta 6$ $(16+295+337+418+730+743+881)$ and $\Delta 7a$ $(16+295+337+418+730+743+881)$ appeared normal. The third construct, $\Delta 7b$ $(16+337+418+514+730+743+881)$, was expressed at high levels on the cell surface, essentially as uncleaved proreceptor. Only the small proportion of $\Delta 7b$ that was correctly processed showed insulin-stimulated autophosphorylation [50].

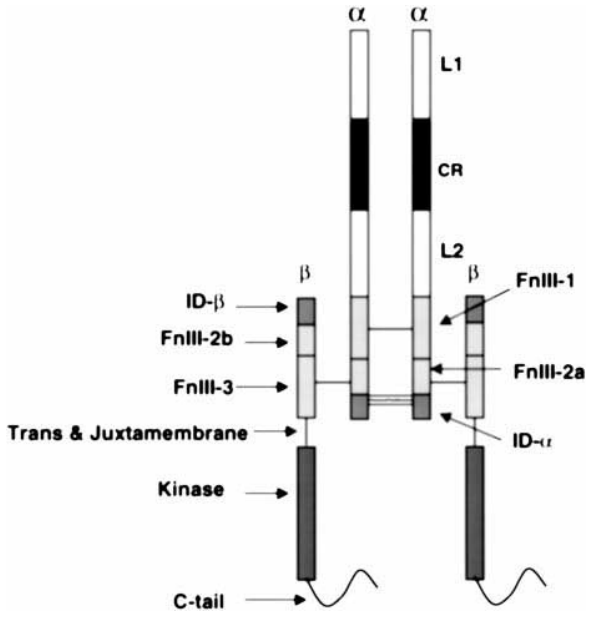

Figure 3. Cartoon of the IGF-1R dimer showing the distribution of domains across the α and β chains and the approximate location of the α–β disulfides and the α–α dimer disulfide bonds. Based on [84].

Domain organization and evolutionary relationships of IGF-1R and related receptors

Comparative sequence analyses have revealed that many proteins, particularly eukaryotic extracellular proteins, are composed of a number of different, sometimes repeated, structural units. In the case of the IR subfamily, 11 distinct regions have been identified in each monomer (figs 3 and 4). The N-terminal half of the IR and IGF-1R ectodomains were shown to contain two homologous domains (L1 and L2), separated by a cys-rich region (Cys^{148} to Cys^{298} in IGF-1R) containing 25 or 27 cysteines in three repeating units [53]. In a later analysis, the boundaries of the L1 and L2 domains were extended by 39 and 41 residues, respectively, and the cys-rich region (Cys^{152}-Cys^{298} in IGF-1R) and the nature of the repeat units were redefined [54]. The C-terminal halves of the IGF-1R, IR and IRR ectodomains consist of three fibronectin type III (FnIII) domains, the second of which contains a large insert domain of ~120–130 residues [55–58]. Intracellularly, each IGF-1R monomer contains a tyrosine kinase catalytic domain (residues 973–1229) flanked by two regulatory regions—a juxtamembrane region, residues 930–972, and an 108-residue C-tail, residues 1230–1337—that contains the phosphotyrosine binding sites for signalling molecules (fig. 1). The C-terminal boundary of the kinase domain of IGF-1R is Phe^{1229} or shorter, not Ile^{1236} [59], since IGF-1Rs truncated at 1229 [60] and 1243 [61] are still catalytically active as judged by receptor autophosphorylation, the phosphorylation/activation of cellular substrates and mitogenic responses to IGF-1. The 3D structure of the hIR catalytic domain has been described for both the inactive [62] and active [63] states. Phe^{1229}, which is 10 residues downstream from the conserved CysTrp sequence located at 1218–1219 (fig. 1), appears to be very close to the catalytic domain boundary when compared with the 3D structure of the cyclic AMP (cAMP)-dependent protein kinase [62, 64]. Tyrosine kinases with short C-terminal tails that end 25 residues downstream of the conserved CysTrp sequence are *v-kit* [65], *flk*, *fes* and *fps* tyrosine kinases [66].

The IR family is related to another class of cell-surface receptor, the epidermal growth factor receptor (EGFR). The EGFR ectodomain has a similar arrangement of

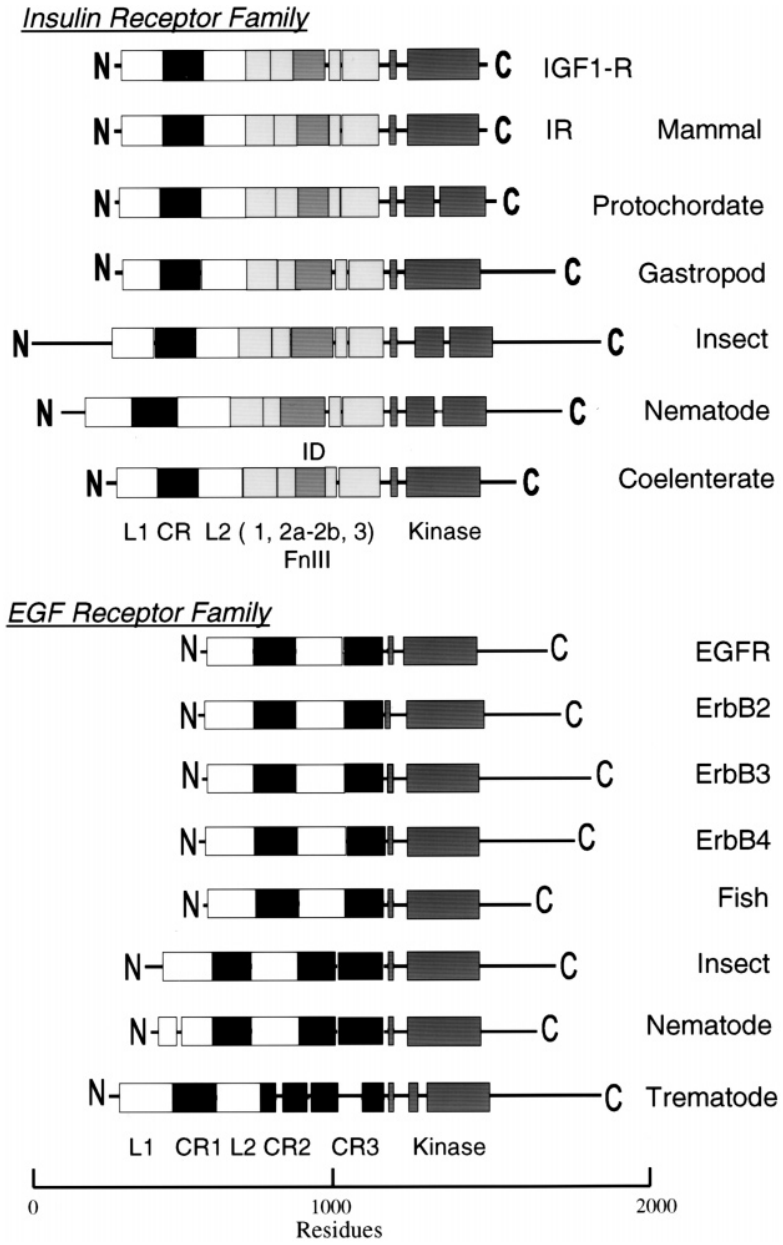

Figure 4. Domain organization within the insulin and EGF receptor families. Both families are tyrosine kinases and contain the common arrangement of L1/cys-rich/L2 domains in their extracellular regions. The primitive IR proteins contain additional domains at their N and C termini. The primitive EGFRs contain a third cys-rich region before the transmembrane domain. Based on [102].

L1/cys-rich/L2 domains followed by a second cys-rich region rather than the multiple fibronectin type III domains found in the IR subfamily [67, 68]. Like the IR subfamily, EGFR members have a single transmembrane region and a tyrosine kinase catalytic domain flanked by regulatory juxtamembrane and C-tail sequences (fig. 4). The EGFRs do not form disulfide-linked dimers, and the ligands, such as EGF and transforming growth factor α (TGF-α), are structurally unrelated to IGFs [69].

Representatives of the IGF-1R/IR receptor family have been characterized in some of the simplest multicellular animals, including cnidarians (polyps and jellyfish) [70], nematodes [71], gastropods [72] and insects [73–75]. As

summarized in figure 4, some of the primitive receptors have additional domains when compared with their mammalian counterparts. For example, the IGF-1R/IRs from *Caenorhabditis elegans* (nematode) and *Drosophila melanogaster* (insect) have additional sequences at their N- and C-termini, whereas the EGFRs from *D. melanogaster* [76], *C. elegans* [77] and *Schistosoma mansoni* (trematode) [78] have further duplications of the cys-rich sequences at the end of their ectodomains (fig. 4). Despite these differences, functional activity is retained within a receptor class, as illustrated by the ability of porcine insulin to activate the IR from the mosquito *Aedes aegypti* and to stimulate the secretion of ecdysteroids from mosquito ovaries [75]. The emergence of distinct IR and IGF-1R genes appears to coincide with the evolutionary transition from protochordates to vertebrates. The protochordate amphioxus contains only a single IR-like receptor cDNA in contrast to the hagfish, considered to be the most primitive extant vertebrate, which appears to contain two IR/IGF-1R-like cDNAs [79].

Secondary structure

The α chains of IGF-1R and IR have a total of 38 or 37 cysteine residues, respectively, whereas their respective β chains have three or four extracellular and five or six intracellular cysteine residues, respectively (fig. 1). Alkylation of IR indicates there is one free thiol group per β chain [80–82]. Mild reduction of IR cleaves a small number of disulfide bonds (the class I disulfides), giving rise to disulfide-linked $\alpha\beta$ monomers which can still bind ligand [83]. More vigorous reduction cleaves the α–β bond(s) (the class II disulfides), leading to free α and β chains [83].

Some of the disulfide bonds have been established by chemical analysis of IR. In the L domains, Cys^{8} is linked to Cys^{26} in the L1 domain [84], and Cys^{435} is linked to Cys^{468} in the L2 domain [85]. Sequence alignments suggest the homologous pairs of Cys residues at the end of the L1 domain (Cys^{126} and Cys^{155}) and the start of the L2 domain (Cys^{312} and Cys^{333}) are also disulfide-bonded [54], as confirmed in the 3D structure [86]. None of the disulfide bonds in the cys-rich region have been established chemically except for the additional disulfide bond Cys^{266}–Cys^{274}, in the large loop of sixth cys-rich module of hIR [87]. Sequence profile analyses [54] suggest that this region consists of a series of disulfide-linked modules similar to those found in the tumour necrosis factor (TNF) receptor [88] and subsequently seen in laminin [89]. The arrangement of these disulfide-bonded modules was experimentally verified when the 3D structure of the first three domains of IGF-1R was solved [86].

Cys^{524}, in the first FnIII domain, was shown to form a dimer disulfide bond with Cys^{524} in the second monomer [85]. However, mutation of Cys^{524} to Ala [90] or Ser [91, 92] resulted in an IR that was still dimeric, indicating that more than one disulfide bond is involved in dimer formation. Labelling of monomeric IR had shown that there are at least two cysteines per monomer involved in class I disulfide bonds [81, 82]. The additional dimer disulfides of IR were shown, by chemical analysis, to involve the triplet of Cys residues at positions 682, 683 and 685 in the insert domain of IR [84]. It was not possible to determine which one of the three, or whether all three Cys residues, are involved in dimer disulfides. The sequence around this triplet resembles those found in the hinge region of antibodies [93] where multiple disulfide bonds occur. It is interesting to note that the IGF-1 receptor has an additional Cys seven residues upstream of the Cys triplet in the insert domain and lacks the Cys residue equivalent to 884 in hIR (fig. 1). *Drosophila* IR [73, 74] also lacks the cysteine residue equivalent to Cys^{884} in hIR as well as the equivalent of one of Cys^{682} or Cys^{683}. The two reports differ with regard to the presence or absence of the cysteine residue equivalent to the hIR Cys^{524}, known to form one of the dimer bonds in hIR [85].

There is only a single α–β disulfide link in the hIR, between Cys^{647} in the first FnIII repeat and Cys^{872} (exon 11 + isoform) in the second FnIII repeat [84], which is consistent with the mutagenesis data [92, 94] and the predictions of Ward et al. [54]. It differs from the predictions made by Schaefer et al. [95], who suggested disulfide links between the four β-chain cysteines, 798, 807, 872 and 884 (exon 11 + numbering). Their suggestion was based on alignment of the FnIII domains of the hIR with those of the GH receptor [96] and would not allow for the presence of the known α–β disulfide linkage. The structural implications of the disulfide bond between Cys^{647} and Cys^{872} are that the two FnIII domains are aligned side by side [54], not end to end, as is the more common configuration [97].

An intrachain disulfide linkage between the β-chain residues Cys^{798} and Cys^{807} (exon 11 + numbering) in the predicted F and G strands of the third FnIII domain was confirmed [84]. Interestingly, the reported sequence for rat IR [98] shows a Ser residue at the position equivalent to Cys^{807} in human IR. The remaining Cys residue in hIR, Cys^{884} (exon 11 + isoform), which has no counterpart in hIGF-1R (fig. 1) or hIRR, has been shown to exist as a buried free thiol [84]. Alkylation of the β-chain thiol of the native receptor takes place readily in the absence of disaggregating agents [81], whereas alkylation of Cys^{884} in the isolated ectodomain requires denaturation, suggesting that it is not the free thiol identified in the native receptor [84].

3D structure of the L1/cys-rich/L2 domains of the IGF-1R

Recently the 3D structure of the L1/cys-rich/L2 domain fragment of the IGF-1R was solved [86]. As shown in figure 5, the molecule adopts an extended bilobal structure (approximately $40 \times 48 \times 105$ Å) with the L domains at either end. The cys-rich region runs two-thirds the length of the molecule, making contact along the length of the L1 domain but having very little contact with the L2 domain. This leaves a space at the centre of the molecule of approximately 24 Å diameter and of sufficient size to accommodate the ligands IGF-1 or IGF-2. The space is bounded on three sides by the regions of IGF-1R which are known to contribute to ligand binding, based on studies of chemical cross-linking, receptor chimeras, and natural or site-specific mutants [86]. The EGFR ectodomain would be expected to have similar overall architecture and a potential binding pocket large enough to accommodate ligands such as EGF and TGF-α. To date there are few data regarding the involvement of residues lining this putative EGFR pocket and ligand binding.

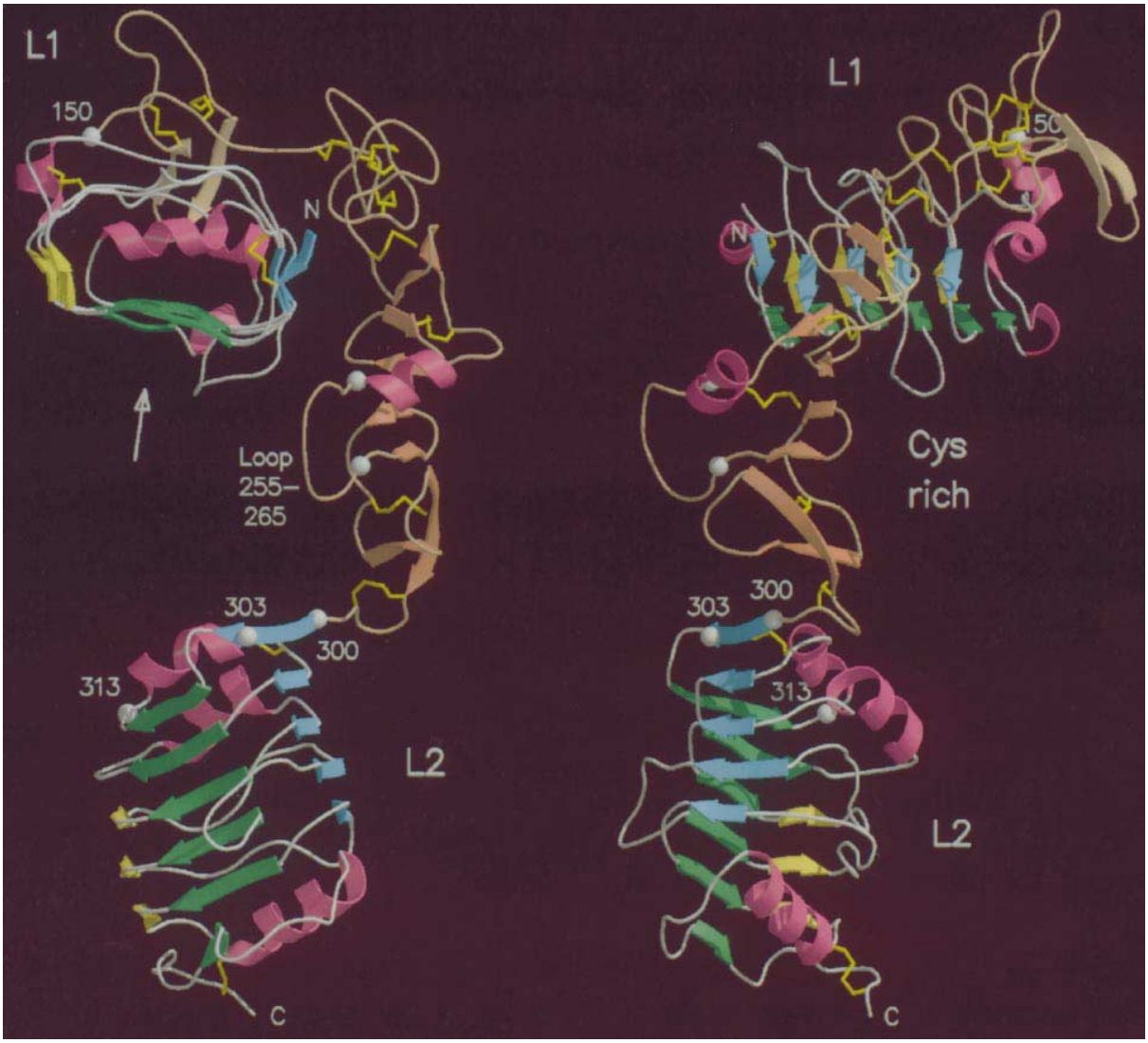

Figure 5. Polypeptide fold for residues 1–459 of the hIGF-1R. In the left-hand panel, the L1 domain is at the top viewed from the N-terminal end. In the right-hand view, the model has been rotated clockwise 90°. Helices are depicted as curled ribbons and β strands as broad arrows. Based on [86].

The L domains

Each L domain (residues 1–150 and 300–460) adopts a compact shape ($\sim 24 \times 32 \times 37$ Å) being formed from a single-stranded, right-handed β helix, capped at each end by short α helices and a disulfide bond. The body of each L domain looks like a loaf of bread with three flat sides (β sheets) and an irregular top (fig. 5). The two domains are superimposable [86]. The repetitive nature of the β helix is reflected in the sequence where a fivefold repeat, centred on a conserved glycine, had been identified by sequence analyses [53]. The structure, however, revealed that the L domains comprise six helical turns and a fold that was quite unexpected [86].

A notable difference between the L1 and L2 domains is found at their C-terminal ends. The indole ring of Trp^{176}, from the first module in the cys-rich region, is inserted into a pocket in the hydrophobic core of L1 formed by residues Ile^{98}, Gly^{99}, Leu^{100} and Leu^{103} from the fifth turn and Val^{125}, Trp^{127} and Ile^{130} from the sixth turn of L1 [86]. The sequence motif of residues which form the Trp pocket in L1 does not occur in L2 of the IR/IGF-1R family. However, in EGFR, which has an additional cys-rich region after the L2 domain, the motif can be found in both the L1 and L2 domains, and the Trp is conserved in the first module of both cys-rich regions [86].

Comparison of the L domains with other right-handed β-helix structures such as pectate lyase [99], ribonuclease inhibitor [100] and the p22 tailspike protein [101] reveals some striking similarities as well as differences. The other β-helix domains are considerably larger (up to 16 β-helical turns) and often contain many, and sometimes quite substantial, insertions. Although the sizes of the helix repeats are similar (here 24–25 residues vs 22–23 for pectate lyase), the type of helix is best characterized by its cross-section. The IGF-1R L domains have a slightly skewed rectangular cross-section like the ribonuclease inhibitor, whereas the other β-helix proteins have a triangular or V-shaped cross-section. In the hydrophobic core a common feature is the stacking of aliphatic residues from successive turns of the β helix. Near the C-terminus of each L domain of IGF-1R there is a short ladder of asparagines on the third β sheet [86], reminiscent of the long Asn ladder observed in pectate lyase [99]. On the opposite side of the L domains, the β strand of the first β sheet and the Gly turns (between the first and second β sheets) in each rung correspond to the sequence motif identified previously [53]. This feature has no counterpart in the other β-helix domains. In most cases only the N-terminal end of the other β-helix domains is capped by an α helix, but L domains are capped at both ends by α helices and have a disulfide bond at each end anchoring the termini. Thus, although the L domains are built with some principles common to the other β-helix domains, they appear to constitute a new type of domain.

The cys-rich domain

As anticipated [54], the cys-rich domain is composed of modules with disulfide bond connectivities resembling parts of the TNF receptor [88] and laminin [89] repeats. The first module sits at the end of L1 domain, whereas the remaining seven form a curved rod running diagonally across L1 and reaching to L2 (fig. 5). The strands in modules 2–7 run roughly perpendicular to the axis of the rod in a manner more akin to laminin than to the TNF receptor, where the strands run parallel to the axis. The modular arrangement of the IGF-1R cys-rich domain is different to other cys-rich proteins for which structures are known [86]. The first three modules of IGF-1R have a common core, containing a pair of disulfide bonds, but show considerable variation in the loops. These modules have been referred to as C2 (two disulfide bonds) [102]. The connectivity of the cysteines is the same as the first part of an EGF motif (Cys 1–3 and 2–4). Modules 4–7 have a different motif, a β finger, seen previously in residues 2152–2168 of fibrillin [103]. Each is composed of three polypeptide strands, the first and third being disulfide-bonded and the second and third forming a β ribbon. These have been referred to as C1, because of the single disulfide bond [102]. The β ribbon of each β finger (or C1) module lines up antiparallel to form a tightly twisted eight-stranded β sheet [86]. Module 6 deviates from the common pattern with the first segment being replaced by an α helix followed by a large loop that is implicated in ligand binding (see below). As modules 4–7 are similar, it is possible that they arose from a series of gene duplications. The final module is a disulfide-linked bend of five residues.

The fact that the C1 and C2 cys-rich modules found here are grouped according to type implies that these are the minimal building blocks of cys-rich domains found in many proteins. Although it can be as short as 16 residues, the β-finger (C1) motif is clearly distinct and capable of forming a regular, extended structure. Thus cys-rich domains such as laminin [89] and fibrillin [103] can be considered not as modified EGF repeats but as a series of repeat units each composed of a small number of C1 and C2 modules as illustrated in figure 6. This is further suppported by the observation that EGFRs from insects (*D. melanogaster*), nematodes (*C. elegans*, *C. vulgaris*) and trematodes (*S. mansoni*) contain a third cys-rich region, with a similar repeat pattern of C1 and C2 modules. In *S. mansoni* there are large insertions between two of the modules (fig. 4). The combination of the second and third cys-rich repeats in these primitive organisms could also be viewed as five repeats of the C2-C1-C1 set of modules (fig. 6).

TNFR	**[C1--C2]**$_{4\text{-}6}$
preproEGF	**[C2--C1]**$_{9}$
Fibrillin	**[C2--C1]**$_{5+5+4+12+2+7+5+7}$
Laminin	**[C2--C1--C1]**$_{12\text{-}16}$
IR	**C2--C2--C2--C1--C1--C1--C1--C1**
EGFR-1st:	**C2--C2--C2--C1--C1--C1--C1--C1**
EGFR- 2nd:	**[C2--C1--C1]**$_{2}$**--C2**
EGFR- 3rd *(D. melanogaster C.elegans, S. mansoni)*	**C1--C1--[C2--C1--C1]** $_{2}$

Figure 6. Disulfide-bonded modules in cys-rich domains of different proteins. The C1 modules contain a single disulfide bond, whereas the C2 domains contain two disulfide bonds with 1–3, 2–4 connectivity. The repeating units and the number of repeats are shown by the square brackets. Based on [102].

The fibronectin type III domains

The FnIII domain is one of the most common structural modules found in many proteins, including membrane-anchored receptors. 3D structures have been reported for several such domains and have the fold and topology shown in figure 7. They are especially common in extracellular matrix proteins such as collagen, fibronectin and tenascin as well as in the extracellular portions of cell-surface adhesion proteins, haemopoietic cytokine receptors and membrane-bound tyrosine kinase and tyrosine phosphatase receptors. The FnIII domain is relatively small ($\sim$ 100 residues) and has a fold similar to that of immunoglobulins but with a distinctive sequence motif. The domain consists of a seven-stranded β sandwich in a three-on-four (EBA:GFCC′) topology. Its main functions appear to be to mediate protein-protein interactions, including ligand binding and to act as spacers to correctly position functionally important regions of extracellular proteins. O'Bryan et al. [55] were the first to describe the existence of FnIII domains in members of the IR subfamily following their cloning and characterization of the tyrosine kinase *axl*. Their sequence alignments and descriptions covered the two C-terminal FnIII domains in the IR subfamily, and these were given structural assignments following comparisons with the FnIII modules present in the growth hormone receptor [95]. Recently it has been shown that members of the IR subfamily contain an additional FnIII domain in the region previously referred to as the connecting domain [56–58]. This first FnIII domain (equivalent to residues 461–579 in IGF-1R) is 118–122 residues long, whereas the second FnIII domain (equivalent to 580–798 in IGF-1R) has a major insert of 120–130 amino acids. The third FnIII domain (equivalent to residues 799–901 in IGF-1R) is of normal size.

There are differences, among the different authors, in the residues assigned to the seven β strands in each of these FnIII domains, with the regions of greatest disagreement being the locations of the C′ and E strands in all three modules (table 1). Cys^{514} (Cys^{524} in hIR), which is involved in one of the dimer disulfide bonds, is predicted to be in the C–C′ loop [56, 57] or the C′–E loop [58], Cys^{633} (Cys^{647} in hIR), which is involved in the α–β disulfide bond, is predicted to lie in the C′–E loop [57, 58] or the E strand [95]. Cys^{849} (Cys^{860} in hIR), the other partner in the α–β disulfide bond, is predicted to be located in the C′–E loop [58] or the C′ strand [57, 95]. Residues forming part of the epitope for the monoclonal antibody 83–14 (i.e. those differing between mouse IR and human IR) are predicted to be in the

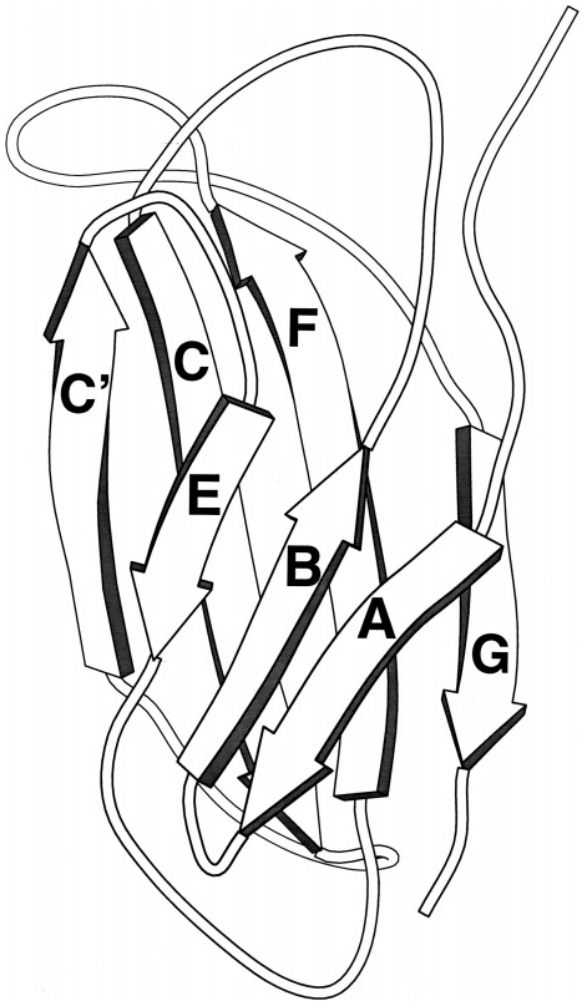

Figure 7. Polypeptide fold of the 10th type III repeat of fibronectin [383]. The seven β strands that comprise the two-sheet sandwich are labelled A, B, C, C′, E, F and G.

Table 1. Comparison of alignments of the three fibronectin type-III domains in the hIGF-1R.

FnIII-1							
A	B	C	C′	E	F	G	Ref.
462–466	475–480	492–499	**520–524**	**535–538**	547–554	572–574	[56]
461–467	476–482	492–498	**519–523**	**526–531**	545–553	565–573	[57]
463–469*	474–480	492–500	505–510	518–522	546–554	563–569	[58]
nd	nd	nd	nd	nd	nd	nd	[95]
FnIII-2							
A	B	C	C′	E	F	G	
nd	nd	nd	nd	nd	nd	nd	[56]
585–591	598–604	613–619	627–631	**754–759**	767–775	784–793	[57]
587–593	596–602	613–621	624–629	636–639	768–776	782–788	[58]
586–593	595–602	609–616	619–627	**632–636**	772–780	781–788	[95]
FnIII-3							
A	B	C	C′	E	F	G	
nd	nd	nd	nd	nd	nd	nd	[56]
806–812	819–825	834–840	**848–854**	857–862	869–877	885–895	[57]
808–814	817–823	834–841	843–848	855–859	870–878	885–891	[58]
807–814	816–823	830–837	845–853	859–865	869–881	885–897	[95]

The seven β strands in each FnIII module are A, B, C, C′, E, F, G. Regions of greatest divergence between the four reports are shown in bold.

Table 2. Monoclonal antibodies to IGF-1R and IR.

Antibody	Epitope	Effect on binding	Other effects	Reference
IR				
3D7	191–297	no effect		[108, 112]
5D9	469–593	inhibits		[104, 108, 112]
18-44	765–770	inhibits	activates IR	[113, 115, 118, 119]
25-49	469–593	inhibits	activates IR	[108, 113, 115, 119]
47-9	469–593	inhibits	no activation	[108, 113, 115, 119]
83-7	191–297	no effect	weak activation	[108, 113, 115, 119]
83-14	469–593	inhibits	activates IR	[108, 113, 115, 116, 118]
MA-5	469–593	inhibits	mimics some responses	[108, 114, 384]
MA-10	469–593	inhibits	no activation	[108, 114]
MA-20	469–593	inhibits	mimics some responses	[104, 108, 114]
MC51	469–593	inhibits		[104, 108, 112]
IGF-1R				
αIR-3	217–274	inhibits		[22, 104]
4-52	62–184	enhances		[105]
16-13	62–184	enhances		[105]
17-69	514–586	inhibits		[105, 110]
24-31	283–440	no effect		[105, 110]
24-55	440–586	inhibits		[105, 110]
24-60	184–283	inhibits		[105, 110]
25-57	440–586	inhibits		[105, 110]
26-3	283–440	enhances		[105, 110]

C′–E loop [56] at the top of the first FnIII domain (fig. 7) or the E–F loop at the bottom of this domain [57, 58]. This antibody has been used to locate the FnIII domain in the hIR ectodomain dimer, as discussed below.

Anti-receptor monoclonal antibodies

The characteristics of several commonly used monoclonal antibodies (Mabs) to hIGF-1R and hIR are listed in table 2. Antibodies αIR-1, αIR-2 and αIR-3 were raised against a human placental IR preparation with αIR-1 preferentially precipitating hIR, whereas αIR-2 and αIR-3 react more strongly with hIGF-1R [22]. The epitope for αIR-3 has been mapped to the region 223–274 in IGF-1R [104]. An additional 20 Mabs have been produced against hIGF-1R which did not react with hIR nor with rat IGF-1R [105]. All are conformationally dependent and fall into seven epitope groups based on competitive binding [105] to a series of

IGF-1R chimeras [106–108]. Some of the epitopes have been refined based on binding reactions to receptor chimeras [109, 110] or to truncated IGF-1R/1-462 [111]. Additional Mabs have been generated against placental hIR [112–114]. In one set, only 3 of the 37 antibodies, 3D7, 5D9 and MC51, bound ectodomain epitopes [112], whereas the second set all appeared to target the ectodomain [113, 114]. These antibodies have been extensively characterized with respect to their effects on insulin binding [112–115], their capacity to mimic insulin action [114–117] and the approximate location of their epitopes [104, 108, 113, 118, 119] as summarized in table 2.

Comparison of the human and rodent sequences for IR [27, 28, 120] and IGF-1R [26, 121] indicate potential residues involved in these epitopes. There are eight sequence differences between mouse and human IRs in the region 191–297, which contains the epitopes for Mabs 3D7 and 83-7. These are at residues 210 and 218 in the third cys-rich module, 236 in the fourth cys-rich module and 264, 267, 271 and 272 in the large loop in the sixth cys-rich module [86]. Residue 218 is unlikely to be part of the epitope as it is adjacent to an N-linked glycosylation site at 215. In addition, the four residues in module 6 of the cys-rich region can be discounted as major contributors since Mab83-7 could still immunoprecipitate the hIR-based, cysteine-loop exchange chimera, hIR_CLX, in which residues 260–277 were replaced with residues 253–266 of the hIGF-1R [122].

There are five sequence differences between mouse and hIRs in the region 467–593, which contains the epitopes for Mabs 5D9, 25-49, 47-9, 83-14, MA-5, MA-10, MA-20 and MC51. These are at residues 477, 536 and 545 (hIR numbers), including an insert of two residues between 544 and 545 in the mouse sequence. This region is predicted to be a FnIII module with 477 located in the A strand, 536 located in the C′–E loop [56], the E strand [57] or the E–F loop [58], and the double insert and sequence difference at position 545 located in the C′–E loop [56] or the E–F loop [57, 58].

The third epitope on hIR that has been characterized is a short linear sequence, PEEHRP, at residues 765–770 (exon 11+ numbering) in the insert domain, 30 residues downstream of the α–β cleavage site (fig. 1). There is a single sequence difference, at 765, in this peptide epitope for Mab 18-44.

The epitopes for the anti-IGF-1R antibodies fall into four regions (table 2). There are only two sequence differences in the region 62–184, which contains the epitopes for Mabs 4-52 and 16-13. These are at 125, which is buried in the core of the L1 domain, and 156, which is exposed in the first cys-rich module [86]. There are 13 sequence differences in the region 184–283, which contains the epitope for Mab 24-60, seven of which are in the region 217–274 that contains the epitope for Mab αIR3. These differences are at residue 188 in the second cys-rich module, residues 210, 211, 214–217 in the third module, residue 227 in the fourth module, residue 237 in the fifth module and residues 256, 257, 263 and 264 in the loop of the sixth cys-rich module. This region of IGF-1R is homologous to residues 191–294 in hIR, which contains the epitopes for Mabs 3D7 and 83-7. The third epitope region described for IGF-1R comprises residues 283–440, and contains the epitopes for Mabs 24-31 and 26-3. There are nine sequence differences between the human and rat sequences in this region, which extends from the seventh cys-rich module to the end of the fifth rung in the L2 domain (fig. 5). Two of these differences are at residues 285–286 in the seventh cys-rich module, whereas most of the other changes are located on one edge (the first β sheet) of the L2 domain. These sequence differences are at residues 302–303 in the first strand of the first β sheet; residue 326 in the second strand of the first β sheet; residue 405 in the irregular loop at the back of the fourth rung; and residues 411–413 in the fifth strand of the first β sheet [86]. The final epitope region of hIGF-1R which has been described is residues 440–586, which contains the binding sites for Mabs 24-55 and 24-57. The shorter fragment, residues 514–586, contains the epitope for Mab 17-69. There are four sequence differences between the human and rat IGF-1R sequences and all occur in the region predicted to be the first FnIII module of the IGF-1R. These differences are at residue 465 at the start of the A strand, residue 471 at the end of the A strand [58] or in the A–B loop [56, 57] and residues 531–532, predicted to lie either in the C′–E loop [56] at the top of the module, or at the end of the E strand [57], or in the E–F loop [58] at the bottom of the module (table 1, fig. 7).

The IGF-1R and IR ectodomain dimers

How are these 22 domains organized in the dimeric, native receptor? The first clues have come from electron microscopy (EM) of single-molecule images of the hIGF-1R ectodomain and, more particularly, the hIR ectodomain and its complexes with three different monoclonal antibody-derived Fab fragments [123]. These images show that the hIGF-1R and hIR ectodomains resemble a U-shaped prism of approximate dimensions $90 \times 80 \times 120$ Å. The images show clearly the dimeric structure of these ectodomains, with the length of the images ~ 80 Å along each bar, and the width of ~ 90 Å across the two bars. The width of the cleft (assumed membrane-distal) between the two side arms is ~ 30 Å, sufficient to accommodate ligand [123].

Fab molecules from the monoclonal antibody 83-7 bound hIR half-way up one end of each side arm in a diametrically opposite manner, indicating a twofold axis of symmetry normal to the membrane surface [123]. Mab 83-7 recognizes an epitope between residues 191 and 297 in the cys-rich region of hIR [108, 119]. Examination of the location of the sequence differences between mouse and human IRs (discussed above) and the EM images suggests residues 210 in the third cys-rich module and 236 in the fourth cys-rich module form part of the 83-7 epitope. They are located at one corner of the fragment in line with the centre of the L1 domain (fig. 5). This suggests that the L1/cys-rich/L2 fragment spans the cleft between the parallel bars rather than lying within each parallel bar. Fabs 83-14 and 18-44, which, as discussed above, have been mapped respectively to the first FnIII repeat (residues 469–592) and residues 765–770 in the insert domain [118], bound near the base of the prism at opposite corners [123].

The single-molecule images, together with the 3D structure of the first three domains of hIGF-1R [86], suggest that the ectodomain dimer is organized into two layers. The L1/cys-rich/L2 domains are suggested to occupy the upper (membrane-distal) region of the U-shaped prism with the FnIII domains, the insert domains (and the disulfide bonds involved in dimer formation) located predominantly in the membrane-proximal region [123]. The nature of the interaction between the two L1/cys-rich/L2 fragments in the ectodomain dimer is not clear from these EM studies. However, some clues to these domain associations may be gained by examining the surface properties of the L1/cys-rich/L2 fragment. Surfaces that are involved in the dimer interface would be expected to have complementary properties. In addition, the surfaces involved in either ligand binding or the interface with the bottom layer of fibronectin type III and insert modules would be expected to be more conserved than those regions that are exposed on the surface of the dimer. Figure 8A shows the amino acid sequence conservation mapped on to the molecular surface of the first three domains of the IGF-1R. Quite noticeably, there is significantly higher sequence conservation on one face of the IGF-1R molecule (the left-hand panel of fig. 8A) than on the opposite face (right-hand panel). Since sequence conservation is highly correlated with biological functions such as the association with other proteins or protein domains, one can infer that the face of IGF-1R shown on the left-hand panel is more likely to be involved in such functions than the opposite face. In addition, the face of the L1 domain that points towards the central cavity (the second, green, β sheet in fig. 5) is very highly conserved. This surface has been implicated in some of the binding interactions of the IGF-1R with insulin and IGF-1 [86].

Figure 8B shows the electrostatic potential of the IGF-1R L1/cys-rich/L2 domains mapped on to the molecular surface in the same three orientations as in figure 8A. Two observations are quite striking. First, the cys-rich domain has an overwhelmingly large negative electrostatic potential. Second, the external (facing the reader) surface of the L1 and L2 domains have opposing and qualitatively complementary electrostatic potentials (negative and positive, respectively), consistent with an antiparallel association in the dimer [123].

High-resolution data are required to establish the precise arrangement of the 14 modules that make up the ectodomain dimer and the way they interact with the respective ligands to generate signal transduction. Recently whole receptors solubilized from human placental membranes have been examined by electron cryomicroscopy and 3D reconstruction performed using a library of 700 images [124]. Gold-labelled insulin was used to locate the insulin-binding domain. The images seen were compact and globular, measuring 150 Å in diameter. Some domainlike features became evident at intermediate-density thresholds, which indicated a strong twofold vertical rotational symmetry. When this symmetry was applied to the reconstruction, some structural features became evident. The overall model showed the L1/cys-rich/L2 domains arranged in an antiparallel manner and at an angle to each other when viewed from the side. The six FnIII domains and the two L2 domains are placed in a central band with the two tyrosine kinase domains at the base of the model [124]. The images described in these studies [123, 124] are substantially different from the 'T'-, 'X'- or 'Y'-shaped objects reported for recombinant ectodomain [95], detergent-solubilized whole receptors or vesicle-reconstituted whole receptors [125–127].

Ligand binding

Conformational changes and two affinity states

Insulin binding to detergent-solubilized whole hIR induces conformational changes, as assessed by a decrease in the Stokes radius from 9.1 to 7.5 nm and an increase in the sedimentation coefficient (S20.w) from 10.1 S to 11.4 S, which are correlated with the onset of autophosphorylation of the kinase domain [128]. The 9.1-nm form of the $(\alpha\beta)^2$ IR is restored following dissociation of the ligand, without reduction in the kinase activity of the now autophosphorylated cytoplasmic domains. In contrast, solubilized $\alpha\beta$ half-receptors do not show altered conformation on binding insulin, nor is their kinase activity induced [128].

Similarly, there are marked differences in the insulin-binding characteristics of the $(\alpha\beta)^2$ dimer versus the $\alpha\beta$ monomer. The $(\alpha\beta)^2$ dimer displays complex binding

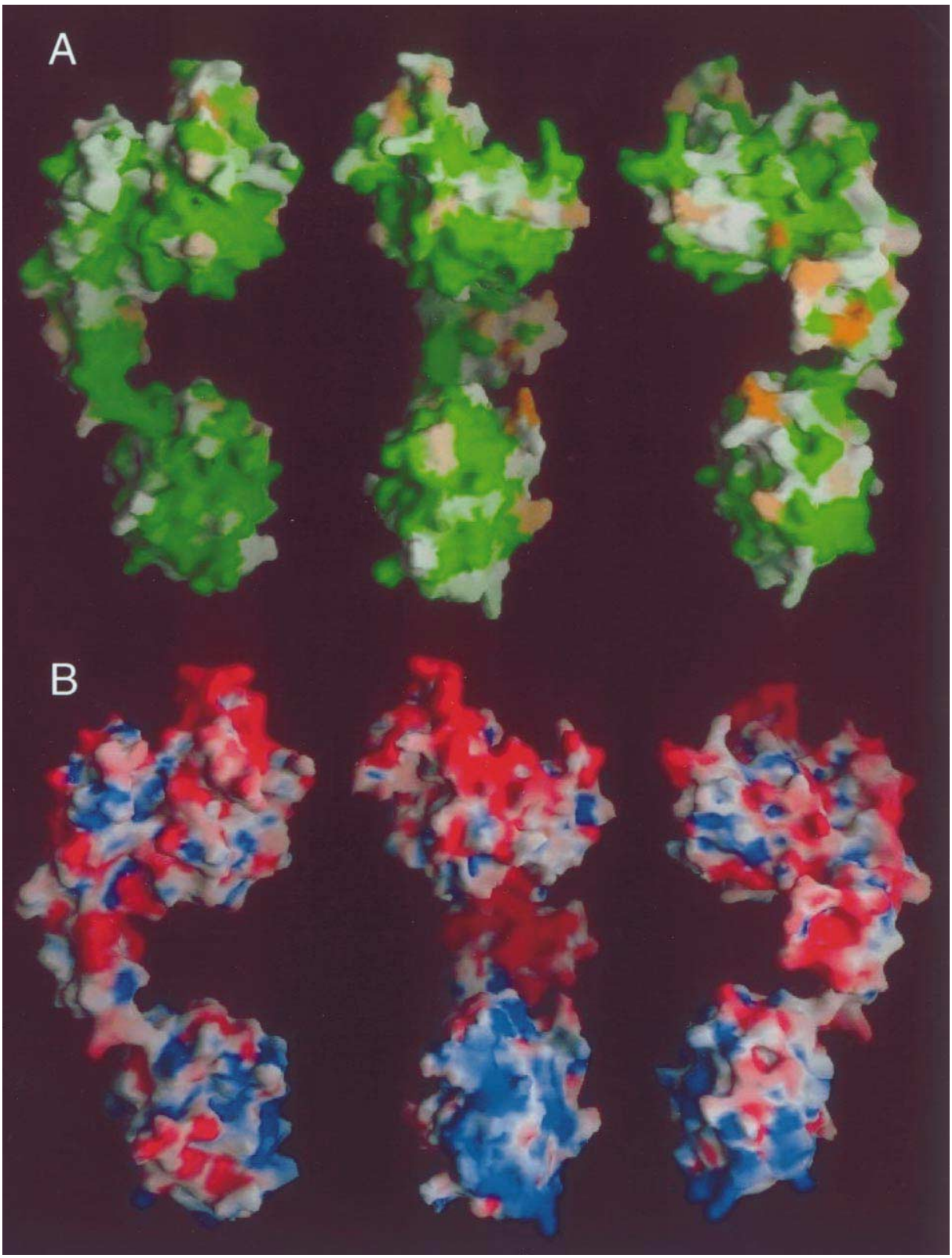

Fig. 8.

characteristics including curvilinear Scatchard plots, indicative of two binding states (high and low affinity) and negative cooperativity [128–131]. In contrast, the $\alpha\beta$ half-receptors exhibit linear Scatchard plots consistent with a simple one-class insulin binding state which has relatively low affinity [128]. Collectively these data indicate that interaction between the two $\alpha\beta$ monomers is required for the generation of high-affinity binding and ligand-induced signal transduction.

IGF-1R or IR half-receptors can be induced to reassociate to form the corresponding native receptor dimers by incubation with Mn/MgATP or their specific ligand [132], suggesting structural communication between the kinase domain and the ligand binding sites [80, 133]. IR/IGF-1R hybrid receptors can be formed from mixtures of half-receptors by incubation with Mn/MgATP or a combination of both insulin and IGF-1 [132]. The binding characteristics of IR/IGF-1R hybrids revealed that they more closely resembled IGF-1R than IR [134], suggesting that insulin binding is more sensitive to specificity determinants in the second half-receptor moiety than IGF-1 binding. The IR half-receptor in the hybrid is equally as good as the IGF-1R half-receptor in the wild-type IGF-1R in allowing high-affinity IGF-1 binding to be generated, whereas the opposite is not the case. Insulin binding to the hybrid is significantly poorer than insulin binding to the IR holoreceptor [134], suggesting key contacts are not provided by the IGF-1R component of the hybrid.

Although ectodomain constructs are $(\alpha\beta')^2$ dimers, they resemble the $\alpha\beta$ half-receptors in their binding characteristics and do not show high-affinity binding [135]. In contrast, hIR constructs which terminate after the transmembrane domain [136] or have either the Fc (244 amino acids) or λ (103 amino acids) immunoglobulin domains [137], or the 33-amino acid leucine zipper from the GCN4 transcriptional activator of *Saccharomyces cerevisiae* [P. A. Hoyne et al., unpublished] attached to the C-terminal end of the ectodomain, exhibit curvilinear Scatchard plots similar to those found with the wild-type receptor. These data imply that one function of the transmembrane region is to bring the C-termini of the adjacent monomer chains into close proximity to facilitate the conformational changes required for the generation of high-affinity binding and its accompanying phenomenon of negative cooperativity.

Insights into the molecular basis of negative cooperativity can be obtained from the *Salmonella typhimurium* aspartate receptor [138, 139]. This receptor is a transmembrane homodimer with two identical binding sites that undergoes conformational change on binding ligand, resulting in the activation of a protein kinase (CheA) leading to signal transduction [140]. In the ligand/receptor complex, only one site is occupied by aspartate, which makes contacts with residues from both monomers. The effect of aspartate binding is to bring the two monomers slightly closer together and make the second (empty site) more crowded so that it cannot accommodate ligand [138, 139].

This may serve as a good model for negative cooperativity in the IGF-1R/IR family. The whole receptor $(\alpha\beta)^2$ dimer, like the aspartate receptor, binds only one ligand molecule with high affinity. A cross-linking model has been proposed to explain this behaviour where, in the high-affinity state, insulin makes contact with distinct regions of the two monomers in the hIR dimer [129–131, 141]. Negative cooperativity follows if the receptor dimer has internal symmetry [130] and binding of ligand in one site causes conformational change that precludes ligand binding at the second site. Structural support for internal symmetry has come from the EM studies [123, 124]. Fluorescence spectroscopy measurements of hIR on ligand binding suggest the effect of insulin binding is to move the $\alpha\beta$ halves into closer proximity [133].

Chemical cross-linking with derivatized ligands

Photoreaction of hIR with an iodinated, LysB29-derivative of insulin labelled a 23-kDa fragment comprising residues 205–316 [142]. This fragment extends from the end of second module of the cys-rich region to the start of the L2 domain (figs 1 and 5). In contrast, insulin substituted at B29 with a different photoaffinity reagent labelled a 14-kDa tryptic fragment suggested to comprise approximately 100 amino acids from the L1 domain beginning at Leu^{20} [143]. The presence of potential glycosylation sites at Asn^{16}, Asn^{25}, Asn^{78} and Asn^{111}, which would contribute to the molecular weight,

Figure 8. Structural features of the first three domains of the IGF-1R. In this figure, the right image shows the structure in the same orientation as Fig. 1 in [86], whereas in the centre and left images the structure has been rotated about the y-axis by 90° and 180°, respectively. (*A*) The amino acid sequence conservation mapped on to the molecular surface of the first three domains of the IGF-1R. The conservation was calculated with the program consindex [385]. The matrix 'gcggap' (a rescaled version of the default Dayhoff matrix from the program GAP of the GCG Wisconsin package) was used to compute the conservation from a multiple sequence alignment of the human, pig, mouse, rat, chick and frog IGF-1R sequences and the human, mouse and rat IR sequences. The colour spectrum in the figure ranges from dark green, signifying very high conservation to orange, representing poor conservation. (*B*) The electrostatic potential mapped on to the molecular surface of the L1/cys-rich/L2 domains of IGF-1R. The electrostatic potential was calculated with the program DelPhi [386] as implemented within the program GRASP [387]. The PARSE3 charge set [388] was used to place atom-centred charges on the molecule. Positive and negative electrostatic potentials are depicted with blue and red colours, respectively.

suggests the tryptic fragment is shorter and probably extends no further than Arg^{86} or Lys^{102}. Insulin derivatized at the B1 position, when bound to soluble hIR ectodomain, labelled an 18-kDa fragment beginning at Gly^{390} (in middle of the L2 domain) and estimated to extend to Arg^{488} [144] in the first FnIII domain (fig. 1). Insulin, with a photoactivable amino acid, benzoylphenylalanine, at the B25 position, could be covalently cross-linked to the hIR and induce autophosphorylation [145]. Gel-shift analysis showed that receptor activation occurred with one insulin molecule per hIR dimer. When these were converted into half-receptors, only 50% were labelled. The contact site for B25 Phe in hIR has been shown to be in the 15-residue peptide sequence, Thr^{704} to Lys^{718} (exon 11 + isoform), near the end of the α chain [146]. Both groups concluded that B25 Phe is involved in the formation of the high-affinity binding state [145, 146].

Receptor chimeras

The results of the studies with IR/IGF-1R chimeras are summarized in table 3 and fig. 9 and indicate that the determinants of specificity for insulin and IGF-1 binding reside in different regions of the two receptors. In the studies with whole receptor chimeras [109, 110],

Table 3. Location of binding determinants in IR/IGF-1R chimeric receptors and ectodomains.

Construct A	Insulin binding (cpm bound)	IGF-1 binding (cpm bound)	Ins/IGF1	Reference
IR	13,000	1,800	7.2/1.0	[104]
IGF1R	300	13,500	1.0/45	[104]
IR/1–211	5,000	3,000	1.7/1.0	[104]
IR/1–217	7,500	2,200	3.4/1.0	[104]
IR/1–273	1,800	8,000	1.0/4.4	[104]
IR/1–282	2,700	10,000	1.0/3.7	[104]
IR/1–435	1,200	7,800	1.0/6.5	[104]
IGF1R/1–735	14,000	4,000	3.5/1.0	[104]
IGF1R/1–452	7,000	4,800	1.5/1.0	[104]
B	**K_d (relative value)**	**K_d (relative value)**		**Reference**
IR	2.3×10^{-10} (1.00)	110×10^{-10} (0.014)		[109, 110]
IGF-1R	160×10^{-10} (0.014)	1.6×10^{-10} (1.00)		[109, 110]
IR/1–131	0	0		[109]
IR/132–315	1.7×10^{-10} (1.35)	1.1×10^{-10} (1.45)		[109]
IR/1–315	70×10^{-10} (0.03)	1.0×10^{-10} (1.60)		[109]
IR/316–514	0	0		[110]
IR/1–131,316–514	0	0		[110]
IR/1–514	230×10^{-10} (0.01)	1.5×10^{-10} (1.07)		[110]
IGF1R/1–137	0	0		[110]
IGF1R/138–325	0	0		[110]
IGF1R/326–524	1.5×10^{-10} (1.53)	0.7×10^{-10} (2.29)		[110]
IGF1R/1–325	0	0		[110]
IGF1R/1–137,326–524	3.6×10^{-10} (0.64)	2.4×10^{-10} (0.67)		[110]
IGF1R/1–524	2.7×10^{-10} (0.85)	240×10^{-10} (0.007)		[110]
C	**IC50 (relative value)**	**IC50† (relative value)**		**Reference**
sIR	8.0×10^{-9} (1.00)	800×10^{-9} (0.001)		[106, 147]
sIGF1R	400×10^{-9} (0.02)	0.4×10^{-9} (1.00)		[106, 107, 147, 148]
sIR/1–62	400×10^{-9} (0.02)	200×10^{-9} (0.002)		[106]
sIR/1–180	400×10^{-9} (0.02)	100×10^{-9} (0.004)		[106]
sIR/1–284*	600×10^{-9} (0.008)	0.8×10^{-9} (0.50)		[147]
sIR/184–279	0.4×10^{-9} (20.0)	1.0×10^{-9} (0.40)		[106]
sIGF1R/1–27	56×10^{-9} (0.14)	1.3×10^{-9} (0.31)		[148]
sIGF1R/28–68	21×10^{-9} (0.38)	0.39×10^{-9} (1.02)		[148]
sIGF1R/38–68	23×10^{-9} (0.35)	0.17×10^{-9} (2.35)		[107, 148]
sIGF1R/38–50	14×10^{-9} (0.57)	0.28×10^{-9} (1.43)		[107]
sIGF1R/42–50	200×10^{-9} (0.04)	0.15×10^{-9} (2.67)		[107, 148]
sIGF1R/1–68	2.0×10^{-9} (4.00)	1.8×10^{-9} (0.20)		[106, 148]
sIGF1R/1–83	4.0×10^{-9} (2.00)	2.0×10^{-9} (0.20)		[106]
sIGF1R/1–191	4.0×10^{-9} (2.00)	4.0×10^{-9} (0.10)		[106]

* Based on K_d values.
† The amount of unlabelled ligand required to reduce binding of iodinated ligand to receptors by 50%.

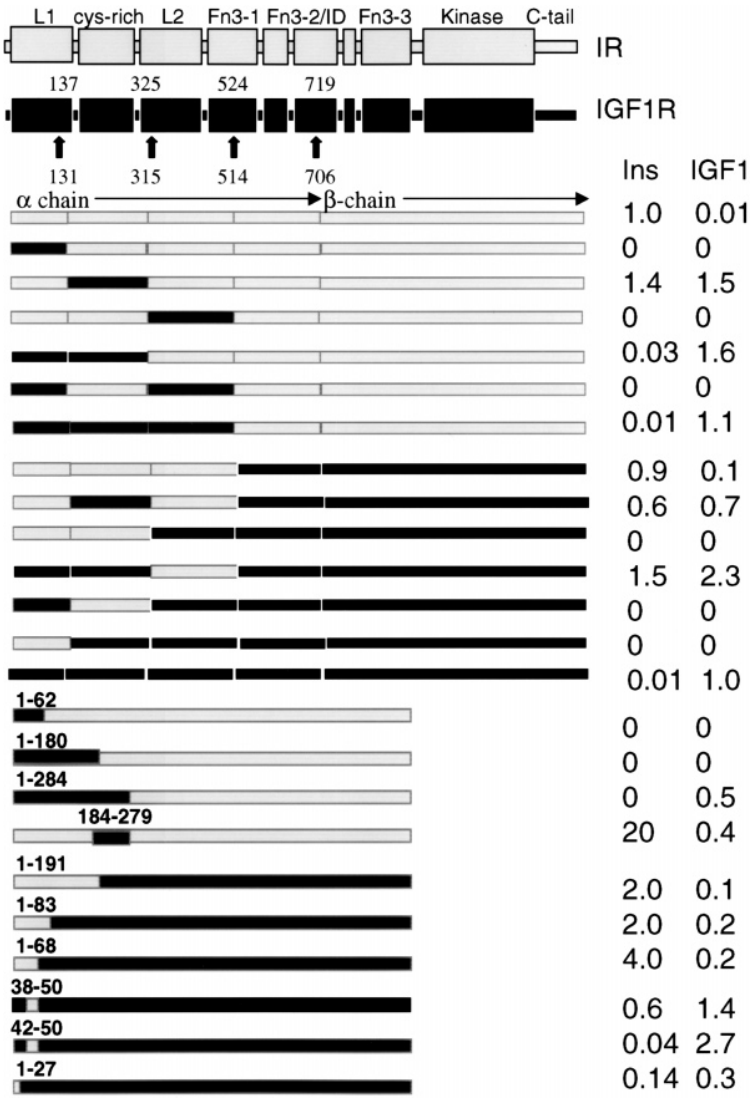

Figure 9. Schematic summary of IR/IGF-1R chimeras. The domain organization of the receptors is shown at the top. The approximate locations of the fragment boundaries exchanged in the whole receptor chimeras are shown, as are the α–β cleavage sites for hIR and hIGF-1R. The fragment composition of each chimera is shown by solid and open boxes. The residues substituted in the ectodomain IR-based or IGF-1R-based chimeras are indicated above each construct. The relative binding affinities of the parent receptors and the various chimeras are shown opposite each construct on the right hand side. Based on [106, 107, 109, 110, 147, 148].

three fragments from the N-terminal region of hIR and hIGF-1R were joined in different combinations in hIR and hIGF-1R backgrounds. In this review the hIR-derived fragments are referred to as 1, 2 and 3, and the hIGF-1R-derived fragments as I, II and III, to enable related chimeras such as 1-II-3-hIR and 1-II-3-IGF1R to be readily compared. The data shown in table 3 and fig. 9 show that fragment II, residues 131–315 in the IGF-1R (cys-rich plus flanking regions from L1 and L2), is a prime requirement for IGF-1 binding. In contrast, fragments 1 and 3 (residues 1–137 in the L1 domain of hIR and residues 325–524, comprising most of the L2 domain and part of the first FnIII domain of hIR), are important determinants of insulin binding. The supporting data are as follows: (i) chimera 1-2-3-IGF1R resembles hIR and binds insulin with high affinity; (ii) chimeras I-II-III-IR and I-II-3-IR resemble the IGF-1R and bind IGF-1 preferentially; (iii) chimeras 1-II-3-IR and 1-II-3-IGF1R, which contain all three optimal elements for binding IGF-1 and insulin,

bind both ligands well; (iv) chimeras 1-2-III-IR, 1-2-III-IGF1R and I-2-3-IR, which lack two of the specific, ligand-binding regions and chimeras I-2-III-IR and I-2-III-IGF1R, which lack all three, bind neither ligand. Two of the IGF-1R-based chimeras showed unexpected characteristics: chimera 1-II-III-IGF1R failed to bind IGF-1 despite containing fragment II; and I-II-3-IGF1R bound both ligands with high affinity despite the lack of the hIR-derived fragment 1 (residues 1–137). The differential binding specificities of the hIR-based chimeric receptors containing residues 1–217 of IGF-1R and 1–274 of IGF-1R (table 3A) add further support to the importance of the cys-rich region in IGF-1 binding [104]. Additional support for the importance of the L2-FnIII-1 region in insulin binding comes from antibody competition studies. Many autoantibodies and several monoclonal antibodies that inhibit insulin binding recognize an epitope in the region 450–601, which overlaps residues 326–524 [108]. In addition, an hIR-based chimera where residues 450–601 were replaced with the corresponding residues from hIRR showed decreased insulin binding [108].

The studies with ectodomain chimeras provide similar findings to those obtained with whole-receptor chimeras (table 3C, fig. 9). The hIR-based chimera containing IGF-1R residues 1–284 resembled the IGF-1R ectodomain and showed high affinity for IGF-1 and poor binding for insulin [147]. The chimeras with smaller IGF-1R fragments, either 1–180 or 1–62, lack the appropriate specificity determinants for either ligand and showed poor binding of both ligands as expected [106]. The ectodomain chimera with IGF-1R residues 184–279 showed high affinity for both ligands, particularly insulin. The region controlling IGF-1 specificity has been further narrowed to the 14 residues (amino acids 253–266) in the variable loop of module 6 of the cys-rich region. The chimeric receptor where residues 260–277 of hIR were replaced with residues 253–266 from hIGF-1R was not significantly different from wild-type hIR in terms of insulin binding affinity, but was more amenable to displacement of radiolabelled ligand by IGF-1 than the parent hIR [122].

The importance of the N-terminal region in insulin binding was confirmed by examining a series of IGF-1R-based chimeric ectodomains [106] where the N-terminus contained decreasingly smaller proportions (191, 83 and 68 residues, respectively) of hIR-derived sequences. As summarized in table 3C, all showed similar binding affinities, binding insulin with comparable affinity to wild-type hIR while retaining relatively high (10–20%) binding affinity for IGF-1 [106, 148]. The 1-68/63-1337 IR/IGF-1R whole receptor chimera displayed relative ligand affinities similar to the corresponding ectodomain construct, validating the use of ectodomain constructs in such studies [148]. IGF-1R chimeras with residues 1–137 or 1–325 from hIR failed to bind insulin or IGF-1 [110]. There are at least two determinants in the N-terminal 68 residues of hIR responsible for the 200-fold increase in insulin binding, since the 1–27 and 28–68 chimeras displayed 8-fold and 20-fold increases in insulin affinity, respectively, compared with IGF-1R [148]. The 28–68 region of hIR contains 15 sequence differences from IGF-1R at residues 32, 34, 38, 39, 41–43, 46, 48–50, 55, 56, 59 and 67 (fig. 1). The differences at 32 and 34 in hIR appear unimportant, as the 38–68 chimera showed enhanced binding affinities, comparable to the 28–68 chimera [148]. Similarly, the differences at 55, 56, 59 and 67 are not important since a 38–50 chimera still exhibited enhanced affinity for insulin [107], and an IGF-1R with mutations $Thr^{49}Ile$, $Val^{50}Met$, $Glu^{53}Asp$ and $Ala^{61}Tyr$ resembled the IGF-1R and bound insulin poorly. Enhanced insulin binding was retained in a chimera in which only residues 38–43 corresponded to hIR, whereas a 42–50 chimera showed only a twofold increase in insulin affinity [107, 148].

Residues 38–43 are predicted to lie in the second rung of the L1 β-helix domain [86] at the edge of the putative binding pocket (fig. 5). The region 223–274 in IGF-1R, implicated in IGF-1 specificity, contains major sequence differences when compared with hIR (fig. 1). It corresponds to modules 4–6 in the cys-rich region and includes a large and somewhat mobile loop [residues 255–263, mean B(C^{α} atoms) = 57 $Å^2$] which extends into the central space (see fig. 5). In hIR, this loop is four residues bigger, differs totally in sequence and is stabilized by an additional disulfide bond [54, 87]. The improvement in IGF-1 binding by the hIR cys-loop exchange chimera, hIR_CLX, suggests that the larger loop of hIR may serve to exclude IGF-1 from the hormone binding site but allows the smaller insulin molecule to bind [122]. It is interesting to note that the mosquito IR homologue, which has a loop two residues larger than the mammalian IRs, also appears to bind insulin but not IGF-1 [75]. The region 326–524 in IR, also implicated in insulin binding, starts in the middle of the first rung of the L2 β-helix domain [86] and extends to Cys^{514} in the middle of the first Fn III domain (fig. 1).

Point mutations in the ectodomain

As chimeras only address residues which differ between the two receptors, a more precise analysis of binding determinants can be obtained from single-site mutants. Most of these studies have been carried out on hIR and include analyses of mutant receptors from patients as well as receptors generated by site-specific mutagenesis. The naturally occurring mutations of hIR fall into five classes: impaired biosynthesis of full-length receptor

(class 1), impaired transport to the plasma membrane (class 2), decreased binding affinity (class 3), defective tyrosine kinase (class 4) and accelerated receptor degradation (class 5). These mutations have been described in detail [149–152].

L1 domain mutations. The Asn[15]Lys mutation was found in a patient with severe insulin resistance, and was associated with defects in IR processing, cell-surface expression and a fivefold reduction in insulin affinity [153]. Asn[15] was identified as a major determinant of insulin binding after alanine scanning mutagenesis of the L1 domain [154]. Alanine mutations made at 47 single sites and six residue pairs in the L1 domain showed that 14 changes in four discontinuous peptide segments (table 4) disrupted insulin binding [154]. The first segment involved residues Asp[12], Ile[13], Arg[14] and Asn[15] with the latter two mutations having major effects (250–700-fold reductions) on insulin binding. Arg[14] appears to interact directly with the C-terminus of insulin since the truncated superanalog X92 binds the Arg[14]Ala mutant only eightfold lower than the wild-type receptor [155]. Mutations in the second segment (residues Gln[34], Leu[36], Met[38], Phe[39] and Glu[44]) caused a 3- to 25-fold reduction; one in the third segment (Phe[64]) had a major ($>$ 700-fold reduction) effect, whereas mutations in the fourth segment (residues Phe[89], Asn[90] and Tyr[91]) caused only a 3- to 6-fold reduction in binding (table 4).

Phe[39], and to a lesser extent Arg[42] and Pro[43] in the second segment described above, had been shown previously to be the hIR residues responsible for increased insulin binding by the IR/IGF-1R chimeras [107]. The reciprocal mutation, Phe[39]Ser in hIR, decreased insulin binding eightfold [107]. Recently, two further natural mutants have been described which involve residues Asp[59] and Leu[62] in the third segment [152]. The Asp[59]Gly mutant receptor showed a fourfold reduction in insulin binding but normal levels of expression on the cell surface. In contrast the Leu[62]Pro mutant showed normal insulin affinity, but major defects in folding, oligomerization and transport to the cell surface [152].

Table 4. Effect of mutations on ligand binding by hIR.

Mutation	K_d (nM)	Relative affinity (%)
Wild-type receptor	1.4	1.00
D12A	9.12	0.154
I13A	16.31	0.085
R14A	$>$1000	$<$0.0014
N15A	352	0.004
Q34A	18.10	0.077
L36A	14.10	0.099
M38A	4.43	0.316
F39A	35.6	0.039
E44A	4.84	0.289
F64A	$>$1000	$<$0.0014
Y67A	3.22	0.435
F89A	5.17	0.271
N90A	8.83	0.159
Y91A	3.85	0.364

Based on [154].

Residues in segment four have been investigated by other workers with similar findings for the Phe[88]Ala mutation (no effect) and the Phe[89]Ala mutation (threefold reduction) [109]. Changing Phe[89] to Tyr, the residue present in hIGF-1R (fig. 1), had no effect on binding [109], whereas the Phe[89]Leu mutations (the residue present in hIRR) obliterated insulin binding capacity [156]. The Phe[88]Leu [156] and Arg[86]Asn single mutants, and the Ser[85]Thr/Arg[86]Asn and Ser[85]Trp/Arg[86]Lys double mutants [157], were normal and comparable to the double Ala mutant [154]. Two mutations associated with leprechaunism, Arg[86]Pro and Leu[87]Pro, occur in this region of the receptor [157, 158]. The Arg[86]Pro mutant hIR failed to bind insulin or stimulate growth, but did cause a significant increase in basal glucose transport in CHO cells [159]. The importance of Leu[87] is indicated by the fourfold enhancement of insulin binding by a Leu[87]Ile mutant and the sixfold reduction in ligand binding by the Leu[87]Ala mutant [158]. Other natural mutations of the hIR L1 domain are the Ile[119]Met class 2 mutation associated with leprechaunism and Rabson-Mendenhall syndrome [160], and the Lys[121] deletion (Leprechaun Rochester) which displayed normal insulin binding but elevated basal levels of autophosphorylation and blunted insulin responsiveness [161].

Alanine scanning mutagenesis has been carried out in the L1 domain of the IGF-1R [162] at residues equivalent to 10 of the 14 amino acids identified as important contributors to insulin binding in hIR [154]. Mutations of Asp[8], Asn[11] and Phe[58] in hIGF-1R resulted in a three- to fourfold reduction in affinity for IGF-1, whereas the other seven mutations were without effect [162]. This is in marked contrast to the effects of these mutations on insulin binding to the hIR [154]. Of the 10 IGF-1R mutations, only 1, Arg[10]Ala, had a significant effect on insulin binding as measured by insulin displacement of labelled IGF-1 or labelled insulin superanalog X92 [162].

As shown in figures 1 and 10, the four segments identified in the alanine scan [154] are located at similar positions on the first four turns of the L1 β helix. These residues, along with the naturally occurring mutants Asp[59] and Leu[62] [152], form a footprint for insulin binding on the first half of the second β sheet, which faces into the central cavity formed by the L1/cys-rich/L2 domains. Residues further along the sheet, which are conserved in IGF-1R and were not subjected to alanine mutagensis, could also be important [86]. The two leprechaun-associated mutations, Ile[119]Met and the Lys[121]

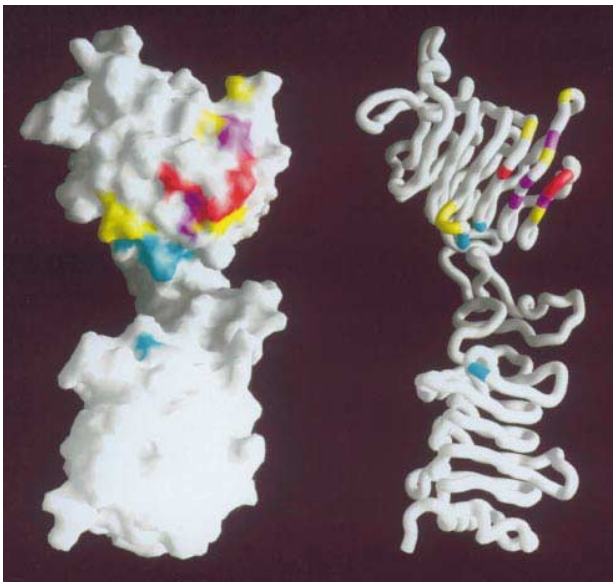

Figure 10. Mutations plotted on 3D structure of hIGF-1R L1/cys-rich/L2 fragment. The Ala scan mutations [154] are colour-coded: red, >300-fold reduction in affinity for mutations at residues 14, 15 and 64 (hIR numbering); magenta, 10–100-fold reduction in affinity for residues 13, 34, 36 and 39; yellow, 3–9-fold reduction in affinity for residues 12, 38, 44, 67, 89, 90 and 91 (see table 4). The natural mutants $Asp^{59}Gly$ [152], $Arg^{86}Pro$ [159], $Leu^{87}Pro$ [158] and $Ser^{323}Leu$ [166] are shown in cyan. Residue 15 in hIR is also a site for a natural mutation $Asn^{15}Lys$ [153]. The image on the right-hand side is the structure in the same orientation displayed as a 'worm' depiction with the position of the mutations indicated.

deletion, involve residues in a similar location on the fifth rung of the L1 β helix (figs 1 and 10).

Cys-rich domain mutations. Apart from the chimera studies discussed earlier, there has been very little investigation of the contributions of specific residues in the cys-rich region to ligand binding. A $Pro^{243}Arg/Pro^{244}Arg/His^{246}Asp$ triple mutation showed two- to threefold higher insulin affinity than normal receptors, and enhanced tyrosine kinase and amino acid transport responses [163]. The small magnitude of the effects of these substantial mutations has led to the conclusion that these residues are not directly involved in insulin binding [164]. Five class 2 IR mutations which affect posttranslational processing and assembly map to this region [151]. A patient with a 3-bp deletion removing Asn^{281} in module 6 of the cys-rich region had an hIR with impaired insulin binding but constitutive activation of the tyrosine kinase [165]. Cells expressing this receptor were desensitized to insulin stimulation of glycogen and DNA synthesis, suggesting that Asn^{281} plays a critical role in the inhibitory constraint exerted by the extracellular α subunit over the intracellular kinase activity.

L2 domain mutants. The mutation $Ser^{323}Leu$ has been reported in two severely insulin-resistant patients with Rabson-Mendenhall syndrome [166]. The mutant IR is processed normally and transported to the plasma membrane but has very low binding affinity for insulin, indicating that Ser^{323} forms part of the insulin binding site or stabilizes its conformation. While the Leu^{323} mutant homodimer cannot bind insulin, a heterodimeric receptor comprising subunits derived from Leu^{323} and a C-terminal truncation of the wild-type receptor binds insulin with high affinity [167]. One explanation for these observations is that the high-affinity site for insulin includes regions provided by both half-receptors, similar to the interactions seen in the growth hormone ligand/receptor complex [96]. The mutant receptor is only deficient in one of the components of the high-affinity site and can form high-affinity complexes as a hybrid receptor. An alternative explanation is that the high-affinity site is formed within one half-receptor (here the $\Delta 43$ moiety), but only when it is associated as a receptor dimer to enable the domain rearrangements (conformational changes) required for high-affinity binding to take place. It is not possible to discriminate between the active or passive models for high-affinity binding, but it is interesting to note that the $Ser^{323}Leu$ mutant receptor can bind and be activated by the monoclonal antibody Mab 83-14 in a manner similar to normal IR [168].

As shown in figure 5, the axis of the L2 domain is perpendicular to that of the L1 domain, and the N-terminal end of its β helix is presented to the putative hormone binding site. The equivalent residue to Ser^{323} in hIGF-1R, Ser^{313}, lies on this face of the L2 domain. Structurally, Ser^{313} lies in the middle of a region, residues 309–318, which is conserved in both hIGF-1R and hIR (residues 319–328). The surrounding region, 332–345 of hIGF-1R, is also quite well conserved in both receptors (fig. 1) but not in hIRR [29]. The distance from this face (first rung) of the L2 to the second β sheet of the L1 domain is about 30 Å (fig. 5). Thus L1 and L2 appear too far apart to bind IGF-1 or insulin. However, it has been pointed out [86] that in the crystal structure there is a deep cleft between part of the cys-rich domain (residue 262) and L2 (residue 305), and this cleft is occupied by a loop from a neighbouring molecule. Thus it is probable that the position of the L2 domain in the receptor structure or the hormone-receptor complex may adopt a different position with respect to the cys-rich domain than that found in the crystal. The movement required to bring L2 sufficiently close to L1 is small, namely a rotation of approximately 25° about residue 298 [86].

Other mutations in the L2 domain that have been described in hIR are the three class 2 natural mutations

Gly^{366}Arg, Phe^{382}Val and Trp^{412}Ser, which all display defective proreceptor processing and transport to the plasma membrane but no reductions in insulin affinity [151]. The Gly^{366}Arg receptor can undergo insulin-induced autophosphorylation, whereas the Phe^{382}Arg and Trp^{412}Ser mutants do not [150, 169–171], indicating a defect in their ability to undergo postbinding conformational change. The Phe^{382}Val receptor was shown to be constrained in a conformation that is not changed on insulin binding [172], compared with the changes seen in the exposure of epitopes in the intracellular domain following ligand binding to the wild-type receptor [173]. The Phe^{381}Val and Phe^{382}Leu mutations showed only slight impairments in intracellular processing and transport of hIR and normal insulin-induced internalization responses [174].

The final L2 domain mutations are the two hIR class 5 mutants, Lys^{460}Glu and Asn^{462}Ser, located in the sixth turn of the L2 domain, at the bottom of the third β sheet (fig. 5). Both mutations lead to enhanced receptor degradation. The authors suggest that Lys^{460} is not part of the insulin binding site but is located at the interface between adjacent receptor monomers in the hIR dimer [153], a suggestion supported by the 3D structure of the L1/cys-rich/L2 fragment of IGF-1R. The Lys^{460}Glu mutant and a Lys^{460}Asp receptor have enhanced affinity for insulin and significantly reduced dissociation of bound insulin under acid conditions in the endosome following internalization [153]. This persistent insulin binding inhibits receptor recycling and promotes the targeting of the mutant receptor for degradation within the lysosome. A range of other mutations (Arg, Val, Met, Thr and Gln) at residue 460 had no effect on sensitivity to acid pH.

First and second FnIII domain mutants. There are no reported mutations for the first or second FnIII domains of hIR or hIGF-1R, apart from the investigations of Cys mutations on the disulfide bond arrangements of the receptor [90–92, 94].

Insert domain mutants. Alanine scanning mutagenesis of the region 704–717 of the exon 11+ hIR ectodomain has confirmed its importance in insulin binding [175]. This region was implicated in ligand binding by chemical cross-linking of a derivatized insulin to the whole receptor [146]. In addition, this region produced a 70-kDa minimal binding fragment that bound insulin with an affinity that was similar to the full-length hIR ectodomain when fused directly to the end of the L1/cys-rich/L2 domain fragment of hIR, [176]. A similar minireceptor fragment has been produced for the hIGF-1R and has a binding specificity comparable to the full-length hIGF-1R ectodomain [177]. Only 4 of the 14 residues in this region of hIR (Asp^{707}, Val^{712}, Pro^{716} and Arg^{717}) could be mutated to Ala without compromising insulin binding [175]. The mutations at Thr^{704}, Phe^{705}, Glu^{706}, His^{710} and Val^{713} each resulted in a fall in affinity of more than 1000-fold, whereas mutation of Tyr^{708}, Leu^{709} and Phe^{714} caused 80–100-fold reductions in insulin binding affinity. Since these studies were carried out on soluble ectodomain, which exhibits only low-affinity binding, the authors [175] conclude that these residues are involved in binding site 1 [130]. Nine of these mutant receptors were examined for their capacity to bind the high-affinity insulin analogue X92 [155]. In general the decreases in affinities were comparable with insulin and X-92, the exceptions being the Thr^{704}Ala, and to a greater extent, the His^{710}Ala mutant ectodomains, which bound X92 better than insulin [155].

Mutations of seven of these residues in the corresponding region of hIGF-1R had much lower effects on IGF-1 binding, except for Phe^{701}Ala. The Leu^{696}Ala and Val^{702}Ala mutants showed normal IGF-1 binding, whereas the Phe^{692}Ala, Glu^{693}Ala, His^{697}Ala and Asn^{698}Ala mutants showed falls in affinity of only 3.6- to 5.6-fold [162]. The Phe^{701}Ala mutant resulted in a receptor with undetectable IGF-1 binding [162], in contrast to the mutation of this residue in hIR, which decreased insulin binding by 140-fold [175]. The relative levels of insulin binding by these seven IGF-1R mutants were similar to those found with the corresponding hIR mutants [162].

The importance of Phe^{701} was reinforced in the studies of hIR- or hIGF-1R-based chimeric minireceptors. Swapping the carboxy-terminal domains (16 amino acids) with that from human hIRR completely abolished insulin and IGF-1 binding by either hIR- or hIGF-1R-based minireceptor chimeras, whereas chimeras involving the carboxy-terminal domains of hIR or hIGF-1R were little affected [177]. Sequence comparisons of hIRR, hIR and hIGF-1R suggest the substitution of Thr for Phe at the position equivalent to 701 in IGF-1R is responsible for this loss of ligand binding by the chimeras containing the carboxy-terminal peptide from the hIRR α chain.

Third FnIII domain mutants. Linkage analysis of a Japanese family suggested that the mutation Thr^{831}Ala (exon +11 numbering) is possibly responsible for the onset of non-insulin-dependent diabetes mellitus [178]. As shown in figure 1 and table 1, this residue is predicted to occur in the A strand of the third FnIII repeat. The other two mutations in this third FnIII module are Thr^{910}Met, in the G strand, and Asp^{919}Glu, five residues after the end of the G strand (fig. 1, table 1), both of which are leprechaun class 4 mutations, with defective kinase activity [150]. The structural basis for these effects, if real, is not known.

Ligand mutations

Insulin

There is a very large literature on the structure-function relationships of insulin and IGFs [130, 179–183] and their respective receptors. The sequence alignment and secondary structure assignments for insulin, IGF-1 and IGF-2 are shown in figure 11. The initial receptor-binding site on insulin was identified as Val^{B12}, Tyr^{B16}, Phe^{B24}-Phe^{B25}-Tyr^{B26} and Asn^{A21} from the dimer interface, and Gly^{A1}, Glu^{A4} and Tyr^{A19} on an adjacent face [184]. Additional residues such as Thr^{A8} were also suggested because of the wide range in biological potency of insulins from different species [184]. The binding sites were extended to include Gly^{B23} from the dimer interface and Gln^{A5} on the adjacent face [156, 185].

Alanine scanning mutagenesis of insulin showed that the mutations at Gly^{B23}, Phe^{B24}, Ile^{A2}, Val^{A3} and Tyr^{A19} were the most disruptive in terms of receptor binding [181]. These residues form a patch in the insulin molecule, part of which is covered by the C-terminus of the B chain. However, the C-terminus of the B chain is thought to move following receptor binding [186, 187], potentially exposing this hydrophobic region. Structural insights into such a change have been gained from a Tyr^{B16}Glu, Phe^{B24}Gly, desThr^{B30} insulin where the B20–25 region is rearranged so that the last five residues B26–B30 move sufficiently to expose Gly^{A1}, Ile^{A2} and Val^{A3} [188]. The low biological activity (2000-fold lower) of an A-chain analogue lacking the A6–A11 disulfide bond further confirmed the importance of the N-terminal region (A1–A8) of the A chain [189]. This analogue exhibited localized, segmental unfolding in an otherwise native structure. Its poor biological activity suggests that this segment, a site of clinical mutation causing diabetes mellitus, functions as a preformed recognition α helix [189]. Other residues important for insulin binding are the hexamer surface, including His^{B10}. Arguments supporting alternate proposals for the sequential order of binding of these different sites has been reviewed [130].

IGFs

There have been fewer studies on the IGFs and the regions involved in receptor binding. Relative to insulin, IGF-1 and -2 contain two extra regions, the C region between the B and A domains and a D peptide at the C-terminus (fig. 11). Replacing residues 1–16 of IGF-1 with the first 17 residues of the insulin B chain, which involves 10 sequence differences (fig. 11), gave only a twofold reduction in IGF-1R binding [190]. This can be compared with the >1000-fold reduction in binding to IGFBPs or the 100-fold reduction in binding IGF-2R [190]. Changing just two of these residues in IGF-1, to the corresponding sequence in insulin (Gln^{15}Tyr-Phe^{16}Leu), increased affinity for IR by 10-fold, had no change in affinity for IGF-1R or IGF-2R, and reduced affinity for the serum binding proteins by fourfold [190]. A different double mutant, Glu^{3}Gln-Thr^{4}Ala, showed fourfold reduction in binding to IGFBPs but normal IGF1 binding to IR, IGF-1R and IGF-2R [190]. The Glu^{3}Ala mutation decreased BP-1 binding 34-fold but had no effect on binding to IGFBP-3 [191]. Systematic alanine scanning mutagenesis of IGF-1 revealed no side-chain specificity determinant for IGFBP-3, but did establish that the polypetide backbone between residues 3 and 4 was important for IGF-1 binding to IGFBP-3 [191]. The quadruple mutant showed a dramatic (600-fold) reduction in affinity for the binding proteins, but otherwise resembled the (Gln^{15}Tyr-Phe^{16}Leu)-IGF-1 ligand with respect to 10-fold enhanced IR binding and normal IGF-1R and IGF-2R binding [190]. This identifies residues critical for IGFBP interactions and suggests that the insulin residues Tyr^{16} and Leu^{17} are important for IR binding but not IGF-1R binding. The importance of the N-terminal residues in IGFBP interactions are further supported by the improved potencies of des(1–3)IGF-1 and des(1–4)IGF-1 [192].

Residues in the IGF-1 A region are important for binding to the type 2 receptor and to the IGFBPs, but not the type 1 receptor. Replacing the A-domain residues 42–56 of IGF-1 with A1–15 of insulin, together with a Thr^{41}Ile mutation (eight differences, fig. 11) had no effect on IGF-1R binding, reduced IGF-2R binding 20-fold and increased IR binding by 7-

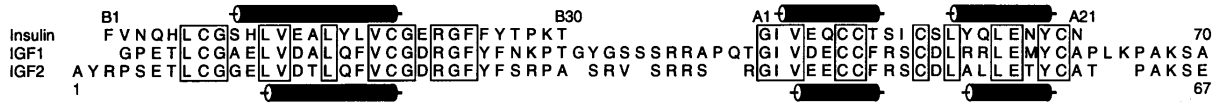

Figure 11. Sequence alignments of human insulin and human IGF-1 and IGF-2. The secondary structural assignments are depicted as cylinders for α helices and arrows for β strands. The assignments for insulin are shown above the sequences, whereas the assignments for IGF-2 are below. The secondary structure has been assigned using the 3D structures of insulin [183] and IGF-2 [33].

fold [193]. The areas of greatest divergence between the A domains of insulin and the IGFs are residues 49–51 and 55–56 (fig. 11). Replacement of residues 49–51 with the corresponding sequence from insulin had no effect on IGF-1R or IR binding but reduced IGF-2R binding by 20-fold [193] and IGFBP interactions 20–1000-fold [194]. Thus the determinants for type 1 receptor binding are different than those required for interaction with the type 2 receptor or serum BPs. Differential effects on binding protein interactions are also found; the mutation $Phe^{49}Ala$ reducing BP-1 binding by 100-fold but IGFBP-3 binding by only 4-fold [191]. The double mutant $Arg^{55}Tyr$ and $Arg^{56}Gln$, where the residues in IGF-1 are replaced by the corresponding residues in the insulin sequence, had no effect on IGF1-R or IR binding but resulted in a sevenfold increase in affinity for the type 2 receptor [193]. The triple exchange mutant $Arg^{50}Ser$, $Arg^{55}Tyr$ and $Arg^{56}Gln$ in a recombinant construct gave a similar result, with no effects on IGF-1R or IR binding [195]. The structure of IGF-1 is very sensitive to amino acid substitutions, with the loss of binding due to either direct effects on receptor interactions or indirect effects on ligand structure [196]. Residues implicated in IGF-1R binding are Phe^{23} and Tyr^{24} in the B region, Tyr^{31} in the C domain and Tyr^{60} in the A region of IGF-1 [197, 198], and the corresponding residues Phe^{26} and Tyr^{27} in IGF-2 [199, 200]. Tyr^{24} and Phe^{25} can be changed to the Phe.Tyr sequence found in insulin without affecting IGF-1R, IGF-2R and IR binding [197]. Tyr^{24}, Tyr^{31} and Tyr^{60} are protected from iodination when bound to IGF-1R [201]. Residues Phe^{23}, Tyr^{24} and Tyr^{31} form part of a hydrophobic patch on the surface of IGF-1, whereas Tyr^{60} is largely buried and unlikely to interact directly with the IGF-1R. Similarly, the reduction in IGF-1R binding by a $Phe^{16}Ala$ mutant is concluded to be due to the loss of structural integrity rather than direct binding interactions [196]. The mutations $Ala^{8}Leu$ and $Met^{59}Phe$, on opposite sides of the IGF-1 molecule, decreased binding to IGF-1R and IR ectodomains 5- to 6-fold and 17- to 28-fold, respectively [202]. Asp^{12}, which is adjacent to Ala^{8} on the IGF-1 surface, appears to contribute to IGF-1R binding, as the $Asp^{12}Ala$ mutant showed a fourfold reduction in affinity [196]. In addition, an $Ala^{62}Leu$ mutant showed eightfold and twofold reductions in binding to the IGF-1R and IR ectodomains, respectively [202]. The B-domain helix mutants of IGF-1, $Val^{11}Ala$, $Val^{11}Thr$, $Gln^{15}Ala$ and $Gln^{15}Glu$, showed smaller (1.5- to 3-fold) reductions in affinity, which have been correlated with structural defects such as the loss of α-helix content in the mutant ligand [196, 203]. Finally, the A-chain helix mutant of IGF-2, $Val^{43}Leu$, showed a 16-fold reduction in IGF-1R binding, and a 220-fold reduction in IR binding, but normal binding to the IGF-2R [200]. The corresponding mutant in IGF-1 has not been examined.

One notable feature of IGF-1 and -2 is the large number of charged residues and their uneven distribution over the surface. Replacement of the C region of IGF-1 by a four Gly linker reduced affinity for IGF-1R by 40–100-fold, with a 10-fold or no reduction in binding to IR [204, 205]. An IGF-1 analogue in which residues 29–41 of the C region have been deleted (mini IGF-1) showed no affinity for either receptor, indicating that the C region of IGF-1 contributes directly to the free energy of binding to the IGF-1R [205]. These authors suggested that binding of IGF-1 to the IGF-1R resembles insulin/IR binding, and involves a conformational change in which the C-terminal B-region residues are displaced from the body of the molecule to expose the underlying A-region residues. It has been shown that deletion of the C-region of IGF-1 results in a substantial tertiary structural rearrangement that can account for the loss of receptor affinity [206]. Truncation of the nearby D peptide in IGF-2 reduced IGF-1R binding sixfold [199], whereas the corresponding truncation of IGF-1 had no effect [200]. Ala mutants have implicated Arg^{21}, Arg^{36} and Arg^{37} in IGF-1R binding, whereas the Arg^{50}, Arg^{55} and Arg^{56} mutations had smaller effects [207], in agreement with the IGF-1/insulin sequence exchanges involving Arg^{50}, Arg^{55} and Arg^{56} [193, 195]. The region of the receptor responsible for recognizing $Arg^{36}Arg^{37}$ was shown to be the cys-rich region 217–284 [195]. The putative binding site of the receptor, which incorporates these residues, has a sizeable patch of acidic residues in the corner where the cys-rich domain departs from L1. Other acidic residues which are specific to this receptor are found along the inside face of the cys-rich domain and the loop (residues 255–263) extending from module 6. Thus it is possible that electrostatics play an important part in IGF-1 binding, with the C region binding to the acidic patch of the cys-rich region near L1, and the acidic patch on the other side of the hormone directed towards a small patch of basic residues (residues 307–310) on the N-terminal end of L2 [86].

It is instructive to compare the distribution of the electrostatic potential on the IGF-1R (fig. 8B), with that on the ligand, IGF-1 (fig. 12). The bottom panel shows the electrostatic potential mapped on to the molecular surface of IGF-1, shown in three different orientations. The orientations are shown in the top panel, with the IGF-1 molecule represented by a backbone 'worm'. The first orientation has the loop of the 'C region' (residues 29–41) of this 'wedge'-shaped IGF-1 molecule pointing downwards and the face of IGF-1 containing the N- and C-termini of the molecule facing the reader. Rotation by 180 degrees about the y-axis (so now the face containing the N- and C-termini are facing away from the reader) yields the second orientation, whereas the final orientation shows the view from the 'top' of the

'wedge'. The IGF-1 residues implicated in binding to the receptor (residues Ala^{8}, Asp^{12}, Phe^{23}, Tyr^{24}, Tyr^{31}, Arg^{36}, Arg^{37}, Ile^{43} but not Arg^{21}, Met^{59} or Ala^{62}) are predominantly in the 'C-region' loop and on the surface facing the reader in the middle view (i.e. that opposite the face containing the N- and C-termini). It can be seen that the 'bottom' of the IGF-1 'wedge' containing Arg^{36} and Arg^{37} shows electrostatic potential complementarity to the cys-rich domain of IGF-1R. This complementarity may have an orientational role in the binding of the ligand to its receptor. The 3D structure of IGF-1 used in these calculations was from the nuclear magnetic resonance (NMR) ensemble of structures solved by Cooke et al. [208], and it should be noted that the C-region loop is highly flexible in the experimental structure.

Signal transduction: substrates and effectors

It is not surprising, given the diverse physiological functions regulated by IGF-1, that a diverse (and expanding) repertoire of intracellular proteins has been identified as substrates/effectors of ligand-activated IGF-1R signalling. Many of these are shared not only with the IR, but also with other receptor tyrosine kinases and members of the cytokine receptor superfamily. With respect to the IGF-1R, this area has been reviewed a number of times, most recently and comprehensively by LeRoith et al. [209]. As such, we will only identify the major components of the signalling pathways activated by IGF-1, although their participation in terms of the cellular outcomes of growth, differentiation and apoptosis will be highlighted in the following sections.

Ligand-induced activation of the IR kinase requires the sequential phosphorylation of three conserved tyrosines within the activation loop of the catalytic domain [210]. The deletion of the corresponding three residues of the IGF-1R, at positions 1131, 1135 and1136, renders the kinase domain inactive [211–213], although the role played by individual tyrosines in the biological activity of the receptor is subject to conjecture. The subsequent phosphorylation of other tyrosines within the cytoplasmic domain of the receptor provides 'docking' sites for the recruitment of other proteins as an obligatory step for initiating the signalling cascade. The residues in-

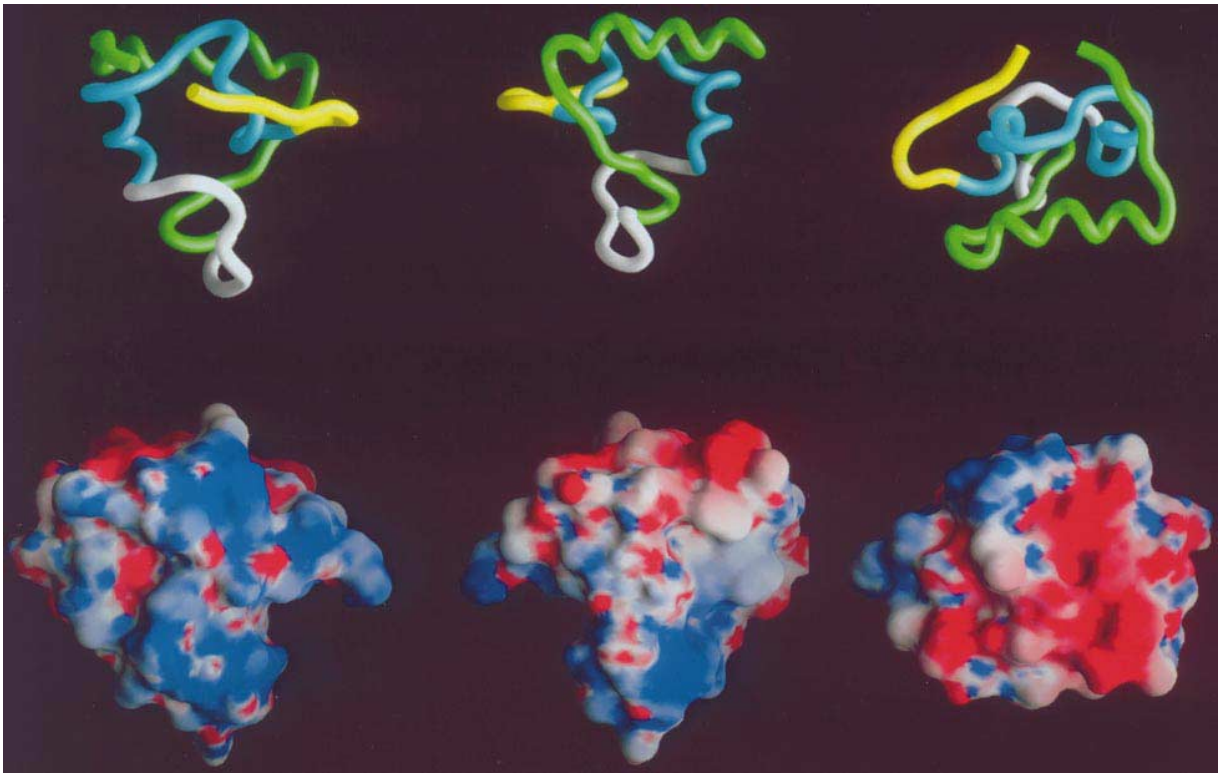

Figure 12. Electrostatic potential of IGF-1. Bottom panel: the electrostatic potential mapped on to the molecular surface of IGF-1. Top panel: 'Worm' representation of the backbone of IGF-1 in the same orientation. The colour coding is B domain, green; C domain, white; A domain, blue; D domain, yellow. The left-hand image has been rotated 180° about the y-axis to give the centre image, whereas the right-hand image is a view of the centre model from the top (see text for details).

volved in binding signal transduction molecules are summarized in table 5. The two best-characterized pathways associated with IGF-1R activation are based around two distinct adaptor proteins, Shc and the insulin receptor substrates (IRSs). There are three isoforms of Shc, derived by both alternate splicing and multiple translational initiation of transcripts from a single gene [214], whereas there are at least four genes (*Irs-1–4*) that encode the IRS proteins, of which IRS-1 and IRS-2 are the best characterized [209]. IRS-1, IRS-2, Shc and by extrapolation IRS-3 bind to the same phosphotyrosine (P-Tyr^{950}), within the juxtamembrane region of the IGF-1R cytoplasmic domain, using functionally related phosphotyrosine-binding (PTB) domains [215–219]. In the case of Shc and IRS-1, the phosphorylation of individual residues of the tyrosine cluster within the catalytic domain may differentially influence binding [216], whereas an additional domain has been identified in IRS-2 that can bind the IGF-1R in a phosphotyrosine-dependent manner, independent of P-Tyr^{950} [218]. There are, however, subtle differences in the subsequent tyrosine phosphorylation of these adaptors: for example, while maximal phosphorylation of IRS-1 is observed within 1–2 min of IGF-1 stimulation, for Shc the time frame is 5–10 min [220, 221]. Indeed, it appears that receptor internalization may be required before maximal phosphorylation of Shc is achieved [221].

The tyrosine phosphorylation of Shc allows complexing with a second adaptor protein, Grb2, which in turn facilitates the activation of the GTP-binding protein Ras via a complex with the guanine nucleotide exchange factor Sos. The activation of Ras initiates a now classical phosphorylation cascade through the intermediary kinases c-Raf and MEK that culminates in the activation of the serine-threonine mitogen-activated protein (MAP) kinases, extracellular signal-related kinase (ERK)1 and ERK2 (reviewed in [131]). A variety of cytoplasmic and nuclear proteins that include growth factor receptors, transcription factors, kinases and other enzymes are phosphorylated by the activated MAP kinases: these in turn mediate a cellular response that may be metabolic, mitogenic or differentiative in nature [222]. The activation of the MAP-kinase pathway by IGF-1 may also involve the participation of IRS-1; Grb2 can bind directly to tyrosine phosphorylated IRS-1 via its Src homology 2 (SH2) domain following IGF-1 treatment of different cell types [223, 224]. IRS-2 and Grb2 can also form a complex in neuroblastoma cells in response to IGF-1, although this association is not linked to the activation of the MAP kinase pathway [225].

While the IRS proteins exhibit similar general features that include an N-terminal pleckstrin homology (PH) domain followed by a PTB domain, there is extensive heterogeneity within the C-terminal domain, which contains multiple potential tyrosine phosphorylation sites. The number of potential sites varies from 13 in IRS-3, a protein of some 60 kDa, to over 20 in IRS-1, a protein of 160–180 kDa [226, 227]. Many of these tyrosine residues are displayed within the context of motifs that are known to favour interaction with SH2-containing proteins [228]. It is therefore not surprising that growth factor stimulation of different cell types promotes the

Table 5. Cellular proteins interacting directly with the cytoplasmic domain of the IGF-1R.

Protein	Function	Site(s) of interaction*	Reference
IRS-1	adaptor	P-Tyr^{950} and kinase domain	[215,216]
IRS-2	adaptor	P-Tyr^{950} and other residues	[218]
IRS-3	adaptor	P-Tyr^{950}†	[219]
Shc	adaptor	P-Tyr^{950}	[215–217]
Grb10	adaptor	amino acids 1229–1245	[245]
CrkII	adaptor	P-Tyr^{943} and P-Tyr^{950}	[238]
SH2-B	adaptor	P-Tyr^{950} and P-Tyr^{1316}	[243]
p85	regulatory subunit of PI 3-kinase	P-Tyr^{1316} and other residues	[239, 240, 242]
p55γ	regulatory subunit of PI 3-kinase	kinase-dependent	[246]
GAP	GTPase-activating protein	P-Tyr^{950}	[240]
Syp/SHP-2	tyrosine phosphatase	P-Tyr^{1316}	[240]
CSK	cytoplasmic tyrosine kinase	P-Tyr^{943} and P-Tyr^{1316}	[244]
Jak-1	cytoplasmic tyrosine kinase	kinase-dependent	[248]
SOCS-1	suppressor of cytokine receptor signalling	kinase-dependent	[247]
SOCS-2	suppressor of cytokine receptor signalling	kinase-dependent	[247]
14.3.3β	adaptor 'scaffolding' proteins	P-Ser^{1283}	[250]
14.3.3ζ	adaptor 'scaffolding' proteins	P-Ser^{1283}	[250]
14.3.3ε	adaptor 'scaffolding' proteins	P-Ser^{1272} and P-Ser^{1283}	[249]

* Refers to defined site(s) within the cytoplasmic domain of the IGF-1R that mediate the interaction with cellular proteins. All interactions described require a functional receptor kinase; where no specific residue/region has been defined, the interaction is referred to as 'kinase-dependent'.

† By extrapolation from studies done with the IR, and in keeping with the results obtained for other members of the IRS family, it is likely that P-Tyr^{950} mediates the association between the IGF-1R and IRS-3.

binding of a diverse array of SH2-containing proteins with one or more IRS isoforms. These include the p85 regulatory subunit of phosphoinositol (PI) 3-kinase, the adaptor proteins Grb2, Shc and Nck, the tyrosine phosphatase Syp/SH-PTP2 and the cytoplasmic tyrosine kinase Fyn [209, 219].

The utilization of the PI 3-kinase signalling pathway by IGF-1 is of particular relevance. The initial description of the recruitment and subsequent activation of PI 3-kinase by the ligand-activated IGF-1R, either by direct binding [229] or via IRS-1 [230], came at a time when a role for IGF-1 in the protection of cells and tissues from programmed cell death was being established. There are in fact three discrete classes of PI 3-kinases that can be distinguished on the basis of structure and in vitro lipid substrate specificity, with one or more classes being found in a range of eukaryotic organisms [231]. IGF-1 activates class I PI 3-kinases, a function it shares with other growth factor receptor tyrosine kinases. Exactly how the generation of 3-phosphoinositides by PI 3-kinase ties in with the pleiotropic cellular responses elicited by the activation of this enzyme is only starting to be unravelled, although modular structures have been identified in different proteins that can bind these lipid substrates in vitro [232]. The best-characterized substrates of PI 3-kinase signalling are the Akt/PKB serine/threonine kinases, which comprise three known isoforms that are targets of IGF-1 and insulin signalling, amongst other growth factors [233]. The proposed model for the activation of Akt/PKB kinases involves the binding of 3-phosphoinositides to the kinase, triggering translocation to the cell membrane. The subsequent activation of AKT/PKB is catalyzed by phosphorylation by the lipid-activated, serine-threonine kinase, 3-phosphoinositide-dependent kinase 1 (PDK1) [233]. Following activation, Akt/PKB orchestrates a spectrum of biological responses including metabolic responses, proliferation and inhibition of apoptosis.

The apparent redundancy in the ability of the IRS proteins to bind an overlapping set of cellular substrates is offset, in part, by some differences in the developmental and tissue-specific profiles of expression of individual family members, as well as in their level of expression in different cell types [209]. There are, however, examples of isoform-specific recognition of substrate proteins. Following IGF-1 stimulation, members of the Crk family of adaptor proteins preferentially associate in a phosphotyrosine-dependent manner with IRS-4, but not IRS-1 or -2 [234]. The Crk proteins represent a nexus between activation of the IGF-1R and the integrin signalling pathway, forming complexes with the focal adhesion adaptor proteins paxillin and $p130^{Cas}$ [235, 236]. Paxillin, $p130^{Cas}$ and the cytosolic focal adhesion tyrosine kinase $p125^{Fak}$ are rapidly tyrosine-phosphorylated in response to IGF-1 [236, 237], with the latter study indicating a requirement for PI 3-kinase and an intact actin cytoskeleton to effect these responses.

One of the Crk family members, Crk-II, can bind directly to the juxtamembrane region of the activated IGF-1R, via $P\text{-}Tyr^{943}$ and $P\text{-}Tyr^{950}$, in in vitro assays [238]. In fact, a variety of technical approaches (yeast two-hybrid, fusion protein pull downs, in vitro phosphorylation) have been used to show that many of the signalling effector molecules activated by IGF-1 stimulation can interact directly with the C-terminus of the ligand-activated IGF-1R in an SH2-dependent manner (see table 5). These include the p85 subunit of PI 3-kinase and Syp/SH-PTP2, which bind $P\text{-}Tyr^{1316}$ [229, 239–242], the guanosine triphosphatase (GTPase) activating protein GAP, which binds $P\text{-}Tyr^{950}$ [229, 240], the adaptor SH2-B which binds $P\text{-}Tyr^{950}$ and $P\text{-}Tyr^{1316}$ [243], and the C-terminal src kinase CSK, which binds $P\text{-}Tyr^{943}$ and $P\text{-}Tyr^{1316}$ [244]. Other proteins known to bind directly to the cytoplasmic domain of the kinase-active receptor include the adaptor Grb10 [217, 241, 245], the p55 regulatory subunit of PI 3-kinase [246], and both positive (the tyrosine kinase Jak1) and negative (SOCS-1 and SOCS-2) effector proteins of cytokine receptor signalling [247, 248].

Yet another family of cytoplasmic proteins providing a link between the IGF-1R and multiple signal transduction pathways are the 14.3.3 proteins. The binding of three 14.3.3 isoforms (β, ε and ζ) to the cytoplasmic tail of the IGF-1R requires a functional tyrosine kinase catalytic domain, although the specific residues with which they complex are phosphoserines (table 5). The binding of the ε isoform requires phosphorylation of either Ser^{1272} or Ser^{1283} [249], whereas the β and ζ isoforms are dependent on a phosphorylation of Ser^{1283} [250]. The actual biological role of the 14.3.3 proteins has been the subject of some conjecture. They are highly conserved and found in a diverse range of eukaryotic organisms, where they have been implicated in the regulation of a variety of cellular events including neurotransmitter biosynthesis, vesicular trafficking, cell-cycle progression [251] and protection from apoptosis [252].

Proliferation and differentiation

The development of mouse foetuses lacking IGF-1Rs appears first affected between days 11.0 and 12.5 of embryogenesis [2]. The resulting growth retardation, which is characterized by widespread organ hypoplasia, does not result from a cessation of cell division but rather an extended transition through all phases of the cell cycle [13]. As such, the IGFs have been described as 'efficiency factors' for maintaining normal growth rates [2]. Exactly what role the different signalling pathways

activated by the IGF-1R play in this process is not entirely clear. Mice homozygous for a targeted disruption of *Irs-1* display growth-retardation in utero and during postnatal life of varying severity, survive to adulthood and are fertile [253, 254]. While these animals do exhibit insulin resistance, they do not develop the fatal diabetic phenotype that is found in mice deficient for IRS-2 [255]. On the other hand, IRS-2-deficient mice grow normally in utero and show only mild growth retardation postnatally [255], whereas *Irs-3*-null animals grow normally with glucose homeostasis being unaffected [256].

Collectively, these studies indicate that IRS-1 plays a dominant role in mediating mitogenic signalling by the IGFR-1R in vivo, but that alternative pathways exist to rescue *Irs-1*-null mice from the early postnatal lethality that characterizes IGF-1R null mice [1]. In this respect it is of interest that IRS-4, when overexpressed in mouse fibroblasts, can augment the proliferative response to IGF-1 [257].

Primary and established fibroblast cell lines from IRS-1-deficient mice proliferate poorly in response to IGF-1 when compared with wild-type cells [258]. The principal defect stems from the absence of IRS-1-activated PI 3-kinase, which accounts for some 70% of PI 3-kinase activity in normal cells. The activation of PI 3-kinase via IRS-2 by IGF-1 is normal in IRS-1-deficient cells, as is the formation of Shc-Grb2 complexes and the activation of the MAP kinase pathway. Blocking PI 3-kinase activity in wild-type cells completely ablates IGF-1-induced proliferation [258]. Whereas IGF-1-stimulated proliferation of MCF7 breast cancer cells has been reported to be dependent on the activation of PI 3-kinase and not MAP kinase [259], a subsequent study in the same cell type points to the participation of both pathways in the mitogenic response [260]. In contrast, PI 3-kinase is dispensable for the mitogenic actions of IGF-1 on foetal brown adipocytes [261], where the activation of the Ras-MAP kinase pathway via IRS-1/Grb2 and Shc/Grb2 signalling complexes appears pivotal [224, 262]. In these cells, two serine-threonine kinase-dependent signalling pathways are activated downstream of Ras, one involving Raf-1 and the second targeting protein kinase C ζ, although the relative contribution of each to the proliferative response is not known [263]. The microinjection of antibodies against other participants in the MAP kinase cascade such as Shc [264], Syp/SH-PTP2 [265] and Ras [220] also inhibits IGF-1 stimulation of DNA synthesis in fibroblasts.

Microinjection of dominant-negative forms of the Grb10 adaptor protein can inhibit IGF-1-stimulated proliferation in fibroblasts, whereas overexpression of the wild-type protein enhances the mitogenic response to IGF-1 and other growth factors [266, 267]. However, overexpression of Grb10 has also been reported to significantly impair the proliferative response to IGF-1 in a transformed fibroblast line that synthesizes elevated levels of IGF-1R [268]. Grb10 appears to be another example of a kinase-anchoring protein, as it constitutively associates with Raf-1, and following insulin stimulation binds another component of the MAP kinase pathway, the dual tyrosine /threonine kinase MEK [269].

Mutagenesis of the IGF-1R has delineated multiple domains that participate in mitogenic signalling. Kinase-defective receptors, derived either by mutation of the ATP-binding lysine or the triple tyrosine cluster within the catalytic domain, are not mitogenic [211, 213, 270]. Receptors bearing a mutation of the Shc/IRS-1-docking Tyr^{950} were originally described as being unable to deliver a mitogenic signal [271], although a recent study from the same group indicates that these receptors are in fact mitogenic under defined culture conditions [272]. There are, however, other residues within the C-terminus of the IGF-1R, in particular Tyr^{1250} and Tyr^{1251}, whose mutation adversely affects proliferation independently of any impact on the well-recognized signalling pathways [273]. The effect of these and other mutations on the relationship between proliferation and transformation is discussed in more detail below.

One of the more intriguing aspects of the phenotype of *Igf-1r*-null mice is the apparent lack of an overt effect on cellular differentiation [1]. IGF-1 can stimulate the differentiation of a variety of primary and established cultured cells including preadipocytes, myoblasts, osteoclasts, and cells of neural and haemopoietic origin [reviewed in 274, 275]. However, many other growth factors can also drive cellular differentiation in vitro; it would appear that the IGFs play a redundant role behind other cellular factors with respect to many facets of embryonic morphogenesis in vivo. There are exceptions to this generalization. In *Igf-1r*-null mice, pancreatic islets fail to form, and there is a marked reduction in the number of insulin-secreting β cells [276]. A similar phenotype is seen in *Irs-2* mutant animals but is not as pronounced in *Irs-1*-null mice. Mice carrying inactivating deletions in both *Igf-1r* and *Irs-2* have less than 2% of the β cells found in wild-type animals, indicating that there is a preferential coupling of signalling between the IGF-1R and IRS-2 in β-cell neogenesis [276]. There is also a critical requirement for IGF-1 for terminal end bud formation and ductal morphogenesis during mammary gland development in postnatal mice [277].

From a number of in vitro studies, there is clear evidence for the bifurcation of signalling pathways in the decision of different cell types to differentiate or prolif-

erate in response to IGF-1. The activation of PI 3-kinase, mediated via IRS-1 and IRS-2, is tied to the IGF-1-induced differentiation of foetal brown adipocytes [261]. In myoblasts, the ability of IGFs to stimulate differentiation or proliferation correlates with the activation of the PI 3-kinase or Ras-MAP kinase pathways, respectively [278]. Similarly, the promotion of macrophage differentiation by IGF-1 is dependent on PI 3-kinase [279]. In contrast, overexpression of IRS-1 inhibits, whereas elevated levels of Shc promote, the differentiation of 32D cells along the granulocytic pathway in response to IGF-1 [280]. The contradictory nature of much of the data emerging from the role of the IGF-1R in activating different signalling routes to effect proliferation, or differentiation, in different cell types is best summarized in a recent review by Florini et al. To quote, 'it seems there are no simple generalisations that can be used to forecast the signalling pathway that will be involved in any response to the IGFs' [281].

Apoptosis

The precise physiological function(s) played by the IGF-1R in regulating the normal pattern of programmed cell death observed during development is not known. There is indirect evidence linking perinatal apoptosis of rat pancreatic islet cells with a decline in the local expression of IGF-2 [282]. In this respect, it is of some interest that the failure of β-cell neogenesis in mice that carry a double deletion of both *Igf-1r* and *Irs-2* is accompanied by islet cell apoptosis [280]. Experimentally, the ligand-activated IGF-1R promotes cell survival in response to a range of apoptotic stimuli including hypoxia [10], growth factor depletion [11], *c-myc* overexpression [12], TNF-α [283], chemotherapeutic agents [284] and ultraviolet B irradiation [285]. The potent nature of the antiapoptotic signal, together with the realization that this aspect of receptor function may represent an Achille's heel for tumour growth, has provoked a concerted effort to elucidate the participating signalling pathways.

The activation of PI 3-kinase by IGF-1 has emerged as the dominant route by which a variety of cells including haemopoietic [286], neural [287], fibroblast [285], smooth muscle [288] and germ cell [289] lineages are protected from apoptotic stimuli. One substrate/effector activated by PI 3-kinase following IGF-1 treatment is Akt/PKB [285, 287, 290, 291], the activation of which is recognized as a common denominator of antiapoptotic signalling. Akt/PKB can phosphorylate Bad, a proapoptotic member of the Bcl-2 family of proteins [290, 292]. Serine-phosphorylated Bad is sequestered into a complex with 14.3.3ζ, thereby preventing its heterodimerization with, and functional inactivation of, antiapoptotic members of the Bcl-2 family, including Bcl-X_L and Bcl-2 [293]. That IGF-1 can stimulate serine phosphorylation of Bad, via the PI 3-kinase-dependent activation of Akt/PKB, to effect cell survival has been established independently by several groups [290, 291, 294]. What is interesting, however, is that under certain conditions where there is overexpression of mutated IGF-1R, signalling pathways other than PI 3-kinase may be recruited that lead to Bad phosphorylation. In a murine haemopoietic cell line (32D) that lacks both IRS-1 and IRS-2, activation of the IGF-1R can protect against apoptosis through two alternative pathways, one involving the activation of MAP kinase and another that results in the mitochondrial translocation of Raf [294]. Both pathways result in Bad phosphorylation, which in turn correlates with cell survival. These observations are consistent with a number of other studies that highlight the participation of other signalling pathways, in particular those encompassing members of the MAP kinase superfamily [295–297], in IGF-1-mediated protection from programmed cell death.

Not surprisingly, there are multiple other targets of IGF-1 within the apoptotic cascade. In neural and haemopoietic cell lines, the loss of Bcl-2 that accompanies apoptosis is blocked by IGF-1 [298, 299]. Conversely, IGF-1 can stimulate elevated expression of Bcl-X_L mRNA and an accompanying increase in protein [300, 301]. In at least two instances, the maintenance or enhancement of antiapoptotic protein expression depends on PI 3-kinase [301, 302]. The ligand-activated IGF-1R may also attenuate, in part, the execution of cell death signalling by blocking the induction of proapoptotic members of the Bcl-2 family, such as Bax, which can occur in response to apoptotic stimuli [303, 304]. Some very recent work provides a molecular basis by which IGF-1 might regulate the balance between pro- and antiapoptotic proteins at a cellular level. On the one hand, IGF-1 can induce the phosphorylation/activation of the nuclear transcription factor cAMP-response element-binding protein (CREB) via the p38 MAP kinase pathway: CREB in turn can bind to and transactivate the *Bcl-2* promoter [305]. In a contrasting mode of action, IGF-1 inhibits stretch-induced apoptosis in myocytes by the functional inactivation and downregulation of the tumour suppressor p53, a transcriptional activator of *Bax* and other genes that participate in programmed cell death in myocytes [306]. This is achieved through the induction of Mdm, a cellular protein that forms a complex with p53, thereby inhibiting p53 DNA binding and accelerating p53 degradation (reviewed in [307]). The result is a marked decrease in the level of Bax synthesized, whereas there is an accompanying increase in the level of Bcl-2.

IGF-mediated protection from programmed cell death is also associated with the modulation of activity of the principal effector arm of apoptosis, the family of cysteine proteases called caspases. The autocatalytic activation of the caspases, which normally reside in the cell as proenzymes with little activity, leads to the cleavage of a variety of substrate proteins, culminating in cell death [308]. The activation of the IGF-1R during exposure to apoptotic stimuli can inhibit the proteolytic cleavage and activation of different members of the caspase family [298, 303, 309]. This is accomplished, in part, through signalling via the PI 3-kinase pathway [298, 301]. Although the specific mechanism(s) of inhibition have not been elucidated, it has recently been found that IGF-1 can block the release of mitochondrial cytochrome c that occurs as part of the apoptotic sequelae to *c-myc* overexpression [310]. Once released into the cytosol, cytochrome c plays an essential role in orchestrating the sequential activation of different caspases that culminates in apoptosis [311]. Importantly, IGF-1 cannot protect cells from apoptosis once cytochrome c enters the cytoplasm, reinforcing an earlier observation that IGF-1 lowers the probability of apoptosis occurring in susceptible cells, but has no effect on the kinetics of apoptosis once the process is initiated [312]. It is likely that the ability of the activated IGF-1R to prevent cytochrome c release ties in with the ability of members of the Bcl-2 family to regulate the physiological integrity of mitochondria [311].

Cellular transformation

As indicated earlier, functional IGF-1Rs are an obligate requirement for normal cellular proliferation, both in vivo and in vitro. Under serum-free conditions, endogenous levels of receptor are unable to deliver a mitogenic signal to embryonic fibroblasts, and require the activation of other growth factor receptors, for example EGFR, before proliferation occurs [313]. It has been argued that the main function of these other growth factors is to establish an autocrine loop around the IGF axis, whereby sufficient IGF-1 and IGF-1R are induced to allow progression through the cell cyle [15]. Indeed, EGF enhances the expression of IGF-1 mRNA in fibroblasts [313], whereas platelet-derived growth factor (PDGF) is a transcriptional activator of the IGF-1R gene promoter [314]. Constitutive overexpression of the IGF-1R sidesteps this requirement for other growth factors, allowing ligand-dependent proliferation under serum-free conditions [13, 315]. This cellular phenotype is dependent, at least in mouse 3T3 fibroblasts, upon a minimum number of receptors of between 15,000 and 22,000 per cell; above 30,000 receptors per cell a second phenotype indicative of cellular transformation becomes apparent [316]. These results extend a number of earlier studies describing a link between the forced overexpression of the IGF-1R, or a fusion protein containing the cytoplasmic domain thereof, and the oncogenic transformation of cell lines [14, 317].

Given the demonstrated oncogenic potential of other receptor tyrosine kinases, it is not surprising that the IGF-1R behaves as a cellular oncogene when overexpressed. What is surprising, however, is the effect that the presence or absence of this receptor has on the transforming function of a variety of viral and cellular oncogenes. Embryonic fibroblasts derived from *Igf-1r*-null mice cannot be transformed, as judged by their ability to display anchorage-independent growth, by simian virus 40 (SV40) T antigen [318], Ha-Ras [13], EGFR [319], PDGFRβ [320], bovine [321] and human papillomavirus oncoproteins [322], the Ewing's sarcoma fusion protein [323] or an activated *c-src* [324]. Restoration of IGF-1R expression reverses this effect. Part of this dependence on IGF-1R expression may involve oncogene-mediated activation of an IGF autocrine loop [325]. It should be pointed out that just as the IGF axis is not absolutely required for cell growth per se, there are oncogenes such as *v-src* and a GTPase-deficient mutant of $G_{\alpha 13}$ that transform *Igf-1r*-null fibroblasts efficiently [324, 326].

Can these experimental findings linking the IGF axis with cellular transformation be extended to human cancers? A range of different human tumour cell lines express receptors for IGF-1 and respond mitogenically to this growth factor [327]. The question arises as to whether this is a characteristic of the process of tumour development alone, or is acquired as a result of adaptation to life in vitro. Alternatively, it may simply reflect the fact that expression of the IGF-1R, and its ligand(s), is widespread across both normal and tumour cells generally. A number of recent epidemiological studies have now highlighted a positive correlation between elevated levels of plasma IGF-1 and the risk of a number of common human cancers. A prospective study of breast cancer identified elevated plasma IGF-1 as a significant risk factor in premenopausal women under 50, more so if the IGF-1 levels were adjusted for plasma IGFBP-3 concentrations [328], a finding consistent with the conclusions of two other studies [329, 330]. Similarly, a case-control study on prospectively collected plasma samples revealed that men whose IGF-1 levels fell in the upper quartile had a greater than twofold relative risk of developing prostate cancer compared with those in the lowest quartile [331]. When adjusted for the circulating level of IGFBP-3, elevated levels of which are associated with a reduced relative risk, the risk factor jumped to 4.3. A similar relationship between IGF-1 and IGFBP-3 has also been iden-

tified as a risk factor for lung cancer [332]. While these results suggest an increase in the bioavailibility of IGF-1 may be a predisposing factor for the development of malignancy, exactly how this participates at a cellular level to promote risk is not known. The hypersecretion of IGF-1 in transgenic mice is not typically associated with enhanced susceptibility to malignancy, except where specific tumour-promoting agents are used [333]. Hypersecretion of IGF-2, however, can lead to an increase in a variety of malignancies in transgenic mice [334, 335]. Conceivably, two receptors may be the target of IGF-2 action: the IGF-1R, and the alternatively spliced insulin receptor (exon 11 −) that delivers a mitogenic signal following IGF-2 binding and is, interestingly, preferentially expressed in breast and other tumour tissues [5, 336]. The reactivation of endogenous IGF-2 expression has also been identified as a contributing factor for tumour growth and development in transgenic mice expressing SV40 T antigen in pancreatic β cells [337].

Changes in the IGF sensitivity of target tissues, perhaps through an increase in the number of cell-surface IGF-1Rs, may contribute to aberrant proliferation. The expression of IGF-1R is elevated 10- to 14-fold in malignant breast cancer tissue [338, 339] and exhibits higher receptor autophosphorylation and kinase activity than receptors from normal breast tissue [339]. Although overexpression of the IGF-1R has been implicated in cellular radioresistance in vitro and local breast cancer recurrence in vivo [340], others have reported a low-risk status for breast cancer where IGF-1Rs are overexpressed [338]. Elevated levels of IGF-1R are found in a number of tumour types including sarcoma [341], melanoma [342] and pancreatic carcinoma [343]. One feature of the last study is the overexpression of IGF-1 transcripts in 8 out of 12 tumours examined, on average a 32-fold increase in expression compared with normal tissue. In 6 of 12 tumours examined, IGF-1R mRNA was elevated on average fourfold. Together, the data indicate that in some tumours, an autocrine loop may be a contributing factor to malignancy. In a very recent study, overexpression of IGF-1R was significantly associated with an aggressive phenotype in a subset of synovial sarcomas, characterized by a high incidence of lung metastases [344]. In an experimental parallel, enhanced expression of the IGF-1R in transfected murine lung carcinoma cells resulted in an increase in metastatic potential [345].

From a human perspective, it is enticing to consider the role played by two cellular tumour suppressor genes in the regulation of expression of members of the IGF axis. The transcription factor p53 has been tagged 'the guardian of the genome' for the pivotal role it plays in regulating the cellular response to a variety of 'insults' including DNA damage, hypoxia and oncogene expression [346]. The consequences of p53 activation can include growth arrest and apoptosis, hence the high frequency of p53-inactivating mutations and deletions in human tumours. Wild-type p53 represses transcription from the *Igf-1r* gene promoter, whereas mutant forms of p53 stimulate transcription, leading to corresponding changes in the biosynthesis of receptor protein [347, 348]. Downregulation of endogenous IGF-1R by wild-type p53 is linked to the induction of apoptosis in haemopoietic cells following growth factor withdrawal [349]. Conversely, wild-type but not mutant forms of p53 transcriptionally activate *Igfbp-3* [350, 351], whereas wild-type p53 represses *Igf-2* promoter activity [352]. Given the interacting nature of these proteins, it is apparent how the balance of autocrine/paracrine signalling through the IGF pathway may be perturbed by mutations in the *p53* gene. The product of the Wilms' tumour suppressor locus, WT1, is also a transcription factor whose mutation/deletion is implicated in the aetiology of a subset of paediatric malignancies of the kidney [353]. WT1 binds to and inhibits transcription from promoter elements for *Igf-1r* [354] and *Igf-2* [355]. Transfection of wild-type WT1 into a WT1-null cell line downregulates *Igf-1r* transcription and subsequent cell-surface expression, and inhibits IGF-1 stimulation of proliferation and anchorage-independent growth [356].

Multiple domains within the cytoplasmic tail of the IGF-1R contribute to the transformed phenotype, yet for most of these domains the corresponding target signalling pathway remains obscure. Not surprisingly, receptors lacking a tyrosine kinase domain do not transform, and indeed behave as dominant-negative receptors for ligand-dependent transformation in vitro, and tumorigenesis in vivo [357]. Mutation of all three tyrosines (Tyr^{1131}, Tyr^{1135}, Tyr^{1136}) within the activation loop of the kinase domain ablates kinase activity, preventing receptor autophosphorylation [211], and blocks IGF-1-dependent cellular proliferation and anchorage-independent growth of transfected 3T3 fibroblasts derived from *Igf-1r*-null mice, as does a single mutation of Tyr^{1136} [213]. Surprisingly, while the mutation of either Tyr^{1131} or Tyr^{1135} has little effect on the proliferative response to IGF-1, anchorage-independent growth is ablated. Pairwise mutations of tyrosines within this cluster impair the mitogenic response to IGF-1 and tumorigenesis [358]. The mutation of a distal tyrosine residue, Tyr^{1316}, that is conserved in both the IGF-1 and insulin receptors, impairs proliferation in vitro and tumour formation in vivo, with only minimal effects on receptor and IRS-1 tyrosine phosphorylation [359]. Similarly, individual mutations of either Tyr^{1250} or Tyr^{1251} have been reported to have little effect on receptor or substrate (IRS-1, Shc) phosphorylation, or ligand-induced activation of PI 3-kinase and MAP

kinases, yet profoundly inhibit proliferation and anchorage-independent growth of transfected fibroblasts [273]. The Tyr^{1251} mutation in particular disrupts the actin cytoskeleton and the cellular localization of vinculin, a component of focal adhesion plaques. In contrast, others have reported that mutation of either of these residues has no effect on the proliferative response to IGF-1, and only the Tyr^{1251} mutation is nontransforming [271]. Some of these results may be related to subtle differences in the cell type used, and the levels of exogenous receptor expressed.

A number of other IGF-1R mutations have been described that separate mitogenic signalling from cellular transformation. These include deletion of the C-terminal 108 amino acids to Phe^{1229} [60], the mutation of one of the defined binding sites for the 14-3-3 proteins (the serine quartet at 1280–1283) [360], and recently mutations at either Tyr^{950}, the consensus IRS-1/Shc target residue (table 5), and the amino acid quartet Arg^{1289}, His^{1290}, His^{1293} and Lys^{1294} [272]. The three latter mutations also retain the ability to protect cells, to varying degrees, from anoikis, a form of apoptosis that occurs under anchorage-independent conditions [272]. Where studied, the introduction of these mutations had negligible effect on the activation of the major effector pathways by the IGF-1R. For a number of these mutations, no specific effector pathways have been identified. Collectively, the data imply that no one pathway is dominantly associated with the appearance of the transformed phenotype following overexpression and ligand-induced activation of the IGF-1R. Instead, the manifestation of tumorigenic conversion within the experimental systems used requires the activation, and cooperation, of a number of intracellular signalling cascades.

The IGF-1R as a therapeutic target in cancer

A variety of experimental strategies have been employed to block either IGF-1R function, or expression, in a spectrum of different tumour cell lines derived from a number of species. These have included the use of neutralizing antibodies [361, 362] and dominant-negative receptors [357], antisense and triple-helix-forming oligonucleotides [363, 364], and antisense expression vectors delivered by transfection [365–367] or adenovirus [368]. Generally, these approaches have been successful at retarding mitogenic and anchorage-independent cell growth in vitro, and inhibiting tumour development in vivo. Nonetheless, tumour-specific targeting of the IGF-1R in a clinical setting raises the same challenges as any other 'tumour antigen', namely efficiency of delivery and persistence of modulating agents, accessibility of target tissues, and relative levels of receptor expression between malignant and normal tissues. There are, however, a number of unexpected results that have emerged from these studies that may suggest therapeutic potential.

Downregulation of receptor expression using antisense techniques renders a number of different tumour cell types exquisitely sensitive to apoptosis when grown in biodiffusion chambers in vivo, effectively resulting in complete cell death within a 24-h time frame [369]. In contrast, the effect on both cell growth and cell death in vitro is much less dramatic. These results imply that the requirement for a functional IGF axis is more stringent in vivo in terms of cell survival. The induction of massive apoptosis clearly provides one explanation why antisense-targeted tumour cells are nontumorigenic in vivo [363, 366]. Intriguingly, attempts to reproduce the same effect on cell death in vivo by overexpression of a number of full-length dominant-negative forms of the IGF-1R have been unsuccessful [370], although stable expression of a myristilated form of the receptor encompassing only the C-terminal 108 amino acids promotes apoptosis in vivo and abrogates tumorigenesis [371]. There is no doubt, however, that expression of other full-length dominant-negative forms of the receptor can be antitumorigenic in vivo [273, 359].

One exciting aspect of this work has been the finding that one of the transfected tumours expressing antisense IGF-1R transcripts, a rat C6 glioblastoma, can elicit a protective host response following injection into syngeneic rats that protects against a coinjection of what would be an otherwise tumorigenic inoculum of wild-type cells [363]. Furthermore, injection of C6 IGF-1R antisense cells causes regression of established tumours. The transfected C6 cell line is itself nontumorigenic and undergoes apoptosis in vivo [369]. The transient implantation of transfected cells at a subcutaneous location has been shown to stimulate an antitumour response in the brain, resulting in tumour regression [372]. In a series of studies with striking parallels, injection of stably transfected C6 cells, rendered nontumorigenic following transfection of an antisense IGF-1 vector, prevented the formation of both subcutaneous and brain tumours induced by injection of unmanipulated parental cells, and caused regression of established brain tumours [373]. Protection was specific for the glioblastoma cell line: no cross-protection was found following injection of syngeneic B-104 neuroblastoma tumour cells. Injection of antisense-transfected B-104 did not protect against tumour formation by parental cells [373], although abrogation of tumorigenicity and induction of tumour immunity has been obtained using a similar vector and murine teratocarcinoma cells [374].

A role for cytotoxic T cells in the cross-protection initiated by C6 transfectants has been reported: cyto-

toxic T cell proliferative responses were noted only in cells taken from mice challenged with IGF-1R antisense cells, whereas antisense cells did not induce regression of established tumours in athymic mice and only slightly retarded tumour growth of wild-type tumours [375]. The transfectants themselves were weakly tumorigenic, suggesting that surveillance by cytotoxic T cells alone was not sufficient for complete antitumour immunity. A subsequent paper demonstrated that whereas untransfected C6 cells lacked cell-surface expression of major histocompatibility class I antigens and the potent T-cell-stimulatory B-7 coreceptor, antisense IGF-1 transfectants expressed class I and B-7 molecules on 11 and 17% of cells, respectively [376]. In contrast, transfection of the B-104 neuroblastoma line used in earlier studies failed to induce expression of these molecules. This strongly implies that a functional IGF-1 axis may compromise the immunological recognition of some tumours by downregulating the elements critical for tumour antigen recognition.

In a particularly intriguing study, Whitesell et al. have largely utilized transient expression of an antisense IGF-1R construct, both in vitro and in vivo, to modulate tumour phenotype [377]. Thus, electroporation of mouse N2A neuroblastoma cells in vitro gives rise to the expected effects—retardation of cellular growth, loss of anchorage-independent growth and apoptosis leading to cell necrosis. Transfected cells are not tumorigenic: strikingly, inhibition of tumorigenicity in vivo is obtained by direct intratumoural injection of plasmid DNA over successive days, with a regression rate of 50%. Surviving animals were 'immune' to a subsequent inoculation of parental tumour cells. Similar studies performed in *scid* mice, which lack B and T cells, do not result in tumour regression. In discussing their data, the authors highlight how the mechanism of tumour cell death in vivo may influence the immune response to tumour-specific antigens, leading to antitumour immunity. Necrotic cell death, as opposed to apoptotic cell death, is associated with the induction of heat-shock proteins, high immunogenicity and the induction of tumour immunity [378]. Indeed, the expression of antisense IGF-1 receptor transcripts is associated with both apoptotic and necrotic cell death of N2A cells [377], with the authors commenting that unpublished observations have revealed the induction of heat-shock proteins in dying neuroblastoma cells.

Of some relevance from a structural perspective are the results of experiments performed with a soluble form of the receptor containing the first 481 amino acids of the ectodomain followed by four unrelated residues. This isoform of the receptor, designated 486/STOP, behaves as a strong dominant-negative when expressed in a number of different tumour cell lines, inhibiting anchorage-independent growth in vitro, and inducing apoptosis and blocking tumorigenesis in vivo [379, 380]. Expression of 486/STOP in breast cancer cells was inhibitory in assays of cell adhesion and invasion, and blocked tumour metastasis in nude mice, although the incidence of tumour formation was unaffected [381]. A number of these effects relate to the fact that this isoform is secreted, leading to so-called bystander effects [380, 381]. In particular, injection of wild-type tumour cells into nude mice together with either 486/STOP-expressing tumour or non-tumour cell lines at different cell ratios led to a striking reduction in tumour size, and in some cases prevented tumour formation [380]. Tumour growth was also inhibited by injecting conditioned medium from 486/STOP-secreting cells into tissue sites adjacent to those that had received a previous inoculation of tumour cells. As provocative as these results are, little has been reported about the possible mode of action of the 486/STOP mutant, with the exception of the original study which indicated that ligand-induced phosphorylation of endogenous IGF-1R and IRS-1 was impaired by coexpression of the secreted mutant [379]. Two possible explanations were put forward for these observations. One was that 486/STOP was acting as a classical dominant-negative and competed with endogenous receptors for ligand binding. The alternative was that 486/STOP was capable of interaction with endogenous receptors, leading to a change in their conformational status and their responses to IGF-1 [379]. Attempts to demonstrate coprecipitation of 486/STOP with endogenous receptors were unsuccessful, and the effects of immunodepletion of 486/STOP protein from conditioned medium from 486/STOP-secreting cells has not been reported. Direct binding of IGF-1 or IGF-2 has not been reported for 486/STOP. The smallest ligand-binding fragment described so far is the IGF-1R minireceptor which comprises the L1/cys-rich/L2 domains of IGF-1R fused to the last 16 amino acids of the α chain [178]. The shorter L1/cys-rich/L2 fragment (residues 1–462) does not bind ligand [111]. The extra 19 residues in 486/STOP, which are predicted to extend to the end of the B strand in the first FnIII domain, have little sequence identity with the α chain C-terminus (fig. 1). However, direct binding of ligand by 486/STOP protein cannot be ruled out and needs investigation.

Concluding remarks

The weight of experimental and, more recently, epidemiological evidence points to deregulated signalling through the IGF-1R as a contributing factor in the pathogenesis of some cancers. As the list of oncogenic

factors expands, especially with the assembly of the complete portfolio of human genes, 'tumour profiling' of candidate oncogenes will undoubtedly emerge as a requisite step in determining the nature of therapeutic intervention. With respect to the IGF-1R, a number of experimental strategies, particularly those based on antisense technology, offer clinical promise. The ongoing refinement of the crystal structure of the IGF-1R presents opportunities for the development of organically derived, small-molecule antagonists of receptor function. This augments the parallel approach of developing selective inhibitors of the catalytic tyrosine kinase domain as described for the fibroblast and vascular endothelial families of growth factor receptors [382].

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