Different Pathways of Postreceptor Desensitization following Chronic Insulin Treatment and in Cells Overexpressing Constitutively Active Insulin Receptors*

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We have reported previously that substitution of the transmembrane domain of the insulin receptor with that of the erbB-2 oncogene (IRerbV−E) results in constitutive activation of the insulin receptor kinase. Compared to NIH3T3 cells overexpressing wild-type insulin receptors (IRwt), cells overexpressing IRerbV−E displayed a decrease in IRS-1 protein content by 55%, but basal tyrosine phosphorylation of IRS-1 was increased. This resulted in an increased association of IRS-1 with the p85 subunit of phosphatidylinositol 3-kinase, increased phosphatidylinositol 3-kinase activity in anti-IRS-1 immunoprecipitates, constitutive activation of the p70 S6 kinase, and an increased association of Grb2 with Shc in the absence of ligand. However, Grb2 association with IRS-1 could not be detected in the basal or insulin-stimulated states, and mitogen-activated protein kinase (MAPK) activity could not be stimulated by insulin, epidermal growth factor, or platelet-derived growth factor.

In contrast to IRerbV−E, the insulin receptor content and its tyrosine phosphorylation were significantly decreased in IRwt cells chronically stimulated (>24 h) with insulin. With decreased IRS-1 content, tyrosine phosphorylation of IRS-1 was decreased by over 75%, leading to decreased IRS-1-associated PI 3-kinase and Grb2. In addition, Grb2 association with Shc and activation of MAPK and the p70 S6 kinase were insensitive to insulin stimulation. By contrast, association of Grb2 with Shc and activation of MAPK, but not the p70 S6 kinase, could be stimulated by epidermal growth factor or platelet-derived growth factor. These data suggest that there are multiple levels of postreceptor desensitization to insulin action. These are used somewhat differently in these two different models, probably due in part to the difference in receptor down-regulation.

The insulin receptor (IR)1 is an integral membrane protein comprised of two extracellular α-subunits, which bind insulin, and two transmembrane β-subunits, which contain a tyrosine kinase activity that is activated following insulin binding (1). Activation of the receptor tyrosine kinase appears to be essential for most, if not all, insulin actions (1). In addition to insulin binding, the receptor-associated tyrosine kinase activity can be activated by tryptic cleavage of the α-subunit or removal of the α-subunit by in vitro mutagenesis, suggesting that the α-subunit acts to repress kinase activity (1). Recently, we have shown that substitution of the insulin receptor transmembrane domain with that from the erbB-2 oncogene (IRerbV−E) also results in a constitutively active insulin receptor kinase (2, 3).

Both in intact animals and in cultured cells, chronic stimulation by insulin induces a desensitization and down-regulation of insulin signaling. This occurs in part through internalization and degradation of the insulin receptor (4, 5), as well as down-regulation of IRS-1 (3). In hyperinsulinemic conditions, tyrosine phosphorylation of IRS-1 and association with PI 3-kinase are also decreased, consistent with the insulin resistance that is present (6, 7). In acute insulin signaling, there may be other levels of feedback inhibition, including serine phosphorylation of the insulin receptor and IRS-1, feedback inhibition of MAPK at the level of Ras and Raf-1 association (8), and dissociation of SOS from Grb2 (9–11). In this study, we have examined the mechanism of down-regulation of insulin signaling and the relationship to the changes of insulin actions using two different cell culture models. The first is NIH3T3 cells overexpressing wild-type human IR (IRwt) and chronically treated by insulin, in which desensitization presumably occurs at the level of the insulin receptor. The second is NIH3T3 cells overexpressing the constitutively active insulin receptor (IRerbV−E), in which desensitization may occur at postreceptor levels, but the level of the activated insulin receptors remains high.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—Insulin was purchased from Boehringer Mannheim, [γ-32P]ATP was from DuPont NEN; polyclonal anti-insulin receptor antibodies, anti-IRS-1 antibodies, anti-p70 S6 kinase antibodies, and anti-MAPK antibodies were prepared as described previously (12). Monoclonal anti-phosphotyrosine antibody (4G10), monoclonal anti-Grb2 antibody, polyclonal anti-Shc antibodies, and polyclonal anti-85 kDa subunit of PI 3-kinase antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The parental mouse NIH3T3 fibroblasts were transfected with the wild-type or mutant insulin receptor expression plasmids as described previously (3). Stable transfectants were selected after selection in the presence of G418 (500 μg/ml) for 8 weeks. Resistant clones were selected by limiting dilution, expanded, and screened further by immunoblotting for insulin receptor expression.

Preparation of Total Cell Lysates and Immunoblotting—Cells were serum-starved for 24 h before experiments. For chronic treatment with insulin, IRwt cells were incubated with 10−6 M insulin for 24 h. Cells were acutely treated with ligands, washed twice with ice-cold phosphate-buffered saline, and harvested in lysis buffer (50 mM HEPES, 10
mm EDTA, 100 mm NaF, 10 mm sodium pyrophosphate, 1 μM Na3VO4, 50 μM okadaic acid, 1% (v/v) Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin at pH 7.5. After centrifugation, supernatants were normalized for protein concentration and subjected to immunoprecipitation and immunoblotting.

For immunoblotting, equal amounts of protein (10–50 μg) were subjected to SDS-PAGE and electroblotted to nitrocellulose filters. The filters were blocked with 5% bovine serum albumin and then incubated with a respective antibody, washed, reacted with anti-rabbit or anti-mouse IgG coupled to peroxidase, and developed with enhanced chemiluminescence reagents as instructed by the manufacturer. For immunoprecipitation, lysates were incubated with primary antibody for 2 h at 4°C and with Sepharose-protein A beads for an additional 1.5 h, followed by three washes with lysis buffer before elution into SDS-PAGE sample buffer. The samples were then loaded onto a SDS-acrylamide gel and subjected to electrophoresis.

**Phosphatidylinositol 3-Kinase Assay**—Confluent cells were treated with or without insulin (10−7 M) for 5 min at 37°C, lysed in buffer containing 1% Nonidet P-40 and immunoprecipitated with anti-phosphotyrosine antibody or anti-IRS-1 antibody and protein A-Sepharose. The immunoprecipitates were washed and subjected to the PI 3-kinase assay as described (13). In parallel, the immunoprecipitates were also subjected to immunoblotting for IRS-1 and the 85-kDa subunit of PI 3-kinase.

**MAPK Assay**—Confluent cells were treated with insulin (10−7 M), EGF (130 ng/ml), or PDGF-BB (25 ng/ml) for 5 min at 37°C. The cell lysates were incubated with anti-MAPK and protein A-Sepharose at 4°C for 3 h. Immunoprecipitates were washed three times with lysis buffer and twice with kinase reaction buffer. The reactions were allowed to proceed for 10 min at 30°C in a final volume of 40 μl containing 40 mM HEPES, pH 8.0, 10 mM MgCl2, 0.25 mg/ml myelin basic protein, 2 μg protein kinase inhibitor (Sigma P-3294), and 50 μM [γ-32P]ATP. The reaction was stopped by the addition of 20 μl of 3 × Laemmli sample buffer containing 300 mM dithiothreitol. The mixtures were boiled for 5 min and subjected to SDS-PAGE. The dried gels were then subjected to PhosphorImager analysis and/or autoradiography.

**RESULTS**

**Basal and Insulin-stimulated Tyrosine Phosphorylation**—As reported previously (2,3), substitution of the transmembrane domain of the wild-type human insulin receptor with that from the oncogenic form of erbB-2 (IRerbV−E) results in constitutive activation of the insulin receptor kinase. This resulted in an increased basal tyrosine phosphorylation of both the β-subunit of the insulin receptor and IRS-1 with little or no further stimulation by insulin (Fig. 1, lanes 1 and 2). However, the tyrosine phosphorylation of the β-subunit of the insulin receptor was decreased compared with that in acutely insulin-stimulated IRwt cells (Fig. 1, lanes 3 and 4). The tyrosine phosphorylation of IRS-1 was also decreased compared with that following acute insulin stimulation of IRwt cells. This was due, at least in part, to the decrease in IRS-1 protein content (see below). The results with IRwt cells treated with insulin for 24 h were somewhat different, with no detectable tyrosine phosphorylation of the insulin receptor in the basal state and only minimally increased IRS-1 phosphorylation. The acute addition of fresh insulin to these cells resulted in a small stimulation of IR phosphorylation but no further increase in tyrosine phosphorylation of IRS-1 (Fig. 1, lanes 1 and 2). However, the tyrosine phosphorylation of IRS-1 was decreased compared with that following acute insulin stimulation of IRwt cells, which was due, at least in part, to the down-regulation of IRS-1 protein content (see below). The results with IRwt cells treated with insulin for 24 h were somewhat different, with no detectable tyrosine phosphorylation of the insulin receptor in the basal state and only minimally increased IRS-1 phosphorylation. The acute addition of fresh insulin to these cells resulted in a small stimulation of IR phosphorylation but no further increase in tyrosine phosphorylation of IRS-1 (Fig. 1, lanes 1 and 2). However, the tyrosine phosphorylation of IRS-1 was decreased compared with that following acute insulin stimulation of IRwt cells, which was due, at least in part, to the down-regulation of IRS-1 protein content (see below).
of the insulin receptors detected by immunoblotting. In addition, both chronic insulin treatment (Fig. 2B) and overexpression of IR<sup>erbV</sup>→E (Fig. 2A) caused a reduction of the levels of IRS-1. IRS-1 was decreased in IR<sup>erbV</sup>→E cells by 55–60% and in chronically insulin-treated IR<sup>wt</sup> cells by 20–30% compared to untreated IR<sup>wt</sup> cells. Interestingly, although chronic insulin treatment of IR<sup>wt</sup> cells did not change the content of Shc (Fig. 2B), the content of all isoforms of Shc (especially of 66 kDa) was increased in IR<sup>erbV</sup>→E cells (Fig. 2A). The levels of the 85-kDa subunit of PI 3-kinase (p85), Grb2, and MAPK were not significantly affected in IR<sup>erbV</sup>→E cells (Fig. 2A) or in chronically insulin-stimulated cells (Fig. 2B). The content of other constituents of the insulin signaling pathway, including mSOS, SHPTP2, Ras, and MEK1, was also unaffected (data not shown).

**Tyrosine Phosphorylation of IRS-1 and Association with PI 3-Kinase and Grb2**—As noted above, in a direct blot of cell lysates with anti-phosphotyrosine, tyrosine phosphorylation of IRS-1 appeared to decrease in both IR<sup>erbV</sup>→E cells and chronically insulin-treated IR<sup>wt</sup> cells as compared with that in IR<sup>wt</sup> cells acutely stimulated with insulin (Fig. 1). In addition, tyrosine phosphorylation of IRS-1 occurred without any treatment in both IR<sup>erbV</sup>→E cells and chronically insulin-treated IR<sup>wt</sup> cells, and acute addition of insulin had little further effect on IRS-1 phosphorylation. To assess the apparent stoichiometry of IRS-1 tyrosine phosphorylation, the phosphorylation of IRS-1 was normalized for IRS-1 protein content. When adjusted for the IRS-1 content, the tyrosine phosphorylation of IRS-1 was increased in the basal state in both IR<sup>erbV</sup>→E and chronically insulin-treated IR<sup>wt</sup> cells (23.9 and 34.0%, respectively, compared to acutely stimulated IR<sup>wt</sup> cells versus 6.5% in nonstimulated IR<sup>wt</sup> cells) but showed no further insulin stimulation.

In IR<sup>erbV</sup>→E cells, the constitutively elevated tyrosine phosphorylation of IRS-1 lead to an insulin-independent increase in the formation of the IRS-1-p85 complex and constitutive activation of PI 3-kinase activity (Fig. 3). In IR<sup>wt</sup> cells, insulin stimulated p85 binding and PI 3-kinase activity by 25- and 13-fold, respectively, whereas in cells expressing IR<sup>erbV</sup>→E insulin had no further effect on PI 3-kinase association or activity. In chronically insulin-treated IR<sup>wt</sup> cells, the formation of the IRS-1-p85 complex and PI 3-kinase activity in IRS-1 immunoprecipitates was also increased in the "basal" state. However, this increase was less than that in IR<sup>erbV</sup>→E cells. Further stimulation by insulin was also greatly reduced.

The p85 subunit and PI 3-kinase activity could not be detected in the anti-phosphotyrosine immunoprecipitates in cell extracts from IR<sup>erbV</sup>→E cells or chronically insulin-treated IR<sup>wt</sup> cells (Fig. 3). Compared to IR<sup>wt</sup> cells, IRS-1 associated with the anti-phosphotyrosine precipitates was undetectable in IR<sup>erbV</sup>→E cells and >90% decreased in chronically insulin-treated cells. In contrast to the increased basal association of IRS-1 with PI 3-kinase, there was no detectable IRS-1-Grb2 complex in IR<sup>erbV</sup>→E cells before or after treatment with insulin, whereas in IR<sup>wt</sup> cells, the association of IRS-1 with Grb2 was easily detected following stimulation with insulin (Fig. 4A, left and center panels). In chronically insulin-treated IR<sup>wt</sup> cells, some association between Grb2 and IRS-1 could be detected, although it was decreased compared with that in IR<sup>wt</sup> cells (Fig. 4A, right panel).

In summary, IRS-1 association with PI 3-kinase, but not with Grb2, is preserved in IR<sup>erbV</sup>→E cells despite the decrease in IRS-1 protein amount and its tyrosine phosphorylation. By contrast, both PI 3-kinase and Grb2 associations with IRS-1 appear to be decreased in chronically insulin-treated IR<sup>wt</sup> cells.

**Tyrosine Phosphorylation of Shc and its Association with Grb2 and Activation of MAPK**—As described previously (17) and as shown in Fig. 4B, insulin stimulates the tyrosine phosphorylation of the 46- and 52-kDa isoforms of Shc. Tyrosine phosphorylation of the 66-kDa isoform was absent, consistent with the previous report (17), indicating that the tyrosine-phosphorylated 60-kDa protein in Fig. 1 is not Shc but may be the GAP-associated protein (18).

Tyrosine phosphorylation of Shc leads to its association with the SH2 domains of Grb2. In IR<sup>erbV</sup>→E cells, the tyrosine phosphorylation of Shc and its association with Grb2 was detected even in the basal state, and insulin had no further effect on this interaction. By contrast, in chronically insulin-treated IR<sup>wt</sup> cells, Shc phosphorylation and Grb2 association were not detected in the basal state or following acute insulin treatment. Recent reports indicate that insulin-stimulated activation of MAPK occurs mainly via Shc-mediated, rather than IRS-1-mediated, pathways (19, 20). In IR<sup>wt</sup> cells, after a 5-min incubation with insulin, MAPK activity was stimulated by 3.5-fold (Fig. 5). By contrast, in IR<sup>erbV</sup>→E cells, the basal activity of MAPK was low and was not stimulated by insulin, despite the
fact that the tyrosine phosphorylation of Shc and its association with Grb2 was increased in these cells in the absence of ligand. In chronically insulin-treated IRwt cells, insulin did not activate MAPK, consistent with the absence of the Shc-induced MAPK pathway occurs in both IRerbV plex. These data indicate that desensitization of the insulin-induced MAPK pathway occurs in both IRerbV and chronically insulin-treated IRwt cells. However, in these two settings, the mechanism of insulin resistance appears to be different.

**Insulin Regulation of p70 S6 Kinase**—Recently, it has been shown that several different signaling pathways may lead to phosphorylation and activation of the p70 S6 protein kinase (15). The major pathway for stimulation of p70 S6 kinase by insulin requires activation of PI 3-kinase (12, 16). In IRerbV cells subjected to acute insulin stimulation, the electrophoretic mobility of p70 S6 kinase was decreased, consistent with phosphorylation and activation (Fig. 6). The 85-kDa isoform of p70 S6 kinase was also detected in the blot, and this isoform was also shifted after insulin treatment (Fig. 6). By contrast, in IRerbV cells, both isoforms of p70 S6 kinase were shifted to the slower mobility form in the absence of insulin. The further addition of insulin had no effect. These data coincide with the elevated PI 3-kinase activity observed in the IRS-1 immunoprecipitates from IRerbV cells. In chronically insulin-treated IRerbV cells, the mobility of p70 S6 kinase was not decreased, and acute addition of insulin did not induce a mobility shift, consistent with the lower levels of PI 3-kinase activity recovered in the IRS-1 immunoprecipitates, both in the basal and stimulated states.

**EGF or PDGF Actions in IRerbV Cells and Chronically Insulin-treated IRwt Cells**—To better understand the mechanisms of desensitization, we examined the EGF- and PDGF-stimulated signaling pathways to see whether the desensitization process(es) that affected insulin signaling in IRerbV cells and in chronically insulin-treated IRerbV cells would also occur in other pathways. In IRerbV and IRwt cells, EGF and PDGF activated MAPK to the same extent as insulin (Fig. 5). In addition, in these cells both EGF and PDGF stimulated tyrosine phosphorylation of the 46- and 52-kDa Shc proteins and the formation of Shc-Grb2 complexes (Fig. 4B). However, in IRerbV cells, neither EGF nor PDGF stimulated MAPK activity (Fig. 5). There was also no further increase of the already elevated levels of tyrosine-phosphorylated Shc or in the formation of Shc-Grb2 complexes. These data suggest that at least one site of desensitization is common to the insulin, EGF, and PDGF signaling pathways, and that this step is probably downstream of the formation of Shc-Grb2 complexes. In addition, although EGF-induced tyrosine phosphorylation of EGF receptors was difficult to observe, PDGF-induced tyrosine phosphorylation of PDGF receptors could be clearly observed (data not shown), indicating that overexpression of IRerbV cells did not induce desensitization at the level of the PDGF receptor itself. In contrast to cells expressing IRerbV cells, EGF and PDGF stimulated the formation of Shc-Grb2 complexes (Fig. 4B) and activated MAPK (2.9- and 2.2-fold, respectively), whereas insulin could not (Fig. 5). The tyrosine phosphorylation of 52-kDa Shc was also induced by EGF or PDGF treatment, although it was reduced compared to that in IRwt cells (Fig. 4B). Likewise, PDGF-induced tyrosine phosphorylation of PDGF receptors was also markedly reduced in chronically insulin-treated cells (data not shown). These data suggest that in chronically insulin-treated cells, EGF or PDGF signaling to Shc is partially desensitized but is sufficient to induce the formation of Shc-Grb2 complexes and activate MAPK.

These results are somewhat different from those observed for activation of the p70 S6 kinase. Thus, in IRerbV cells, the electrophoretic mobility of both isoforms of p70 S6 kinase was decreased following EGF or PDGF treatment as well as by insulin (Fig. 6). In IRerbV cells, p70 S6 kinase was constitutively in the slower mobility form and was not further altered by EGF or PDGF treatment. In contrast, neither EGF nor PDGF could induce the mobility shift in chronic insulin-treated IRwt cells, suggesting that chronic insulin treatment induces desensitization of not only insulin but also EGF and PDGF signaling pathway to p70 S6 kinase.

**DISCUSSION**

Chronic insulin stimulation induces down-regulation in various steps in insulin signaling. Insulin has been shown to induce the internalization and degradation of the insulin receptor (4, 5). Insulin is also reported to decrease IRS-1 content in CHO (3) and 3T3-L1 cells (21) in culture. Prolonged exposure to insulin has also been shown to result in a desensitization of the final effectors of the insulin action pathway, such as stimulation of glucose transport (22). In the present study, we have compared desensitization pathways in NIH3T3 cells overexpressing a constitutively activated insulin receptor to those observed in cells overexpressing wild-type insulin receptors chronically treated with insulin. We find that chronic insulin treatment of IRwt cells decreases the content of insulin receptor protein and produces a significant decrease in insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1, although the content of IRS-1 is only moderately decreased. This leads to a decrease in the stoichiometry of insulin-induced tyrosine phosphorylation of IRS-1 as compared with that ob-
served in IR\textsuperscript{wt} cells and a decrease in both IRS-1-associated PI 3-kinase and Grb2. In addition, insulin-induced tyrosine phosphorylation of Shc and its association with Grb2 is also decreased. The decreases in these intermediate pathways lead to a loss in the ability of insulin to stimulate MAPK and p70 S6 kinase. Thus, there is a “broad spectrum” desensitization of insulin action following chronic insulin stimulation both at the level of the insulin receptor and involving several postreceptor mechanisms. Findings in the cells overexpressing the constitutively activated receptor help sort out the role of these various defects.

In IR\textsuperscript{erbV} cells, the basal level of insulin receptor kinase activity is high, and receptors are not down-regulated. Despite a significant decrease in IRS-1 content and in the apparent stoichiometry of IRS-1 tyrosine phosphorylation, IRS-1 is tyrosine-phosphorylated and associates with the 85-kDa subunit of PI 3-kinase in a ligand-independent manner, resulting in constitutively elevated PI 3-kinase activities. In these cells, p70 S6 kinase is also constitutively phosphorylated. These results are consistent with the model in which PI 3-kinase is an upstream regulator of p70 S6 kinase (12, 16). Furthermore, these data also suggest that continuous signals generated from PI 3-kinase downstream to p70 S6 kinase do not significantly alter p70 S6 kinase protein levels or appear to affect any intermediate signals between PI 3-kinase and p70 S6 kinase.

Interestingly, PI 3-kinase activity and the 85-kDa subunit of PI 3-kinase could not be recovered in anti-phosphotyrosine antibody immunoprecipitates in IR\textsuperscript{erbV} cells, although these activities could be detected in the anti-phosphotyrosine immunoprecipitates from IR\textsuperscript{wt} cells after insulin treatment. Moreover, in IR\textsuperscript{erbV} cells, no association between IRS-1 and Grb2 could be detected. These results suggest that tyrosine residues other than the PI 3-kinase binding sites on IRS-1 are not phosphorylated or are quickly dephosphorylated in IR\textsuperscript{erbV} cells. Furthermore, although the apparent stoichiometry of IRS-1 tyrosine phosphorylation is decreased to a similar extent in IR\textsuperscript{erbV} cells compared to that in chronically insulin-treated IR\textsuperscript{wt} cells, more IRS-1-PI 3-kinase complex is formed in IR\textsuperscript{erbV} cells. These findings are consistent with the recent report (25) that a constitutively active insulin-like growth factor 1 receptor (IGF-1R), which is mutated in the transmembrane region (Val\textsuperscript{922}Glu), turns on IGF-1R signaling in CHO cells to an extent similar to that seen with stimulation of wild-type IGF-1R with submaximal concentration of IGF-1. In addition, they also raised the possibility that the Val\textsuperscript{922}Glu mutant of IGF-1R may induce a conformational change of IGF-1R to phosphorylate the PI 3-kinase-binding site on IRS-1 but not some of other sites on IRS-1 (25).

Another possibility for the decreased stoichiometry of IRS-1 tyrosine phosphorylation is an increase in protein-tyrosine phosphatase activities induced by constitutive activation of the insulin signaling pathway. Recently, several protein-tyrosine phosphatases have been shown to be induced by insulin and other growth factors (34), and several protein-tyrosine phosphatases, including PTP CD45 (25), PTP-LAR (26), PTP\textsuperscript{18} (27, 28), and PTP\textsuperscript{e} and PTP\textsuperscript{p} (29), have been reported to inhibit insulin signaling. Consistent with the latter hypothesis, the tyrosine phosphorylation of several proteins, including pp120, the \(\beta\)-subunit of IR, and IRS-1, is decreased in IR\textsuperscript{erbV} cells. It has been reported that in vitro SHPTP2 preferentially dephosphorylates phosphotyrosines of the Grb2 and SHPTP2 binding sites on IRS-1, as compared to the PI 3-kinase binding sites (30). Thus, increased activity of a protein-tyrosine phosphatase possessing substrate specificity similar to SHPTP2 could result in selective desensitization of Grb2-mediated effects in IR\textsuperscript{erbV} cells.

The situation in chronically insulin-treated cells is similar, but not identical, to that in IR\textsuperscript{erbV} cells. For example, in chronically insulin-treated cells, PDGF-induced tyrosine phosphorylation of PDGF receptors is also decreased, in contrast to apparently normal stimulation in IR\textsuperscript{erbV} cells, and there is desensitization of insulin-, EGF-, and PDGF-stimulated p70 S6 kinase. There is also a decrease of tyrosine phosphorylation of some constitutively phosphorylated proteins (for example, pp120) in chronically insulin-treated cells, again raising the possibility that an increase in protein-tyrosine phosphatase activity might play some role in the desensitization. However, in contrast to IR\textsuperscript{erbV} cells, in which Grb2, but not PI 3-kinase, association is markedly decreased, in chronically stimulated cells, Grb2 and PI 3-kinase in anti-IRS-1 immunoprecipitates are decreased to a similar extent. Although this could simply reflect the decrease of insulin receptor kinase activity, the global desensitization of p70 S6 kinase must reflect another mechanism.

In terms of the MAPK pathway, MAPK activation is low and not activated by insulin in either IR\textsuperscript{erbV} or in chronically insulin-treated IR\textsuperscript{wt} cells. However, in IR\textsuperscript{erbV} cells, tyrosine phosphorylation of Shc and its association with Grb2 occurs in the absence of insulin, and neither insulin, EGF, nor PDGF can further increase the Grb2 association of Shc or activate MAPK. These results suggest that some pathway of MAPK activation that is common to insulin, EGF, and PDGF signaling is desensitized downstream of the Shc/Grb2 complex. However, PDGF can fully activate MAPK, despite the lower levels of detectable Shc/Grb2 complexes in IR\textsuperscript{wt} cells, and PDGF may utilize pathways other than Shc for MAPK activation (31). In addition, several recent reports have suggested that there are several potential MEK (MAPK/extracellular signal-regulated kinase) activators, that may be differentially regulated by various growth factors (32). This indicates the complexity and redundancy of pathways for MAPK activation. Nonetheless, PDGF activation of MAPK is also severely compromised in IR\textsuperscript{erbV} cells, suggesting that desensitization may occur at a common site that appears downstream or independent of Shc/Grb2-generated signals. By contrast, in chronically insulin-treated IR\textsuperscript{wt} cells, EGF or PDGF can stimulate tyrosine phosphorylation of Shc in its association with Grb2 and activate MAPK, whereas insulin could not. Although the levels of EGF- and PDGF-stimulated formation of Shc/Grb2 complexes were similar to those observed in IR\textsuperscript{wt} cells, the levels of MAPK activity were slightly less. This suggests that the EGF or PDGF signaling from the receptor to Shc and Grb2 is not lost in these cells, but some desensitization may occur at steps thereafter.

One possibility that may explain these findings is a change in factors leading to deactivation of MAPK. Recently, it has been shown that angiotensin 2 can deactivate MAPK by enhancing MAPK phosphatase 1 (MKP1), a protein-tyrosine phosphatase that dephosphorylates and inactivates MAPK enhancing MAPK phosphatase 1 (MKP1), a protein-tyrosine phosphatase that dephosphorylates and inactivates MAPK (33). MKP1 and other vacinia H-1 gene product–like protein-tyrosine phosphatases have also been shown to be induced by several growth factors (34, 35). Therefore, it is possible that an increased MAPK deactivation activity accounts for the failure of MAPK activation by insulin, EGF, and PDGF in IR\textsuperscript{erbV} cells, and the decreased activation of MAPK by EGF and PDGF in chronically insulin-treated IR\textsuperscript{wt} cells.

In summary, in chronically insulin-treated IR\textsuperscript{wt} cells, desensitization in insulin signaling involves multiple downstream responses but appears to be due mainly to the decrease of the insulin receptor content and insulin receptor kinase activity. However, since tyrosine phosphorylation of PDGF receptors and p70 S6 kinase activation by EGF or PDGF are also compromised, chronic insulin stimulation must induce significant
changes in a postreceptor step(s) common to the insulin, EGF, and PDGF signaling pathways. Overexpression of the constitutively active insulin receptor also induces some profound changes in insulin, EGF, and PDGF signaling pathways that are different from those in chronically insulin-treated cells. In these cells, the activated insulin receptor kinase leads to constitutive activation of IRS-1 PI3-kinase complex formation and p70 S6 kinase. However, IRS-1-Grb2 complex formation cannot be detected, and MAPK activation by insulin, EGF, and PDGF treatment does not occur, although Shc
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