The Dominant Negative Effect of a Kinase-Defective Insulin Receptor on Insulin-Like Growth Factor-I–Stimulated Signaling in Rat-1 Fibroblasts

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To study the interaction between insulin receptor (IR) and insulin-like growth factor-I (IGF-I) receptor (IGF-IR) tyrosine kinases, we examined IGF-I action in Rat-1 cells expressing a naturally occurring tyrosine kinase–deficient mutant IR (Asp 1048 IR). IGF-I normally stimulated receptor autophosphorylation, IRS-I phosphorylation, and glycogen synthesis in cells expressing Asp 1048 IR. However, the Asp 1048 IR inhibited IGF-I–stimulated thymidine uptake by 45% to 52% and amino acid uptake (aminoisobutyric acid [AIB]) by 58% in Asp 1048 IR cells. Furthermore, IGF-I–stimulated tyrosine kinase activity toward synthetic polymers, Shc phosphorylation, and mitogen-activated protein (MAP) kinase activity was inhibited. The inhibition of mitogenesis and AIB uptake was restored with the amelioration of the impaired tyrosine kinase activity and Shc phosphorylation by the introduction of abundant wild-type IGF-IR in Asp 1048 IR cells. These results suggest that the Asp 1048 IR causes a dominant negative effect on IGF-IR in transmitting signals to Shc and MAP kinase activation, which leads to decreased IGF-I–stimulated DNA synthesis, and that the kinase-defective insulin receptor does not affect IGF-I–stimulated IRS-I phosphorylation, which leads to the normal IGF-I–stimulated glycogen synthesis.

THE MAJOR ACTION of insulin is metabolic stimulation, whereas insulin-like growth factor-I (IGF-I) is considered a regulator of cell proliferation.[1] However, they have structurally similar receptors, and these two hormones share these two distinct biological effects.[2,3] Both insulin receptor (IR) and IGF-I receptor (IGF-IR) belong to the tyrosine kinase family and have common pathways for signal transduction.[4] Both receptors are autophosphorylated upon ligand binding and phosphorylate several docking proteins, such as IRS-I and Shc, and activate Ser/Thr kinases, such as mitogen-activated protein (MAP) kinase, S6 kinase, and PI_3 kinase.[5,6] Thus far, no exclusively specific substrates for either hormone's receptors have been identified. Furthermore, the interaction between these receptors after ligand binding is still unclear.[7,8]

IGF-I has recently been introduced to clinical medicine for the treatment of patients with severe insulin resistance.[9] The hypoglycemic effect of IGF-I has been well characterized in vitro and in vivo.[10,11] The adverse effects of IGF-I treatment should be considered because IGF-I, a more potent stimulant than insulin for mitogenesis in many culture cell lines,[1] can accelerate proliferation of endothelial cells and may cause progression of diabetic complications.[12]

The aim of the current investigation was to study the function of IGF-IRs in the presence of mutant IRs with a defective tyrosine kinase, which was found in a type A insulin resistance.[13] In fibroblasts from insulin-resistant patients, IGF-I–mediated biological activities were sometimes blunted, together with a defect in IGF binding.[14,15] The dominant negative effect of the mutant IR on the wild-type IR was previously reported.[16] Because there is structural homology between IRs and IGF-IRs and both receptors form heterodimers, as we previously reported,[17] mutant IRs may exert the dominant negative effect on signal transduction through IGF-IRs. In the current study, we examined IGF-I–stimulated biological activity in cells expressing both IGF-IRs and the kinase-defective Asp 1048 IR to determine whether signal transduction through IGF-IRs was affected by the presence of the mutant IR.

MATERIALS AND METHODS

Materials

IGF-I was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan). [^{125}I]IGF-I (~74 TBq/mmol) and [^{32}P]adenosine triphosphate (ATP) (~185 TBq/mmol) were purchased from Amersham (Buckinghamshire, England). D-[^{14}C]glucose (10.6 Bq/mmol), [methyl-^{3}H]thymidine (3 TBq/mmol), and [methyl-^{3}H]aminoisobutyric acid ([AIB] 329 GBq/mmol) were purchased from Du Pont (Wilmington, DE). Anti–IGF-IR antibodies raised against the α-subunit (anti–IGF-IRα) and β-subunit (anti–IGF-IRβ) were from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biochemistry (Santa Cruz, CA), respectively. Cell culture reagents were purchased from Nissui Seiyaku (Tokyo, Japan) and GIBCO (Gaithersburg, MD).

Cell Culture and Transfection

Rat-1 fibroblasts grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) were transfected with cDNA coding for Asp 1048 mutant IR including exon 11 in a RLDN vector by the calcium phosphate coprecipitation method.[13] The RLDN vector, carrying G418-resistant gene, was kindly provided by Dr Allan R. Shatzman of Smith-Kline (King of Prussia, PA). Geneticin (GIBCO)-resistant clonal cell lines were selected by insulin binding studies. Several cell lines stably expressing Asp 1048 IRs were obtained. Rat-1 cells transfected with RLDN vector alone and selected by Geneticin were used as control Rat-1 cells. A clonal Asp 1048 IR cell (clone 12) with 2×10^5 mutant IRs per cell was overtransfected with cDNA coding human IGF-IR, which was

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kindly provided by Dr Jonathan Whittaker (Health Science Center, New York). Cotransfected pcDEB vector carrying hygromycin-resistant gene was kindly provided by Dr Hiroshi Hayakawa (University of Kyushu, Fukuoka). Hygromycin B–resistant cell lines were selected by immunoblotting studies with anti–IGF-IRα antibodies, and cells expressing human IGF-IRs (Asp 1048IR/IGF-IR cells) were obtained. The number of IGF-IRs for control Rat-1, Asp 1048 IR (clone 12), and Asp 1048IR/IGFR cells determined based on Scatchard analysis of IGF-I binding studies was 1.5×10^6, 1.6×10^6, and 8.0×10^6 per cell, respectively. The number of IRs in cells expressing wild or mutant receptors was also determined on the Scatchard plot. Both IGF-I and insulin binding to these cell lines remained unchanged during the following studies. All transfected cell lines were cultured in DME/F12 medium with 10% FCS after transfection.

IGF-I Binding and Internalization

Binding and internalization of [^{125}I]IGF-I to the cells was performed as described previously.[18]

Detection of IR/IGF-IR Hybrid

Transfected cells in 10-cm dishes were incubated with 0.07 nmol/L [^{125}I]IGF-I at 4°C for 16 hours. After washing the cells with ice-cold phosphate-buffered saline (PBS), bound IGF-I and IGF-IRs were cross-linked with disuccinimidyl suberate as described previously.[19] Cells were rapidly solubilized in the buffer containing 1% Triton X-100 and protease inhibitors and were immunoprecipitated with monoclonal antihuman specific IR antibody from Immunotech (Marseille, France). Immunoprecipitated and nonimmunoprecipitated cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography to detect the hybrid receptors. Since the antibody immunoprecipitates greater than 90% of labeled human IRs and does not recognize IGF-IRs, any cross-linked IGF-IRs brought down in the immunoprecipitates must exist as human IR/IGF-IR hybrids.[17,19] Furthermore, the antibody did not recognize any rat IRs, and fewer than 5% of total ^{125}I-labeled IGF-IRs were immunoprecipitated by the polyclonal anti-IR antibody.[17] Therefore, we consider the hybrid between rat IR and IGF-IRs present in the transfected cell lines to be negligible in the current study. There was no cross-reactivity of labeled IGF-I to the human IRs in this condition as previously reported.[20] The presence of hybrid receptors was also determined in the absence of the cross-linker and IGF-I. After adjusting the protein concentration, equal amounts of cell lysates from transfected cells grown in a 10-cm dish were immunoprecipitated with either anti–IGF-IRβ or antihuman IR antibody. The immunoprecipitated samples were analyzed by SDS-PAGE and immunoblotting with anti–IGF-IR antibodies and chemiluminescence (ECL; Amersham). The signals on the blots were analyzed by densitometry (EPA 3000; Cosmo Bio, Tokyo, Japan).

Biological Activity

The methods for immunoblotting phosphorylated receptors and determining tyrosine kinase activity toward Glu/Tyr polymers, IGF-I–stimulated glucose incorporation into glycogen, and AIB uptake were previously described.[17-19] Cells were plated in 6-well plates at a density of 1×10^5 cells/well, and glucose incorporation into glycogen and AIB uptake were measured after 7 days of culture. Cells were plated at a density of 1×10^5 cells/well in 24-well plates or 1×10^6 cells/well in 6-well plates for thymidine uptake studies. Since 10% FCS made cells overgrow and cell monolayers float in the 24-well plate, we used 6-well plates to compare the stimulation of IGF-I and serum. After the serum starvation in DMEM containing 0.05% FCS and 40 mmol/L HEPES (pH 7.4) for 24 hours, cells were incubated with various concentrations of IGF-I or FCS (10%) for 20 hours. Labeled thymidine (37 KBq/well) was added to the cells, and thymidine incorporation into the cells for 4 hours was measured as described previously.[19]

Phosphorylation of IGF-IRs, IRS-1, and Shc

Confluent monolayer cells in 6-well plates, starved of serum in DMEM with 0.05% FCS and HEPES 40 mmol/L, pH 7.4, were stimulated with IGF-I (20 nmol/L) at 37°C. After aspirating the medium, cells were immediately lysed in the solubilizing buffer as described previously.[21] Either directly or after immunoprecipitation with antityrosine antibody, proteins in the cell lysates were analyzed by SDS-PAGE followed by immunoblot analysis with antiphosphate tyrosine antibody (PY20) or anti-Shc antibody from Transduction Laboratories (Lexington, KY).

MAP Kinase Activity

MAP kinase activity was measured with the p42/p44 MAP kinase enzyme assay system from Amersham. After starvation of serum for 16 hours in DMEM (0.05% FCS and HEPES 40 mmol/L, pH 7.4), cells in 10-cm dishes were stimulated with IGF-I for 5 minutes at 37°C. The cells were washed once with ice-cold PBS and twice with cell lysis buffer containing 10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L DTT, 1 mmol/L orthovanadate, 1 mmol/L PMSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin, pH 7.4, and then were homogenized in 0.3 mL lysis buffer. Cellular debris was precipitated at 25,000 × *g* for 20 minutes. After adjusting the protein concentration, the cell lysates were used for MAP kinase assay with synthetic peptides and [^{32}P]ATP according to the manufacturer's protocol.

Statistical Analysis

For statistical evaluation of the results, we used one-way ANOVA and Scheffés F test, with P more than .05 considered statistically significant.

RESULTS

Insulin-Stimulated IR Autophosphorylation and Biologic Activity in Asp 1048 IR Cell

As previously described in transient expression experiments in Cos-7 cells,[13] insulin bound to the Asp 1048 IR with similar affinity (data not shown), but did not stimulate Asp 1048 IR autophosphorylation and IRS-I phosphorylation in two different clonal Asp 1048 IR cells (Fig 1A). Accordingly, insulin-stimulated thymidine incorporation and insulin-stimulated glucose uptake (data not shown) were impaired in Asp 1048 IR cells (Fig 1B).

IGF-I Binding and Hybrid Formation

IGF-I bound to Rat-1 cells and Asp 1048 IR cells with similar affinity (Fig 2A). The rate of IGF-I internalization in Rat-1 cells and Asp 1048 IR cells was 17.6% ± 2.1% and 20.2% ± 3.2% of total binding per 20 minutes (mean ± SE, n = 3), respectively. In Asp 1048 IR cells, less than 5% of IGF-IRs formed a hybrid with rat IRs[17] and 30% of labeled IGF-IRs were immunoprecipitated with Asp 1048 IR (Fig 2B). In cells expressing abundant human IGF-IRs by the transfection of IGF-IR cDNA (Asp 1048 IR/IGF-IR cells), total labeled IGF-IRs was increased by fivefold, whereas

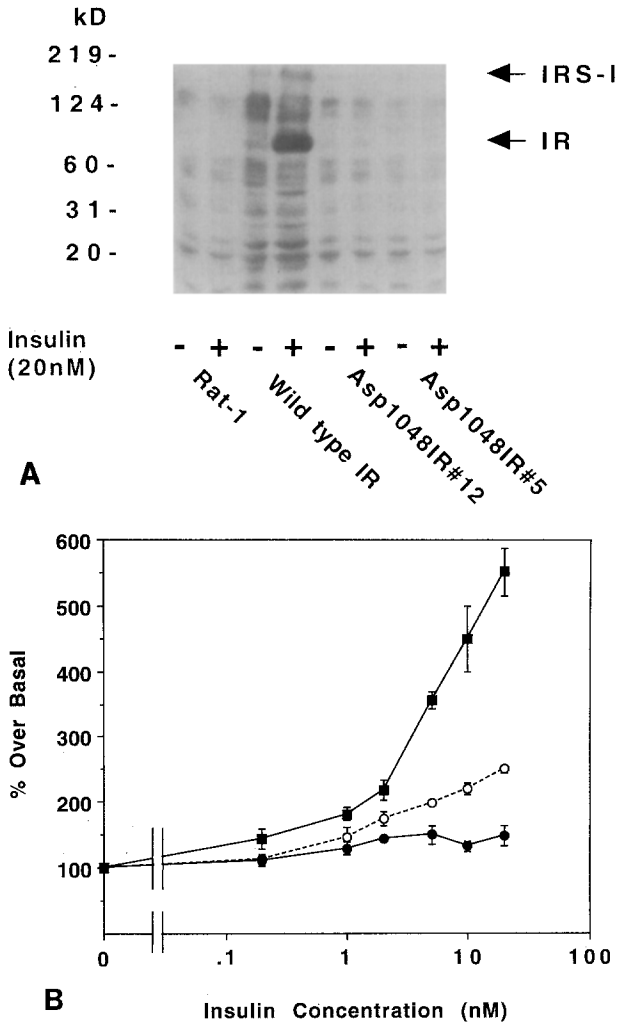


Fig 1. Insulin-stimulated autophosphorylation and thymidine incorporation. (A) Insulin-stimulated autophosphorylation of IRs. Arrows indicate 180-kd IRS-I protein and 95-kd IR β-subunit. (B) Insulin-stimulated thymidine incorporation in transfected cell lines. Rat-1 cells (○), Asp 1048 IR 12 cells (●), and wild-type IR cells (■) were incubated with various concentrations of insulin. Results are shown as % over basal (mean ± SE) from 3 experiments.

immunoprecipitated labeled IGF-IRs with Asp 1048 IR remained unchanged compared with those in the parental Asp 1048 IR cells. Consequently, only 6% of total IGF-IRs immunoprecipitated with Asp 1048 IRs in Asp 1048 IR/IGF-IR cells.

IGF-IRs were also immunoprecipitated with Asp 1048 IR in the absence of the cross-linker. IGF-IRs were immunoblotted with two different anti–IGF-IR antibodies, which recognized either the α-subunit of human IGF-IRs or the β-subunit of IGF-IRs of both rodent and human IGF-IRs. Human IGF-IRs were only detected in cell lysates from Asp 1048 IR/IGF-IR cells, and signal intensity of the IGF-IR β-subunit was increased accordingly. Although a considerable amount of human IGF-IRs were expressed in Asp 1048 IR/IGF-IR cells, the signal intensity of the immunoprecipitated IGF-IR with anti-human IR antibody was comparable to that in the parental Asp 1048 IR cells; 84% ± 12% (mean ± SE, n = 3) of that in the Asp 1048 IR cells. There was no detectable IGF-IR immunoprecipitated with the anti-IR antibody in Rat-1 cell lysates. Therefore, there were hybrids between IGF-IR and Asp 1048 IR in the absence of cross-linkers, but there was no increase in the amount of hybrid receptors by overexpressing human IGF-IR (Fig 3).

IGF-I–Stimulated IGF-IR Autophosphorylation and IRS-I and Shc Phosphorylation

The cells were serum-starved and stimulated with IGF-I. Within 1 minute, autophosphorylation of IGF-IRs occurred in Rat-1 cells and Asp 1048 IR cells (Fig 4). IGF-I stimulated Shc and IRS-I phosphorylation in Rat-1 cells

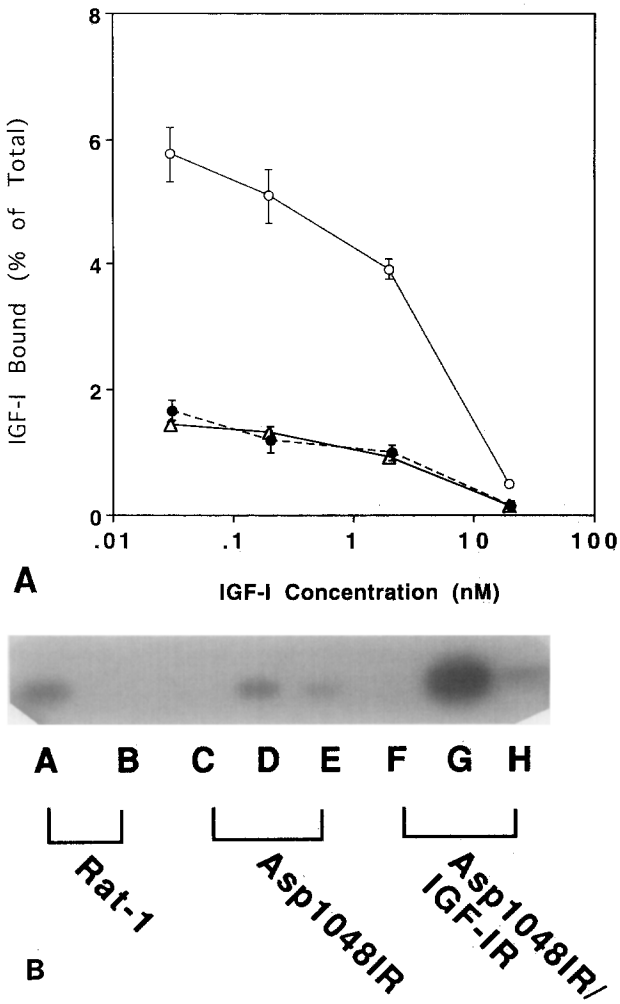


Fig 2. IGF-I binding and hybrid receptor formation in transfected cells. (A) IGF-I binding to Rat-1 cells (Δ), Asp 1048 IR cells (●), and Asp 1048 IR/IGF-IR cells (○) shown as % of total binding per 5 × 10^6 cells. (B) Hybrid of IRs and IGF-IRs in transfected cells. Receptors cross-linked with [^{125}I]IGF-I were immunoprecipitated with antihuman IR antibody (lanes B, E, and H) or control serum (lanes C and F). Samples without immunoprecipitation (lanes A, D, and G) were applied to the gel to show the total number of IGF-IRs. A representative autoradiogram from 2 separate experiments is shown.

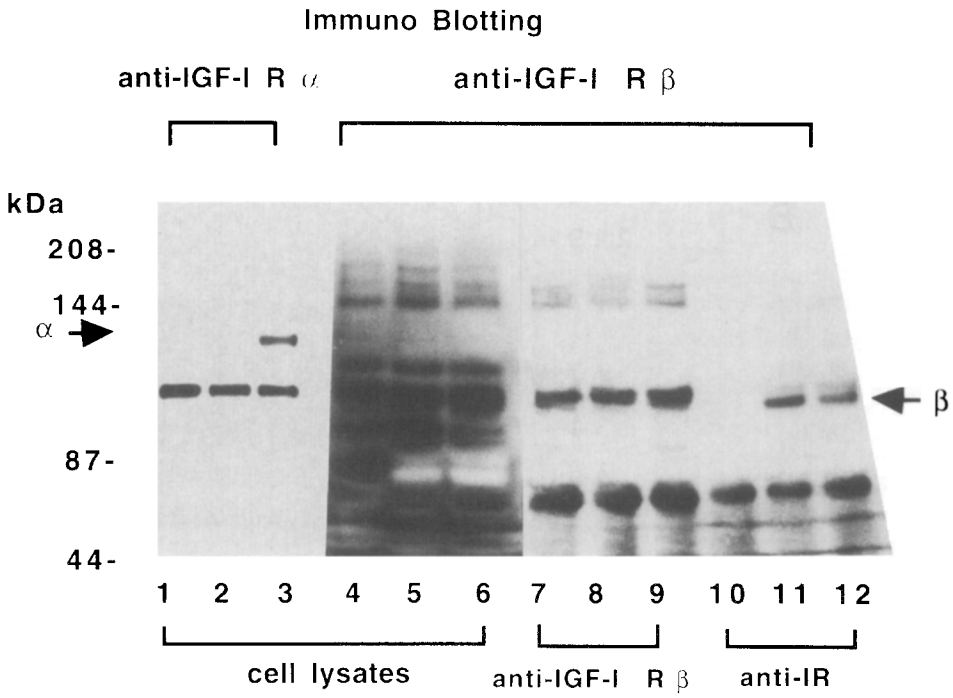


Fig 3. Immunoprecipitated IGF-IRs with anti-IR antibody. Cell lysates or immunoprecipitates with anti–IGF-IR antibody toward β-subunit (anti–IGF-IRβ) or antihuman IR antibody (anti-IR) were analyzed by 5% to 15% gradient SDS-PAGE. Electrotransferred proteins were immunoblotted with anti–IGF-IR antibodies recognizing α-subunit (anti–IGF-IRα) (lanes 1 to 3) or anti–IGF-IRβ (lanes 4 to 12). Cell lysates or immunoprecipitated samples from Rat-1 cells (lanes 1, 4, 7, 10), Asp 1048 IR cells (lanes 2, 5, 8, 11), and Asp 1048 IR/IGF-IR cells (lanes 3, 6, 9, 12) were applied to the gel, and a representative autoradiogram is shown. Arrows α and β indicate position of the α- and β-subunit of IGF-IRs, respectively.

(Fig 5A). IGF-I–stimulated IRS-I phosphorylation was comparable among Rat-1 cells, Asp 1048 IR cells, and Asp 1048 IR/IGF-IR cells (Fig 5B). In contrast to the results of IRS-I phosphorylation studies, IGF-I–stimulated Shc phosphorylation was completely blunted in Asp 1048 IR cells (Fig 5A and C). However, the decreased Shc phosphorylation was restored in Asp 1048 IR/IGF-IR cells. The immunoblotting study with anti-Shc antibody showed that the amount of Shc protein is comparable in these three cell lines (data not shown).

IGF-Stimulated Tyrosine Kinase Activity

The inhibitory effect of Asp 1048 IRs on the IGF-I–stimulated kinase activity of IGF-IRs is shown in Fig 6. The maximal kinase activity of IGF-IRs in Asp 1048 IR cells was decreased to 41% of that in Rat-1 cells. However, in Asp

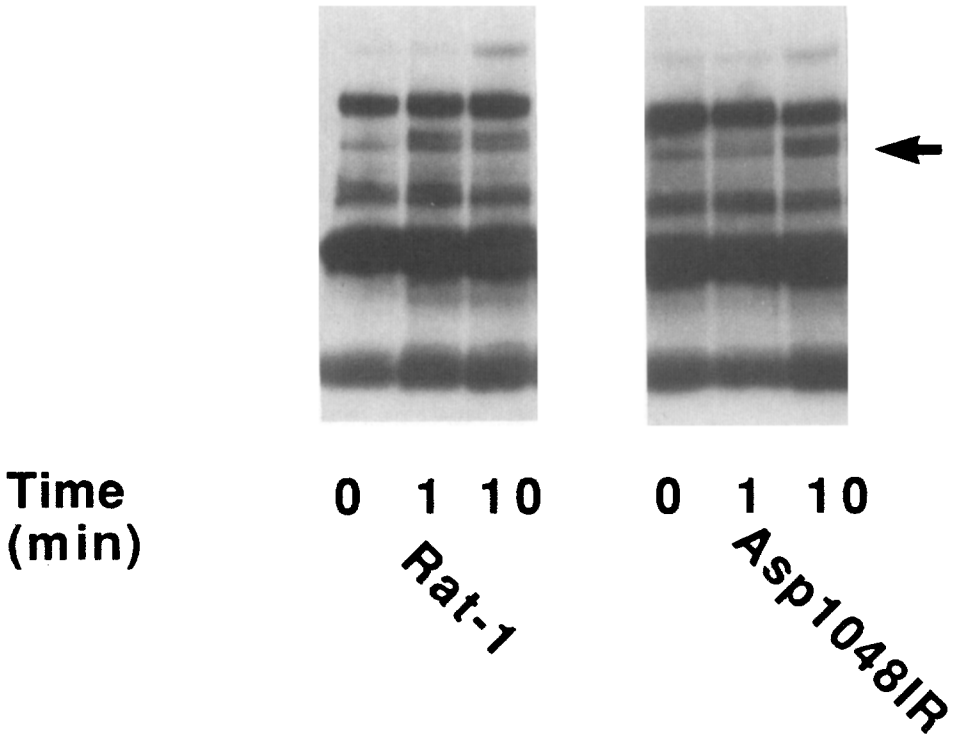
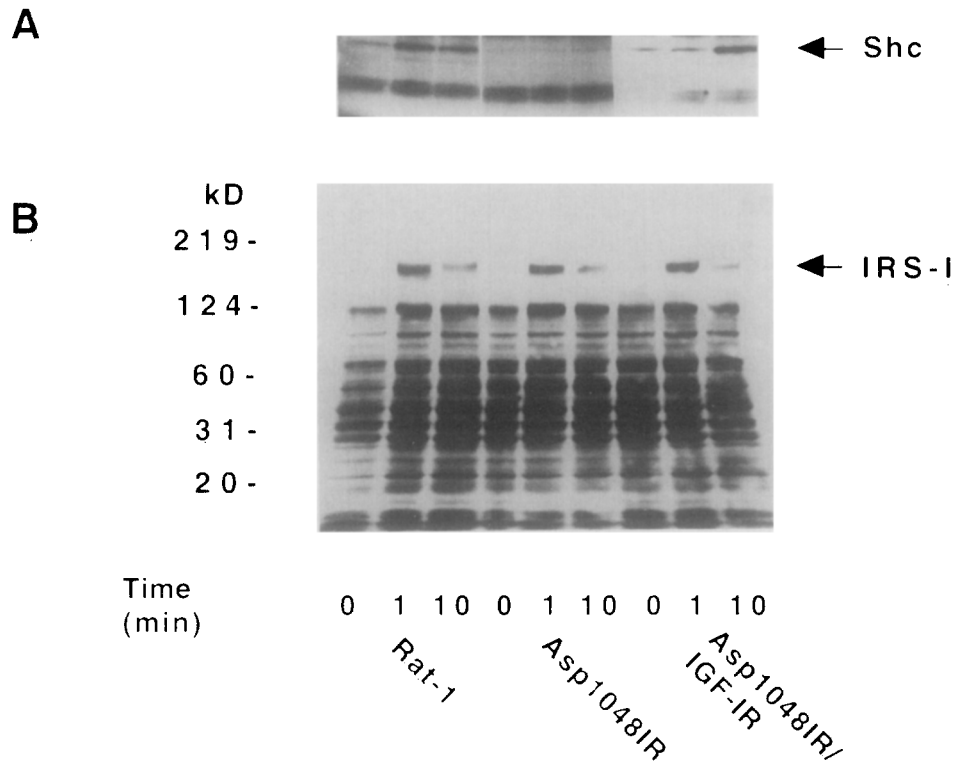


Fig 4. IGF-I–stimulated autophosphorylation in transfected cells. Immunoprecipitates of cell lysates with antiphosphotyrosine antibody were analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibody.



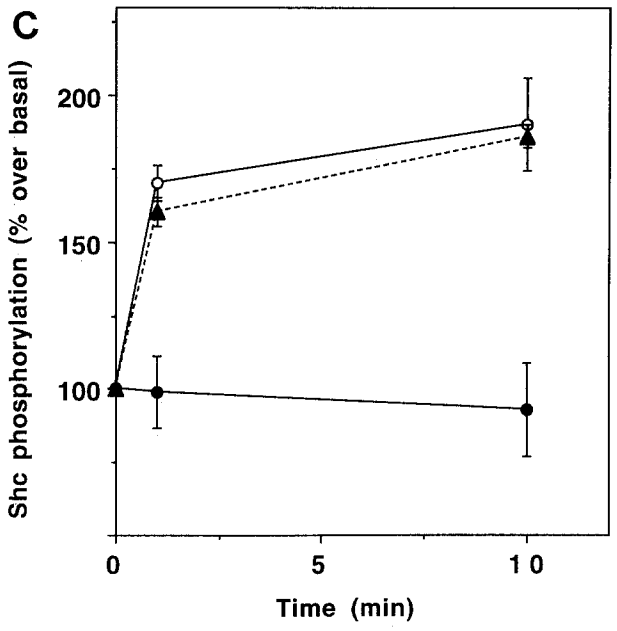


Fig 5. IGF-I–stimulated Shc and IRS-I phosphorylation in transfected cells. (A) IGF-I (20 nmol/L)–stimulated Shc phosphorylation. Tyrosine phosphorylation of Shc in Rat-1 cells, Asp 1048 IR cells, and Asp 1048 IR/IGF-IR cells was measured. Arrow indicates 52-kd Shc protein immunoprecipitated with anti-Shc antibody and detected by antiphosphortyrosine antibody. (B) IRS-I phosphorylation in the cell lysates. A portion of the cell lysates above (1/20 vol) was analyzed without immunoprecipitation by SDS-PAGE and immunoblotting with antiphosphotyrosine antibody. Arrow indicates 180-kd IRS-I protein. (C) Comparison of Shc phosphorylation among 3 cell lines. Signal intensity of phosphorylated Shc protein on the autoradiograms as shown above was analyzed by densitometry. Results in Rat-1 cells (○), Asp 1048 IR cells (●), and Asp 1048IR/IGF-IR cells (△) are shown as % over basal (mean × SE) from 3 experiments.

1048 IR/IGF-IR cells, it was significantly increased to 59% of that in Rat-1 cell receptors to compare with that in Asp 1048 IR cells ($P < .05$).

IGF-Stimulated Biological Effects

IGF-I increased glucose incorporation into glycogen by 2.2-fold over basal levels in Asp 1048 IR cells, which was comparable to the level in Rat-1 cells (Fig 7). In contrast, IGF-I–stimulated thymidine incorporation was markedly decreased in Asp 1048 IR cells compared with Rat-1 cells (Fig 8). The decreased IGF-I stimulation was restored by increasing the number of IGF-IRs by fivefold, as shown in the result from Asp 1048 IR/IGF-IR cells. The dose-response curve of IGF-I stimulation in Asp 1948 IR cells was shifted to the right compared with that in Rat-1 and Asp 1048 IR/IGF-IR cells. Basal and serum-stimulated thymidine incorporation in Rat-1 cells and Asp 1048 IR cells were comparable (Table 1). However, the maximal IGF-I–stimulated thymidine incorporation in Rat-1 cells and Asp 1048 IR cells was 14.1% and 5.3%, respectively.

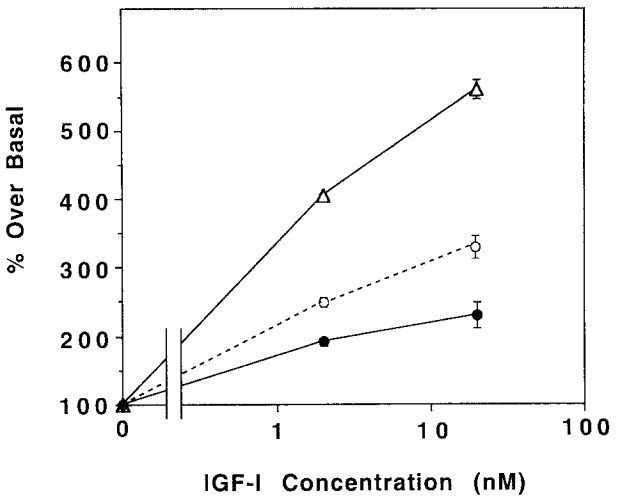


Fig 6. IGF-I–stimulated tyrosine kinase activity toward exogenous substrates Glu/Tyr 4:1. IGF-IRs from Rat-1 cells (△), Asp 1048 IR cells (●), and Asp 1048 IR/IGF-IR cells (○) were incubated with IGF-I. Results are the mean ± SE from 3 experiments. Basal activity in Rat-1 IR, Asp 1048 IR, and Asp 1048IR/IGF-IR is 0.130 ± 0.010, 0.130 ± 0.011, and 0.086 ± 0.009 fmol ATP/mg protein/30 min (mean ± SE, n = 3), respectively.

Therefore, the responsiveness of thymidine incorporation to IGF-I stimulation in Asp 1048 IR cells was severely impaired compared with that in the parental Rat-1 cells.

Furthermore, IGF-I–stimulated AIB uptake was also significantly inhibited in Asp 1048 IR cells compared with Rat-1 cells ($P < .05$) (Fig 9). The inhibitory effect by the Asp 1048 IRs was observed in the two different clonal Asp 1048 IR cells. There was no significance to the inhibitory effect between these two clonal cell lines, probably because

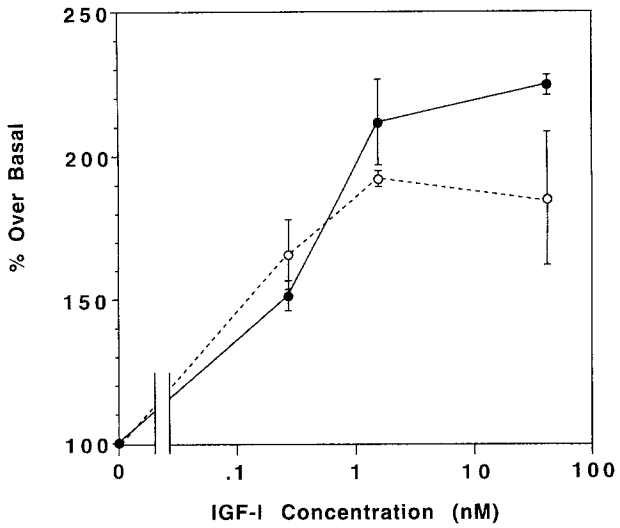


Fig 7. Glucose incorporation into glycogen in transfected cells. Cells postconfluent 2 days were stimulated with various concentrations of IGF-I in the presence of [^{14}C]glucose for 2 hours at 37°C. Labeled glucose incorporated into glycogen was measured. Results are shown as % over basal (mean ± SE, n = 3) in Rat-1 cells (○) and Asp 1048 IR cells (●). Basal values for glucose incorporation into glycogen in Rat-1 cells and Asp 1048 IR cells were 30.8 ± 8.9 and 24.8 ± 1.9 nmol glucose/mg protein (mean ± SE, n = 3), respectively.

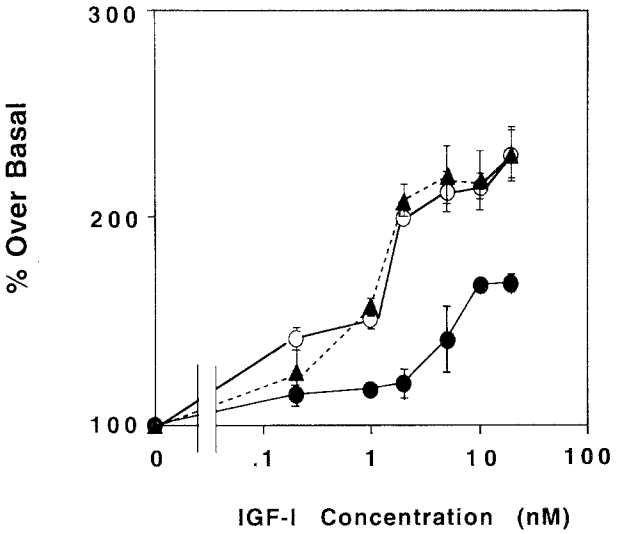


Fig 8. Thymidine uptake in transfected cells. IGF-I–stimulated thymidine uptake in Rat-1 cells (○), Asp 1048 IR cells (●), and Asp 1048 IR/IGF-IR cells (△) in 24-well plates was measured. Results are shown as % over basal (mean ± SE, n = 3). Basal counts in Rat-1 cells, Asp 1048 IR cells, and Asp 1048 IR/IGF-IR cells are 11,534 ± 1,752, 12,579 ± 1,608, and 15,183 ± 3,462 cpm/10^5 cells (mean ± SE, n = 3), respectively.

the number of expressing mutant IRs per cell was similar. The inhibition was significantly ameliorated by the introduction of IGF-IRs, as shown in the result for IGF-I–stimulated AIB uptake in Asp 1048 IR/IGF-IR cells.

IGF-I–Stimulated MAP Kinase Activity

To study further downstream of IGF-I signaling, MAP kinase activity was measured in these transfected cells. IGF-I stimulated MAP kinase activity by 5.3-fold over the basal activity in Rat-1 cells (Fig 10A). The maximal response in IGF-I–stimulated MAP kinase activity was decreased to 53% in Asp 1048 IR cells compared with Rat-1 cells, but it was significantly ameliorated in Asp 1048 IR/IGF-IR cells ($P < .05$) (Fig 10B). There was no difference in the expression of MAP kinase protein among these cell lines (data not shown).

DISCUSSION

The kinase-defective IRs, IGF-IRs, and PDGF receptors showed a dominant negative effect on the wild-type homologous receptors.[16,22-25] However, the mechanism of a dominant negative effect on the heterologous receptors has not been fully investigated. The inhibitory effect of the Asp

Table 1. Comparison of IGF-I– and Serum-Stimulated DNA Synthesis

Cell Line	Basal	IGF-I (20 nmol/L)	Serum (10% FCS)
Rat-1	693 ± 94	3,923 ± 685	27,871 ± 6,069
Asp 1048 IR	558 ± 45	1,756 ± 72*	33,226 ± 4,089
Asp 1048IR/IGF-IR	1,656 ± 81*	6,030 ± 689*	21,289 ± 1,404

NOTE. Thymidine incorporation was measured in cells in 6-well plates. Results are shown as cpm/10^6 cells from 4 experiments (mean ± SE).

*$P < .05$ *v* Rat-1.

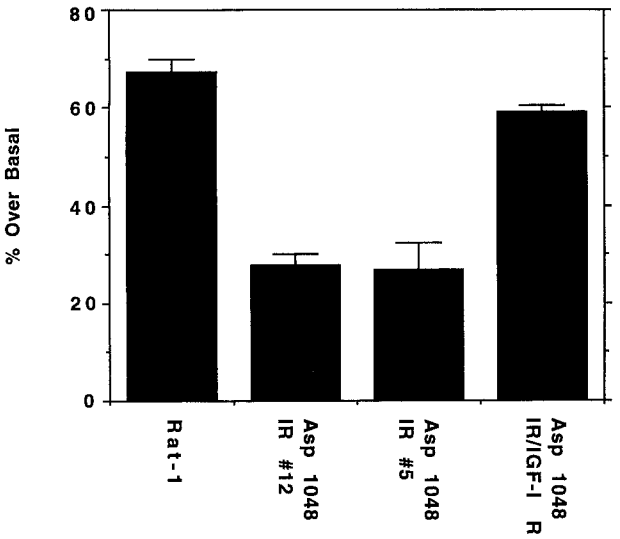


Fig 9. AIB uptake in transfected cells. IGF-I (20 nmol/L)–stimulated AIB uptake in Rat-1 cells, Asp 1048 IR cells (#12) expressing 2 × 10^5 mutant IRs, Asp 1048 IR cells (#5) expressing 1.4 × 10^5 mutant IRs, and Asp 1048 IR/IGF-IR cells expressing 2 × 10^5 mutant IRs and 8 × 10^6 IGF-IRs were studied. Cells postconfluent 2 days were incubated with IGF-I in 6-well plates for 2 hours at 37°C. [^{3}H]AIB incorporated into the cells for 12 minutes was measured. Results are shown as % over basal and mean ± SE from 3 experiments. Basal AIB uptake in Rat-1 cells, Asp 1048 IR #12 cells, Asp 1048 IR #5 cells, and Asp 1048 IR/IGF-IR cells was 1.73, 2.45, 2.62, and 2.51 nmol/mg protein/12 min, respectively.

1048 IR in the current study could be due to the reduction in the number of functionally active IGF-IRs, since an increased number of IGF-IRs leads to augmentation of IGF-I–stimulated biologic actions.[26-28] Hybrid formation between IRs and IGF-IRs might be responsible for the reduction of available functionally active IGF-IRs, since as many as 30% of total IGF-IRs were immunoprecipitated with IRs[19,29,30] (Fig 1B) and hybrid receptors with kinase-

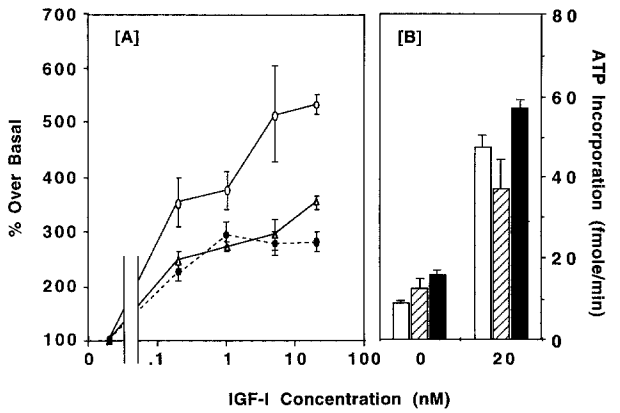


Fig 10. IGF-I–stimulated MAP kinase activity in transfected cells. (A) IGF-I dose-response curve. MAP kinase activity stimulated by various concentrations of IGF-I in cell extracts from Rat-1 cells (○), Asp 1048 IR cells (●), and Asp 1048 IR/IGF-IR cells (△) shown as % over basal (mean ± SE, n = 4). (B) Basal and maximal MAP kinase activity. MAP kinase activity of basal and maximally stimulated samples from Rat-1 cells (□), Asp 1048 IR cells (■), and Asp 1048 IR/IGF-IR cells (▨) are expressed as fmol ATP/mg protein/min (mean ± SE, n = 4).

defective IRs had no tyrosine kinase activity.[20,31,32] Furthermore, the increased number of IGF-IRs caused a decreased ratio of hybrid receptors over total IGF-IRs, ie, the increased number of nonhybrid IGF-IRs, and restored IGF-I–stimulated mitogenesis in Asp 1048 IR/IGF-IR cells. These observations suggest that the hybrid formation inhibits IGF-I actions in Asp 1048 IR cells and contributes to the dominant negative effect of Asp 1048 IRs on signal transduction through IGF-IRs.

Another possibility to explain the inhibitory effect of Asp 1048 IRs is the substrate competition between wild-type IGF-IRs and Asp 1048 IRs. One possible substrate is Shc, which was recently found to be a coupling molecule for the mitogenic signal transduction by insulin and IGF-I.[21,33] Inhibition of tyrosine phosphorylation of Shc in Asp 1048 IR cells can be explained by the contention that the kinase-defective IR or the hybrids with this mutant compete for the limited amount of Shc protein with IGF-IRs and may have a higher affinity for this protein than the homologous IGF-IRs. In contrast, phosphorylation of IRS-I, one of the well-known substrates for both insulin and IGF-I, was not impaired in Asp 1048 IR cells. We confirmed the results by demonstrating that the association of IRS-I with PI_3 kinase was not decreased in cells with Asp 1048 IRs. IGF-I–stimulated PI_3 kinase activity in Asp 1048 IR cells was also comparable to that in Rat-1 cells (data not shown). This normal IGF-I–stimulated phosphorylation of IRS-I in Asp 1048 IR cells was in contrast to the report by McClain et al,[34] in which they demonstrated that a kinase-defective receptor, ie, Ala 1018 IR (A/K 1018 IR), inhibited IGF-I–stimulated mitogenesis and IRS-I phosphorylation. In their experiment, unlike our result (Fig 3), the IRS-I in A/K 1018 IR cells was already phosphorylated without IGF-I stimulation, and it is therefore possible that they underestimated the IGF-I–stimulated phosphorylation of IRS-I. Since both IRS-I and Shc are essential for IGF-I–stimulated mitogenesis,[33,35] both proteins may be the substrates competitively bound by kinase-defective receptors, wild-type IGF-IRs, and hybrids. Because Asp 1048 mutation does not disturb ATP binding to Lys 1018, whereas Ala 1018 mutation destroys the binding site,[13] the specific tertiary structure of the kinase-defective mutant IRs may have a different affinity with these substrates.[36]

In contrast to the restored IGF-I–stimulated biological activity in cells, IGF-I–stimulated tyrosine kinase activity and MAP kinase activity in vitro was still inhibited in Asp 1048 IR/IGF-IR cells. Although the kinase assay in vitro could be much more sensitive than the biological assays in cells,[17,37,38] IGF-I signaling in Asp 1048 IR/IGF-IR cells might not be fully restored. By analyzing the results of biological studies more carefully, we noted that IGF-I–stimulated mitogenesis was significantly improved but still impaired in Asp 1048 IR/IGF-IR cells. Since we observed augmented thymidine incorporation by IGF-I in Rat-1 cells overexpressing IGF-IRs (data not shown), as was reported in NIH-3T3 cells,[26] IGF-I–stimulated mitogenesis in Asp 1048 IR/IGF-IR cells should be augmented compared with levels in Rat-1 cells if there was no inhibitory effect by Asp 1048 IRs. Thus, an overexpression of IGF-IRs that is more than fivefold the levels in Asp 1048 IR cells ameliorates but

might not completely normalize impaired IGF-I signal transduction in Asp 1048 IR/IGF-IR cells.

IGF-I failed to fully stimulate mitogenesis but normally stimulated glycogen synthesis in Asp 1048 IR cells, which is a phenomenon similar to that observed in A/K 1018 IR cells.[34] The spare-receptor theory for insulin action, especially for insulin-mediated glucose metabolism, has been well characterized.[39] Thus, the presence of spare receptors for glucose metabolism but not for mitogenesis in IGF-I action could be one explanation for this discrepancy. Consistent with this assumption, the sensitivity to IGF-I was about 10-fold higher in glycogen synthesis than in thymidine incorporation in Rat-1 cells when we compared the ED_{50} of the dose-response curve for these IGF-I actions. Furthermore, the recent report by Kublaoui et al[40] suggests that only a small fraction of IRs are required to be phosphorylated to elicit full activation of glucose transport in adipocytes. Our results also indicated that even a partial activation of IGF-IR tyrosine kinase was sufficient for IGF-I–stimulated glycogen synthesis and that full activation of the tyrosine kinase was required for maximal IGF-I–stimulated mitogenesis. The impairment of MAP kinase also did not cause the defect in IGF-I–stimulated glycogen synthesis. A recent report also demonstrates the independence of MAP kinase in insulin-induced glucose utilization in 3T3-L1 cells.[41] Taken together, these results might suggest the presence of two different signaling systems diverging from IGF-IRs: the first, which requires maximal tyrosine kinase activity of IGF-IR, is a Shc-MAP pathway leading to mitogenesis, and the second is an IRS-I–PI_3 kinase pathway leading to glucose utilization in the IGF-IR signaling pathway.[42]

The failure to show an increase in the absolute hybrid receptor number in Asp 1048 IR/IGF-IR is an unexpected result. A similar observation is reported by Taouis et al,[43] who coexpressed a 43–amino acid truncated IR and tyrosine kinase–deficient Ile^{1153} mutant IR or an IR with a Leu → Ser^{323} mutation in NIH-3T3 cells.[16,43] They could not detect any heterodimer receptors between these two exogenous IRs. However, the same group also reported a different result, demonstrating that the same mutant Ser^{323} IR formed heterodimers with the coexpressed truncated IR in NIH-3T3 cells in a certain condition and concluding that greater than a fivefold excess number of receptors versus the others in a cell was necessary for the formation of hybrids.[43,44] This theory may explain the current result, because the ratio of IGF-IRs to Asp 1048 IRs is increased from 0.8 to 4.0 in Asp 1048 IR/IGF-IR cells by overexpression of human IGF-IRs to the parental Asp 1048 IR cells, and this ratio may not be high enough to increase the hybrid receptor number in these cells. Exogenous receptors introduced to the cells may require a different machinery for transfer to the plasma membrane compared with the endogenous receptors, making it difficult to form hybrids between exogenous receptors, although the precise mechanism is still unknown.

The unchanged number of hybrids in Asp 1048 IR/IGF-IR cells could be due to underestimation of the hybrid receptor number by the cross-linking method. However, the immunoprecipitation of IGF-IRs with insulin antibody in the absence of the cross-linker was also comparable between Asp 1048 IR cells and Asp 1048 IR/IGF-IR cells. This result was consistent with studies reported previously for the different cells and the different kinase-negative IRs.[29,45,46] Furthermore, we have verified the reliability of the methods used for detection of hybrids by using a cell expressing a mutant IR with a 365–amino acid truncation (Δ365IR).[17,20] The difference in the molecular weight made it possible to clearly distinguish the hybrids with Δ365IR and IGF-IR homodimers by size, and we concluded that the number of cross-linked hybrid receptors and homologous IGF-IRs was proportional to that of unlabeled receptors, because both receptors showed similar affinity for IGF-I.[20] Thereby, we considered the ratio of hybrid receptors to whole IGF-IRs to be decreased in Asp 1048 IR/IGF-IR cells compared with Asp 1048 IR cells, although it is still possible that the absolute number of hybrid receptors was underestimated.

In summary, we have described a dominant negative effect of a mutant IR on the wild-type IGF-IR in Rat-1 cells. The inhibitory effect was apparent in IGF-I–stimulated mitogenesis, but not in glycogen synthesis. Asp 1048 IRs also inhibited IGF-I–stimulated Shc phosphorylation and MAP kinase activity, but did not affect IRS-I phosphorylation. The reduction of the ratio of Asp 1048 IR and IGF-IR hybrids to total IGF-IRs by overexpression of IGF-IRs ameliorated this inhibition. These results suggest that tyrosine kinase–defective IRs could inhibit the function of heterologous IGF-IRs, possibly by formation of the hybrid receptor, and that the dominant negative effect of the Asp 1048 IR on IGF-I signal transduction is specific for mitogenic activity transmitted through the Shc-MAP kinase pathway.

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