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Differential signalling potential of insulin- and IGF-1-receptor cytoplasmic domains

Reiner Lammers[1], Alane Gray, Joseph Schlessinger[2] and Axel Ullrich[1]

Department of Developmental Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 and [2]Rorer Biotechnology, Inc., 680 Allendale Road, King of Prussia, PA 19406, USA

[1]Present address: Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, FRG

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The human receptors for insulin-like growth factor 1 (IGF-1) and insulin, and two chimeric receptors consisting of ligand-binding, extracellular insulin receptor and intracellular IGF-1 receptor structures, have been expressed in NIH-3T3 fibroblasts. All four receptor types were synthesized, processed and transported to the cell surface to form high-affinity binding sites. All normal and chimeric receptors had an active tyrosine kinase which was regulated by homologous or heterologous ligands respectively. In addition, cell surface receptors were internalized efficiently and subjected to accelerated degradation in the presence of ligand. While all four types of receptor stimulated glucose transport with similar efficiency, they displayed significant differences in their mitogenic signalling potentials. Receptors with an IGF-1 receptor cytoplasmic domain were 10 times more active in stimulating DNA synthesis than the insulin receptor. In NIH-3T3 cells overexpressing wild-type and chimeric receptors, maximal growth responses obtained with IGF-1 or insulin alone were equivalent to those obtained with 10% fetal calf serum. We conclude that in the cell system employed the receptors for IGF-1 and insulin mediate short-term responses similarly, but display distinct characteristics in their long-term mitogenic signalling potentials.

Key words: insulin/insulin-like growth factor/receptor/signalling

Introduction

The polypeptide hormones, insulin and insulin-like growth factor 1 (IGF-1), exert their biological effects by binding to distinct transmembrane receptors on the surfaces of target cells. Like their ligands, the receptors for insulin and IGF-1 are also highly homologous (Froesch *et al.*, 1985; Rechler and Nissley, 1985). These receptors belong to a family of cell surface glycoproteins which share a cytoplasmic tyrosine kinase function that is essential for their roles as biological signal transmitters. Insulin and IGF-1 receptors represent subclass II of this family of receptor tyrosine kinases (RTK) (Yarden and Ullrich, 1988a,b). Members of this subclass are synthesized as precursors that are processed and oligomerize, yielding functional, heterotetrameric receptor complexes comprised of disulfide-linked α- and β-subunits. Ligand interaction with the extracellular portions of these receptors activates intracellular tyrosine kinase activity, and generates a biological signal that is thought to be specified by structural determinants in the cytoplasmic domain. Beyond kinase activation, the precise molecular nature of the signal transduction process is poorly understood.

Although the structures of the receptors that mediate the biological effects of insulin and IGF-1 are highly homologous (Ullrich *et al.*, 1986), these proteins appear to play significantly different biological roles. While insulin is known to be a key regulator of physiological processes such as glucose transport and glycogen and fat biosynthesis (Kahn, 1985), IGF-1 is believed to mediate the effects of growth hormone and also to play a role as a paracrine growth factor (Nilsson *et al.*, 1986). Defects related to insulin and its receptor are involved in the pathology of types I and II diabetes (Ward, 1987), yet IGF-1 has been implicated as an autocrine stimulator of malignant tumors (Minuto *et al.*, 1988). Thus, these polypeptides possess distinct biological functions. However, experiments with cultured fibroblast and leukemic cells that express both insulin and IGF-1 receptors suggest some overlap of biological activities (Pepe *et al.*, 1987; Corps and Brown, 1988), which may, to some extent, be a consequence of non-physiological experimental conditions, and/or binding cross-reactivity of ligands and receptors.

To reassess the biological roles of insulin and IGF-1 ligand–receptor systems, we designed an experimental model that employs NIH-3T3 fibroblasts overexpressing either human insulin receptors or human IGF-1 receptors. In addition, we have created receptor chimerae consisting of insulin receptor extracellular ligand binding sequences linked to the intracellular domain of the IGF-1 receptor by the transmembrane domain of either the insulin or IGF-1 receptor. These chimeric receptors allow direct comparison

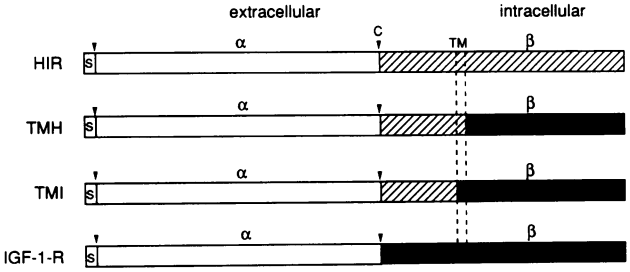

Fig. 1. Schematic structures of normal and chimeric receptor precursors. The schematic structures of the precursors of the human insulin receptor (HIR), insulin-like growth factor-1 receptor (IGF-1-R) and two chimeric receptors that differ from each other by the origin of the transmembrane domain are shown. The signal sequences (S), precursor cleavage sites (C), α- and β-subunits, and transmembrane domains (TM) are indicated. TMH chimera, fusion at the cytoplasmic boundary of the HIR transmembrane domain; TMI chimera, fusion at extracellular boundary of the IGF-1-R transmembrane domain.

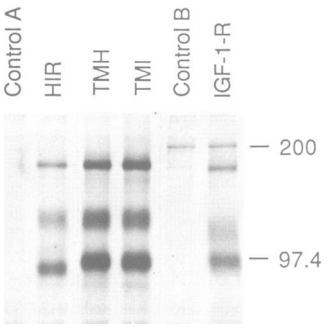

Fig. 2. Immunoprecipitation of biosynthetically labeled receptor molecules expressed in NIH-3T3 cells. Subconfluent cells were labeled overnight with [^{35}S]methionine in methionine-free DMEM containing 10% dialyzed fetal calf serum. After solubilization, receptors were immunoprecipitated with human-specific monoclonal antibodies from parental control or transfected NIH-3T3 cells, and the immunoprecipitates analyzed by SDS–PAGE (7%) under reducing conditions. Insulin receptor antibody 83-14: control A, HIR, TMH, TMI. IGF-1-R antibody αIR-3: control B, IGF-1-R. Molecular weights of size markers are indicated in kilodaltons.

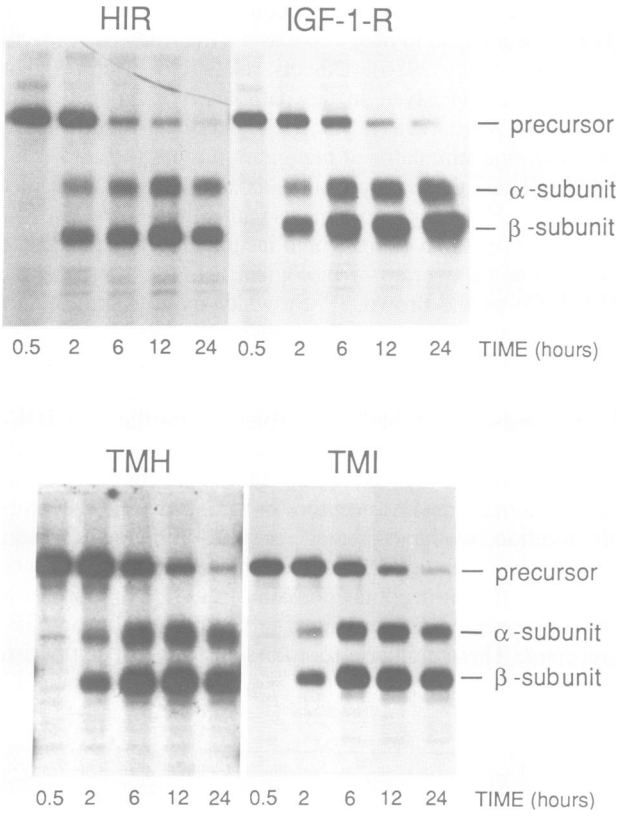

Fig. 3. Processing of human receptor precursors in transfected NIH-3T3 cells. Equal numbers of cells were seeded in 35-mm dishes, pulse-labeled for 30 min with [^{35}S]methionine, washed twice and kept in DMEM with 10% fetal calf serum and 10 mM methionine. At the times indicated, cells were solubilized, and receptors were immunoprecipitated and analyzed by SDS–PAGE (7%) under reducing conditions.

of distinct cytoplasmic domain signalling functions after stimulation with a single ligand, and enable us to investigate a possible role of transmembrane domain sequences in signal definition. We have found that introduction of human receptor cDNA constructs into NIH-3T3 cells results in expression of functional receptors that can mediate both

Table I. Scatchard analysis of ligand binding

Cell line	Ligand	Receptor number ($\times 10^3$)	K_d (nM)
NIH-3T3	insulin	5.4 ± 1.35	2.1 ± 0.55
HIR 3T3	insulin	849 ± 74	1.76 ± 0.32
NIH-3T3	IGF-1	3.4 ± 2.6	0.97 ± 0.89
Human IGF-1-R 3T3	IGF-1	1311 ± 73	0.91 ± 0.13
TMH 3T3	insulin	1490 ± 101	1.81 ± 0.3
TMI 3T3	insulin	1320 ± 93	1.96 ± 0.32

Values for receptor numbers and dissociation constants were determined by cell surface binding of iodinated ligands and Scatchard plot analysis.

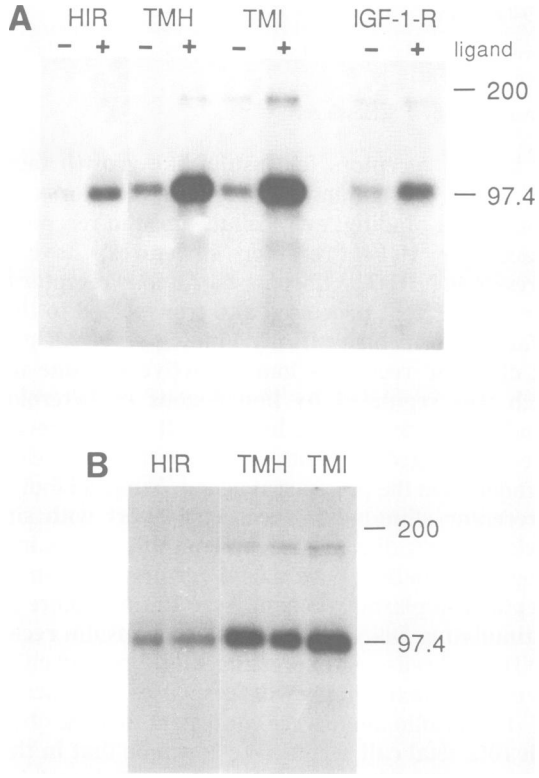

Fig. 4. Ligand-induced autophosphorylation *in vitro*. (**A**) Confluent cells were lysed and immunoprecipitated in the presence or absence of ligand (100 nM). Autophosphorylation was performed in the immunocomplex as described in Materials and methods, and receptors analyzed by 7% SDS–PAGE under reducing conditions. (**B**) *In vitro* specific activity of ligand-stimulated autophosphorylation of receptors possessing an insulin binding domain. Equal amounts of receptors (determined by binding analysis) of two lines expressing HIR, two lines expressing TMH, and one line expressing TMI were immunoprecipitated with antibody 83-14 and used for autophosphorylation as above.

short- and long-term biological effects. Under our experimental conditions, both insulin and IGF-1 receptors mediate similar biological responses in NIH-3T3 fibroblasts, although with significantly different specific activities. Surprisingly, ligand stimulation of overexpressed insulin or IGF-1 receptors resulted in mitogenic responses equivalent to that obtained with fetal calf serum.

Results

A schematic representation of the receptors used in this study is shown in Figure 1. Human insulin receptor (HIR) and

human IGF-1 receptor are synthesized as transmembrane precursor polypeptides that are cleaved at a site in the extracellular domain to generate the α- and β-subunits (Figure 1). The chimerae, TMH and TMI, contain HIR extracellular sequences linked to intracellular sequences of the human IGF-1 receptor. TMH and TMI differ only in the source of their transmembrane domains, which are derived from either HIR or the IGF-1 receptor sequences respectively. Expression vectors containing coding sequences for each of these receptors were used to generate at least two stably expressing, NIH-3T3 cell lines for functional analysis. Representative results obtained with individual cell lines are presented.

Metabolic labeling of transfected cell lines, in conjunction with immunoprecipitation and SDS−PAGE, demonstrated that each of the receptors was correctly synthesized and processed to the mature form. As shown in Figure 2, representative cell lines for each of the different constructs showed polypeptides that corresponded in mol. wt to the unprocessed precursor, and the α- and β-subunits of the parental as well as the chimeric receptors. The slightly higher mol. wt (95 000 versus 90 000 for HIR β-subunit) of the IGF-1 β-subunit is also seen in the chimeric receptors. The differences in size between the HIR (mol. wt 135 000) and IGF-1-R (mol. wt 123 000) α-subunits is likely due to a higher mol. wt of the protein and additional glycosylation sites in the HIR.

To compare the rates of precursor processing and determine receptor turnover characteristics, cells were pulse-labeled for 30 minutes with [^{35}S]methionine and chased in the presence of excess unlabeled methionine (Figure 3). After various times, cells were harvested and receptors analyzed by immunoprecipitation. Overall, the four receptors showed a similar time course of post-translational processing. Processed α- and β-subunits were first detected after 120 min of chase, and between 6 and 12 h most of the precursor had been converted to processed forms. By densitometric analysis, the apparent half-lives of the different precursor molecules ranged from 2.5 to 4 h; the half-life of the mature form of HIR was ~12 h in NIH-3T3 cells, analogous to previous reports for this receptor in other cell lines (7−15 h) (Russell *et al.*, 1987 and refs therein). Interestingly, all receptors containing IGF-1-R cytoplasmic sequences displayed a slower rate of processing ($t_{1/2}$ = 4 h) and also a longer half-life than HIR; due to incomplete precursor processing after 12 h, we were not able to determine their half-lives accurately.

Saturation binding experiments were carried out with [^{125}I]insulin and [^{125}I]IGF-1 to determine the ligand binding characteristics of wild-type human insulin and IGF-1 receptors as well as the hybrid receptors, TMI and TMH, in mouse fibroblasts. Scatchard plot analysis (Scatchard, 1949) indicated the presence of a single affinity class of binding sites for all receptor types (Table I). HIR and the chimerae displayed similar dissociation constants (1.76−2.1 nM). IGF-1 binding displayed a somewhat higher affinity (K_d 0.91 nM). Thus, the cytoplasmic portion of the receptor does not appear to influence ligand affinities. Compared with the parental NIH-3T3 cells, cell lines expressing the various receptors displayed as much as a 300-fold increase in ligand binding sites. This ensures that any biological effects observed upon ligand stimulation are likely to be mediated by the newly introduced human receptors.

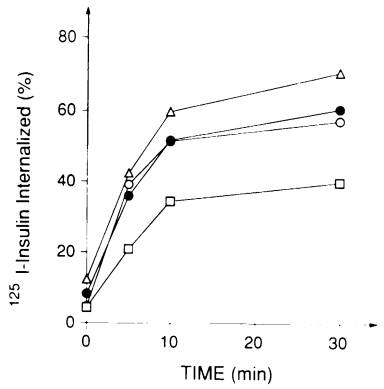

Fig. 5. Internalization of ligand. Confluent cells were cooled to 4°C and incubated for 1 h with 5 nM ligand containing 10^5 c.p.m. iodinated ligand, in DMEM with 0.2% BSA and 50 mM Hepes, pH 7.9. Cells were then transferred to a 37°C waterbath for the time indicated, and cell surface bound and internalized radioactivity was then determined. The averages of two independent experiments performed in duplicate are presented. △, HIR + insulin; ●, TMH + insulin; ○, TMI + insulin; □, IGF-1-R + IGF-1.

Stimulation of autophosphorylation

The biological functions of HIR and IGF-1-R are thought to be mediated by ligand-induced activation of their tyrosine kinase activity (Chou *et al.*, 1987; Ebina *et al.*, 1987; McClain *et al.*, 1987; Yarden and Ullrich, 1988a,b). Ligand stimulation of kinase activity can be detected as an increase in receptor autophosphorylation, both *in vitro* and *in vivo*. As shown in Figure 4A and quantitated by densitometric analysis, insulin stimulated the autophosphorylation of the receptors ~5-fold *in vitro*. The specific activities of HIR and IGF-1-R tyrosine kinases were compared directly by immunoprecipitation with the same 83-14 monoclonal antibody (Taylor *et al.*, 1987) of equal amounts of insulin and chimeric receptors from several independent, stably expressing cell lines, and subsequently analyzing the respective levels of insulin-stimulated autophosphorylation (Figure 4B). Under identical conditions, the chimerae displayed about three times the level of autophosphorylation as HIR. A complementary experiment in which the chimerae and the IGF-1-R were precipitated with an antibody directed against the carboxy terminus of the IGF-1-R confirmed that their kinase activities were similar (data not shown).

Endocytosis and ligand-stimulated degradation

Shortly after ligand binding, receptor−ligand complexes cluster in clathrin-coated pits and are internalized by endocytosis. To compare the time course and efficiency of this process for HIR and IGF-1-R, and to determine whether TMH and TMI chimerae were functional in this regard, each of the cell lines was analyzed for internalization of radiolabeled ligand (Figure 5). Ligand internalization was found to be rapid for all receptors and reached half-maximal levels within 5 min. The efficiency was similar for all four receptor types, ranging between 40 and 70% after 30 min, with HIR being consistently most efficient. Exposure of confluent cells to 100 nM ligand for 16 h did not alter the steady-state number of receptors present on the cell surface (not shown).

To investigate the possible influence of the ligand on the stability of the different receptors, cells were labeled overnight with [^{35}S]methionine and then chased for various

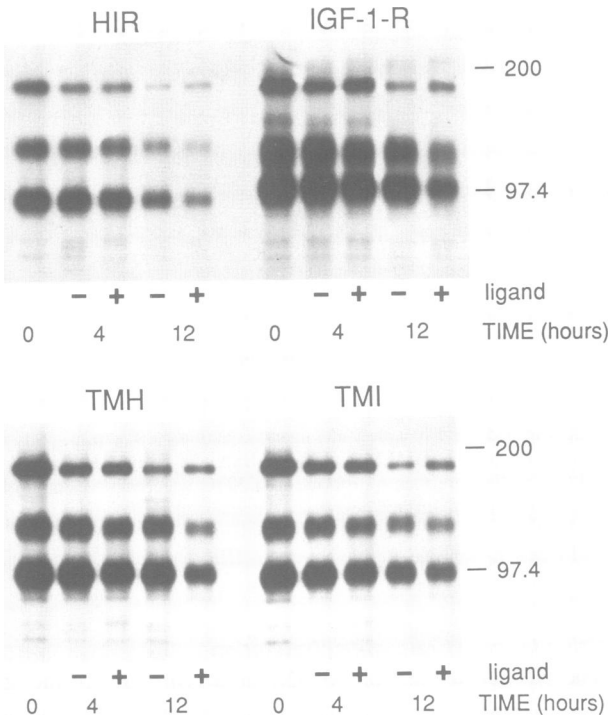

Fig. 6. Influence of the presence of ligand on receptor degradation. Equal numbers of cells were seeded into 6-well dishes, labeled overnight with [^{35}S]methionine, washed twice, and then maintained in DMEM with 0.2% BSA and 10 mM methionine with or without 100 nM insulin (HIR, TMH, TMI expressing lines) or IGF-1 (IGF-1-R expressing line). Cells were harvested at the times indicated and immunoprecipitated receptors subjected to SDS–PAGE (7%, reducing conditions).

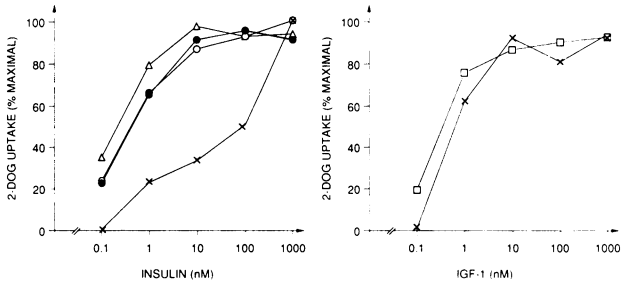

Fig. 7. Stimulation of 2-deoxyglucose uptake. Stimulation of 2-DOG uptake was measured as described in Materials and methods. Each value is plotted as the percentage of the maximal ligand effect and represents the average of three independent experiments performed in quadruplicate. △, HIR; ●, TMH; ○, TMI; □, IGF-1-R; X, NIH-3T3.

times in the presence or absence of either 100 nM insulin or IGF-1. Again, the receptors behaved similarly: after 4 h of incubation, ligands had little effect on receptor levels; however, after 12 h in the presence of ligand, the number of receptors was reduced to half of that detected in cells incubated in the absence of ligand (Figure 6).

Glucose transport

One of the most characteristic actions of insulin is the rapid stimulation of hexose uptake in target cells. In this process, insulin is thought to trigger a redistribution of glucose transporters from an intracellular pool to the cell surface,

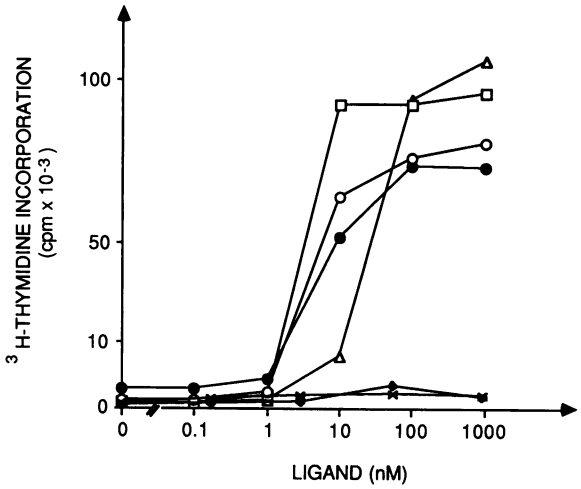

Fig. 8. Stimulation of thymidine incorporation. Confluent cells were starved to quiescence, incubated for 18 h with the concentration of ligand indicated, and then [^{3}H]thymidine (0.5 μCi/well) was added for 4 h. The means of a representative experiment with two parallel measurements are shown for each datapoint. △, HIR + insulin; ●, TMH + insulin; ○, TMI + insulin; □, IGF-1-R + IGF-1; ◆, NIH-3T3 + insulin; X, NIH-3T3 + IGF-1.

Table II. Comparison of maximal [^{3}H]thymidine incorporation values induced by insulin, IGF-1 and fetal calf serum

Cell line	Ligand 1 μM	Receptor number (× 10^3)	% of serum stimulation
NIH-3T3	insulin	5.4	3.8
HIR 3T3	insulin	849	103.1
TMH 3T3	insulin	1490	95
TMI 3T3	insulin	1320	101.1
NIH-3T3	IGF-1	3.4	2.9
IGF-1-R 3T3	IGF-1	1311	120.8

Confluent cells were starved for 24 h in 0.5% serum and then stimulated either with ligand (1 μM) or 10% fetal calf serum for 18 h. [^{3}H]thymidine incorporation was determined after a 4-h pulse and is shown as ligand-stimulated incorporation in percentage of serum-stimulated incorporation. The average values of a representative experiment with two parallel measurements is shown.

and may also increase the V_{max} of the glucose transporter molecule. In freshly isolated fat cells, glucose transport may be stimulated 15-fold. For unknown reasons, however, the response is generally much less pronounced (1.5- to 2-fold) in established cell lines.

In comparison to NIH-3T3 control cells, the glucose transport dose–response curves for insulin were shifted to lower ligand concentrations in cell lines expressing HIR and chimeric receptors. The shift was not as pronounced for higher IGF-1 concentrations but became apparent at 0.1 nM IGF-1. Little difference was observed between the effects of the different receptors tested in this experiment (Figure 7). Cells expressing either of the two chimeric receptors showed insulin-dependent stimulation of glucose transport that was similar to that seen for the IGF-1-R expressing lines. Under similar conditions of overexpression, HIR/3T3 cells generally responded to similar ligand concentrations ($ED_{50} \cong 0.2-0.4$ nM) as cells expressing receptors with IGF-1-R cytoplasmic domains. These results suggest that HIR and IGF-1-R have similar potentials for stimulating glucose transport in NIH-3T3 cells. However, they demonstrate the ligand-dependent capability of the IGF-1-R tyrosine

kinase to stimulate an increase in glucose transport, whether under the control of its own ligand binding domain, or that of the human insulin receptor. Interestingly, glucose transport of NIH-3T3 control cells was more efficiently stimulated by IGF-I ($ED_{50} \cong 0.6$ nM) than by insulin ($ED_{50} \cong 100$ nM). The data indicated that the endogenous mouse insulin receptor was almost inactive for stimulation of this process. Most of the response measured occurred at high, non-physiological insulin concentrations, and was likely due to cross-reaction with IGF-1 receptors. It is not clear whether this finding reflects specific properties of this cultured cell line or a significant cell type characteristic. Nevertheless, expression of HIR in these cells effectively rescued the insulin response.

Mitogenic response

It is generally accepted that insulin is a key regulator of major metabolic processes (Kahn, 1985); however, the role of insulin as a bona fide cellular mitogen has remained a matter of controversy (Rechler and Nissley, 1985). In contrast, IGF-1 is thought to be an important growth factor for a variety of cell types. We investigated the ability of human insulin and IGF-1 receptors to stimulate mitosis in a single cell type, NIH-3T3 fibroblasts, and used the chimeric receptors, TMH and TMI, to compare the mitogenic potential of different cytoplasmic domains after activation by a single ligand. The results are presented in Figure 8 and Table II. Confluent cells were starved to quiescence in DMEM containing 0.5% serum for 24 h. Ligand was then added for 18 h, and DNA synthesis was monitored over a 4-h period after addition of [^{3}H]thymidine. Although the parental NIH-3T3 cells responded only minimally to insulin or IGF-1 stimulation, [^{3}H]thymidine incorporation was strongly stimulated by both ligand−receptor systems in all high-expressing transfected cell lines, with incorporation being similar to that observed in the presence of serum (Table II). After stimulation with the respective ligands, DNA synthesis was induced ~10 times more efficiently in IGF-1-R expressing cells ($ED_{50} \cong 3$ nM) than in HIR 3T3 cells ($Ed_{50} \cong 30$ nM). Most interestingly, the chimeric receptors, TMI and TMH, mediated a mitogenic signal with an ED_{50} more similar to the human IGF-1-R in 3T3 cells ($ED_{50} = 4-5$ nM), despite the presence of an HIR ligand binding domain. These findings demonstrate that the human IGF-1-R displays a higher mitogenic signalling potential than the human insulin receptor in NIH-3T3 cells, and this property is defined by cytoplasmic domain determinants. Nevertheless, the insulin receptor cytoplasmic domain was highly competent to stimulate thymidine incorporation in transfected cells.

Discussion

Expression of human insulin receptor, human IGF-1, and two chimeric receptors, TMH and TMI, in the same mouse NIH-3T3 fibroblast background, permitted a direct analysis of the signalling characteristics of two closely related receptor molecules which possess distinct roles in the development and physiology of mammalian organisms. Although the experimental circumstances created by overexpression of normal and chimeric receptors did not represent normal physiological conditions, we were able to make observations and draw conclusions of general significance. All normal and chimeric receptors investigated in this study were faithfully synthesized, processed and transported to the cell surface, where they formed high-affinity ligand binding sites. In all cases, the binding data were consistent with a single affinity receptor class with K_ds of 1−2 nM. The kinase activity of HIR, IGF-1-R, as well as both chimeric receptors, was regulated by their respective homologous or heterologous ligands. However, the specific activity of phosphate incorporation was significantly lower for insulin receptors relative to IGF-1 receptors. Analysis of the chimeric receptors demonstrated that this property was defined by the cytoplasmic domain itself; thus, the insulin-induced activity of the IGF-1-R tyrosine kinase in TMH and TMI hybrid receptors was approximately three times greater than that observed for an equal amount of insulin receptor. These initial findings provide another example of the feasibility of a chimera approach for functional analysis of receptor characteristics, and demonstrate that heterologous functional domains can co-operate within a chimeric molecule while maintaining at least some of their individual properties. Another important property of cell surface receptors, down-regulation after ligand interaction, was found to be functional in NIH-3T3 cells for wild-type HIR and IGF-1-R, as well for the TMH and TMI chimerae. All of these receptors internalized ligands efficiently and were degraded more rapidly when activated by interaction with insulin or IGF-1.

Stimulation of glucose transport was similar for all of the receptor types. This process was induced efficiently at similar ligand concentrations for HIR as compared with IGF-1-R, TMH and TMI in transfected NIH-3T3 cells (ED_{50} 0.2−0.4 nM). We cannot state with certainty whether the dramatic increase of insulin sensitivity for glucose transport stimulation in HIR 3T3 cells, in comparison with a rather marginal change in IGF-1-R/3T3 lines, reflects a higher signalling potential of HIR or complementation of a mouse receptor incompetence in HIR-transfected NIH-3T3 cells.

While both human IGF-1-R and HIR, as well as chimeric receptors TMH and TMI, elicited similar ligand response curves for glucose transport stimulation in mouse NIH-3T3 cells, their mitogenic signalling activities differed significantly. Neither insulin nor IGF-1 elicited a mitogenic response in parental NIH-3T3 control cells comparable to that of the epidermal growth factor (EGF; not shown), yet overexpression of human IGF-1-R, HIR, and chimeric receptors resulted in a dramatic alteration of this property. Maximal mitogenic responses induced by insulin and IGF-1 exceeded those obtained with 10% fetal calf serum; stimulation levels reached up to 50-fold over background, similar in extent to that achieved with the mitogen EGF in human EGF receptor overexpressing NIH-3T3 cells (not shown). This mitogenic activity, however, was generated at significantly lower ligand concentrations for IGF-1-R, TMH or TMI expressing cells (ED_{50} ~3−5 nM) than for comparable HIR 3T3 cell lines (ED_{50} ~ 30 nM), a finding which documents the dominant role of the cytoplasmic domain. It is likely that the surprisingly strong mitogenic response elicited by insulin in these transfected NIH-3T3 cells is only significant *in vitro*. This experiment demonstrates the mitogenic potential of HIR under experimental conditions, which are unlikely to occur under normal physiologic circumstances.

For both receptors, the ligand concentrations needed to observe a half-maximal increase in DNA synthesis were significantly higher than those needed to stimulate glucose

transport: ~10-fold higher for receptors with an IGF-1-R cytoplasmic domain, and 100-fold higher for HIR. Consistent with this finding, the ED_{50} for IGF-1 mitogenic activity has been shown by others to be lower than that for insulin in several cell lines (Pepe *et al.*, 1987; Corps *et al.*, 1988). In addition, in NIH-3T3 cells expressing a chimeric receptor that consists of HIR extracellular domain sequences linked to the cytoplasmic domain of the EGF-R (IER, H.Riedel *et al.*, submitted), the ED_{50} for ligand-stimulated DNA synthesis was found to be even lower than that observed for the chimeric receptors used in this study, consistent with the more pronounced mitogenic potential of the EGF receptor.

In summary, our experiments support the notion that insulin and IGF-1 receptors have similar, yet distinct, signalling potentials, despite their highly homologous structures. The distinctions between these receptors can even be discerned when expressed in a cellular background that may not be optimal for the generation of the clearly defined responses characteristic of insulin target tissues such as adipocytes. This conclusion is strongly supported by the data obtained with our chimeric receptors, TMH and TMI, which both induced mitogenic responses distinct from the insulin receptor after each of the receptors was stimulated with the same ligand (insulin). Not only were the enzymatic properties of the IGF-1-R kinase preserved in TMH and TMI, but also the signalling characteristics were defined by the cytoplasmic domain. Finally, since we found no significant differences between TMH and TMI, the receptor transmembrane domain appears not to play a role in the definition of the receptor signal.

Materials and methods

Expression plasmid construction

HIR and IGF-1-R cDNA fragments were cloned into an M13mp19 vector digested with *Bam*HI and *Sph*I (bp number according to Ullrich *et al.*, 1985, 1986): *Bam*HI (bp 1926) to *Rsa*I (bp 3042) from HIR and *Sma*I (bp 2736) to *Sph*I (bp 3164) from IGF-1-R. Subsequently, primer-directed deletion using the oligonucleotides ATT TAT CTA TTC CTG/AGG AAA CGA AAT AAC AGC AGG CTG and CCG TCA AAT ATT GCA AAG/TTA ATC ATC GCT CTG CCC resulted in fusion of the extracellular portion of HIR and the intracellular portion of IGF-1-R proteins at the cytoplasmic face of the plasma membrane (TMH) or fusion of the two receptors at the extracellular face of the cytoplasmic membrane (TMI) respectively. The complete cDNAs were reconstituted and, like the cDNAs of HIR and IGF-1-R, cloned into an expression vector containing a neomycin phosphotransferase gene and an expression unit for dihydrofolate reductase (Riedel *et al.*, 1986).

Cell culture

Mouse NIH-3T3 (clone 7) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. DNAs were introduced into the cells by calcium phosphate co-precipitation based on the protocol of Graham and van der Eb (1973). Neomycin-resistant cells were pooled and either subjected to methotrexate-induced gene amplification or, after indirect immunolabeling, submitted to fluorescent-activated cell sorting. Individual clones were isolated and characterized further.

Metabolic labeling and immunoprecipitation

Subconfluent cells were labeled with [^{35}S]methionine (50 μCi/ml) in methionine-free DMEM with 10% dialyzed fetal calf serum. For lysis, 0.3 ml lysis buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl-fluoride and 200 U/ml aprotinin) was added per 10-cm^2 dish. Dishes were incubated for 3 min on ice, the supernatant was collected, kept for 5 min on ice, centrifuged at 12 500 *g* for 2 min and the supernatant used for immunoprecipitation. The supernatant was diluted 1:3 with HNTG (20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol) and incubated with 1 μl antiserum [monoclonal antibodies used were 83-14 against the HIR extracellular domain (Taylor *et al.*, 1987) and α-IR3 against the IGF-1-R extracellular domain (Kull *et al.*, 1983)] and 20 μl of protein A–Sepharose slurry (1:1, swollen and prewashed in HNTG) for 4 h at 4°C. Immunoprecipitates were washed three times in 0.6 ml of HNTG and analyzed by gel electrophoresis.

Receptor autophosphorylation

Ligand stimulation of autophosphorylation was performed by addition of 100 nM ligand to the cell lysate, followed by a 10-min incubation, immunoprecipitation and autophosphorylation in the immunocomplex in the presence of 20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and 20 μM [γ-^{32}P]ATP (25 c.p.m./fmol) with or without ligand at 23°C for 10 min. Gels were fixed for 30 min in 10% acetic acid, 25% isopropanol, incubated for 30 min in Amplify (Amersham), and then dried and exposed.

Proteins were boiled for 5 min in sample buffer and analyzed by electrophoresis on 7% SDS–polyacrylamide gels. For autophosphorylation experiments, gels were dried immediately.

Ligand binding

Confluent cells were suspended after incubation in PBS containing 25 mM EDTA for 1 h at 37°C. Ligand binding was performed at 4°C for 5 h in 100 mM Hepes pH 7.9, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM EDTA, 15 mM NaOAc, 5 mg/ml fatty acid free BSA and 1 mg/ml Bacitracin. [^{125}I]insulin and [^{125}I]IGF-1 were obtained from Amersham, non-radioactive insulin from Eli Lilly (Humulin) and IGF-1 from KabiGen. Data were analyzed by Scatchard analysis using the computer program Scatplot.

Internalization and ligand-induced degradation

Confluent monolayers in 12-well dishes were cooled to 4°C and the medium changed to 0.5 ml DMEM containing 0.2% BSA, 50 mM Hepes, pH 7.9, and 5 nM iodinated ligand (10^5 c.p.m.). After 1 h of binding at 4°C, cells were incubated for various times at 37°C, and then washed three times with cold binding buffer. Externally bound radioactivity was determined using an acid wash protocol (Haigler *et al.*, 1980), and internalized radioactivity by cell lysis in 0.2 mM NaOH, 1% SDS.

To test receptor degradation in the presence of ligand, equal numbers of subconfluent cells in 6-well dishes were labeled with [^{35}S]methionine overnight, washed twice with PBS, and then maintained in DMEM with 0.2% BSA and 10 mM methionine with or without 100 nM ligand. At the times indicated, cells were harvested and receptor degradation monitored by immunoprecipitation and analysis on SDS–PAGE.

Glucose transport and [^{3}H]thymidine incorporation

Glucose transport was assayed as described (McClain *et al.*, 1987), except that preincubation of cells with KRP and ligand was for 20 min, transport was measured for 3 min and assays were carried out in 12-well dishes. [^{3}H]Thymidine incorporation was determined as described by Riedel *et al.* (1988).

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