Insulin and insulin-like growth factor (IGF-I) receptors are heterotetrameric proteins consisting of two α- and two β-subunits and members of the transmembrane tyrosine kinase receptors. Specific ligand binding to the receptor triggers a cascade of intracellular events, which begins with autophosphorylation of several tyrosine residues of the β-subunit of the receptor. The triple cluster in the tyrosine kinase domain of the β-subunit is the earliest and major autophosphorylation site. Previous studies have shown that substitutions of these three tyrosines by phenylalanylamines of both insulin and IGF-I receptors practically abolish any activation of cellular signaling pathways. We have studied the effect of double tyrosine mutations on IGF-I-induced receptor autophosphorylation, activation of Shc and IRS-1 pathways, and cell proliferation and tumorigenicity. Substitution of tyrosines 1131/1135 blocks any detectable autophosphorylation, whereas substitution of tyrosines 1131/1136 or 1135/1136 only reduces autophosphorylation levels in some clones by ~50%. Nevertheless, all the cells expressing IGF-I receptors with double tyrosine substitutions demonstrated markedly reduced signaling through Shc and IRS-1 pathways. In addition, they were unable to respond to IGF-I-stimulated cell growth in culture, and tumor formation in nude mice was abrogated. These data suggest that the presence of tyrosine 1131 or 1135 essential for receptor autophosphorylation, whereas the presence of each of these tyrosines is necessary for a fully functional receptor.

The multiple physiological actions, including cell growth and differentiation of the insulin-like growth factors (IGFs) are mediated by the IGF-I receptor. While the IGF-I receptor and the structurally related insulin receptor are members of the type II receptor tyrosine kinase family, their in vivo biological functions are quite separate. Both the IGF-I and insulin receptors are heterotetrameric proteins composed of two extracellular α-subunits and two membrane-spanning β-subunits linked by disulfide bonds (1–3). Sequences found in the α-subunits of each receptor are important for determining ligand specificity. The amino-terminal and carboxyl-terminal portions of the α subunit of the insulin receptor are critical for high affinity insulin binding, while the cysteine-rich domain of the IGF-I receptor determines high affinity IGF-I binding (4–6). Likewise, the β-subunits contain a number of structurally distinct domains including the extracellular, transmembrane, juxtamembrane, tyrosine kinase, and carboxyl-terminal regions. Binding of ligand to the α-subunit activates the tyrosine kinase activity of the β-subunit resulting in autophosphorylation on distinct tyrosine residues. The triple tyrosine cluster within the kinase domain (1131, 1135, and 1136 tyrosines in the IGF-I receptor and the equivalent residues in the insulin receptor; numbering system of Ullrich et al. (2)) is the earliest and major site of autophosphorylation. Phosphorylation of these three tyrosine residues is necessary for activation of the kinase toward other substrates (7, 8). When the triple tyrosine cluster is substituted by phenylalanine residues, the receptor loses all ligand-induced biological actions (9, 10).

Whereas the function of the triple tyrosine cluster in the tyrosine kinase domain of the insulin receptor has been well characterized (11–13), much less is known about the corresponding tyrosines of the IGF-I receptor. Single substitutions of tyrosine 1131 or 1135 have relatively small effects on receptor and endogenous substrate phosphorylation or cell proliferation (14, 15). In contrast, substitution of tyrosine 1136 apparently has an inhibitory effect on those functions (14). In addition, it has been demonstrated, at least in the case of the insulin receptor, that in intact cells, bis phosphorylation of the kinase domain at Tyr-1158 and either Tyr-1162 or Tyr-1163 comprises 80% of phosphorylated receptors (7, 16–18). Thus, a study involving the substitution of combinations of double tyrosines may be more instructive. To further characterize the role of these tyrosines in IGF-I receptor function, we have performed substitutions of combinations of two tyrosines in the kinase domain of the IGF-I receptor. We transfected NIH-3T3 cells to study receptor autophosphorylation and post-receptor signaling pathways as well as biological functions of the receptor including cell growth and tumorigenicity.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were purchased from New England Biolabs, Boehringer Mannheim, and Life Technologies, Inc. Cell culture media and reagents were purchased from Biofluids, Inc. (Rockville, MD) and Advanced Biotechnologies (Columbia, MD). Insulin-free bovine serum albumin (Fraction V) was obtained from Armour (Kankakee, IL). Monodonal antiphosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology, Inc. Recombinant antiphosphotyrosine RC20H horseradish peroxidase-conjugated, polyclonal anti-Shc, monodonal anti-Grb2, and monodonal anti-PTP1D antibodies were purchased from Transduction Laboratories (Lexington, KY). Recombinant human IGF-I, fetal bovine serum (FBS), monoiodinated 125I-IGF-I, horseradish peroxidase-conjugated anti-mouse immunglobulin, and the ECL detection kit were purchased from Amersham Corp. Prestained high molecular weight protein standards and 3-[4,5-
Table I

| Cell line   | Receptors/cell (×10^6) | K_D (×10^9) |
|-------------|-----------------------|-------------|
| pNeo        | 23                    | 0.96        |
| WT43        | 875                   | 0.38        |
| WT50        | 686                   | 0.40        |
| DYF12 (4)   | 1020                  | 0.37        |
| DYF12 (10)  | 746                   | 0.35        |
| DYF13 (33)  | 627                   | 0.55        |
| DYF13 (42)  | 993                   | 0.43        |
| DT23 (2)    | 531                   | 0.29        |
| DYF23 (46)  | 406                   | 0.21        |

Table I

- Number of IGF-I receptors per cell

Equilibrium IGF-I binding assays were performed as previously described (10). Dissociation constants (K_D) of IGF-I and its receptor as well as the number of cell surface receptors were generated by the ligand program (P. J. Munson, Bethesda, MD). The results are the mean of three different experiments.

### RESULTS

**Expression of Wild-type and Mutant Receptors**—Two separate clones overexpressing each mutant or wild-type IGF-I receptor were used in this study. DYF12 designates substitution of the tyrosine residues with phenylalanine at positions 1131 and 1135. Substitution of tyrosine residues with phenylalanine at positions 1131 and 1135 is designated as DYF12. WT43 and WT50 are clones overexpressing wild-type human IGF-I receptor (15). pNeo is a clone cotransfected with a neomycin-resistant plasmid and the pBPV vector (15). All clones overexpressing IGF-I receptor had similar numbers of cell surface IGF-I receptors as determined by Scatchard analysis (Table I) (21).

**Receptor Autophosphorylation**—One of the first events after IGF-I binding to the IGF-I receptor is the autophosphorylation of tyrosine residues of the β-subunit of the receptor. The triple tyrosine cluster in the tyrosine kinase domain is considered to be the major autophosphorylation site of the IGF-I receptor (10). To study the autophosphorylation capability of the mutant...
receptors, cells were stimulated with 100 ng/ml IGF-I for 1 min at 37 °C. As shown in Fig. 1, IGF-I-induced receptor autophosphorylation is dramatic in cells overexpressing wild-type receptors. In contrast, cells overexpressing a similar number of receptors in which tyrosines 1131 and 1135 have been substituted (DYF12 clones 4 and 10) have undetectable levels of autophosphorylation. Cells overexpressing receptors with tyrosines 1131 and 1136 substitutions (DYF13 clones 33 and 42) or 1135 and 1136 (DYF23 clones 1 and 46) demonstrated clonal variation. Clones 42 (DYF 13) and 46 (DYF 23) showed no significant reduction in autophosphorylation compared with cells expressing wild-type receptors. However, clones 33 (DYF 13) and 1 (DYF 23) demonstrated less than a 50% reduction in autophosphorylation levels. These results suggest a differential role for these three tyrosines in the IGF-I-induced autophosphorylation of the receptor. The presence of either tyrosine 1131 or 1135 is critically important to have detectable levels of autophosphorylation, whereas the presence of tyrosine 1136 is not critical for autophosphorylation.

Phosphorylation of Endogenous Substrates—The two major signal transduction pathways following IGF-I receptor stimulation characterized thus far are those mediated by IRS-1 and Shc. We therefore studied the activation of IRS-1 and Shc pathways by IGF-I in wild-type and double tyrosine mutants. To evaluate IRS-1 pathway activation, cells were stimulated with 100 ng/ml IGF-I for 1 min at 37 °C, and IRS-1 immunoprecipitates were assayed for tyrosine phosphorylation of IRS-1 and Grb2 and PTP1D association. Stripping and reblotting the membranes with a polyclonal anti-IRS-1 antibody confirmed that similar amounts of IRS-1 protein were immunoprecipitated in all samples (data not shown). A typical result of these experiments is presented in Fig. 2. Panel A shows IRS-1 phosphorylation immunoblotted with a phosphotyrosine antibody RC20H. The ability of IGF-I to stimulate IRS-1 tyrosine phosphorylation was reduced in cells expressing the double mutant receptors as compared to wild-type clones. In addition, the IGF-I-stimulated association of PTP1D and Grb2 with IRS-1 occurred to a much lower extent in double tyrosine mutants when compared with stimulated levels in wild-type clones (panels B and C, respectively). This effect was most dramatic in studies of IRS-1/PTP1D association.

To evaluate Shc pathway activation, cells were stimulated with 100 ng/ml IGF-I for 5 min at 37 °C as described above, and Shc immunoprecipitates were assayed for Shc phosphorylation and Grb2 association. Similar amounts of Shc proteins were detected when the membranes were reblotted with a polyclonal anti-Shc antibody (data not shown). A typical experiment is shown in Fig. 3. A and B, respectively. IGF-1 stimulation of tyrosine phosphorylation of the 52- and 46-kDa isoforms of Shc protein (Fig. 3A) was observed in wild-type clones, whereas cells overexpressing the double tyrosine mutant receptors presented very low levels of Shc phosphorylation. Shc-Grb2 association following IGF-I stimulation was similarly diminished in mutant clones with respect to the wild-type clones (Fig. 3B). These results suggest that activation of both the IRS-1 and Shc pathways by IGF-I are at least partially blocked in cells overexpressing double tyrosine mutant receptors as compared with cells overexpressing a similar number of wild-type receptors.

DISCUSSION

The earliest post-binding event following the interaction of insulin and IGF-I with their specific receptors is the autophosphorylation of the triple tyrosine cluster within the β-subunit tyrosine kinase domain (7, 8). Mutational analysis of the insulin receptor tyrosine kinase domain has provided interesting, and at the same time, controversial information about the regulatory role of these three tyrosines in receptor kinase activity. The most dramatic effect has been shown with insulin receptors where these three tyrosine residues (tyrosines 1158, 1162, and 1163) have been mutated to phenylalanine. Insulin-stimulated autophosphorylation and cell signaling by these mutant receptors are impaired (13, 23). Single substitutions of any one of these tyrosine residues with phenylalanine (i.e. Y1158F, Y1162F, or Y1163F) may reduce in vitro autophosphorylation of the receptor β-subunit on the remaining tyrosines and reduce tyrosine kinase activity, although, in contrast, Zhang and Roth (6) reported that the Y1158F mutation had no effect on autophosphorylation or on insulin-stimulated exogenous tyrosine kinase activity. Data on thymidine incorporation is also controversial. In general, however, substitutions of any single tyrosine have only slight effects on insulin-stimulated thymidine incorporation (13, 23). A more substantial reduction in insulin-induced tyrosine kinase activity was observed when double tyrosine substitutions were studied (23). Generally, the triple tyrosine substitution results in even more reduction in thymidine incorporation, although HTC cells expressing the triple tyrosine mutant receptor exhibit normal thymidine incorporation (24). Despite these discrepancies, the data overall are consistent with the idea that autophosphorylation of ty-
Rosines 1158, 1162, and 1163 is essential in autoactivation of the insulin receptor tyrosine kinase toward other substrates. Although IGF-I and insulin receptors are structurally similar, their in vivo biological actions are different. At which level this divergence of function occurs is not yet defined. Thus, analyses of the structural and functional aspects of the IGF-I receptor is of considerable scientific interest. Studies using mutational analyses of the IGF-I receptor are considerably less complete than of the insulin receptor. We and others have reported previously that substitution of the triple tyrosine cluster with phenylalanine has similar effects as seen with the insulin receptor, i.e. essentially all of the functions of the IGF-I receptor were abrogated (9, 10). A single substitution of tyrosine residue 1131 reduces autophosphorylation and receptor internalization, whereas IRS-1 phosphorylation, thymidine incorporation, and cell proliferation were unaffected (15). Li et al. (14) showed that single substitutions of tyrosines 1131 or 1135 did not affect mitogenicity and only slightly reduced autophosphorylation. In contrast, they reported that substitution of tyrosine 1136 abrogated autophosphorylation and cell growth.

In the present study, we show that substitutions of tyrosines 1131 and 1135 reduced β-subunit autophosphorylation to levels
below the level of detectability by our antibody, suggesting that
the presence of either tyrosine 1131 or 1135 is required for full
β-subunit autophosphorylation. Substitutions of 1131/1136 and
1135/1136 affect autophosphorylation to a lesser degree,
suggesting that tyrosine 1136 is not crucial for IGF-I-stimu-
lated autophosphorylation. The crystal structure of the tyro-
sine kinase domain of the human insulin receptor has recently
been characterized (25). Apparently, the phosphorylation of
tyrosine 1162 (the second of the three tyrosines) is a key step in
the receptor kinase activation. The apo structure shows that the
hydroxyl group of tyrosine 1162 is bound in the active site
it from the active site. Our results, in the present study, sug-
gest an important role not only for tyrosine 1135 (equivalent to
tyrosine 1162 in insulin receptor) but also for tyrosine 1131 in
autophosphorylation of the IGF-I receptor.

Of particular interest in our study is the finding that all the
cells expressing double tyrosine substitutions failed to respond
mitogenically to IGF-I stimulation, and tumor formation was
reduced compared to cells overexpressing wild-type IGF-I re-
ciepators, despite the fact that substitution of tyrosines 1131/
1136 or 1135/1136 only slightly reduced receptor autophos-
phorylation. These findings suggest that perhaps a relatively high
threshold of autophosphorylation is required to fully activate
the signaling pathways. The inability of all double mutant
receptors to mediate biological activities could be explained by
the absence of IGF-I-induced tyrosine phosphorylation of the
two major IGF-I signaling pathways, IRS-1 and Shc. IRS-1 is
considered an adapter protein between the insulin and the
IGF-I receptors and the network of their signaling pathways
(26–29). IRS-1 is phosphorylated on multiple tyrosine residues
upon receptor stimulation (30). This provides multiple sites of
interaction for proteins with SH2 (src-homology 2) domains
(31). Several SH2-containing proteins have been shown to as-
so with IRS-1: P13-kinase (32–34), Nck (35), Grb2 (36–41),
and PTP1D (30, 39). PTP1D is a tyrosine phosphatase that
binds to IRS-1 at tyrosine 1172; this binding provides a poten-
tial mechanism for its activation (40). Evidence suggests that
PTP1D is involved in stimulation of mitogenesis, and regula-
tion of Ras and mitogen-activated protein kinase activation (41,
42). Grb2 is thought to stimulate p21ras activity through a
monovalent interaction with p21ras GDP-GTP exchange factor,
mSOS, resulting in stimulation of a serine/threonine phospho-
ylation cascade leading to activation of the mitogen-acted
protein kinase pathway implicated in cell growth and metabo-
lism. Thus, the binding of the Grb2-mSOS complex to IRS-1
after insulin and IGF-I stimulation links insulin and IGF-I
receptor tyrosines and p21ras signaling pathways. An
alternative and possible redundant pathway that links insulin
and IGF-I signaling with p21ras activation is through Shc (43–
47). Shc is tyrosine phosphorylated upon insulin and IGF-I
stimulation (44–46). The phosphorylation of Shc provides a
binding site for Grb2, resulting in the formation of the Shc-
Grb2-mSOS complex and probably leading to activation of the
mitogen-activated protein kinase pathway. Therefore, dimin-
ished stimulation of the IRS-1/PTP1D and IRS-1/Grb2 as well
as Shc-Grb2 pathways by IGF-I in our cells expressing recep-
tors with double tyrosine substitutions is associated with de-
creased IGF-I-stimulated cell growth response of cells express-
ing these receptors. We cannot exclude the possible
involvement of other signaling pathways, such as Crk, that are
also involved in IGF-I-induced signaling (48).

In summary, we have demonstrated that the presence of
both tyrosines 1131 and 1135 of the IGF-I receptor are neces-
sary for full IGF-I-stimulated autophosphorylation of the
β-subunit. However, the absence of any of the tyrosine residues
of the triple tyrosine cluster significantly reduced signal trans-
duction through IRS-1 and Shc activation, thus abrogating IGF-I-stimulated cell growth and IGF-I receptor-mediated tumor formation of transfected fibroblasts.

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