0888-8809/04/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 18(10):2502–2512
Copyright © 2004 by The Endocrine Society
doi: 10.1210/me.2004-0183

Structural Determinants for High-Affinity Binding of Insulin-Like Growth Factor II to Insulin Receptor (IR)-A, the Exon 11 Minus Isoform of the IR

ADAM DENLEY, ERIC R. BONYTHON, GRANT W. BOOKER, LEAH J. COSGROVE, BRIONY E. FORBES, COLIN W. WARD, AND JOHN C. WALLACE

School of Molecular and Biomedical Science (A.D., E.R.B., G.W.B., B.E.F., J.C.W.), The University of Adelaide, Adelaide 5005, Australia; Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Health Sciences and Nutrition (A.D., L.J.C.), Adelaide 5000, Australia; and CSIRO Division of Health Sciences and Nutrition (C.W.W.), Parkville 3052, Australia

The insulin receptor (IR) lacking the alternatively spliced exon 11 (IR-A) is preferentially expressed in fetal and cancer cells. The IR-A has been identified as a high-affinity receptor for insulin and IGF-II but not IGF-I, which it binds with substantially lower affinity. Several cancer cell types that express the IR-A also overexpress IGF-II, suggesting a possible autocrine proliferative loop. To determine the regions of IGF-I and IGF-II responsible for this differential affinity, chimeras were made where the C and D domains were exchanged between IGF-I and IGF-II either singly or together. The abilities of these chimeras to bind to, and activate, the IR-A were investigated. We also investigated the ability of these chimeras to bind and activate the IR exon 11+ isoform (IR-B) and as a positive control, the IGF-I receptor (IGF-1R). We show that the C domain and, to a lesser extent, the D domains represent the principal determinants of the binding differences between IGF-I and IGF-II to IR-A. The C and D domains of IGF-II promote higher affinity binding to the IR-A than the equivalent domains of IGF-I, resulting in an affinity close to that of insulin for the IR-A. The C and D domains also regulate the IR-B binding specificity of the IGFs in a similar manner, although the level of binding for all IGF ligands to IR-B is lower than to IR-A. In contrast, the C and D domains of IGF-I allow higher affinity binding to the IGF-1R than the analogous domains of IGF-II. Activation of IGF-1R by the chimeras reflected their binding affinities whereas the phosphorylation of the two IR isoforms was more complex. (*Molecular Endocrinology* 18: 2502–2512, 2004)

THE INSULIN RECEPTOR (IR) is a transmembrane glycoprotein that mediates the pleiotropic actions of insulin. The myriad of biological roles the IR plays has been investigated using tissue-specific mouse knockout studies. Such studies have shown the IR to be important in neovascularization (1), adipogenesis (2), pancreatic insulin secretion in response to glucose (3), glucose disposal in muscle and adipose (4), and regulation of hepatic glucose synthesis (5). Dysfunctional IRs and/or IR-mediated signaling has been implicated in a wide variety of diseases ranging from type 2 diabetes to cancer, underlying its importance in human pathology (6–8).

Abbreviations: IGF-1R, Type 1 IGF receptor; IGF-I CII, IGF-I with the C domain from IGF-II; IGF-I DII, IGF-I with the D domain from IGF-II; IGF-I CIIDII, IGF-I with the C and D domains from IGF-II; IGF-II CI, IGF-II with the C domain from IGF-I; IGF-II DI, IGF-II with the D domain from IGF-I; IGF-II CIDI, IGF-II with the C and D domains from IGF-I; IR-A, insulin receptor isoform A; IR-B, insulin receptor isoform B; R^-IR-A, R^- cells expressing the human IR-A; R^-IR-B, R^- cells expressing the human IR-B; TBST, 20 mM Tris, 150 mM NaCl, 0.05% (vol/vol) Tween 20.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

The homodimeric IR exists in two isoforms that arise from the alternative splicing of exon 11 in the IR mRNA (9). Exon 11 codes for 12 amino acids (residues 717–728) that are inserted upstream of the third last residue of the extracellular α-subunits of the IR-B isoform. The IR-A (or IR exon 11−) isoform lacks these 12 amino acids. The presence or absence of the exon 11-encoded peptide yields two receptors with unique biochemical properties. Although both isoforms have similar affinity for insulin, the IR-A binds IGF-II with more than 10-fold higher affinity than the IR-B (10–12). The two IR isoforms also display differential kinase activity (13), and the insulin resistance in skeletal muscle, associated with myotonic dystrophy DM1, has been suggested to be caused by an increase in the relative expression of IR-A, the isoform with the lower kinase activity (14). The IR-A isoform exhibits a more rapid internalization and higher recycling rate than the IR-B (15). The isoforms localize to different regions of the plasma membrane (16) and have been shown to differentially regulate insulin and β-glucokinase gene expression via activation of different classes of phosphatidylinositol 3-kinase (17). Given the unique properties of the two isoforms, techniques that change the IR splicing pattern of a cell have been suggested as potential therapeutic strategies (18).

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

The high-affinity interaction of the IR-A with IGF-II, but not IGF-I, is important for normal fetal and cancer cell growth (reviewed in Ref. 19). Mouse knockout models suggest that whereas the developmental growth-promoting activities of IGF-I are exclusively mediated through the IGF-1 receptor (IGF-1R), IGF-II can stimulate growth via not only the IGF-1R but also the IR (20). Mouse fibroblasts, devoid of IGF-1R, transfected to express the IR, were stimulated to proliferate and were protected from apoptosis by insulin and IGF-II but not IGF-I (21). Cancer cells expressing the IR-A migrate and are protected from apoptosis by exposure to IGF-II but not when exposed to the same level of IGF-I (22). IGF-II is overexpressed by many cancer cells such as breast cancer (23), colorectal cancer (24), and sporadic adrenocortical tumors (25) that also express the IR-A as the predominant IR isoform. The expression of the IR-A and IGF-II increases as thyroid cancer progresses to a more malignant dedifferentiated phenotype (26). In addition, IGF-II was as potent as insulin at stimulating ovarian cancer cell proliferation (27). Collectively, these findings suggest that IGF-II binding to the IR-A is important in developmental and cancer biology.

To date, the structural regions of IGF-II that determine its high affinity for the IR-A are unknown and hence are the focus of our studies. IGF-I and IGF-II are small, single chain peptide hormones (70 and 67 amino acids, respectively) that are secreted by most cell types in the human body. The IGF polypeptides consist of four domains in the following order: B, C, A, and D. The solution structures of IGF-I (28, 29) and IGF-II (30, 31) have revealed that the growth factors share a high degree of structural similarity arising from a high degree of overall sequence similarity (Fig. 1A). Both IGF-I and IGF-II are also very similar in structure to insulin; however, mature insulin lacks both a C and D domain. In this study we show that the flexible C and D domains of IGF-I and IGF-II are primarily responsible for their differential abilities to bind to and activate the IR-A.

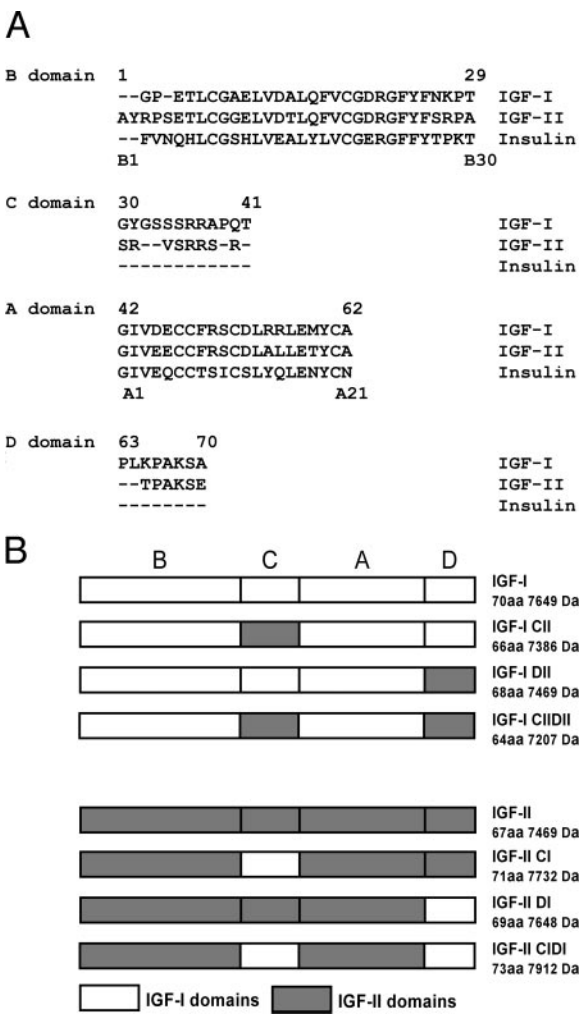

Fig. 1. Sequence Relationship of IGF-I, IGF-II, and Insulin

A, Sequence alignment of human IGF-I, IGF-II, and insulin. Alignment completed using Clustal W, with the numbering of amino acids indicated *above* for IGF-I and *below* for insulin. B, Diagrammatic representation of the domain-exchanged chimeras. Amino acid numbers and molecular weights are given. Each linear representation is divided into the domain structure, B, C, A, D, with all IGF-I domains in *white* and all IGF-II domains in *gray*.

RESULTS

High-Affinity Binding of IGF-II to the IR-A Is Regulated by Its C and D Domains

A total of six chimeric IGFs were produced to analyze the effect of the C and D domains on IR-A binding specificity (Fig. 1B). Structural integrity of the chimeras was confirmed by IGF binding protein 3 (IGFBP-3) binding on the BIAcore (methods are described in Ref. 32). All chimeras had similar IGFBP-3 binding affinities and binding kinetics compared with IGF-I and IGF-II, indicating that swapping the C and D domains did not result in any global structural change (data not shown).

The competition binding curves for insulin, IGF-I, IGF-II, and the six chimeras with the IR-A are shown in Fig. 2, A and C, with the IC_{50} values and relative binding affinities compared with IGF-II listed in Table 1. Our results show that the C and D domains of IGF-II allow high-affinity binding to the IR-A whereas the IGF-I C and D domains do not. The affinity of IGF-II for IR-A is almost 7 times higher than that of IGF-I. The binding characteristics of the chimeras indicate that this difference is due to the IGF-II C and D domains. The IGF-I CII chimera has a 1.9-fold higher binding affinity than IGF-I for IR-A, whereas the IGF-I DII chimera has a 1.5-fold increase in affinity. These contributions are additive as the double chimera binds IR-A almost as well as IGF-II (Table 1). In the converse constructs, exchanging

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

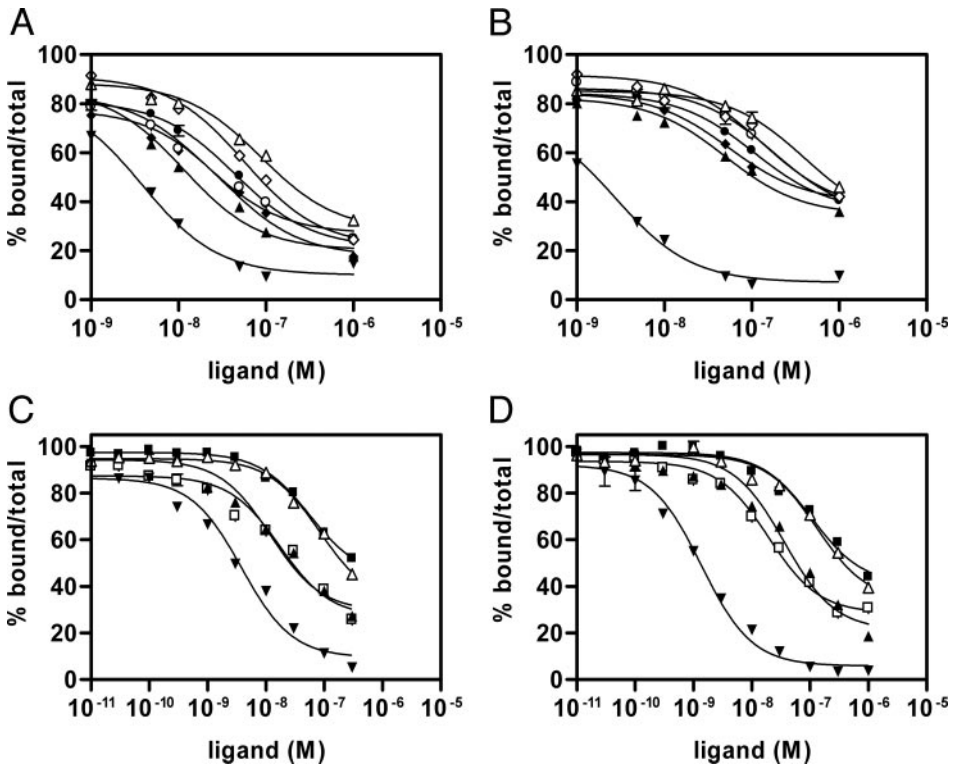

Fig. 2. Competition Binding Curves of Eu-Insulin Binding to Immunopurified Human IR-A or IR-B

Immunocaptured IR-As or IR-Bs were incubated with Eu-insulin in the presence or absence of increasing concentrations of insulin, IGF-I, IGF-II, or IGF chimeras as described in *Materials and Methods*. The *graphs* shown are a representative of three experiments. A and C, Competition for binding to IR-A; B and D, competition for binding to the IR-B. Results are expressed as a percentage of Eu-insulin bound in the absence of competing ligand and the *data points* are means ± SEM of triplicate samples. Errors are shown when greater than the size of the *symbols*. In panels A and B the ligands are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); and IGF-II DI (◆). In panels C and D ligands are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); and IGF-II CIDI (■).

Table 1. Inhibition of Europium-Labeled Insulin for Binding to the IR-A and IR-B by Insulin, IGF-I, IGF-II, and IGF Chimeras

Ligand	IR-A		IR-B	
	IC_{50} (nM)	IC_{50} Relative to IGF-II (%)	IC_{50} (nM)	IC_{50} Relative to IGF-II on IR-A (%)
Insulin	2.8 ± 0.3	654	1.4 ± 0.1	1300
IGF-II	18.2 ± 2.4	100	68 ± 11	27
IGF-II DI	49.3 ± 12.7	37	194 ± 78	9
IGF-II CI	66.3 ± 11.2	27	310 ± 120	6
IGF-II CIDI	106.0 ± 41.3	17	405 ± 98	4
IGF-I	120.4 ± 34.1	15	366 ± 15	5
IGF-I DII	83.2 ± 3.0	22	295 ± 25	6
IGF-I CII	64.0 ± 18.4	28	179 ± 12	10
IGF-I CIIDII	19.5 ± 8.4	93	44 ± 5	41

The IC_{50} relative to that of IGF-II binding to the IR-A is also shown. Values are the means and ± SEM from three independent experiments.

the C or D domains, or both, of IGF-II with those of IGF-I made the chimeras more IGF-I like; their relative IR-A binding affinities being 27%, 37%, and 17%, respectively, that of IGF-II. The double chimera IGF-II CIDI has only slightly higher affinity for the IR-A (1.1-fold) than IGF-I.

The IR-B Binding Specificity of the IGFs Is Also Regulated by Their C and D Domains

The competition binding curves for insulin, IGF-I, IGF-II, and the six chimeras with the IR-B are shown in Fig. 2, B and D, with the IC_{50} values and relative binding

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

affinities compared with IGF-II listed in Table 1. The data show that insulin binds IR-B with 2-fold higher affinity than IR-A whereas IGF-I (3-fold), IGF-II (3.7-fold), and the IGF chimeras (2- to 5-fold) all bind IR-A better than IR-B. Previous reports on the relative affinities of insulin for the two IR isoforms range from IR-B having higher affinity (33), as reported here, to no difference in affinity of insulin for either isoform (12, 34), or to the IR-A isoform having the higher insulin binding affinity (11, 35). Different binding assays and assay conditions could contribute to this variation. The presence of the exon 11-encoded residues had more of a negative effect on IGF-II binding (IC_{50} IR-A: 18.2 nM *vs.* IC_{50} IR-B: 68 nM) than on IGF-I binding (IC_{50} IR-A: 120.4 nM *vs.* IC_{50} IR-B: 366 nM) (Table 1).

Whereas the absolute binding affinities of IGF-I, IGF-II, and the four single chimeras are lower for IR-B compared with IR-A, their relative affinities are similar (Fig. 2B). As summarized in Table 1, the relative order of binding affinity with the IR-A isoform is IGF-II followed by IGF-I CII DII, IGF-II DI, IGF-I CII, IGF-II CI, IGF-I DII, IGF-II CI DI, and IGF-I. The relative order of binding affinity for the IR-B isoform is similar but not identical. In both cases, the four highest affinity ligands contain the IGF-II C domain (denoted as CII) and the four lowest binders contain the IGF-I CI domain. Minor differences between the two IR isoforms are the reversal in the relative positions of the IGF-II DI and the IGF-I CII ligands with IR-B and the equal binding of the IGF-II CI and IGF-I DII ligands on IR-B (equal fifth) compared with their consecutive ranking (fifth and sixth) on IR-A. In contrast to IR-A, the relative affinities of the double chimeras with IR-B fall just outside the range seen with IGF-I and IGF-II. The IGF-I CIIDII chimera, the smallest of the constructs (64 residues), has a higher affinity than IGF-II for binding the IR-B whereas the IGF-II CIDI chimera, the largest of the constructs (73 residues), has a slightly lower affinity than IGF-I for binding the IR-B. The results presented here show that the C and D domains are responsible for the higher affinity of IGF-II for IR-B, compared with IGF-I (Fig. 3).

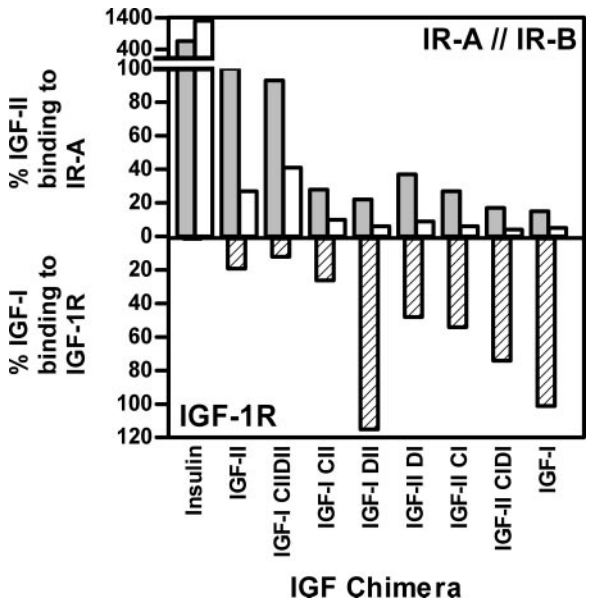

Fig. 3. Summary of Relative IR-A, IR-B, and IGF-1R Binding of Insulin, IGF-I, IGF-II, and IGF Chimeras

In the *upper section of the histogram*, affinities of all ligands for the IR-A as a relative percent of IGF-II binding are shown in *dark gray bars*. Affinities of ligands binding to the IR-B relative to IGF-II binding to the IR-A are shown in *open bars*. In the *lower section* affinities of all ligands for the IGF-1R as a relative percent IGF-I binding are shown in *hatched bars*. SES are not shown; however, for ligands binding to IR-A, SES are between 3.6% and 43%; for IR-B, between 4% and 40%; and for ligands binding to the IGF-1R, between 25% and 44%; absolute values are listed in Tables 1 and 2.

Phosphorylation of the IR-A and IR-B by Stimulation with Chimeric IGFs

The data showing the activation of R^-IR-A or R^-IR-B cells by insulin, IGF-I, IGF-II, and the two double chimeras, IGF-I CIIDII or IGF-II CIDI, are presented in Fig. 4, A and B. As seen with the binding studies (Table 1), insulin was more potent at inducing phosphorylation of IR-B (IC_{50}: 4.1 $\pm$ 0.56 nM) than IR-A (IC_{50}: 18.9 $\pm$ 5.1 nM) although in this case the relative potency was 4-fold higher not 2-fold. Relative to insulin, IGF-II was capable of activating both the IR-A and IR-B to only 40% and 7.2%, respectively. This reflected the IGF-II binding affinity for the IR-A and IR-B relative to insulin. IGF-I showed only a modest ability to stimulate autophosphorylation of either isoform (Fig. 4, A and B). Replacing the C and D domains of IGF-II with those of IGF-I reduced its capacity to activate either IR isoform. The ability of IGF-I and the IGF-II CIDI chimera to phosphorylate the IR-A and IR-B is considerably less than their ability to bind the IR isoforms (Table 1). Conversely, replacing the C and D domains of IGF-I with those of IGF-II resulted in an IGF-I-based chimera that was slightly more active than IGF-II on both IR-A and IR-B. In line with the binding studies, the ability of IGF-II to potently activate the IR-A is due to its C and D domains.

Binding Specificity of the IGFs to IGF-1R Is Also Regulated by the C and D Domains

The competition binding curves for insulin, IGF-I, IGF-II, and the six chimeras with IGF-1R are shown in Fig. 5 with the IC_{50} values and relative binding affinities compared with IGF-I listed in Table 2. The data show that IGF-I had the highest affinity for the IGF-1R whereas insulin bound very poorly (1000-fold lower). The affinity of IGF-II for the IGF-1R was only 18% that of IGF-I, and the single chimeras all fell within that range (Table 2). Replacing the C domain of IGF-I with that of IGF-II reduced the binding affinity for IGF-IR by 75% to a value only slightly higher than that of IGF-II, whereas replacing the D domain of IGF-I caused no

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

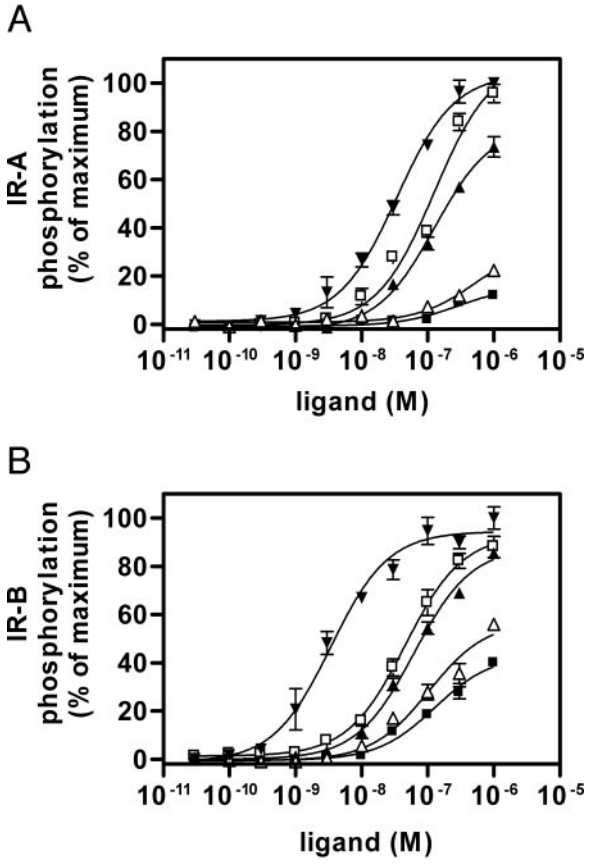

Fig. 4. Activation of the Human IR Isoforms by Insulin, IGF-II, IGF-I, or IGF Chimeras

R^- cells overexpressing the human IR isoforms were serum starved for 4 h followed by stimulation with various concentrations of either insulin, IGF-II, IGF-I, or IGF chimeras for 10 min. Cells were lysed with ice-cold lysis buffer containing phosphatase inhibitors and activated receptors were immunocaptured with the anti-IR antibody 83-7 as described in *Materials and Methods*. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY20 to detect phosphorylated tyrosines. A, IR-A activation by insulin, IGF-I, IGF-II, and IGF double chimeras. B, IR-B activation by insulin, IGF-I, IGF-II, and IGF double chimeras. The *graphs* shown are a representative of three experiments, and data points are means ± SEM of triplicate points. Errors are shown when greater than the size of *symbols*. The ligands are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); and IGF-II CIDI (■).

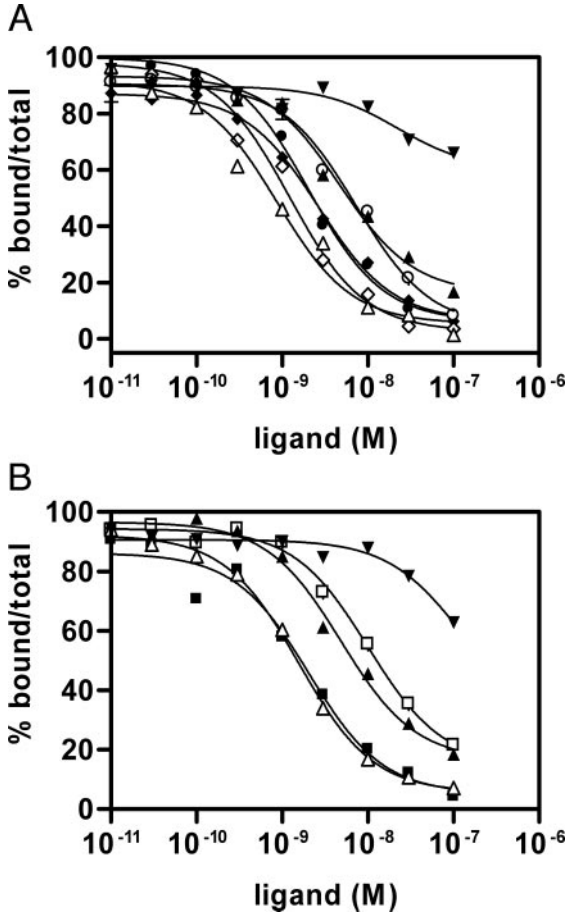

Fig. 5. Competition Binding Curves of Eu-IGF-I Binding to Immunopurified Human IGF-1R

Immunocaptured IGF-1R was incubated with europium-IGF-I in the presence or absence of increasing concentrations of IGF-I, IGF-II, insulin, or IGF chimeras as described in *Materials and Methods*. The *graphs* shown are a representative of three experiments. A, Competition for binding to IGF-1R by IGF-I, IGF-II, insulin, and IGF single chimeras. B, Competition for binding to the IGF-1R by IGF-I, IGF-II, insulin, and IGF double chimeras. Results are expressed as a percentage of europium-IGF-I bound in the absence of competing ligand and the data points are means ± SEM of triplicate samples. Errors are shown when greater than the size of the *symbols*. The ligands in panel A are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); and IGF-II DI (♦). Ligands in panel B are: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); and IGF-II CIDI (■).

reduction in affinity (Table 2). Replacing both the C and D domains of IGF-I with those of IGF-II resulted in a chimera that had a lower binding affinity for the IGF-1R than IGF-II (Table 2). Conversely, incorporating either the C or D domains of IGF-I into IGF-II resulted in proteins that were more IGF-I like. In these chimeras, however, the effects of exchanging either the C domain or the D domain were not significantly different, their binding affinities for IGF-1R being 2.9- and 2.6-fold higher than that of IGF-II, respectively (Table 2). The double chimera, IGF-II CIDI, was further improved and had an affinity that was closer to that of IGF-I (Fig. 5B). A summary of IGF-1R binding by all IGF chimeras is shown in Fig. 3.

Phosphorylation of the IGF-1R by Stimulation with Chimeric IGFs

The activation of the human IGF-1R by insulin, IGF-I, IGF-II, and the six chimeras (Fig. 6, A and B) mirrored the relative binding affinities. The EC_{50} for IGF-I activation of the IGF-1R was 3.9 nM± 0.43 nM. At that same concentration, IGF-II induced phosphorylation

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

Table 2. Inhibition of Europium-Labeled IGF-I for Binding to the IGF-1R by Insulin, IGF-I, IGF-II, and IGF Chimeras

Ligand	IC_{50} (nM)	IC_{50} Relative to IGF-I (%)
Insulin	>100	<1
IGF-I	0.8 ± 0.2	100
IGF-I DII	0.7 ± 0.2	114
IGF-I CII	3.2 ± 1.4	25
IGF-I CIIDII	7.4 ± 2.6	11
IGF-II	4.4 ± 1.1	18
IGF-II DI	1.7 ± 0.5	47
IGF-II CI	1.5 ± 0.5	53
IGF-II CIDI	1.1 ± 0.3	73

The IC_{50} relative to that of IGF-I is also shown. Values are the means and ± SEM from three independent experiments.

to only 35% that of IGF-I (Fig. 6A). Replacing the D domain of IGF-I with that of IGF-II had a negligible effect on IGF-1R phosphorylation, whereas replacing the C domain had a dramatic effect and reduced the potency of this IGF-I based chimera to that of IGF-II. The double chimera, where both the C and D domains of IGF-I were replaced by those of IGF-II, was even poorer than IGF-II at inducing IGF-1R phosphorylation, reflecting the additive effects of these substitutions on IGF-1R binding (Table 2). In the converse IGF-II-based chimeras, the differential effects on phosphorylation were greater than those seen in the binding studies. There was little difference in the increase in IGF-1R binding affinity, relative to IGF-I, between the IGF-II CI (53%) and IGF-II DI (47%) chimeras (Table 2); however, the difference was larger in their ability to stimulate phosphorylation relative to IGF-I (IGF-II CI: 72% *vs.* IGF-II DI: 40% at EC_{50} concentration of IGF-I). The importance of the IGF-I C domain is further illustrated by the fact that the double chimera IGF-II CIDI was only slightly more potent than the single IGF-II CI chimera (IGF-II CIDI: 73% relative to IGF-I). These results illustrate the dominant contribution of the IGF-I C domain to IGF-1R activation.

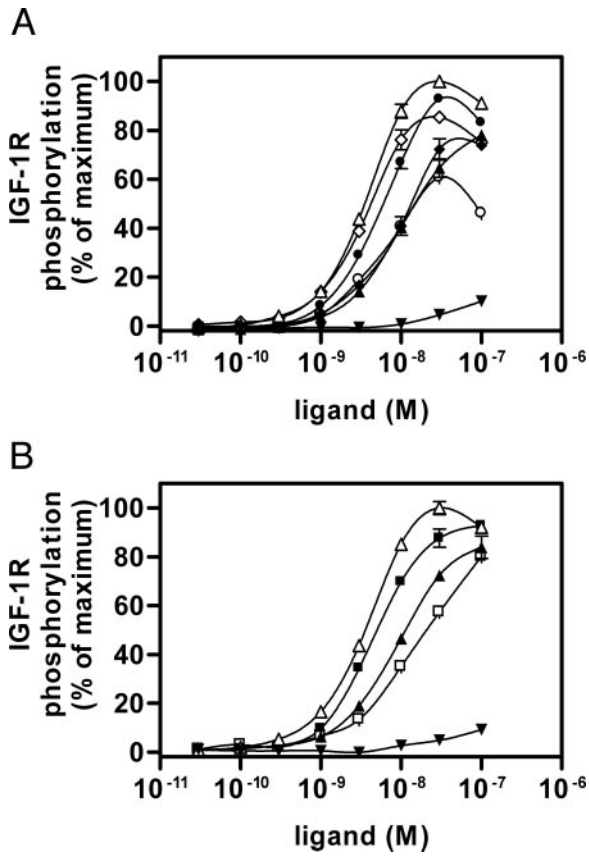

Fig. 6. Activation of the Human IGF-1R by IGF-I, IGF-II, Insulin, or IGF Chimeras

P6 cells overexpressing the human IGF-1R were serum starved for 4 h followed by stimulation with various concentrations of either IGF-I, IGF-II, insulin, or IGF chimeras for 10 min. Cells were lysed with ice-cold lysis buffer containing phosphatase inhibitors, and activated receptors were immunocaptured with the anti-IGF-1R antibody 24-31 as described in *Materials and Methods*. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY20 to detect phosphorylated tyrosines. A, IGF-1R phosphorylation by insulin, IGF-I, IGF-II, and IGF single chimeras. B, IGF-1R phosphorylation by insulin, IGF-I, IGF-II, and IGF double chimeras. The *graphs* shown are a representative of three experiments, and data points are means ± SEM of triplicate points. Errors are shown when greater than the size of *symbols*. The ligands in panel A are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); IGF-II DI (♦). B, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); and IGF-II CIDI (■).

DISCUSSION

The general view, based on numerous studies with whole receptors or soluble ectodomains, is that whereas insulin and IGF-I bind their own receptors with high affinity, they bind the heterologous receptor poorly (<2%) (see Ref. 36). In contrast, IGF-II, but not IGF-I, has been reported to bind the IR-A isoform with an affinity approaching that of insulin (12). The molecular basis for this differing affinity of IGF-I and IGF-II is not known. There are 26 sequence differences between IGF-I and IGF-II (Fig. 1A) with the greatest concentration occurring in the C and D domains, making them prime candidates for this difference in receptor interactions. The IGF-I C domain is four residues larger and differs at a further five positions when compared with the C domain of IGF-II. The D domain of IGF-I is two residues larger and differs in a further two residues from the D domain of IGF-II.

Here we report, for the first time, the structural determinants that allow IGF-II to bind to and potently activate the IR-A. We show that the IGF-II C and D domains allow an IR-A binding affinity near that of insulin. The IGF-I C and D domains prevent high affinity binding and do not allow potent activation of the IR-A. In addition, we show that the C and D domains are also responsible for the higher affinity of IGF-II for

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

the IR-B compared with IGF-I, although the affinities seen with IR-B are lower than those seen with IR-A. For completeness we studied the interactions of these chimeras with the IGF-1R and confirmed previous observations (37) that residues in the C and D domains of IGF-I and IGF-II contribute to IGF-1R binding specificity and play a role in IGF-1R binding and activation.

In this study we report the generation of the first whole-domain chimeras of IGF-I and IGF-II, which have allowed us to investigate the roles of the C and D domains of IGF-I and IGF-II in receptor-binding specificity. As shown in Fig. 3 the C domains and, to a lesser extent, the D domains of the IGFs make major contributions to the IGF binding specificity to the IR isoforms and the IGF-1R. The binding of all of these chimeras to the IR-B isoform is lower than to the IR-A, but the relative trends are similar. The four best binders to both IR isoforms contain the C domain from IGF-II whereas the four ligands that contain the C domain from IGF-I are the worst binders for both isoforms (Fig. 3). The IGF-1R binding affinities of the IGFs studied here in general show the opposite trend to that seen with the IR isoforms, with IGF-I the best ligand and IGF-II much poorer (Fig. 3). These results indicate that the C and D domains of IGF-I and IGF-II play opposing roles in regulating binding specificity to the IR and IGF-1R. Whereas the B and A domains of IGF-I and IGF-II do not appear to be as important in regulating receptor binding specificity, they do make critical contributions to the free energy of receptor binding (38, 39).

It has been reported that the presence of the exon 11-encoded amino acids has little (11, 35) or no effect (12, 34) on insulin binding. Despite this relatively small effect on insulin binding affinity, alanine scanning of the proposed insulin-binding site on both IR isoforms has revealed differences in the energetic contribution of common receptor side chains in the two receptor isoforms (33). This suggests subtly different modes of insulin binding and also that there is significant accommodation for structural differences induced by the extra 12 amino acids to allow almost equal binding affinities for insulin. In this report we show that the presence of the exon 11-encoded sequence does have a significant influence on the binding of IGF-II (12, 40), the single and double chimeras, and IGF-I (Fig. 3). We show for the first time that the presence of the exon 11 peptide has a greater negative effect on IGF-II binding (3.7-fold reduction) than on IGF-I binding (3-fold reduction). However, the absolute affinity of IR-B for IGF-II is still higher than that of IR-A for IGF-I (Table 1).

The 16 amino acids at the C terminus of the IR α-subunits, residues 704–719 in IR-A, are essential for ligand binding as shown by chemical cross-linking (41), mutagenesis, and receptor minimization studies (42–45). In the IR-B, the exon 11-encoded region, which has a negative effect on IGF binding but not on insulin binding, is directly C terminal of these 16 amino acids and may exert its effects by sterically interfering with residues/regions of the IGF molecule that are not present in insulin. Further studies are needed to determine whether IGF-I or IGF-II directly interacts with the exon 11-encoded amino acids. The larger size of the C and D domains of IGF-I may be important in regulating IR binding specificity. The C domain of IGF-I forms a large wedge shape (46) and is four amino acids longer than the IGF-II C domain. The D domain of IGF-I contains two more amino acids, compared with the IGF-II D domain. To determine the nature of these size differences, molecular threading was used to model the structure of IGF-II CI. This allows a comparison of the IGF-II and IGF-I C domains to be made (Fig. 7). The most striking difference is the increased volume of the IGF-I C domain, reflecting the presence of the four extra amino acids not present in the IGF-II C domain. Specifically annotated in the IGF-I C domain are Tyr 31 and Pro 39 with no equivalent residues in the IGF-II C domain. These residues may be sterically hindering the IGF-I C domain interaction with the IR. Supporting our hypothesis is the observation that when the IGF-I C domain was shortened from the native 12 amino acids in two-amino acid decrements to an ultimate length of six amino acids, in a two-chain IGF, the IR binding affinity increased (47).

There are some contradictions in the literature that would suggest that size alone may not be the determinant of IR binding specificity. Kristensen *et al.* (48) demonstrated that inserting the 12-amino acid C domain of IGF-I into insulin to form a single-chain hybrid did not affect IR binding; however Chang *et al.* (49) showed proinsulin, with a 31-amino acid C peptide, binds poorly to the IR. A sequence comparison between IGF-I and IGF-II reveals several charge differences between the IGF-I and IGF-II C and D domains that may influence binding.

In conclusion, we have determined the domains of IGF-II that allow it to bind and activate the IR-A with

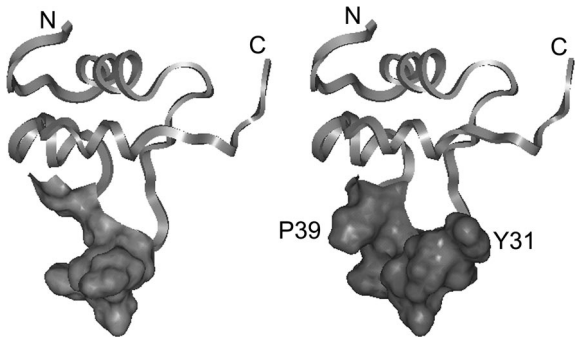

Fig. 7. Comparison of the C Domains of IGF-II and IGF-I

A *ribbon* representation of the nuclear magnetic resonance (NMR) structure of IGF-II with the C domain in surface mode is shown on the *left*. A model of IGF-II CI is shown on the *right*. The sequence of IGF-II CI was entered into SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html) and threaded through the backbone of IGF-II using coordinates from the NMR structure (31). The C domain of IGF-II CI is *highlighted* in surface mode.

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

high affinity. We are currently investigating both domain size and the role of specific residues in the C and D domains in determining IR-A binding specificity. In addition, we are investigating the signaling pathways initiated by IGF-II binding to the IR-A to further define the biological significance of this interaction in cancer. Clearly, a structure of the receptor-ligand complex will ultimately be needed to reveal the molecular details of the high-affinity IGF-II/IR-A interaction.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Geneworks Pty Ltd. (Adelaide, South Australia). Restriction enzymes were from New England Biolabs (Hitchin, UK) or Geneworks Pty Ltd. Long Arg^3IGF-I and human IGF-I were purchased from Gro*Pep* Pty Ltd (Adelaide, South Australia). pGEM-T-Easy Vector system was purchased from Promega Corp. (Madison, WI). Greiner Lumitrac 600 96-well plates were from Omega Scientific (Tarzana, CA). Human insulin was purchased from Novo Nordisk (Bagsværd, Denmark). DELFIA Eu-labeling kit, DELFIA enhancement solution, and europium-conjugated antiphosphotyrosine antibody PY20 were purchased from PerkinElmer (Turku, Finland). Antibodies 83-7 and 24-31 were kind gifts from Professor K. Siddle (Cambridge, UK). P6 cells (BALB/c3T3 cells overexpressing the human IGF-1R) (50) and R^- cells (mouse 3T3-like cells with a targeted ablation of the IGF-1R gene) (51) were a kind gift from Professor R. Baserga (Philadelphia, PA).

Construction of Expression Plasmids Encoding Human IGF-I and IGF-II Chimeras

The chimeras generated are shown in Fig. 1B. They were constructed in halves using two sets of two large complementary template primers (Supplemental Table I published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). A PCR containing gel-purified template primers and two amplification primers (Supplemental Table II published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org) was used to generate each half, which was subsequently TA cloned into the pGEM-T-Easy Vector and then subcloned into the pGH(1–11) expression vector (52). Chimeric IGFs were expressed in *Escherichia coli* JM101 after isopropyl β-D-thiogalactoside induction. Inclusion bodies were isolated as described by King *et al.* (52). Double chimeras were created by ligating appropriate fragments digested from TA cloned vectors into the pGH(1–11) expression vector.

Purification of IGF Chimeras

The procedures for purification of IGF-I, IGF-II, and the six IGF chimeras shown in Fig. 1B were similar to those reported previously (53, 54). All purified proteins were analyzed by mass spectroscopy and N-terminal sequencing and were shown to have the correct masses and to be greater than 95% pure. Quantitation of chimeras was performed by comparing analytical C4 HPLC profiles with profiles of standard Long Arg^3IGF-I preparations (55).

Construction of Cells Expressing the IR-A and IR-B Isoforms

The cDNA encoding the human IR-A and IR-B isoforms was generated as described previously (56, 57). The pECE:hIR-A and hIR-B plasmids were restricted with *Sal*I and *Xba*I to release a 2.9-kb fragment containing the insulin receptor and ligated to *Xho*I/*Xba*I cut pEFIRESneo (58). The exon 11 status of the constructs was confirmed by PCR analysis. R^- cells were transfected with the constructs using Lipofectamine+ (Life Technologies, Inc., Gaithersburg, MD), and stably transfected cells were screened for the IR cDNA by PCR analysis and for IR expression by fluorescence-activated cell sorting analysis using the monoclonal anti-IR antibody 83-7. Cells expressing human IR underwent single-cell sorting to isolate cells expressing similar levels of receptors. These clonal cell lines were used in all subsequent experiments. R^- cells expressing the human IR-A are designated R^-IR-A, and R^- cells expressing the human IR-B are designated R^-IR-B.

Binding Analysis of Chimeras to IR Isoforms and IGF-1R

Receptor binding affinities were measured using an assay similar to that measuring epidermal growth factor binding to the epidermal growth factor receptor (59). R^-IR-A, R^-IR-B, and P6 cells were used as sources of IR-A, IR-B, and IGF-1R, respectively. Cells were lysed with lysis buffer [20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM EGTA (pH 7.5)] for 1 h at 4 C. Lysates were centrifuged for 10 min at 3500 rpm and then 100 μl was added per well to a white Greiner Lumitrac 600 plate previously coated with anti-IR antibody 83-7 (60) or anti-IGF-1R antibody 24-31 (61). Neither capture antibody interferes with receptor binding by insulin, IGF-I (60, 61), or IGF-II (Cosgrove, L. J., unpublished results).

Europium-labeled receptor grade human insulin and human IGF-I were prepared as instructed by the manufacturer (DELFIA Eu-labeling kit, Perkin Elmer). Briefly, 0.43 mM peptide was incubated with 2 mM labeling reagent in a 30-μl reaction (0.1 M Na_2CO_3, pH 8.5), at 4 C for 2 d. The reaction was terminated with 0.05 M Tris-HCl, 0.15 M NaCl (pH 7.5), and unbound europium was removed by size exclusion chromatography in the termination buffer (Superdex 75, Amersham Pharmacia Biotech, Uppsala, Sweden). Approximately 100,000 fluorescent counts of europium-labeled insulin or europium-labeled IGF-I were added to each well along with various amounts of unlabeled competitor and incubated for 16 h at 4 C. Wells were washed with 20 mM Tris, 150 mM NaCl, 0.05% (vol/vol) Tween 20 (TBST), and DELFIA enhancement solution (100 μl/well) was added. Time-resolved fluorescence was measured using 340-nm excitation and 612-nm emission filters with a Polarstar Fluorimeter (BMG Lab Technologies, Mornington, Australia). IC_{50} values were calculated, using Prism 3.03, by curve-fitting with a one-site competition model. The baseline used to calculate all IC_{50} values was set at the % bound/total value of the highest competing insulin concentration.

IR and IGF-1R Phosphorylation Assays

Receptor phosphorylation was detected essentially as described by Chen *et al.* (62). R^-IR-A, R^-IR-B cells, or P6 cells were plated in a Falcon 96-well flat-bottom plate at 2.5×10^4 cells per well and grown overnight at 37 C, 5% CO_2. Cells were washed for 4 h in serum-free medium before being treated with one of either insulin, IGF-II, IGF-II, or chimera in 100 μl DMEM with 1% BSA for 10 min at 37 C, 5% CO_2. Lysis buffer containing 2 mM Na_3VO_4 and 1 mg/ml NaF was added to cells, and receptors from lysates were captured on 96-well plates precoated with antibody 83-7 or 24-31 and blocked with 1× TBST/0.5% BSA. After overnight incubation at 4 C,

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

the plates were washed with 1× TBST. Phosphorylated receptor was detected with europium-labeled antiphosphotyrosine antibody PY20 (130 ng/well, room temperature, 2 h). DELFIA enhancement solution (100 μl/well) was added, and time-resolved fluorescence was detected as described above.

Acknowledgments

We thank Ms. Kerrie McNeil for her assistance in the protein purification; Dr. Tim Adams and Mr. Peter Hoyne for their detailed discussions on the europium-based competition assay; and Mr. Chris Cursaro for N-terminal sequencing and mass spectrometry analyses. We thank Professor Ken Siddle for providing monoclonal antibodies to the IR and IGF-1R and Professor Renato Baserga for providing P6 and R^- cells.

Received April 29, 2004. Accepted June 7, 2004.

Address all correspondence and requests for reprints to: John C. Wallace, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide 5005, Australia. E-mail: john.wallace@adelaide.edu.au.

Present address for B.E.F.: Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Health Sciences and Nutrition, Adelaide 5000, Australia.

This work was supported equally by The University of Adelaide and CSIRO Division of Health Sciences and Nutrition.

REFERENCES

1. Kondo T, Vicent D, Suzuma K, Yanagisawa M, King GL, Holzenberger M, Kahn CR 2003 Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization. J Clin Invest 111: 1835–1842
2. Entingh AJ, Taniguchi CM, Kahn CR 2003 Bi-directional regulation of brown fat adipogenesis by the insulin receptor. J Biol Chem 278:33377–33383
3. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR 1999 Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329–339
4. Lauro D, Kido Y, Castle AL, Zarnowski MJ, Hayashi H, Ebina Y, Accili D 1998 Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue. Nat Genet 20:294–298
5. Michael MD, Kulkarni RN, Postic C, Previs SS, Shulman GI, Magnuson MA, Kahn CR 2000 Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol Cell 6:87–97
6. De Meyts P, Whittaker J 2002 Structural biology of insulin and IGF1 receptors: implications for drug design. Nat Rev Drug Discov 1:769–783
7. Sesti G 2000 Insulin receptor variant forms and type 2 diabetes mellitus. Pharmacogenomics 1:49–61
8. Sesti G, Federici M, Lauro D, Sbraccia P, Lauro R 2001 Molecular mechanism of insulin resistance in type 2 diabetes mellitus: role of the insulin receptor variant forms. Diabetes Metab Res Rev 17:363–373
9. Seino S, Bell GI 1989 Alternative splicing of human insulin receptor messenger RNA. Biochem Biophys Res Commun 159:312–316
10. McClain DA 1991 Different ligand affinities of the two human insulin receptor splice variants are reflected in parallel changes in sensitivity for insulin action. Mol Endocrinol 5:734–739
11. Yamaguchi Y, Flier JS, Yokota A, Benecke H, Backer JM, Moller DE 1991 Functional properties of two naturally occurring isoforms of the human insulin receptor in Chinese hamster ovary cells. Endocrinology 129: 2058–2066
12. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A, Vigneri R 1999 Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19:3278–3288
13. Kellerer M, Lammers R, Ermel B, Tippmer S, Vogt B, Obermaier-Kusser B, Ullrich A, Haring HU 1992 Distinct α-subunit structures of human insulin receptor A and B variants determine differences in tyrosine kinase activities. Biochemistry 31:4588–4596
14. Savkur RS, Philips AV, Cooper TA 2001 Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nat Genet 29:40–47
15. Vogt B, Carrascosa JM, Ermel B, Ullrich A, Haring HU 1991 The two isotypes of the human insulin receptor (HIR-A and HIR-B) follow different internalization kinetics. Biochem Biophys Res Commun 177:1013–1018
16. Uhles S, Moede T, Leibiger B, Berggren PO, Leibiger IB 2003 Isoform-specific insulin receptor signaling involves different plasma membrane domains. J Cell Biol 163: 1327–1337
17. Leibiger B, Leibiger IB, Moede T, Kemper S, Kulkarni RN, Kahn CR, de Vargas LM, Berggren PO 2001 Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic β cells. Mol Cell 7:559–570
18. Sazani P, Kole R 2003 Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J Clin Invest 112:481–486
19. Denley A, Wallace JC, Cosgrove LJ, Forbes BE 2003 The insulin receptor isoform exon 11-(IR-A) in cancer and other diseases: a review. Horm Metab Res 35: 778–785
20. Louvi A, Accili D, Efstratiadis A 1997 Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol 189:33–48
21. Morrione A, Valentinis B, Xu SQ, Yumet G, Louvi A, Efstratiadis A, Baserga R 1997 Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci USA 94:3777–3782
22. Sciacca L, Mineo R, Pandini G, Murabito A, Vigneri R, Belfiore A 2002 In IGF-I receptor-deficient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A. Oncogene 21:8240–8250
23. Quinn KA, Treston AM, Unsworth EJ, Miller MJ, Vos M, Grimley C, Battey J, Mulshine JL, Cuttitta F 1996 Insulin-like growth factor expression in human cancer cell lines. J Biol Chem 271:11477–11483
24. Renehan AG, Jones J, Potten CS, Shalet SM, O'Dwyer ST 2000 Elevated serum insulin-like growth factor (IGF)-II and IGF binding protein-2 in patients with colorectal cancer. Br J Cancer 83:1344–1350
25. Gicquel C, Bertagna X, Schneid H, Francillard-Leblond M, Luton JP, Girard F, Le Bouc Y 1994 Rearrangements at the 11p15 locus and overexpression of insulin-like growth factor-II gene in sporadic adrenocortical tumors. J Clin Endocrinol Metab 78:1444–1453
26. Vella V, Pandini G, Sciacca L, Mineo R, Vigneri R, Pezzino V, Belfiore A 2002 A novel autocrine loop involving IGF-II and the insulin receptor isoform-A stimulates growth of thyroid cancer. J Clin Endocrinol Metab 87:245–254
27. Kalli KR, Falowo OI, Bale LK, Zschunke MA, Roche PC, Conover CA 2002 Functional insulin receptors on human epithelial ovarian carcinoma cells: implications for IGF-II mitogenic signaling. Endocrinology 143:3259–3267

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

28. Cooke RM, Harvey TS, Campbell ID 1991 Solution structure of human insulin-like growth factor 1: a nuclear magnetic resonance and restrained molecular dynamics study. Biochemistry 30:5484–5491
29. Sato A, Nishimura S, Ohkubo T, Kyogoku Y, Koyama S, Kobayashi M, Yasuda T, Kobayashi Y 1993 Three-dimensional structure of human insulin-like growth factor-I (IGF-I) determined by 1H-NMR and distance geometry. Int J Pept Protein Res 41:433–440
30. Terasawa H, Kohda D, Hatanaka H, Nagata K, Higashihashi N, Fujiwara H, Sakano K, Inagaki F 1994 Solution structure of human insulin-like growth factor II; recognition sites for receptors and binding proteins. EMBO J 13:5590–5597
31. Torres AM, Forbes BE, Aplin SE, Wallace JC, Francis GL, Norton RS 1995 Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. J Mol Biol 248:385–401
32. Carrick FE, Forbes BE, Wallace JC 2001 BIAcore analysis of bovine insulin-like growth factor (IGF)-binding protein-2 identifies major IGF binding site determinants in both the amino- and carboxyl-terminal domains. J Biol Chem 276:27120–27128
33. Whittaker J, Sorensen H, Gadsboll VL, Hinrichsen J 2002 Comparison of the functional insulin binding epitopes of the A and B isoforms of the insulin receptor. J Biol Chem 277:47380–47384
34. Surinya KH, Molina L, Soos MA, Brandt J, Kristensen C, Siddle K 2002 Role of insulin receptor dimerization domains in ligand binding, cooperativity, and modulation by anti-receptor antibodies. J Biol Chem 277: 16718–16725
35. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A, McClain DA 1990 Functionally distinct insulin receptors generated by tissue-specific alternative splicing. EMBO J 9:2409–2413
36. Adams TE, Epa VC, Garrett TP, Ward CW 2000 Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 57:1050–1093
37. Bayne ML, Applebaum J, Underwood D, Chicchi GG, Green BG, Hayes NS, Cascieri MA 1989 The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. J Biol Chem 264:11004–11008
38. Bayne ML, Applebaum J, Chicchi GG, Miller RE, Cascieri MA 1990 The roles of tyrosines 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor. J Biol Chem 265: 15648–15652
39. Hodgson DR, May FE, Westley BR 1996 Involvement of phenylalanine 23 in the binding of IGF-1 to the insulin and type I IGF receptor. Regul Pept 66:191–196
40. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A 2002 Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem 277:39684–39695
41. Kurose T, Pashmforoush M, Yoshimasa Y, Carroll R, Schwartz GP, Burke GT, Katsoyannis PG, Steiner DF 1994 Cross-linking of a B25 azidophenylalanine insulin derivative to the carboxyl-terminal region of the α-subunit of the insulin receptor. Identification of a new insulin-binding domain in the insulin receptor. J Biol Chem 269: 29190–29197
42. Mynarcik DC, Yu GQ, Whittaker J 1996 Alanine-scanning mutagenesis of a C-terminal ligand binding domain of the insulin receptor α subunit. J Biol Chem 271:2439–2442
43. Mynarcik DC, Williams PF, Schaffer L, Yu GQ, Whittaker J 1997 Identification of common ligand binding determinants of the insulin and insulin-like growth factor 1 receptors. Insights into mechanisms of ligand binding. J Biol Chem 272:18650–18655
44. Brandt J, Andersen AS, Kristensen C 2001 Dimeric fragment of the insulin receptor α-subunit binds insulin with full holoreceptor affinity. J Biol Chem 276: 12378–12384
45. Kristensen C, Andersen AS, Ostergaard S, Hansen PH, Brandt J 2002 Functional reconstitution of insulin receptor binding site from non-binding receptor fragments. J Biol Chem 277:18340–18345
46. Brzozowski AM, Dodson EJ, Dodson GG, Murshudov GN, Verma C, Turkenburg JP, de Bree FM, Dauter Z 2002 Structural origins of the functional divergence of human insulin-like growth factor-I and insulin. Biochemistry 41: 9389–9397
47. DiMarchi RD, Fan L, Long HB 1997 IGF-I superagonists. Indianapolis, IN: Eli Lilly and Company; 1–17 (U.S. Patent No. 5,622,932)
48. Kristensen C, Andersen AS, Hach M, Wiberg FC, Schaffer L, Kjeldsen T 1995 A single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the insulin receptor. Biochem J 305:981–986
49. Chang SG, Kim DY, Choi KD, Shin JM, Shin HC 1998 Human insulin production from a novel mini-proinsulin which has high receptor-binding activity. Biochem J 329: 631–635
50. Pietrzkowski Z, Lammers R, Carpenter G, Soderquist AM, Limardo M, Phillips PD, Ullrich A, Baserga R 1992 Constitutive expression of insulin-like growth factor 1 and insulin-like growth factor 1 receptor abrogates all requirements for exogenous growth factors. Cell Growth Differ 3:199–205
51. Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A, Baserga R 1994 Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol 14:3604–3612
52. King R, Wells JR, Krieg P, Snoswell M, Brazier J, Bagley CJ, Wallace CJ, Wallace JC, Ballard FJ, Ross M, Francis GL 1992 Production and characterization of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substituted for Glu3, following their expression in *Escherichia coli* as fusion proteins. J Mol Endocrinol 8:29–41
53. Shooter GK, Magee B, Soos MA, Francis GL, Siddle K, Wallace JC 1996 Insulin-like growth factor (IGF)-I A- and B-domain analogues with altered type 1 IGF and insulin receptor binding specificities. J Mol Endocrinol 17: 237–246
54. Lien S, Milner SJ, Graham LD, Wallace JC, Francis GL 2001 Linkers for improved cleavage of fusion proteins with an engineered α-lytic protease. Biotechnol Bioeng 74:335–343
55. Milner SJ, Francis GL, Wallace JC, Magee BA, Ballard FJ 1995 Mutations in the B-domain of insulin-like growth factor-I influence the oxidative folding to yield products with modified biological properties. Biochem J 308: 865–871
56. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter WJ 1986 Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45: 721–732
57. Hoyne PA, Cosgrove LJ, McKern NM, Bentley JD, Ivancic N, Elleman TC, Ward CW 2000 High affinity insulin binding by soluble insulin receptor extracellular domain fused to a leucine zipper. FEBS Lett 479:15–18
58. Hobbs S, Jitrapakdee S, Wallace JC 1998 Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1α promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun 252:368–372
59. Mazor O, Hillairet de Boisferon M, Lombet A, Gruaz-Guyon A, Gayer B, Skrzydelsky D, Kohen F, Forgez P,

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024

Scherz A, Rostene W, Salomon Y 2002 Europium-labeled epidermal growth factor and neurotensin: novel probes for receptor-binding studies. Anal Biochem 301: 75–81

60. Soos MA, O'Brien RM, Brindle NP, Stigter JM, Okamoto AK, Whittaker J, Siddle K 1989 Monoclonal antibodies to the insulin receptor mimic metabolic effects of insulin but do not stimulate receptor autophosphorylation in transfected NIH 3T3 fibroblasts. Proc Natl Acad Sci USA 86:5217–5221
61. Soos MA, Field CE, Lammers R, Ullrich A, Zhang B, Roth RA, Andersen AS, Kjeldsen T, Siddle K 1992 A panel of monoclonal antibodies for the type I insulin-like growth factor receptor. Epitope mapping, effects on ligand binding, and biological activity. J Biol Chem 267:12955–12963
62. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS, Frystyk J 2003 A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. Am J Physiol Endocrinol Metab 284:E1149–E1155

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/18/10/2502/2747524 by guest on 24 April 2024