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The Role of Insulin Receptor Kinase Domain Autophosphorylation in Receptor-mediated Activities

ANALYSIS WITH INSULIN AND ANTI-RECEPTOR ANTIBODIES*

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The role of specific tyrosine autophosphorylation sites in the human insulin receptor kinase domain (Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}) was analyzed using *in vitro* mutagenesis to replace tyrosine residues individually or in combination. Each of the three *single-Phe*, the three possible *double-Phe* a *triple-Phe* and a *triple-Ser* mutant receptors, stably expressed in Chinese hamster ovary cells, were compared with the wild-type receptor in their ability to mediate stimulation of receptor kinase activity, glycogen synthesis, and DNA synthesis by insulin or the human-specific anti-receptor monoclonal antibody 83-14. At a concentration of 0.1 nM insulin which produced approximately half-maximal responses with wild-type receptor, DNA synthesis and glycogen synthesis mediated by the three *single-Phe* mutants ranged from 52 to 88% and from 32 to 79% of the wild-type receptor, respectively. The corresponding figures for the *double-Phe* mutants averaged 15 and 6%, whereas the *triple-mutants* were unresponsive in both assays. The level of biological function approximately paralleled the insulin-stimulated tyrosine kinase activity in the intact cell as estimated by tyrosine phosphorylation of the insulin receptor and its endogenous substrate pp185/IRS-1. Interestingly, all mutants showed a marked decrease in insulin-stimulated receptor internalization. Anti-receptor antibody stimulated receptor kinase activity and mimicked insulin action in these cells. In general, the impairment of the metabolic response was greater and impairment of the growth response was less when antibody was the stimulus. These experiments show that the level and specific sites of autophosphorylation are critical determinants of receptor function. The data are consistent with a requirement for the receptor tyrosine kinase either as an obligatory step or a modulator, in both metabolic and growth responses, and demonstrate the important role of the level of insulin receptor kinase domain autophosphorylation in regulating insulin sensitivity.

Insulin exerts a broad range of pleiotrophic actions at the cellular level. The exact molecular pathways of intracellular signaling activated by the insulin receptor which result in these diverse biological responses remain unclear (1–4). Considerable evidence suggests that the tyrosine protein kinase activity intrinsic to the receptor plays an essential role in mediating both acute metabolic effects and longer term growth-promoting effects of insulin (5–7). The insulin receptor kinase is activated by insulin binding and amplified by a series of autophosphorylation reactions (8, 9). The activated kinase then acts on one or more intracellular substrates, which are believed to initiate a regulatory phosphorylation cascade (10) or interact non-covalently with other signaling molecules (11). In addition, conformational changes in the receptor, dependent on the binding of insulin or ATP or autophosphorylation, might influence non-covalent interactions with other proteins (12–15). Thus it is possible that multiple signals are generated by the receptor, which initiate pathways leading to different bioeffects or synergize in producing the full biological response.

The pattern of receptor autophosphorylation is complex, involving at least 5 tyrosine residues (9, 16, 17). Within the kinase domain proper are 3 tyrosines (Tyr^{1158}, Tyr^{1162}, Tyr^{1163})[1] whose phosphorylation is required for amplification of the kinase activity (9, 18, 19). Phosphorylation of two additional sites close to the C terminus (Tyr^{1328}, Tyr^{1334}) does not appear to directly influence kinase activity (20, 21). Although the exact role of individual phosphorylation sites is unknown,

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[1] Two forms of the insulin receptor have been identified representing alternate splice variants of a single gene with or without exon 11 (55, 56). This results in an inframe addition of 36 bases and 12 amino acids to the C-terminal region of the α-subunit. Both forms have been shown to have equivalent kinase activity (57). All experiments were performed with the exon 11 minus form of the receptor. The amino acids are numbered, however, based on the full-length sequence including exon 11. For reference to other publications the following conversion table can be used.

Amino Acid	Receptor Form	
	−Exon 11	+Exon 11
Lys	1018	1030
Tyr	1146	1158
Tyr	1150	1162
Tyr	1151	1163
Tyr	1316	1328
Tyr	1132	1334

studies with receptors mutated at one or more of these sites have suggested the existence of divergent signaling pathways for metabolic and mitogenic effects, dependent on the phosphorylation of specific tyrosines (22–24). By contrast, some studies have suggested that insulin signaling is normal in mutant receptors lacking the 3 regulatory tyrosines (25) or an ATP binding domain (26).

The role of tyrosine phosphorylation has also been probed by using receptor antibodies which mimic a wide range of insulin effects (27–29). Some studies have found that antibodies exert their insulin-like effects with little stimulation of receptor autophosphorylation or kinase activity (30–33). Furthermore, mutant receptors lacking autophosphorylation sites, which are unresponsive to insulin, have been reported to respond normally to stimulation by antibodies (34). These observations have raised questions as to the role of the receptor kinase in cellular signaling and have led to the inference that a conformation or aggregation state of receptors, induced either by insulin-stimulated autophosphorylation or antibody-mediated cross-linking, may be more important than kinase activation.

To investigate the significance of individual autophosphorylation sites and the level of autophosphorylation on receptor kinase activation and signaling, we have prepared a series of eight different mutant receptors containing all possible single, double, and triple mutants of tyrosines 1158, 1162, and 1163, and expressed these mutant receptors by transfection of Chinese hamster ovary (CHO)[2] cells. We have shown that these mutant receptors display a progressive reduction in insulin-stimulated receptor autophosphorylation and kinase activity. In the present report we describe the ability of these mutant receptors to mediate metabolic and growth responses to insulin and insulin-mimetic receptor antibodies and their capacity to internalize insulin.

MATERIALS AND METHODS

Cell Lines and Culture—The transfected CHO cell lines expressing wild-type and mutant insulin receptors are as described elsewhere (23). Briefly, point mutations were introduced into the human insulin receptor cDNA by oligonucleotide-directed mutagenesis to replace Tyr^{1158}, Tyr^{1162}, Tyr^{1163}, $Tyr^{1158/1162}$, $Tyr^{1158/1163}$, $Tyr^{1162/1163}$, and $Tyr^{1158/1162/1163}$ with phenylalanine. The first three mutants are referred to as the *single-Phe* group, the next three as the *double-Phe* group, and the last as the *triple-Phe* mutant. An additional mutant in which all 3 tyrosine residues were replaced with serine (*triple-Ser*) was also constructed, since serine could serve as an alternate phosphate acceptor. Wild-type and mutant receptor sequences in the vector pSG5 (Stratagene) were expressed in CHO cells by co-transfection with the neomycin resistance plasmid pSVneo using calcium phosphate-mediated gene transfer. Cells expressing insulin receptor constructs were enriched by fluorescence-activated cell sorting before cloning at limiting dilution and selection of lines matched with regard to the level of receptor expression. Cells were maintained in Ham's F-12 medium (GIBCO) containing 10% fetal bovine serum (Sigma) in an atmosphere of 5% CO_2, 95% air. Cultures were passaged by trypsinization and split 1:20 every 5–7 days. Experiments were performed on cells which had been passaged <20 times.

Receptor Antibody—The monoclonal antibody Ab 83-14, specific for the human insulin receptor (35), was purified from ascites fluid by precipitation with 42% saturated ammonium sulfate followed by chromatography on protein A-Sepharose (36). We have previously shown that this antibody reacts with determinants in the α-subunit of the receptor (37), inhibits insulin binding and mimics insulin action in both isolated adipocytes (28) and in transfected cells (33), and efficiently immunoprecipitates receptor in the absence or presence of insulin (17).

[2] The abbreviations used are: CHO, Chinese hamster ovary; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate: PBS, phosphate-buffered saline; αPY, anti-phosphotyrosine antibody; Ab, antibody.

Insulin Binding—Subconfluent cells on 24-well plates were incubated for 3 h at 15 °C with 0.06 μCi of ^{125}I-insulin (DERC Core Laboratory, Joslin Diabetes Center) together with unlabeled insulin or antibody (10^{-11} to 10^{-6} M) in 0.3 ml of binding buffer (Krebs-Ringer-Hepes, pH 7.8, containing 1 mg/ml bovine serum albumin). Incubations were terminated by aspirating the binding buffer and washing cells 3 times with 1 ml of ice-cold PBS. Cells were solubilized in 0.5 ml of 1 M NaOH for determination of radioactivity by γ-counting.

Insulin Receptor and pp185 Tyrosine Phosphorylation—Insulin and anti-insulin receptor antibody Ab 83-14 were used to stimulate CHO cells expressing wild-type or mutant insulin receptors. Confluent cells on six-well plates were incubated for 30 min at 37 °C with various concentration of insulin or antibody in Ham's F-12, pH 7.4, containing 1 mg/ml bovine serum albumin. The medium was removed, and the reaction terminated by the addition of 0.3 ml of 2 × Laemmli sample buffer containing 100 mM dithiothreitol and boiling for 1 min. The samples were then sonicated and proteins separated on 7% SDS-PAGE gels. Proteins were transferred to nitrocellulose and blocked overnight at 4 °C in 20 mM Tris, 150 mM NaCl, 3% bovine serum albumin, and 0.01% Tween 20, pH 7.4. The nitrocellulose blots were incubated with anti-phosphotyrosine (αPY) antibody in blocking solution for 2 h at 23 °C, after which the nitrocellulose was washed 4 times in 20 mM Tris, 150 mM 0.01% Tween 20. The bound antibody was detected by incubation with ^{125}I-protein A (ICN 68038) for 1 h at 23 °C in blocking buffer (0.1 μCi/ml). Finally, the nitrocellulose was washed 4 times with wash buffer, and the membrane was autoradiographed. Quantitation was carried out by laser densitometry and counting (^{125}I) in the excised band.

Insulin Internalization—Internalization of surface-bound insulin was determined by modification of procedures described previously (38). Confluent cells on 24-well culture plates were incubated for 3 h at 4 °C with 0.15 μCi of ^{125}I-insulin in 0.3 ml of Ham's F-12 medium, pH 7.8, containing 10 mg/ml bovine serum albumin. Unbound insulin was removed by a single wash with ice-cold PBS, pH 7.4, containing 10 mg/ml bovine serum albumin, and the cells were incubated at 37 °C for various times. Cells were then washed twice with ice-cold PBS adjusted to pH 3, and once with PBS, pH 7.4, both containing 10 mg/ml bovine serum albumin, and solubilized in 0.5 ml of 1 M NaOH for determination of radioactivity by γ-counting. Residual cell-associated radioactivity under these conditions was taken to represent internalized insulin. Total insulin binding was determined in cells which had not been incubated at 37 °C and had been washed three times with PBS, pH 7.4, containing 10 mg/ml bovine serum albumin.

Biosynthetic Labeling—Confluent monolayers of cells on 10-cm culture dishes (Nunc) were labeled by incubation for 16 h at 37 °C in 5 ml of methionine-free RPMI 1640 medium (GIBCO) containing 1 mCi of [^{35}S]methionine (ICN 51006). The cells were then solubilized in 50 mM Tris, pH 7.4, containing 1% Triton X-100 and protease inhibitors. Human insulin receptors were immunoprecipitated with Ab 83-14 (17), and precipitated proteins separated under reducing conditions by 7% SDS-PAGE. Gels were Coomassie-stained, destained, dried, and autoradiographed.

Metabolic Responses—Incorporation of [^{14}C]glucose into glycogen was determined in confluent monolayers on 12-well culture plates. Cells were equilibrated in culture medium containing 25 mM Hepes, pH 7.4, 2.5 mM glucose, and 0.1% bovine albumin for 3 h at 37 °C in an ungassed incubator. This medium was then replaced with 0.5 ml of the same medium containing insulin or Ab 83-14. After 30 min, 50 μl of medium containing 0.5 μCi of [U-^{14}C]glucose (Amersham Corp., CFB 96) was added, and incubations were continued for 90 min. Cells were then washed 3 times in ice-cold PBS and solubilized in 0.5 ml of 20% KOH. The soluble extract was transferred to glass tubes containing 1 mg of glycogen and incubated in a boiling water bath for 30 min. Glycogen was precipitated with 66% ethanol and collected by filtration on glass fiber discs for determination of radioactivity by liquid scintillation counting.

Incorporation of [^{3}H]thymidine into DNA was carried out as previously described (23) with minor modifications. Subconfluent cells on 12-well culture plates were serum-starved for 30 h (unless otherwise indicated) in Ham's F-12 medium containing bovine serum albumin (1 mg/ml), prior to addition of insulin or antibody for another 15 h. The medium was then replaced with 1 ml of fresh medium supplemented with 25 mM Hepes, pH 7.4, and containing 1 μCi of [*methyl*-^{3}H]thymidine (Du Pont-New England Nuclear, NET 027). After 90 min the cells were washed 3 times in ice-cold PBS and solubilized in 1 ml of 0.1% SDS. Trichloroacetic acid was added to a

final concentration of 10%, and the precipitate collected by filtration on glass fiber discs for determination of radioactivity by liquid scintillation counting.

RESULTS

Expression of Wild-type and Mutant Receptors—Scatchard analysis of binding data showed that mock-transfected cells (CHO-Neo) contained approximately 30,000 surface insulin receptors/cell. A clone of cells expressing wild-type human insulin receptors (CHO-HIRc) was selected in which the number of surface receptors was increased 30-fold to approximately 10^6/cell. Clones of cells expressing mutant receptors were selected which contained as nearly as possible the same number of receptors as in CHO-HIRc. When insulin binding data were fitted to a two-site model, the average dissociation constants for high and low affinity sites in all cell lines were 0.6 and 55 nM, respectively, and did not differ significantly among the cell lines. Receptor structure, analyzed by metabolic labeling, immunoprecipitation, SDS-PAGE, and autoradiography, confirmed that in all the transfected cell lines the expressed receptors were processed normally to mature α and β-subunits (Fig. 1).

Insulin and Antibody 83-14 Show Distinct Sensitivity in CHO-HIRc Cells—Ab 83-14, like insulin, reacts with the insulin receptor α-subunit (36, 38). The affinities of insulin and Ab 83-14 for the human receptor expressed in CHO cells were compared in terms of their ability to inhibit ^{125}I-insulin binding. Half-maximal inhibition of binding was obtained with 2 nM antibody or 2 nM insulin in cells expressing wild-type receptor (Fig. 2). Similar data were obtained with a clone expressing a mutant receptor (not shown).

The potencies of insulin and Ab 83-14 were compared for stimulation of glucose incorporation into glycogen and thymidine incorporation into DNA. In CHO-HIRc cells, the half-maximally effective concentration of insulin for stimulation of DNA synthesis was 0.03 nM and for glycogen synthesis was 0.07 nM (Fig. 2). The response curves for insulin in the CHO-HIRc cells were dose-shifted to the left more than 30-fold relative to responses in CHO-Neo cells; ED_{50} values were 1 and 10 nM (data not shown; see also Ref. 23). Moreover, the maximum stimulation by insulin in CHO-HIRc cells was achieved at low fractional occupancy of receptors (Fig. 2). These data indicate that the wild-type human receptors were efficiently coupled to intracellular signaling systems in these cells.

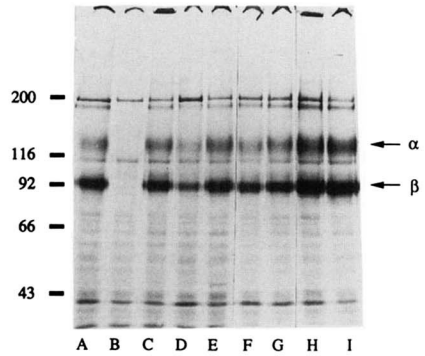

FIG. 1. **Biosynthetic labeling of mutant receptors in transfected cells.** Confluent 10-cm dishes of CHO cells transfected with wild-type or mutant insulin receptor cDNA were metabolically labeled with [^{35}S]methionine as described under "Materials and Methods.' The insulin receptors were immunoprecipitated with monoclonal antibody (83-14) and separated by SDS-PAGE. The stained, destained, and dried gel was autoradiographed for 24 h. The *tracks* show cells transfected with the following: *A*, wild-type receptor, CHO-HIRc; *B*, no human receptor, CHO-Neo (neomycin resistance plasmid only); *C*, IR^{F1158}; *D*, IR^{F1162}; *E*, IR^{F1163}; *F*, $IR^{F1158,1162}$; *G*, $IR^{F1162,1163}$; *H*, $IR^{F1158,1162,1163}$; *I*, $IR^{S1158,1162,1163}$.

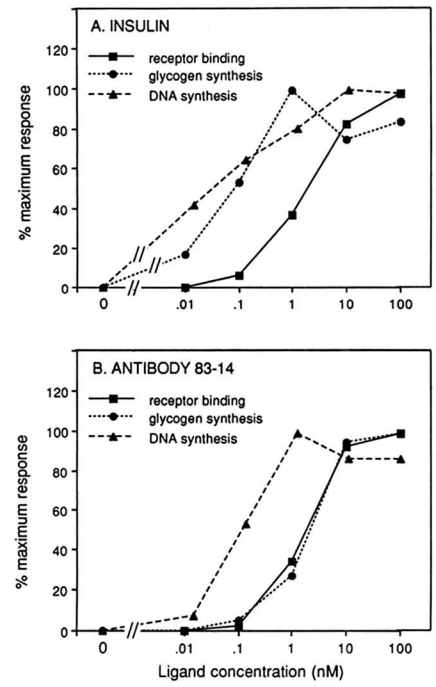

FIG. 2. **Concentration dependence of effects of insulin and antibody 83-14 on CHO-HIRc cells.** Incorporation of [^{14}C]glucose and [^{3}H]thymidine was determined during incubation of cells for 90 min at 37 °C following preincubation with insulin or antibody as described under "Materials and Methods." Inhibition of binding of ^{125}I-insulin (0.06 μCi) was determined during incubation of cells for 3 h at 15 °C with unlabeled insulin or antibody as described under "Materials and Methods." Data for [^{14}C]glucose and [^{3}H]thymidine incorporation are plotted as percent of maximum stimulation for each agonist (mean of six independent experiments). Data for ^{125}I-insulin binding are plotted as percent of maximum inhibition of binding of (mean of triplicate determinations within a single typical experiment). *Panel A*, insulin effects. Maximum responses were as follows: inhibition of insulin binding (100%), stimulation of glucose incorporation into glycogen (125%; 2.25-fold), stimulation of thymidine incorporation into DNA (175%; 2.75-fold). *Panel B*, antibody 83-14 effects. Maximum responses were as follows: inhibition of insulin binding (70%), stimulation of glucose incorporation into glycogen (100%; 2.00-fold), stimulation of thymidine incorporation into DNA (128%; 2.28-fold).

Effects of Ab 83-14 on CHO-HIRc cells were very similar to those induced by insulin in both bioassays; however, the half-maximally effective concentrations of antibody were 0.1 nM for DNA synthesis and 2 nM for glycogen synthesis. Thus, the dose-response curve for antibody-stimulated DNA synthesis was left-shifted 20-fold relative to the antibody binding curve and was similar to the curve for insulin-stimulated DNA synthesis, whereas the response curve for antibody-stimulated glycogen synthesis was almost superimposable on that for binding and was not left-shifted. Even allowing for the fact that binding and metabolic assays were not carried out under identical conditions, these results indicate that receptor occupancy by antibody is not efficiently coupled to activation in these cells, at least as regards signaling for acute metabolic effects. In spite of the high level of receptor expression, there are few "spare receptors" for antibody-stimulated glycogen synthesis in CHO-HIRc cells. These data are in contrast to previous studies, which have shown that Ab 83-14 exerts metabolic effects on human adipocytes and transfected mouse fibroblasts with a potency similar to insulin (28, 33).

Insulin and Anti-insulin Receptor Antibody Stimulation of

Insulin Receptor and pp185 Phosphorylation—The ability of insulin and Ab 83-14 to activate the insulin receptor kinase was studied using CHO cells expressing each of the of mutant insulin receptors constructs (*single-Phe*, *double-Phe*, and *triple-Phe*), as well as the wild-type and ATP binding site (A^{1030}) mutants. As previously reported, stimulation of CHO-HIRc cells by insulin led to a dramatic increase in tyrosine phosphorylation of the insulin receptor β-subunit and the endogenous protein substrate, pp185 (Fig. 3*A*). Ab 83-14 also stimulated receptor and pp185 phosphorylation but was approximately 10-fold less potent on a molar basis. This experiment confirms previous work (50, 51), showing that this anti-receptor antibody does stimulate receptor kinase activity but appears to be less effective than insulin at equivalent levels of binding inhibition. Insulin-stimulated tyrosine phosphorylation of the insulin receptor and pp185 in CHO cells expressing the *single-Phe* mutants was reduced 50–70% when compared with the CHO-HIRc cells when detected by αPY blotting (Fig. 3, *B* and *C*). The greatest reduction was with the IR^{F1162} mutant; the least was with the IR^{F1163} mutant. Insulin-stimulated phosphorylation of the *double-Phe* mutants was reduced to 20% of the wild-type control level. The *triple-mutants* were reduced even further, to the level of the kinase-inactive mutant IR^{A1030} and mock-transfected CHO-Neo cell line. Except for the IR^{F1163} mutant, Ab 83-14 (10^{-7} M) stimulated tyrosine phosphorylation of the insulin receptor β-subunit and pp185 in CHO cells expressing the mutant insulin receptor species was at or only slightly above the level of detection of the αPY blotting. It is difficult, however, to compare this reduction directly to insulin, since Ab 83-14 stimulation of phosphorylation in cells with wild-type receptor was only about 20–35% of that observed with insulin stimulation (Fig. 3, *B* and *C*).

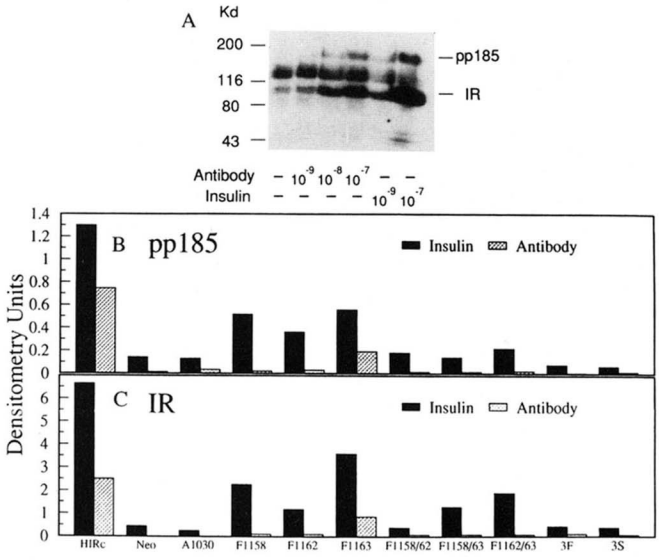

FIG. 3. **Insulin and anti-insulin receptor antibody 83-14 stimulation of insulin receptor β-subunit and pp185 tyrosine phosphorylation.** Tyrosine phosphorylation of the insulin receptor β-subunit and pp185 were measured in CHO-HIRc cells by αPY blotting using ^{125}I-protein A following 30 min of insulin or antibody 83-14 stimulation at 37 °C at the concentrations listed in the figure. Cells were extracted, proteins separated, transferred to nitrocellulose, and detected by αPY blotting as described under "Materials and Methods." The autoradiograph of such an experiment is seen in *panel A*. Tyrosine phosphorylation of the insulin receptor β-subunit (*panel C*) and pp185 (*panel B*) were measured in CHO cells expressing the mutant insulin receptor by αPY blotting following 30 min of insulin (10^{-7} M) or antibody 83-14 (10^{-7} M) stimulation at 37 °C. Autoradiographs were quantified by scanning laser densitometry using a Molecular Dynamics computer densitometer.

Glycogen Synthesis in Cells Expressing Mutant Receptors—Maximum stimulation of glucose incorporation by insulin (10^{-6} M) was very similar in all cell lines studied, generally in the range 2.0–2.7-fold over basal (Table I). However, the concentration-dependence of the insulin stimulation varied between cell lines. As previously reported (23), mutation of Tyr^{1158} to Phe had little or no effect on the ability of insulin to stimulate [^{14}C]glucose incorporation into glycogen (Fig. 4*A*). All other mutations affecting autophosphorylation sites significantly impaired the capacity of receptors to mediate insulin-stimulated glycogen synthesis (Fig. 4*B*). The reduction in glycogen synthesis at 1 nM insulin was ~45% in cells expressing the IR^{F1162}, ~55% for cells with IR^{F1163}, 55–60% of each of the *double-Phe* mutants, and ~65% for cells expressing the *triple-Phe* and *triple-Ser* mutants. The residual response with the triple mutants was not significantly different from that in mock-transfected CHO-Neo or cells transfected with the kinase-inactive IR^{A1030} mutant, and probably reflects stimulation mediated through endogenous hamster insulin receptors. A similar pattern was observed using 0.1 nM insulin for stimulation, although all values were lower.

Ab 83-14 stimulated glucose incorporation in CHO-HIRc cells, but as noted in Fig. 2 above, 10-fold higher concentrations were required to produce a similar level of response (Fig. 4*C*). The *single-Phe* mutants and the *double-Phe* mutant $IR^{F1162,1163}$ all showed stimulations by antibody which were above those in IR^{A1030} cells, but were reduced more than 50% compared with wild-type. In contrast, responses of the *double-Phe* $IR^{F1158,1162}$, *triple-Phe*, and *triple-Ser* mutants showed an additional impairment and differed little if at all from IR^{A1030} or mock-transfected cells (Fig. 4*C*). The changes in antibody response generally paralleled the change in insulin response except for IR^{F1158}, which was impaired with antibody and normal with insulin. A slight stimulation in the glucose incorporation into glycogen was observed in kinase-inactive IR^{A1030} cells, although the stimulation was less than 20% of that in CHO-HIRc.

DNA Synthesis in Cells Expressing Mutant Receptors—Thymidine incorporation into DNA varies considerably

TABLE I

Maximum stimulation of glycogen synthesis and DNA synthesis

Incorporation of [^{14}C]glucose into glycogen and [^{3}H]thymidine into DNA was determined as described under "Materials and Methods," both basally (no insulin or serum present) and in the presence of a maximally effective stimulus (1 μM insulin for glycogen synthesis; 10% fetal bovine serum for DNA synthesis). Basal incorporation varied between experiments and cell lines in the range of 200–300 cpm (glucose) and 2000–8000 cpm (thymidine). Results are the mean ± S.E. of fold stimulation for the number of experiments shown (triplicate determination of basal and stimulated incorporation within each experiment).

Insulin receptor construct	Glycogen synthesis		DNA synthesis	
	-fold stimulation by 1 μM insulin	(*n*)	-fold stimulation by 10% serum	(*n*)
HIRc	2.42 ± 0.20	(14)	2.73 ± 0.19	(16)
Neo control	2.27 ± 0.13	(10)	1.89 ± 0.16	(11)
A1030	2.25 ± 0.11	(6)	1.79 ± 0.11	(6)
F1158	2.04 ± 0.06	(8)	1.90 ± 0.08	(9)
F1162	2.32 ± 0.12	(4)	2.81 ± 0.30	(4)
F1163	2.35 ± 0.10	(3)	3.73 ± 0.87	(3)
F1158/1162	2.05 ± 0.13	(4)	4.43 ± 0.61	(4)
F1162/1163	2.38 ± 0.41	(4)	2.96 ± 0.26	(4)
F1158/1162/1163	2.71 ± 0.07	(4)	4.76 ± 0.60	(4)
S1158/1162/1163	3.18 ± 0.86	(4)	2.64 ± 0.18	(5)

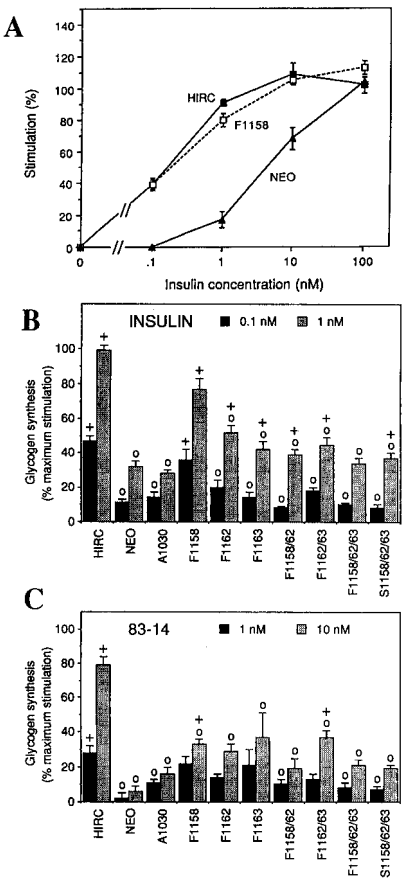

FIG. 4. **Insulin-stimulated [^{14}C]glucose incorporation into glycogen.** [^{14}C]glucose incorporation into glycogen during a 2-h incubation with insulin, were measured in CHO cells transfected with wild-type insulin receptor cDNA, IR^{F1158} mutant receptor cDNA, or neomycin resistance plasmid only (*panel A*). Stimulation is shown as percent increase above basal (no insulin) levels. Results are mean ± S.E. of triplicate observations. Similar results were obtained in three independent experiments. Incorporation of [^{14}C]glucose into glycogen was determined in the presence of 0.1 nM and 1 nM insulin (*panel B*) or 1 and 10 nM antibody 83-14 (*panel C*), as described under "Materials and Methods." Data (mean ± S.E.) are calculated as percent of maximum stimulation (increment over basal in the presence of 1 mM insulin). *Open circle* (O) indicates a response which is significantly less ($p < 0.03$) than the corresponding response in HIRc; *cross* (+) indicates a response which is significantly greater ($p < 0.03$) than the corresponding response in IR^{A1030}. Values for maximum stimulation and number of independent experiments with each cell line are as given in Table I.

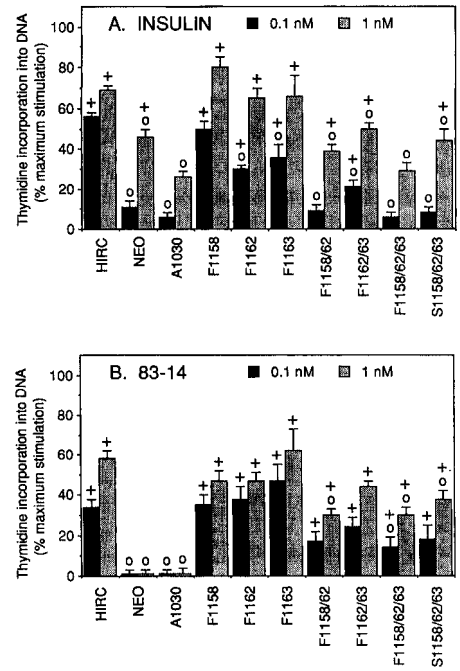

FIG. 5. **Stimulation of DNA synthesis in cells transfected with mutant insulin receptors.** Incorporation of [^{3}H]thymidine into DNA was determined in the presence of 0.1 nM and 1 nM insulin (*panel A*) or 0.1 nM and 1 nM antibody 83-14 (*panel B*), as described under "Materials and Methods." Data (mean ± S.E.) are calculated as percent of maximum stimulation (increment over basal in the presence of 10% fetal bovine serum). O, indicates a response which is significantly less ($p < 0.03$) than the corresponding response in HIRc; +, indicates a response which is significantly greater ($p < 0.03$) than the corresponding response in IR^{A1030}. Values for maximum stimulation and number of independent experiments with each cell line are as in Table I.

among clonal cell lines. In this series of experiments the maximum stimulation of thymidine incorporation by serum with different cell lines ranged from 1.8- to 4.8-fold over basal (Table I). This variability was largely due to differences in basal rather than stimulated rates of incorporation and probably reflected different effects of serum starvation between the clones of cells. To facilitate comparison between mutants, data for insulin or antibody stimulation of thymidine incorporation into DNA were therefore normalized as a percentage of the maximum stimulation produced by 10% serum with each cell line.

Compared with wild-type receptors, the *single-Phe* mutants showed only a modest impairment of insulin-stimulated DNA synthesis. Thymidine incorporation at low insulin concentrations (10^{-10} M) was decreased 10–40% when compared with wild-type receptor under conditions of short term (30 h) serum starvation (Fig. 5*A*). The *double-Phe* mutant $IR^{F1162/1163}$ also exhibited some stimulation above that seen with CHO-Neo and IR^{A1030} cells at 10^{-10} M insulin, whereas at this low concentration, the $IR^{F1158,1162}$ *double-Phe* and $IR^{F1158,1162,1163}$ *triple-Phe* mutants did not. At a high insulin concentration (10^{-9} M), all cell lines, including the mock-transfected cells showed significant stimulation of DNA synthesis probably as a result of insulin acting through the small number of endogenous insulin receptors or insulin-like growth factor-I receptors.

To further assess the mutants, full insulin dose-responses were carried out under conditions of long term (72 h) serum starvation; this facilitates the detection of small stimulations above basal at low insulin concentrations (Fig. 6). Focusing on the lowest insulin concentrations (10^{-11} to 10^{-10} M), all three *single-Phe* mutants showed diminished responses with the most normal response in IR^{F1163} cells. Half-maximally effective insulin concentrations for *single-Phe* mutants were intermediate between cells expressing the wild-type receptor and the kinase-inactive IR^{A1030} mutant. The *double-Phe* and *triple-Phe* mutants generally showed responses similar to, or even less than, those in IR^{A1030} cells across the full range of insulin concentrations.

In cells expressing functional human insulin receptors, Ab 83-14 stimulates DNA synthesis in a manner almost equipotent with insulin (Fig. 2). Thymidine incorporation was completely unresponsive to stimulation by this human-specific anti-receptor antibody in both CHO-Neo and IR^{A1030} cells, consistent with the notion that an active receptor kinase or ATP binding site is required for antibody stimulation of DNA synthesis. By contrast, antibody stimulation of the *single-Phe* mutations was almost identical to that of the wild-type receptor (Fig. 5*B*). The *double-Phe*, *triple-Phe*, and *triple-Ser* mu-

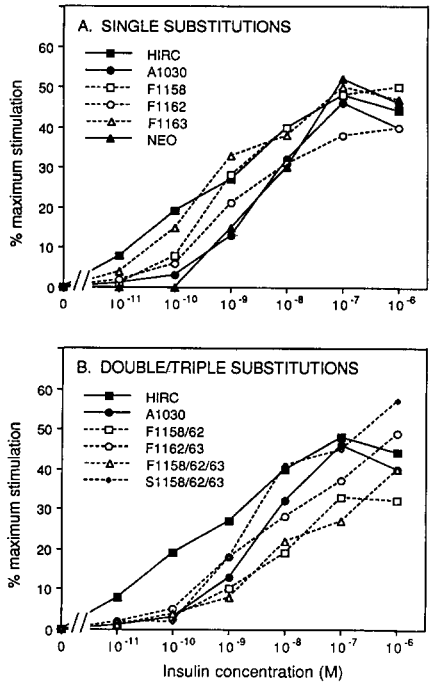

FIG. 6. **Dose responses for insulin-stimulated thymidine incorporation in long term serum-starved cells.** Subconfluent cells in 24-well plates were serum-starved for 72 h prior to the addition of various concentrations of insulin in serum-free medium for 15 h. Incorporation of [^{3}H]thymidine into DNA was then measured for 1 h as described under "Materials and Methods." Data are calculated as percent of maximum stimulation (increment over basal in the presence of 10% fetal bovine serum) and are means of triplicate observations within a single experiment. Basal incorporation was 500–2000 cpm for different cell lines, and maximum stimulation by serum was 10–40-fold.

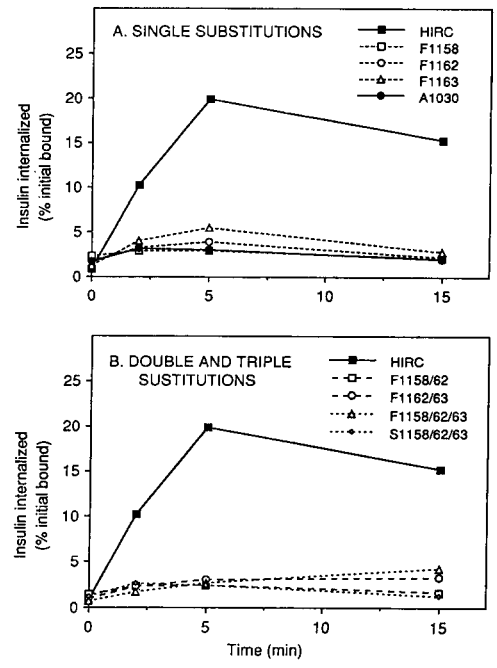

FIG. 7. **Internalization of insulin in cells transfected with mutant insulin receptors.** Confluent CHO cells on 24-well plates were incubated with ^{125}I-insulin at 4 °C, washed to remove unbound insulin, and then incubated for various times at 37 °C as described under "Materials and Methods." At each time point, surface-bound insulin was removed by washing cells at pH 3, and cells were then solubilized for determination of internalized insulin (defined as the amount of acid-resistant radioactivity associated with the cells). Internalized insulin is expressed as percent of initial binding.

tants also mediated substantial stimulation of DNA synthesis by antibody, amounting to 50–60% of that seen in CHO-HIRc cells. All mutants therefore showed relatively better retention of function for antibody-stimulated DNA synthesis than for insulin-stimulated DNA synthesis or antibody-stimulated glycogen synthesis.

Insulin Internalization in Cells Expressing Mutant Receptors—Previous studies have provided conflicting evidence on the role of tyrosine kinase activity and autophosphorylation in the insulin-induced internalization of the insulin receptor. Mutation of Lys^{1030} in the ATP binding site severely impairs both short term endocytosis of insulin and long term down-regulation of receptors in transfected Rat-1 or CHO fibroblasts (7, 40) but does not affect down-regulation in HTC hepatoma cells (41). Moreover internalization of wild-type receptor in Fao hepatoma cells proceeds normally even when autophosphorylation is inhibited by 90% following depletion of intracellular ATP (42).

In the present study we have followed the internalization of a single cohort of surface-bound insulin as a measure of initial translocation of the occupied receptor in transfected cells. Under these conditions, the wild-type receptor (CHO-HIRc) internalized 20% of bound insulin within 5 min, whereas the kinase-inactive IR^{A1030} internalized only 2–3%. All *single-*, *double-*, and *triple-Phe* mutants showed markedly decreased insulin internalization (Fig. 7). Only in IR^{F1162} and IR^{F1163} cells was there evidence of significant internalization above the level of IR^{A1030} cells. These observations confirm and extend previous work demonstrating severe impairment of insulin internalizaiton by the IR^{F1158} and IR^{F1162,1163} mutants (23, 44).

DISCUSSION

The aim of this work was to examine the role of major tyrosine autophosphorylation sites in controlling the activity and specificity of signaling by the insulin receptor. We focused our attention on three sites in the tyrosine kinase domain (Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}) which are believed to be crucial for modulating kinase activity and receptor function (16–19). It has been previously demonstrated that tris-phosphorylation of this cluster of tyrosines is necessary for full kinase activation *in vitro* (9, 18, 19), although in intact cells a substantial fraction (40–90%) of receptors remain in the bis-phosphorylated state even under conditions of maximum insulin stimulation (9, 16, 17, 42). An important role for individual tyrosine residues has been suggested by studies in which replacement of Tyr^{1158} or Tyr^{1162} and Tyr^{1163} with phenylalanine has been shown to reduce the insulin-stimulated tyrosine kinase activity *in vitro* and support differential signaling in growth and metabolic pathways (22, 23). Individual sites might contribute to the specificity of signaling either because of their influence on overall kinase activity (9) or because of a more subtle involvement in the interaction with individual substrates (11).

In this study we have systematically investigated the ability of eight different mutant receptors involving all possible combination of tyrosine residues in the kinase domain to mediate effects of insulin or anti-insulin receptor antibody on phosphorylation of the insulin receptor β-subunit and an endogenous substrate, stimulation of glycogen and DNA synthesis, and stimulation of insulin receptor internalization. A clear finding of this work is that receptor signaling potential becomes progressively impaired as phosphorylation sites are removed. The *single-Phe* mutants all exhibit a modest reduction in metabolic and growth-stimulating activity, while the *double-Phe* and *triple-Phe* mutants show substantial or com-

plete loss of function. Within these generalizations some specificity was apparent in terms of differential effects of mutations on glycogen synthesis and DNA synthesis, and there were also differences in the responsiveness of cells to insulin and anti-receptor antibody. These more subtle differences, as well as similar observations in other studies, however, should be interpreted cautiously in view of the clonal nature of the cells used in these studies.

Cells expressing receptors mutated at only 1 of the 3 regulatory tyrosines showed only moderate decreases in metabolic and growth responses to insulin relative to wild-type receptor. With the IR^{F1158} cells, at low insulin concentrations, stimulation of DNA synthesis was more affected than stimulation of glycogen synthesis, whereas the converse was the case with IR^{F1163} cells. This is similar to our previous findings with IR^{F1158} (23), although the differential signaling is of a smaller degree in the present studies. This quantitative change may reflect differences in methodology or in data analysis, since in the previous work responses were calculated as a percentage stimulation above basal. Taken together as a group, the mean responses of the *single-Phe* mutants at 0.1 nM insulin were 32% for glycogen synthesis and 64% for DNA synthesis of the wild-type responses, when the background seen in untransfected and IR^{A1030} cells was subtracted. These results indicate that substantial metabolic and growth responses are possible with receptors which have only two of the three potential regulatory autophosphorylation sites. Although autophosphorylation of *single-Phe* mutants was decreased to a greater extent than could be accounted for by loss of a single phosphorylation site and maximum tyrosine kinase activity averaged <50% of wild-type, insulin-stimulated kinase activity remained approximately 70% of wild-type. Thus trisphosphorylation of receptors is not essential for signal transduction, even though it is required for full receptor kinase activation and maximal biological response (9). This finding is consistent with our previous demonstration that only a small fraction of receptors achieve the tris-phosphorylated, fully activated state in normal cells (9), and suggests that insulin receptor signaling maybe modulated by the degree of receptor autophosphorylation. Interesting all three *single-Phe* mutants exhibited markedly impaired insulin internalization, indicating that normal phosphorylation is required for receptor internalization in these cells.

Cells expressing receptors with each of the *double-Phe* mutations showed little or no increase in responsiveness to insulin relative to CHO-Neo or IR^{A1030} cells in either metabolic or growth effects, or insulin internalization. As a group, metabolic and growth responses of the *double-Phe* mutants at 0.1 nM insulin averaged 6 and 15% of wild-type values, respectively, broadly in line with the tyrosine kinase activity in these mutants (45). This is contrast with the previous study by Debant and co-workers (22), who reported normal mitogenic signaling by receptors with the double mutation $IR^{F1162,1163}$, despite defective acute metabolic responses and internalization (43, 45). In this study the $IR^{F1162,1163}$ mutant displayed an elevated basal kinase activity which was not increased by insulin *in vitro* (46), although there was evidence of insulin stimulation *in vivo* (47). In our hands, $IR^{F1162,1163}$ cells consistently showed a marked impairment in both insulin-stimulated tyrosine phosphorylation and both metabolic and growth responses. The exact cause of this discrepancy is not clear.

Receptors in which all 3 tyrosines were replaced by either phenylalanine or serine displayed no significant responses to insulin relative to the kinase-inactive IR^{A1030}. The absence of responses which we observed with *triple-Phe* and *triple-Ser* receptors is in line with the lack of significant insulin-stimulated tyrosine kinase activity and autophosphorylation in these mutants (48), but is in contrast to studies (25, 32) that claimed significant activity of receptors with the *triple-Phe* mutation. Whether these mutant receptors could mediate any other biological responses of insulin remains to be determined.

Polyclonal and monoclonal antibodies to the insulin receptor initiate a wide range of insulin-like bioeffects in a variety of cell types (27–33). Some reports have suggested that the metabolic effects produced by these antibodies can occur without activation of the receptor kinase (30–33). In the present study, we found that the anti-receptor monoclonal antibody 83-14 showed a similar concentration dependence to insulin for receptor binding and growth stimulation but was significantly less potent in stimulating insulin receptor kinase activity and eliciting metabolic stimulation in CHO-HIRc cells. This is consistent with several studies which indicate that anti-receptor antibodies do stimulate receptor autophosphorylation and activate the kinase in intact cells, albeit more slowly and to a lesser extent than insulin (49–51). The lack of spare receptors for the antibody-induced metabolic response (but not the growth response) and the difference in the extent and time course of kinase activation by antibody and insulin suggest some differences in mechanism of action of antibody and insulin (50, 51). The possible role of differential receptor aggregation and/or conformational changes induced by the two types of ligands needs additional study.

The assessment of antibody-induced metabolic and growth responses of mutant receptors was facilitated by the fact that the anti-receptor antibody used was human-specific (35), and unlike insulin elicited no responses via endogenous hamster insulin receptors or insulin-like growth factor receptors, as indicated by the lack of stimulation in the CHO-Neo cells. Thus, cells expressing mutant receptors generally appeared more defective with antibody than with insulin for stimulation of glycogen synthesis, both in terms of sensitivity and maximal response. This largely reflects the weaker stimulation of receptor tyrosine kinase and the lack of spare receptors for this effect of antibody. Surprisingly, a small, but consistent, response to antibody was observed in kinase-inactive IR^{A1030} cells. Although this was less than 20% of the response in CHO-HIRc cells, it raises the question as to whether it is possible to have a weak effect of antibody which may not require receptor kinase activity. Alternatively, the antibody may react with mutant receptors which are combined with normal hamster receptors in hybrid structures. Such hybrids between human and endogenous receptors have been demonstrated in transfected cell lines (52, 53). It has been shown that a kinase-inactive IR^{A1030} mutant incorporated *in vitro* into a hybrid with wild-type receptors is transdominant with respect to activation by insulin (54), but it may be competent to produce a small signal upon aggregation by antibody. In addition, single clones of each cell line were used which may involve some variation in responsiveness due to the individual cell background.

All of the *single-Phe* mutants and the *double-Phe* $IR^{F1162,1163}$ showed a greater metabolic responses to antibody stimulation than did IR^{A1030}, but were considerably less active than wild-type receptors. We could not confirm a previous report (34) that anti-receptor antibody elicited a normal response by cross-linking of $IR^{F1162,1163}$ receptors, which insulin was less active. The responses of $IR^{F1158,1162}$ and $IR^{F1158,1162,1163}$ to antibody were only very slightly greater than those of the kinase-inactive IR^{A1030}. The relative metabolic responses of tyrosine-mutant receptors to antibody thus broadly paralleled the responses to insulin in qualitative terms, consistent with a

requirement for tyrosine kinase activity which was more effectively stimulated by insulin than by antibody in the short term.

In contrast to the situation for metabolic stimulation, mutant receptors were more responsive to antibody than to insulin for stimulation of DNA synthesis. The *single-Phe* mutants all showed antibody-stimulated thymidine uptake identical to wild-type receptor, and the *double-Phe* and *triple-Phe* mutants also mediated a substantial effect of antibody on DNA synthesis. These responses were surprising in view of the low levels of antibody stimulation of receptor tyrosine kinase in mutant receptors. The apparently greater responsiveness of these receptors to antibody than to insulin is in part a reflection of the absence of background response from endogenous CHO receptors, although this is unlikely to be the sole explanation for the difference. The possibility cannot be ruled out that antibody is able to elicit significant activity from mutant receptors by mechanisms which do not depend directly on kinase activity, but which are modulated in extent by the kinase activity. It is also possible that antibody induces signal activation in the autophosphorylation-defective receptors by virtue of persistent occupancy, inasmuch as it is likely that antibody, unlike insulin, will not dissociate from the receptor at the pH encountered in the endosomal system. Again, additional complexity may arise from the ability of antibodies to cross-link receptors and from the presence of hybrids between the human and endogenous hamster receptors (52–54).

In summary, we have shown that insulin receptors lacking any single autophosphorylation site still exhibit substantial capacity to mediate effects of both insulin and anti-receptor antibody on glycogen and DNA synthesis. However, the same mutants are clearly defective in their ability to internalize insulin. Loss of additional autophosphorylation sites results in more severe impairment of receptor function. It is uncertain what the activity of a hybrid receptor which incorporating a tyrosine-mutant half and wild-type half will be, and whether this might differ for insulin and antibody stimulation. It is therefore difficult to predict the possible contribution of hybrid receptors to metabolic and growth responses. We conclude that although tris-phosphorylation of the kinase domain is essential for full kinase activation and receptor internalization, it is not obligatory for signaling metabolic or growth effects. Insulin stimulation and the modulatory effect of the overall level of receptor phosphorylation on tyrosine kinase activity are nevertheless major determinants of receptor function. The present data do not provide clear evidence for specific roles of individual phosphorylation sites in divergent signaling pathways, although some small differences in signaling of the two major insulin action pathways does occur with the IR^{F1158} receptor. It remains possible that the differences between metabolic and growth responses are dependent on the extent of kinase activation. A role for receptor kinase activity in mediating or modulating both metabolic and growth effects of insulin appears clear.

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