THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 23, Issue of June 10, pp. 16242-16246, 1994
Printed in U.S.A.

Internalization of the Constitutively Active Arginine 1152 → Glutamine Insulin Receptor Occurs Independently of Insulin at an Accelerated Rate*

(Received for publication, February 2, 1994, and in revised form, March 18, 1994)

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Signals controlling the insulin receptor endocytotic pathway have been investigated using the R1152Q insulin receptor mutant (M). This mutant receptor exhibits high levels of insulin-independent kinase activity, impaired autophosphorylation, and lack of an insulin stimulatory effect on both auto- and substrate phosphorylation. NIH-3T3 fibroblasts expressing M receptors displayed a 2.5-fold higher ^{125}I-insulin internalization rate than wild type (WT) but lacked insulin-induced receptor internalization and down-regulation. Cell surface recycling of internalized receptors also occurred at a higher rate in M cells and was unaffected by insulin. Cell preincubation with 35 mM Tris, which inhibits the insulin receptor degradative route, elicited no effect on M receptor recycling but inhibited that of WT by 40%. In contrast, the energy depleter 2,4-dinitrophenol, which inhibits normal insulin receptor retroendocytosis, impaired M receptor recycling 4-fold more effectively than that of WT. The release of internalized intact ^{125}I-insulin was 6-fold greater in M than in WT fibroblasts and was almost completely inhibited by dinitrophenol, whereas insulin degradation by M cells was 4-fold decreased as compared with WT. Thus, internalization and recycling of the constitutively active Gln^{1152} receptor kinase occur in the absence of autophosphorylation. However, tyrosine phosphorylation appears to be required for proper sorting of endocytosed insulin receptors.

Insulin receptor internalization and intracellular itinerary play a key role in controlling the biological action of insulin (1–3). Although constitutive, non-ligand-dependent internalization and turnover also occur (3–5), most intact insulin receptors enter the cells upon insulin binding and kinase activation (3, 4, 6). Kinetic arguments and studies with inhibitors indicated that, at least in adipocytes, a sorting mechanism partitions incoming receptors into either one of two major intracellular routes (7–9). First, a degradative route leads to intracellular catabolism and release of degraded ligand in addition to returning the majority (70–80% in adipocytes) of internalized receptors at the cell surface. Second, a retroendocytotic pathway determines the release of intact insulin as well as further receptor recycling.

The signals in the insulin receptor which control and specify its endocytotic pathway are still unclear. The iuxtamembrane domain of the receptor has been found to contain sequences (Gly-Pro-Leu-Tyr and to a lesser extent Asn-Pro-Gln-Tyr) that are necessary for normal endocytosis (10, 11). Unlike the low density lipoprotein and transferrin receptors, however, these sequences are not sufficient for endocytosis (3). Intact receptor tyrosine kinase activity is also required for autophosphorylation and normal endocytosis (12, 14). Thus, ATP-binding mutants of the insulin receptor do not undergo autophosphorylation or endocytosis (5, 15, 16). Whether this requirement depends on the fact that autophosphorylation itself is a signal directing receptor routing, whether kinase-dependent events different from autophosphorylation are more relevant, and whether both events are important in specifying separate steps in receptor itinerary across the cell are currently unsettled.

In the present work, we have addressed this problem by studying the internalization and intracellular processing of the Gln^{1152} mutant insulin receptor. As reported previously (17, 18), this receptor exhibits high levels of insulin-independent kinase activity but does not autophosphorylate. This allowed us to study independently the role of autophosphorylation and kinase activity in controlling different steps of the receptor endocytotic pathway.

EXPERIMENTAL PROCEDURES

Materials—Construction of the Gln^{1152} insulin receptor mutant and cells overexpressing the wild type insulin receptor (WT_2[1] clone) and the Gln^{1152} receptor (M_1 and M_2 clones) have been described previously (17). Cell culture media were purchased from Life Technologies Inc. ^{125}I-Insulin was from Amersham (Milano, Italy), and all other materials were from Sigma (Milano, Italy).

Insulin Internalization—Confluent monolayers were washed three times with 5.0 ml of washing buffer (100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 15 mM CH_3COONa, 10 mM glucose, 1% bovine serum albumin, pH 7.4) and incubated at 4 °C for 4 h in 3.0 ml of the same buffer containing 30 pM ^{125}I-insulin. Unlabeled ligand was removed by rinsing monolayers with ice-cold washing buffer, and cells were incubated further with washing buffer at 37 °C for the indicated

* This work was supported in part by the Progetto Finalizzato FATMA, the Progetto Finalizzato Biotecnologie e Biostrumentazione, the Progetto Finalizzato Invecchiamento of the Consiglio Nazionale delle Ricerche, and by the Exchange Program Between Italian Scientists and the National Institutes of Health funded by the Italian Departement of Education (Ministero della Ricerca). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a travel grant from the Associazione Leonardo di Capua.

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[1] The abbreviations used are: WT, wild type; M, R1152Q insulin receptor mutant; DNP, 2,4-dinitrophenol.

times. At each time point studied, the incubation medium was removed, and the cells were washed three times with ice-cold phosphate-buffered saline, 1% bovine serum albumin, pH 3.0, and twice more with washing buffer. Control experiments showed that this procedure removed >95% of surface-bound ligand at the end of the 4-h incubation at 4 °C in both the WT and the mutant receptor cells. Cells were then solubilized with 1.0 ml of 1 N NaOH at 37 °C. Acid washes and solubilized cells were counted in a γ-counter. Specific internalization rates represent the slope of the line relating internalized to surface-bound insulin and were determined as described previously (22).

Insulin Binding and Receptor Down-regulation—The insulin binding assay was performed as described in (15). Radioactivity remaining bound in the presence of 1 μM unlabeled insulin was considered nonspecifically bound and was subtracted from the total bound radioactivity to yield specific binding. The assays for short and long term receptor down-regulation were performed according to the method described in (24). Briefly, confluent monolayers were incubated at 37 °C in serum-free Dulbecco's modified Eagle's medium containing 1% bovine serum albumin, 10 mM HEPES, with or without 100 nM insulin. Surface-bound insulin was removed by acid washing as described above followed by two additional washes with phosphate-buffered saline. The insulin binding activity of the cells was then determined by incubation with binding buffer containing ^{125}I-insulin for 4 h at 14 °C. For long term (14 h) down-regulation studies an identical procedure was adopted except that down-regulation was performed in medium supplemented with 5% serum.

Receptor Internalization—The assay for receptor internalization was performed according to the method of McClain *et al.* (16). Cells were incubated with 100 nM insulin at 37 °C for the indicated times and acid washed as described above. Cells to be used for quantification of the total receptor pool were solubilized in 600 μl of solubilization buffer (16). Cells to be used for quantification of intracellular receptors were treated with 1 mg/ml trypsin at 4 °C for 15 min and solubilized. This treatment inactivates > 98% of cell surface WT and mutant receptors. ^{25}I-Insulin binding to the solubilized receptors was assayed as described in (16) and analyzed according to Scatchard (25).

Receptor Recycling—Receptor recycling was assayed as described in (21). Briefly, cells were incubated with 100 nM insulin at 37 °C for 1 h, acid washed, and then treated with trypsin as described above. Upon further rinsing with washing buffer, cells were incubated for the indicated times at 37 °C in binding buffer (15), acid washed three more times, rinsed with washing buffer, and finally assayed for ^{125}I-insulin binding as described above.

Preloading of Radiolabeled Insulin into the Cells and Measurement of Release and Composition—Cells were exposed to 2 ng/ml ^{125}I-insulin for 30 min at 37 °C and then rapidly cooled to 4 °C to prevent further ligand uptake and processing. Extracellular and surface-bound insulin was removed by repeated acid washes as described above, and cells were warmed and incubated in binding buffer at 37 °C. After the indicated times, aliquots of the incubation medium were removed to determine the amount of ^{125}I-insulin released from the cells. Degraded insulin was assessed by determining the trichloroacetic acid solubility of ^{125}I-insulin products, whereas the radioactive material precipitable in 10% trichloroacetic acid was considered intact insulin (9).

RESULTS

Internalization of the Gln^{1152} *Insulin Receptor*—Gln^{1152} insulin receptor internalization was studied in M_1 and M_2 fibroblasts. These cell clones (1.2 and 2.3 × 10^4 Gln^{1152} surface receptors/cell, respectively), the WT receptor expressing clone (WT_2; 2.5 × 10^4 surface receptors/cell), and the autophosphorylation-defective but constitutively active Gln^{1152} receptor kinase have been described elsewhere (17, 18). Initially, internalization of insulin was analyzed. The cells were exposed to a tracer concentration (0.03 nM) of ^{125}I-insulin at 37 °C, and cell-associated radioactivity was quantified upon acid wash. As shown in Fig. 1 (*upper panel*), cells expressing Gln^{1152} receptors progressively internalized ^{125}I-insulin so that by 40 min of incubation, 60% of the cell-associated radioactive material was inside the cells. Insulin appeared more extensively internalized in M_1 and M_2 fibroblasts than in cells expressing a similar number of WT receptors (WT_2). Also, the initial internalization rate was 2.5-fold higher in cells expressing the mutant receptors (Fig. 1, *lower panel*).

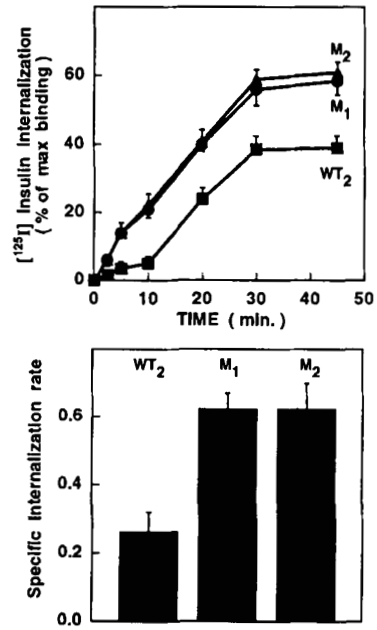

FIG. 1. **^{125}I-Insulin internalization in NIH-3T3-transfected cells.** *Upper panel*, cells expressing WT or Gln^{1152} (M_1, M_2) receptors were preincubated for 4 h at 4 °C with labeled insulin (^{125}I-insulin surface binding to WT_2, M_2, and M_1 cells was 910 ± 50, 870 ± 30, and 500 ± 45 dpm/mg of protein, respectively) and were subsequently warmed at 37 °C for the indicated times. Internalization of insulin was quantified at each time point as described under "Experimental Procedures." *Lower panel*, specific internalization rates were determined from the slope of the line relating internalized to surface-bound insulin at early times after warming the cells (1–10 min) as described in (22). All results are expressed as the mean ± S.D. of four duplicate experiments.

To investigate more directly the internalization of Gln^{1152} receptors, down-regulation studies were performed. As shown in Fig. 2 (*upper panel*), preincubation of the two clones expressing the Gln^{1152} receptors with 100 nM insulin for up to 14 h did not result in any significant loss in cell surface receptors. At variance, preincubation of WT cells for 0.5, 1, and 14 h reduced subsequent ^{125}I-insulin binding by 19, 25, and 28%, respectively. Also, in WT cells, a rapid increase in trypsin-resistant binding activity (intracellular receptors) was observed upon the addition of insulin, reaching maximum levels at 20–30 min (Fig. 2, *lower panel*). Based on Scatchard analysis of binding data (25), the number of intracellular receptors increased from 3.0 to 10.5 × 10^3, *i.e.* from 11 to 37% of the total receptor pool (surface plus intracellular receptors) (Table I). In agreement with down-regulation experiments, no significant change in intracellular receptors was measurable at any time upon insulin stimulation of the mutant clones (Fig. 2, *lower panel*). In these cells, intracellular receptors represented approximately 35% of the total receptor pool (Table I). It appeared therefore that, although unable to feature increased internalization in response to the ligand, Gln^{1152} receptors were capable of internalizing insulin more efficiently than WT.

Intracellular Routing of the Gln^{1152} *Insulin Receptor*—These intriguing observations could be explained by postulating that, in addition to the high levels of insulin-independent kinase activity, Gln^{1152} receptors also exhibit constitutively accelerated internalization. In this case, to maintain a constant cell surface receptor complement, the Gln^{1152} receptor would also be expected to feature constitutively accelerated cell surface recycling. To address this issue, cells were first exposed to trypsin to degrade surface receptors, and the reappearance of insulin binding activity (receptor recycling) was then quantified over the time. In the absence of insulin, this occurred at a slow rate with the WT cells. Only about 10% of the initial binding (*i.e.*

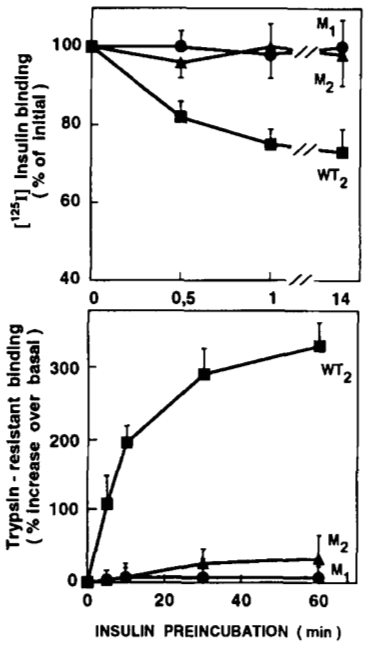

FIG. 2. **Insulin-induced receptor down-regulation and internalization in transfected cells.** *Upper panel*, WT_1, M_1, and M_2 cells were incubated with 100 nM insulin for the indicated times and then washed extensively to remove all free and bound insulin. Specific ^{125}I-insulin binding was then determined as described under "Experimental Procedures." *Lower panel*, cells were incubated with insulin at 37 °C for the indicated times (at this temperature ^{125}I-insulin surface binding after 30 min of incubation with the labeled alone was 930 ± 60, 890 ± 70, and 480 ± 80 dpm/mg of protein in WT_2, M_2 and M_1 cells, respectively) and then trypsin treated for 10 min at 4 °C. Upon Triton X-100 solubilization, trypsin-resistant binding (intracellular receptors) was quantified by determining ^{125}I-insulin binding as described under "Experimental Procedures." Basal binding indicates intracellular receptors at time 0. Data are the mean ± S.D. of four duplicate (*upper panel*) and three triplicate (*lower panel*) experiments.

TABLE I
Receptor distribution in WT and M cells

Cells	Total I_R[a] (× 10^{-3})	Surface I_R (× 10^{-3})	Intracellular I_R	
			Basal (× 10^{-3})	+Insulin (× 10^{-3})
WT_2	28.0	25.0	3.0	10.5
M_2	36.0	23.0	13.0	13.2
M_1	18.8	12.0	6.8	7.0

[a] I_R, insulin receptors (number/cell).

binding measured with no previous trypsin exposure) was reconstituted by incubation for 30 min at 37 °C (Fig. 3, *upper panel*). At variance, in the two mutant clones analyzed, > 60% of the initial binding was reconstituted at this time. These amounts were unchanged by preincubation of the cells with 250 μM cycloheximide for 45 min. Protein synthesis was inhibited by 99% at this concentration of cycloheximide, and no major fraction of biosynthetic insulin receptor was imported to the cell surface in this amount of time (data not shown). Preincubation of the cells with insulin for 30 min before receptor trypsin cleavage produced no change in the kinetics of mutant receptor recycling but enhanced that of the WT receptor by 3–4-fold (Fig. 3, *lower panel*). Thus, consistent with a constitutively accelerated internalization, the Gln^{1152} receptors also featured rapid and insulin-independent plasma membrane recycling.

Upon entering cells, normal insulin receptors return to the plasma membrane mainly through a Tris-inhibitable pathway.

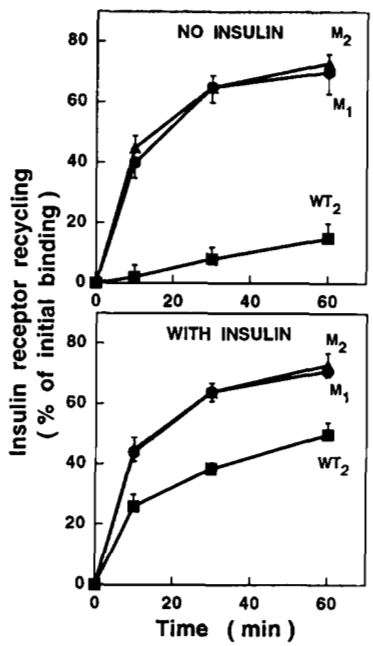

FIG. 3. **Cell surface recycling of the Gln^{1152} insulin receptor.** WT_1, M_1, and M_2 cells were preincubated with (*lower panel*) or without (*upper panel*) insulin for 30 min at 37 °C, cooled at 4 °C, acid washed to remove extracellular and receptor-bound insulin, and trypsin treated for a further 10 min. Cells were then warmed at 37 °C for the indicated times, and the reappearance of ^{125}I-insulin binding was measured at each time point. The initial insulin binding was determined on control cells maintained at 37 °C and not treated further. Results are expressed as the mean ± S.D. of four duplicate experiments.

Smaller amounts of internalized receptor are also rapidly recycled through an energy- dependent retroendocytotic mechanism (7–9). Whether the same intracellular routing is involved in returning Gln^{1152} receptors to the cell surface was investigated by performing recycling experiments in the presence of Tris or the energy depleter 2,4-dinitrophenol (DNP). As shown in Fig. 4, very little WT receptor returns to the plasma membrane in the absence of insulin-induced internalization (*bars A–C*). This small amount was not significantly changed by either one of the two inhibitors. Consistent with previous findings (7–9), insulin-stimulated receptor recycling was inhibited by 40% when insulin exposure was followed by incubation with Tris (*bars D* and *E*). DNP incubation of the cells following exposure to insulin inhibited WT receptor recycling by no more than 15% (*bar F*). Different from the WT receptor however, both insulin-dependent and -independent Gln^{1152} receptor recycling were not inhibited by Tris, whereas DNP produced 40–70% inhibition (Fig. 4; *middle* and *bottom panels*). Thus, DNP-inhibited retroendocytosis appeared to be involved more extensively in recycling Gln^{1152} than WT internalized insulin receptors, whereas the normal routing through the Tris-sensitive dissociative pathway appeared to be skipped by the mutant receptor.

Insulin Release and Degradation by Gln^{1152} Cells—Preferential involvement of DNP-sensitive retroendocytosis in recycling Gln^{1152} receptors has been more extensively investigated by analyzing the release of degraded and intact insulin by mutant cells. Normal insulin receptor retroendocytosis releases intact insulin at the cell surface in addition to recycling receptors, whereas routing insulin receptors through the Tris-sensitive mechanism also leads to intracellular insulin catabolism and release of degraded products. Thus, if the mutant receptor preferentially recycled through retroendocytosis, one would also predict more extensive release of intact insulin in cells expressing Gln^{1152} than WT receptors. As shown in Fig. 5

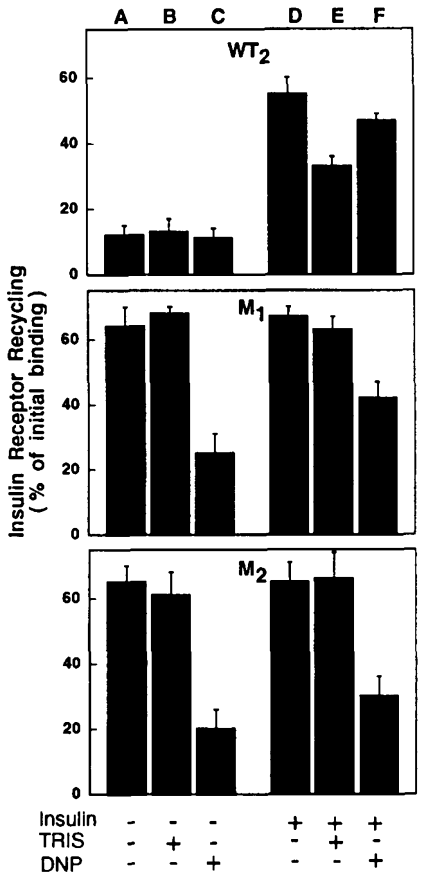

FIG. 4. **Effect of Tris and DNP on insulin receptor recycling.** WT_1, M_1, and M_2 cells were exposed to insulin for 30 min at 37 °C and then incubated with 35 mM Tris or 2 mM DNP for an additional 15 and 5 min, respectively. Cell surface recycling of the insulin receptors was then quantified as described in the legend to Fig. 3. The results are expressed as the mean ± S.D. of four triplicate experiments.

(*lower panel*), trichloroacetic acid-precipitable intact ^{125}I-insulin was more rapidly released into the medium after being internalized in mutant than in the WT cells. After 60 min the total amount of insulin released by the mutant cells was 5-fold greater than normal. By contrast, insulin degradation by WT cells was 4–5-fold less than by the mutants (Fig. 5, *lower panel*). As was the case for receptor recycling, native insulin release by mutant cells was largely inhibited by DNP (Fig. 6). No such effect was measured in WT cells. Thus, once entered into the cells, Gln^{1152} receptor-bound insulin seemed to skip the degradative pathway and to be largely returned to the cell exterior by DNP-inhibitable retroendocytosis.

DISCUSSION

Internalization sequences in the immediate submembranous domain of the insulin receptor appear to be necessary for endocytosis (10, 11), but neither their simple presence nor their exposure within the cell is sufficient for signaling internalization (3, 19). In a wide variety of cell types, kinase activity is also necessary for allowing insulin receptor to enter into the cells (5, 12, 14–16). In addition, the role of kinase activity in insulin receptor routing across the cell is still unsettled. In previous work, uncoupling the specific role of receptor phosphorylation and kinase activation in controlling insulin receptor internalization and intracellular itinerary has been difficult because autophosphorylation and kinase activation are causally related. In the present report, we have addressed this issue by using the naturally occurring Gln^{1152} insulin receptor mutant which is autophosphorylation-defective but features constitutively elevated kinase activity (17, 18).

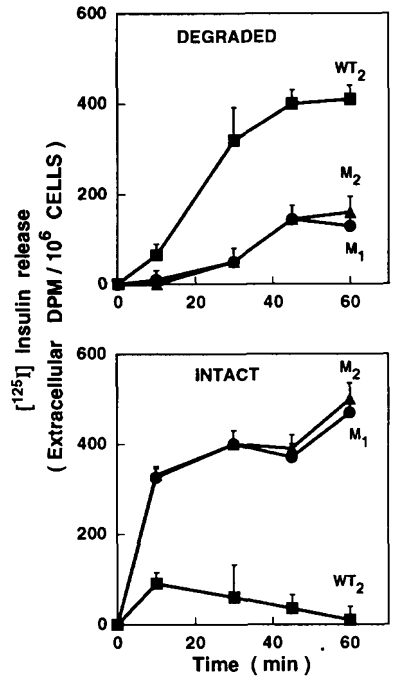

FIG. 5. **Release of degraded and intact insulin by transfected cells.** ^{125}I-Insulin was preloaded into the cell interior by exposing cells to 2 ng/ml ^{125}I-insulin for 30 min at 37 °C. After acid wash of the cells at 4 °C to remove extracellular and surface-bound insulin, cells were warmed at 37 °C, and aliquots of medium were taken at the indicated times to determine the composition of released radioactivity. Trichloroacetic acid-precipitable radioactivity was taken as representative of intact ^{125}I-insulin released into the medium; trichloroacetic acid-soluble radioactivity represented degraded insulin. Released insulin, either intact or degraded, is normalized per 10^6 cells and plotted as dpm. Results are expressed as the mean ± S.D. of three duplicate experiments.

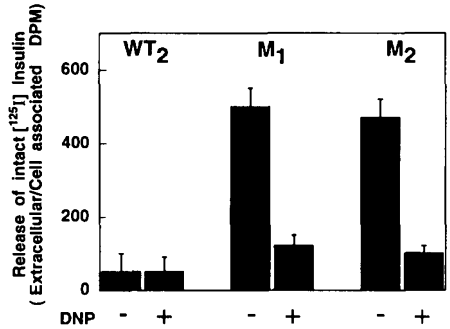

FIG. 6. **Effect of DNP on the release of intact insulin by Gln^{1152} cells.** Cells were incubated with ^{125}I-insulin for 30 min at 37 °C and then treated with 2 mM DNP for an additional 5 min. The release of intact insulin by the cells was then determined as described in the legend to Fig. 5. Results are expressed as the mean ± S.D. of three duplicate experiments.

We have shown that the Gln^{1152} insulin receptor internalizes insulin and recycles to the cell surface faster than insulin-unstimulated WT receptors. In agreement with previous studies (10, 20), these findings indicate that phosphorylated receptor tyrosines need not be recognized by the cell machinery for driving receptor internalization. Although most cells use a coated pit-dependent mechanism to internalize insulin receptors (3, 19), pharmacologic and kinetic evidence support the existence of more than one pathway of internalization (23). Therefore, one might speculate that Gln^{1152} and WT receptors enter into the cells through different ports. This is a less likely possibility, however, since treatments that disrupt insulin receptor internalization via coated pits (2) impaired insulin in-

ternalization by mutant and WT cells to a similar extent (data not shown).

Coincident with the constitutive kinase activity, the Gln^{1152} insulin receptor internalization and recycling also were independent on insulin. The Gln^{1152} substitution might have induced a conformational change in the receptor which mimics that induced by ligand binding and autophosphorylation and is necessary to expose the internalization domain of the normal insulin receptor. Alternatively, as suggested by Carpentier *et al.* (19), kinase-dependent events in the receptor signaling cascade other than autophosphorylation, such as phosphorylation of proteins in the endocytotic machinery, may be necessary to allow receptor concentration into the coated pits.

In addition to its ability to enter into the cells and to be recycled independently of insulin, the Gln^{1152} insulin receptor did not follow a normal intracellular itinerary to return to the cell surface. The above conclusion is based on the observation that Tris, which inhibits normal insulin receptor recycling through the degradative pathway (7–9), had no effect on the Gln^{1152} insulin receptor recycling. On the other hand, DNP, which inhibits insulin receptor retroendocytosis (7–9), had little effect on WT receptor and intact insulin recycling while quenching both the mutant recycling and the mutant-mediated release of intact insulin by the cell. Thus, once entered into the cells, the autophosphorylation-defective but kinase-competent Gln^{1152} insulin receptors were largely retroendocytosed rather than sorted into the dissociative compartment that initiates recycling of most normal insulin receptors to the cell surface (7–9). The argument might be raised that because mutant receptors are undergoing constitutive endocytosis the normal intracellular route has already been saturated at the base line, accounting for the spillover use of the retroendocytotic pathway. However, the higher specific internalization rates of mutant receptors, the lack of down-regulation, and the fact that the cells express relatively few receptors argue against this possibility. In addition, raising the concentrations of insulin to which the mutant cells are exposed up to 10-fold proportionally increased both the amount of that released intact and that degraded (not shown). In the adipocytes, increasing the rate of entry of insulin-receptor complexes with maximally effective concentrations of insulin diverted part of the complexes from the degradative to the retroendocytotic pathway and led to a 13% increase in the amount of insulin released intact and a similar sized decrease in that degraded (8). Therefore, the constitutive increase in the Gln^{1152} receptor internalization rate cannot completely account for such a large increase in the release of intact insulin (5-fold) and decrease in that of degraded insulin (4-fold) as that observed in the mutant cells.

Previous studies by Marshall *et al.* (9) have shown that treatment of cells with the specific tyrosine phosphatase inhibitor vanadate, at concentrations that maximally increase receptor tyrosine phosphorylation, shunted incoming receptors from a retroendocytotic to a degradative pathway. In agreement with these findings, the data in the present report indicate that the autophosphorylation-defective but internalizing Gln^{1152} insulin receptor undergoes an opposite sorting alteration. Thus, tyrosine phosphorylation appears to be relevant in specifying the intracellular routing of incoming insulin receptors.

In conclusion, although kinase activity is required for the insulin receptor internalization, tyrosine phosphorylation may be a signal that directs its further intracellular itinerary.

Acknowledgments—We are grateful to Drs. E. Consiglio and G. Salvatore for continuous support during the course of this work. We also thank Dr. S. M. Aloj for a critical reading of the manuscript.

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