Intracellular Signaling by a Mutant Human Insulin Receptor Lacking the Carboxyl-terminal Tyrosine Autophosphorylation Sites*

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We have recently characterized a mutant insulin receptor (Y/F2) in which the two tyrosines in the carboxyl terminus (Tyr1316, Tyr1325) were mutated to phenylalanine. Compared with wild type receptors, the Y/F2 receptor exhibited markedly enhanced sensitivity to insulin-stimulated DNA synthesis with normal insulin-stimulated glucose uptake (Takata, Y., Webster, N. J. G., and Olefsky, J. M. (1991) J. Biol. Chem. 266, 9135–9139). In this paper, we present further evidence for the divergence of the metabolic and mitogenic signaling pathways utilized by the insulin receptor. The mutant receptor showed normal sensitivity and responsiveness for insulin-stimulated glucose incorporation into glycogen. The insulin sensitivity for phosphorylation of two substrates (pp180 and pp220) was the same in both Y/F2 cells and HIRc cells. Phosphotyrosine content, however, was greater in Y/F2 cells than in HIRc cells, especially in the basal state. Insulin stimulated S6 kinase activity 2–6-fold, with an ED_{50} of ~10 nM in Rat 1 cells and 0.5 nM in HIRc cells. The sensitivity to insulin was enhanced in Y/F2 cells with an ED_{50} of 0.1 nM. These effects were insulin-specific, since insulin-like growth factor (IGF)-1-stimulated mitogenesis was normal. In summary: 1) Y/F2 receptors exhibit normal metabolic and enhanced mitogenic signaling; 2) the enhanced mitogenic signaling is specific for the insulin receptor in the Y/F2 cells, since IGF-1-stimulated mitogenesis is normal; 3) Y/F2 cells display increased endogenous substrate phosphorylation and augmented insulin-stimulated S6 kinase activity placing these responses among insulin’s mitogenic effects; and 4) these results are consistent with the concept that the COOH-terminal tyrosine residues of the insulin receptor are normally inhibitory to mitogenic signaling.

The insulin receptor is a member of the receptor tyrosine kinase class of cell surface molecules (1). As such, it is a multifunctional protein mediating a variety of biological effects. A number of functional domains within the heterotetrameric insulin receptor structure has been identified already (2). The cytoplasmic domain of the insulin receptor β subunit contains the tyrosine kinase catalytic region and also contains sequences which interact with endogenous substrates as well as other potential downstream coupling, or signaling, molecules. As with other tyrosine kinases, the COOH terminus of the insulin receptor β subunit appears to have an important regulatory role in modulating the overall signaling functions of the insulin receptor (3). Indeed, recent studies have demonstrated that insulin binding, as well as autophosphorylation, can induce conformational changes in the COOH-terminal region (4). The fact that the insulin receptor COOH terminus is poorly homologous to other receptor tyrosine kinases, including the IGF-I receptor, suggests that these insulin receptor-specific COOH-terminal sequences are important for insulin-specific signaling (5). In earlier studies, we have shown that deletion of the insulin receptor COOH terminus leaves ligand binding and tyrosine kinase functions intact, compromises metabolic signaling, but enhances mitogenic signaling (6–8). Recently, Kadowaki et al. (9) have prepared a similar mutant insulin receptor, deleting the COOH-terminal 38 amino acids. In a preliminary report (9), their findings confirm the enhanced insulin-stimulated mitogenic signaling mediated by the truncated receptor. On the other hand, Myers et al. (10) have studied a COOH-terminal truncated insulin receptor and were unable to demonstrate enhanced mitogenic stimulation by insulin (10).

Within the COOH terminus are two tyrosine residues (Tyr1316, Tyr1325) which are autophosphorylated in response to insulin (11). To explore the role of these two tyrosines in the function of the COOH terminus, we constructed a mutant insulin receptor in which both residues were changed to phenylalanine by site-directed mutagenesis (Y/F2 receptor). We found that the ligand binding and tyrosine kinase activities of the Y/F2 receptor were normal and that its ability to signal metabolic events was unchanged (12). In contrast, the Y/F2 receptor demonstrated markedly enhanced mitogenic signaling properties (12). Taken together, these data suggested that the COOH-terminal tyrosines normally serve to inhibit, or restrain, the growth signaling properties of the insulin receptor, and when these residues are mutated or deleted, this inhibitory effect is relieved, allowing greatly enhanced mitogenic signaling upon ligand binding. To further explore the regulatory role of the COOH-terminal tyrosines, we have now conducted additional studies with the Y/F2 receptor examining its ability to interact with other downstream signaling pathways. Since this receptor displays enhanced mitogenic signaling properties, this approach may be useful in identifying the additional components of the insulin action cascade which are in the mitogenic signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—The generation of stable clonal cell lines expressing wild type insulin receptors (HIRc) and mutant insulin receptors, in which tyrosines 1316 and 1322 were replaced with phenylalanines (Y/F2), has been described (12, 13). Cells were maintained in DMEM/
Insulin Signaling by a Y/F2 Receptor

F-12 medium (GIBCO), deficient in hypoxanthine and thymidine, and supplemented with 10% fetal calf serum and 500 nM methotrexate. Two HIRc cell lines expressing 2.7 × 10⁵ (HIRc270) and 1.2 × 10⁵ (HIRc 1.2 M) insulin receptors and one Y/F2 cell line expressing 3.8 × 10⁶ receptors were used unless noted otherwise (12). A-14 monolayers and 125I-IGF-I were generally provided by the Lilly. d-[14C]Glucose (287 mCi/mmol), [3H]thymidine (83 Ci/mmol), and [32P]ATP labeled in the γ position (6000 Ci/mmol) were from Du Pont-New England Nuclear. 125I-Protein A (30 mCi/µg) was from Amerham Corp. Electrophoresis and protein assay reagents were from Bio-Rad. A monoclonal anti-insulin receptor antibody was kindly provided by Dr. Kenneth Siddle (University of Cambridge, Cambridge, United Kingdom).

d-[14C]Glucose Incorporation into Glycogen—Confuent cell monolayers in 35-mm dishes were incubated in glucose-free medium for 4 h. Cells were stimulated by insulin for 2 h at 37 °C with 5 mM glucose and 1 µCi of [14C]glucose. Monolayers were rinsed with phosphate-buffered saline and then solubilized for 30 min at 37 °C with 1 ml of 30% KOH solution. After boiling the sample for 30 min with 4 mg of carrier glycogen, glycogen was precipitated by the addition of 2 ml of carrier glycogen. Cell pellets were washed three times with 25% KOH solution followed by the addition of 2 ml of aqueous 2 N HCl. After boiling the sample for 30 min, the assay was terminated by the addition of 4 ml of 5% TCA, and the samples were precipitated by centrifugation at 150,000g for 2 h, and the supernatant was aspirated. Two HIRc cell lines expressing 2.7 × 10⁵ receptors were used unless noted otherwise (12). A-14 monolayers and 125I-IGF-I were generally provided by the Lilly. d-[14C]Glucose (287 mCi/mmol), [3H]thymidine (83 Ci/mmol), and [32P]ATP labeled in the γ position (6000 Ci/mmol) were from Du Pont-New England Nuclear. 125I-Protein A (30 mCi/µg) was from Amerham Corp. Electrophoresis and protein assay reagents were from Bio-Rad. A monoclonal anti-insulin receptor antibody was kindly provided by Dr. Kenneth Siddle (University of Cambridge, Cambridge, United Kingdom).

RESULTS

Insulin-stimulated Glucose Incorporation into Glycogen—An early metabolic response of target cells to insulin is an increase in the rate of glucose transport. We have reported previously that insulin stimulates the uptake of 2-deoxyglucose in Y/F2 cells with a similar sensitivity to insulin (12). Insulin also enhances glycogen synthase activity in fibroblast cell lines. As seen in Fig. 1, insulin stimulates glucose incorporation into glycogen in parental Rat 1 fibroblasts with an ED₅₀ value of ~2 nM. In contrast, the dose-response curves are left shifted to the same degree in Y/F2 and HIRc cells (ED₅₀ ~0.2 nM). Comparable findings were observed in HIRc1.2M cells. These data indicate that the Y/F2 receptor functions normally with respect to insulin-stimulated metabolic signaling.

DNA Synthesis—We have reported previously that the ability of insulin to stimulate mitogenesis is greatly enhanced in Y/F2 cells compared with HIRc cells (12). For example, the ED₅₀ for insulin stimulation of DNA synthesis was 0.1 nM in Y/F2 cells compared with 0.6 nM in HIRc cells expressing comparable numbers of wild type receptors (8). We show now that this augmentation is restricted to the insulin signaling pathway. The dose-response curves for IGF-I-stimulated mitogenesis are comparable among Rat 1, HIRc, and Y/F2 cell lines (Fig. 2). It should be noted that each of these cell lines expresses approximately 1 × 10⁶ endogenous IGF-I receptors (Ref. 22 and data not shown). The data for insulin and IGF-I stimulation are summarized in Table I.

Hybrid Insulin-IGF-I Receptors—In the Rat 1 cells, the IGF-I receptor is more potent for mitogenic signaling than the insulin receptor (Table I). Insulin stimulation in the Y/F2 cells, however, approaches the same sensitivity as for IGF-I. This observation leads to the suggestion that the insulin effect may be mediated by the IGF-I receptor β-subunit through the formation of hybrid receptors. Hybrid heterotetrameric receptors consisting of one-half α-β insulin receptor and one-half α-β IGF-I receptor have been described recently, particularly in transfected cells overexpressing insulin receptors (23). Although the functional role of these hybrid receptors is unknown, it is possible that they mediate the insulin mitogenic signal and that a difference in the formation of insulin-IGF-I receptor hybrids exists in Y/F2 compared with HIRc cells. To assess this issue, we measured the proportion of hybrid receptors in both cell lines. HIRc and Y/F2 cells

FIG. 1. Insulin-stimulated glucose incorporation into glycogen. Dose-response curves for the incorporation of labeled glucose into glycogen are shown. Results are presented as the percent of maximal response for Rat-1 (●). For HIRc (○) and Y/F2 (Δ) (n = 6). The basal and maximal activities are 8.0 ± 3.3 and 13.9 ± 1.6 in Rat 1, 12.2 ± 3.8 and 18.5 ± 3.2 in HIRc270 cells, and 12.2 ± 3.2 and 24.3 ± 3.7 in Y/F2 cells (mean ± S.E., nmol/200 µg of protein/2 h), respectively.

The abbreviations used are: DME, Dulbecco's modified Eagle's; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; IGF, insulin-like growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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either of two antibodies specific for the insulin receptor. Since the cross-linked receptors were immunoprecipitated with disuccinimidyl suberate. The cells were then solubilized, and these antibodies are insulin receptor-specific and do not recognize IGF-I receptors, any cross-linked IGF-I brought down by either a monoclonal (lanes A and C) or Y/F2 cells (lanes E and F).

TABLE 1
DNA synthesis in transfected cell lines
Comparison of sensitivity to insulin and IGF-I in transfected cell lines is shown. Dose-response curves for insulin stimulation (10) or IGF-I stimulation (Fig. 2) were used to calculate the ED_{50} values in each cell line.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Insulin</th>
<th>IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 1</td>
<td>4.6</td>
<td>0.3</td>
</tr>
<tr>
<td>HIRc (270)</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>HIRc (1.2M)</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Y/F2 (380)</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Fig. 2. IGF-I-stimulated thymidine incorporation in Rat 1 cells and transfected cell lines. Dose-response curves for IGF-I stimulation of thymidine incorporation are shown. Results are mean (±S.E.) of three experiments for Rat-1 (■), HIRc270 (○), HIRc1.2M (□), and Y/F2 (△) cell lines and are presented as percent of maximal stimulation. The basal (cpm) and maximal (percent of serum stimulation) activities are 3778 ± 1823, 60.6 ± 8.8% in Rat 1, 6856 ± 227, 106.3 ± 4.8% in HIRc270, 2753 ± 521, 68.4 ± 15.4% in HIRc1.2M, and 3950 ± 2371, 89.1 ± 4.0% in Y/F2 cells (mean ± S.E.), respectively.

Insulin-stimulated phosphorylation of endogenous substrates. Cell extracts from 5 × 10^6 cells were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Phosphotyrosine-containing proteins were detected by an anti-phosphotyrosine antibody and ^{125}I-protein A. A representative autoradiogram is shown.
played normal ligand binding and tyrosine kinase properties as substrate. Insulin stimulated phosphate incorporation into phenylalanine (Y/F2 receptor). This Y/F2 receptor diminished as the insulin concentration increased, but under all conditions the phosphorylation state of these two endogenous substrates was greater in the Y/F2 cells. Similar observations were obtained in a separate Y/F2 clonal cell line (data not shown).

S-6 Kinase Activity—Binding of insulin to its receptor causes the activation of a number of serine/threonine kinase, including S6 kinase, which phosphorylates the S6 ribosomal protein. Several mitogens and oncogenes are known to stimulate S6 kinase activity and, therefore, S6 kinase activity is presumed to be in the growth-signaling pathway (24, 25). Consequently, we measured insulin-stimulated S6 kinase activity in Rat 1, Y/F2, and HIRc cells, using rat 40 S ribosomal protein as substrate. Insulin stimulated phosphate incorporation into this protein by 2-6 fold; stimulation could be observed after 5 min of insulin incubation reaching a maximum by 30 min which is maintained for up to 60 min (data not shown). The insulin dose-response curves were analyzed after a 30-min incubation. insulin stimulates S6 kinase activity in a dose-dependent manner (Fig. 6A). In the parental Rat 1 cells, the E_{50} value is approximately 10 nM (Fig. 6B). Insulin sensitivity was enhanced in two HIRc cell lines (expressing 2.7 × 10^5 or 1.2 × 10^6 receptors/cell, respectively) with E_{50} values of 0.5 ± 0.06 nM. Interestingly, insulin sensitivity was even greater in Y/F2 cells with an E_{50} value of 0.1 ± 0.01 nM (p < 0.01 compared with HIRc cells). It should be noted that these Y/F2 cells express 3.8 × 10^6 receptors/cell. As can be seen, maximal S6 kinase activity was similar in all cell lines (Fig. 6B).

DISCUSSION

The insulin receptor is a member of the tyrosine kinase family which includes a variety of oncogenes, protooncogenes, and growth factor receptors (1). For several of these proteins, the carboxyl-terminal domains exert regulatory control over the tyrosine kinase activity (2). Earlier, we have shown that deletion of the 43 COOH-terminal amino acids of the insulin receptor alters biological signaling, indicating that the insulin receptor COOH terminus has regulatory functions. Within the COOH terminus two tyrosine residues (Tyr^{1316}, Tyr^{1322}) become autophosphorylated following insulin binding. Recently, we have prepared and studied a mutant insulin receptor in which the two COOH-terminal tyrosines were converted to phenylalanine (Y/F2 receptor). This Y/F2 receptor displayed normal ligand binding and tyrosine kinase properties and led to normal insulin stimulation of glucose transport (12). As a further confirmation, we show here that a second metabolic effect, namely glucose incorporation into glycogen, is stimulated normally by insulin in Y/F2 cells. In contrast, the Y/F2 receptor displayed a strikingly enhanced ability to transmit a mitogenic signal (12). This led to the suggestion that the COOH-terminal tyrosines normally exert an inhibitory, or restraining, effect on ligand-mediated mitogenic signaling and that when these residues are mutated (12) or deleted (8), this inhibition is released, leading to enhanced growth signaling. The current results are consistent with this notion and also help elucidate the mechanisms underlying this effect. Regardless of the precise mechanism, it is apparent that the Y/F2 receptor couples into the signaling pathway more efficiently than the wild type receptor. This provides a potentially important paradigm allowing one to identify the downstream components of the insulin action cascade which are in the mitogenic versus the metabolic pathway. We show here that Y/F2 cells display enhanced sensitivity for S6 kinase stimulation. This indicates that the kinases involved in S6 phosphorylation lie along the mitogenic signaling pathway distinct from metabolic action. These results do not necessarily mean that S6 kinase stimulation is necessary for insulin-induced DNA synthesis. It is possible that stimulation of the "mitogenic pathway" initiates a number of biological effects which are part of the overall cellular growth signaling program but not all of which lead directly to de novo DNA synthesis. Indeed, there is already some evidence to support this contention as an insulin receptor-Ros chimera protein can mediate insulin stimulation of S6 kinase activity, whereas no induction of mitogenesis occurs (26).

The most likely point of divergence of the metabolic and mitogenic pathways is at the level of the receptor itself, relating to the way the Y/F2 receptor interacts or couples with the next downstream signaling molecule. Starting with this premise, we examined endogenous substrate phosphorylation in these cells. We observed an increase in the phosphorylation state of two phosphoprotein targets of the insulin receptor, pp180 and pp220, in both the absence and presence of insulin. Possibly, one or both of these substrates is linked to mitogenic signaling, and the increase in their phosphorylation state would be consistent with the enhanced growth-promoting effects of insulin in the Y/F2 cells. While either of these phosphoproteins is potentially a direct substrate of the insulin receptor, we have been unable thus far to demonstrate direct physical association of either with the insulin receptor by co-precipitation methods (data not shown). The increased phosphorylation of these substrates in Y/F2 cells in the basal state raises the possibility that the Y/F2 cells do not arrest as well as the HIRc cells, perhaps due to differences in

**Fig. 5. Quantification of phosphotyrosine-containing proteins in transfected cells.** Western blots with anti-phosphotyrosine antibodies were analyzed by densitometry. A, the ratio of phosphorylation for each substrate between the Y/F2 and HIRc270 cells is plotted for each insulin concentration. Data for insulin receptor (IRβ) β-subunits (G), pp180 (●), and pp220 (○) are shown (mean ± S.E., n = 4). B, basal (solid bar) and maximal (hatched bar) phosphotyrosine content for the three substrates in Y/F2 and HIRc cells are expressed as percent of the maximal response in Y/F2 cells.
endogenous IGF-I production by the cells. However, in a previous paper (12) we estimated the percentage of the cells that were growth-arrested by using a bromodeoxyuridine in situ labeling method and found no significant difference between HIRc and Y/F2 cells. Furthermore, we could not detect any significant IGF-I secretion into the starvation medium in both cell lines (data not shown) (15). Therefore, it is unlikely that the differences in endogenous substrate phosphorylation in the Y/F2 and HIRc cells reflect differences in their starvation behavior, but rather the Y/F2 receptor couples more efficiently to these substrates. Interestingly, pp180 and pp220 phosphorylation was enhanced in the Y/F2 cells, even in the basal state. Since no increase in basal S6 kinase or glycogen synthesis activity was observed in the Y/F2 cells, this finding might be interpreted to indicate that these endogenous substrates are unrelated to these two biologic effects. On the other hand, the stoichiometry of coupling between proximal substrates and downstream effectors is almost entirely unknown, and, therefore, any interpretation of this relationship would be entirely speculative.

It is known that when the insulin receptor is overexpressed in host cells, hybrid heterotetramer molecules can form between the insulin receptor and endogenous IGF-I receptors leading to heterotetramers comprised of one-half insulin receptor and the other half IGF-I receptor in a β-insulin receptor-β-IGF receptor structure (23, 27, 28). Interestingly, the enhanced mitogenic signaling in Y/F2 cells was restricted to insulin stimulation, since the sensitivity for IGF-I stimulation of mitogenesis was the same in Y/F2, HIRc, and untransfected Rat 1 fibroblasts. Since the IGF-I receptor is more potent with respect to growth promoting activity than the insulin receptor, it seemed possible that enhanced formation of Y/F2-IGF-I receptor hybrids might explain the enhanced insulin-induced mitogenic signaling in Y/F2 cells. However, our results demonstrated that the proportion of hybrids formed in the Y/F2 cells was comparable with that in HIRc cells, arguing against a role of hybrids in the enhanced Y/F2 mitogenic signaling. All three of these cell lines express approximately equal numbers of IGF-I binding sites. Since in Y/F2 and HIRc cells a large number of the IGF-I binding sites (60-70%) is contained within insulin-IGF-I receptor hybrids, whereas in Rat 1 cells (which express few endogenous insulin receptors) essentially all the IGF-I binding sites are IGF-I receptor homodimers (either wild type or Y/F2), possess a comparable ability to mediate the IGF-I mitogenic signal. This would suggest that within the hybrid receptors, once IGF-I binds, the mitogenic signals are transmitted largely through the IGF-I receptor half.

From a functional standpoint, the Y/F2 receptor mutation transforms the insulin receptor from a weak to a relatively potent mitogenic signaling receptor. Thus, its biological profile is similar to the IGF-I receptor. Interestingly, the carboxyl terminus of the insulin and IGF-I receptors shows the least sequence similarity, and the positions in the IGF-I receptor COOH terminus (1310, 1316) analogous to the tyrosine positions (1316, 1322) of the insulin receptor are phenylalanine and tyrosine, respectively. Since the native IGF-I receptor has potent mitogenic signaling activity and since position 1310 is already phenylalanine within the IGF-I receptor, this suggests that conversion of tyrosine 1316 within the Y/F2 receptor may be the more important substitution. With this line of reasoning, the absence of a tyrosine at position 1310 of the IGF-I receptor would be important for efficient mitogenic signaling, and its presence at position 1316 of the insulin receptor would be inhibitory to mitogenic signaling. It would be interesting to determine whether mutation of phenylalanine 1310 within the IGF-I receptor to tyrosine would lead to inhibition of its growth signaling properties.

Recently, Kadowaki et al. (9) have studied an identical Y/F2 receptor transfected into Chinese hamster ovary cells. A preliminary report of their findings indicates that their results are comparable to our previous findings in Rat 1 fibroblasts (12), i.e. normal insulin-induced metabolic signaling but strikingly enhanced insulin-mediated mitogenic activity.
Our current results assessing glycogen synthesis again confirm the fact that mutation of the COOH-terminal tyrosines does not alter the metabolic signaling pathway. This indicates that these residues, or their phosphorylation state, are not involved in this aspect of insulin's biological activity. Baron et al. (4) have recently demonstrated that insulin binding, as well as autophosphorylation, leads to a conformational change in the receptor COOH terminus. Taken together, these findings suggest that the COOH-terminal conformational change is either unrelated to autophosphorylation of the COOH-terminal tyrosine residues or the conformation change is unrelated to biological signaling.

In summary, Y/F2 insulin receptors display enhanced mitogenic signaling properties despite normal metabolic signaling effects. This demonstrates the divergence of these two signaling pathways, most likely at the level of the insulin receptor itself. Endogenous substrate phosphorylation (pp180 and pp220) as well as S6 kinase activation are also enhanced in Y/F2 cells, indicating that these biological effects are part of the same pathway responsible for mitogenesis. These results are consistent with the idea that the COOH-terminal tyrosines are normally inhibitory to insulin receptor mitogenic signaling and that their deletion, or mutation, allows the insulin receptor to interact more efficiently with the downstream signaling molecules which participate in the mitogenic response.

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