We showed previously that upon insulin stimulation of an insulin receptor overexpressing cell line, most of the p21ras was rapidly converted into the GTP bound state (Burgering, B. M. T., Medema, R. H., Maassen, J. A., Van de Wetering, M. L., Van der Eb, A. J., McCormick, F., and Bos, J. L. (1991) EMBO J. 10, 1103–1109). To determine whether this process also occurs in cells expressing physiologically relevant numbers of insulin receptors, insulin stimulated Ras-GTP formation was quantitated in Chinese hamster ovary (CHO)-derived cell lines expressing varying numbers of insulin receptors. In the parental CHO9 cells, expressing only 5.10⁴ insulin receptors, insulin stimulation for 3 min increased Ras-GTP levels with 10%. Upon increasing the number of insulin receptors in these cells, Ras-GTP levels increased almost proportionally until a plateau value of 60% is reached at high receptor numbers. These data show that receptor overexpression is not a prerequisite for insulin-stimulated Ras-GTP formation. The yield of Ras-GTP generated is 0.2–1.0 mol/mol autoprophosphorylated insulin receptor in CHO9- and NIH3T3-derived cell lines, respectively. These values argue against signal-amplifying processes between the insulin receptor and p21ras.

To determine whether receptor autophosphorylation is required for Ras-GTP formation, NIH3T3 cells overexpressing insulin receptors were stimulated with a monoclonal antibody which activates the receptor and subsequent glucose transport without inducing detectable autophosphorylation. Also, CHO cells expressing the mutant Ser¹²⁰⁰ receptor, which has markedly impaired tyrosyl autophosphorylation but is capable of mediating insulin-stimulated metabolic effects in CHO cells, were used. In both cases, no Ras-GTP formation was observed.

Furthermore, Rat-1-derived cell lines expressing mutant p21ras, which is permanently in the active GTP-bound form, still responded to insulin by increasing the glucose uptake. These results support our hypothesis that Ras-GTP formation is activated by the tyrosyl-phosphorylated insulin receptor and suggest that an active Ras-GTP complex does not mediate metabolic signaling.

The insulin receptor is a member of the large family of receptors with tyrosine kinase activity (1). These receptors induce, in general, mitogenic responses (2). In addition, insulin receptors stimulate metabolic processes like the synthesis of glycogen and protein and the transport of glucose and some amino acids across the plasma membrane (3–6).

The post-receptor processes mediating the signals from tyrosine-kinase receptors to internal targets are largely unknown, although recently some proteins have been identified which associate with these receptors. These proteins include phosphatidylinositol 3-kinase (7–10), GAP¹ (11), and phospholipase C-γ (12). In addition, some of these proteins are phosphorylated on tyrosine residues upon receptor activation (11–13). These proteins may bind to the phototyrosiner moiety of the activated receptors via SH2 domains (src homology region 2) in their structures.

The p21ras proteins belong to the family of small GTP-binding proteins that cycle between an active GTP-bound conformation and an inactive GDP-bound conformation (14). The proteins play a role in signal transduction, although the extracellular factor(s) that can activate p21ras were until recently unknown. Using cells overexpressing normal Hras, we obtained evidence that insulin might signal through p21ras to induce gene expression and mitogenicity (15). Recently, we showed that in NIH3T3 cells overexpressing the human insulin receptor, insulin can induce a rapid accumulation of the GTP-bound conformation of the ras protein (16). Furthermore, we showed that insulin-induced gene expression, i.e. the fos promoter, can be inhibited by interfering mutants of the ras protein (17), indicating that the ras protein mediates insulin-induced gene expression.

These results were obtained with cells having non-physiological high levels of insulin receptor. In this study we have investigated the relationship between insulin receptor numbers/cell and the activation of p21ras.

To gain insight whether a signal-amplifying process operates between the insulin receptor and the actual conversion of p21ras into the GTP-bound state, the amount of Ras-GTP was quantitated in relation to the number of activated insulin receptors.

As Ras-GTP is thought to be active in mitogenic signaling whereas insulin stimulates both metabolic and mitogenic effects in cells, we have additionally studied the involvement of

*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 1754 solely to indicate this fact.

¹To whom correspondence should be addressed: Lab. of Protein Synthesis and Hormone Regulation, Sylvis Laboratory, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. Tel.: 31-71-276127; Fax: 31-71-276125.

* The abbreviations used are: GAP, GTPase activating protein; CHO, Chinese hamster ovary; 2-DOG, 2-deoxyglucose; DMEM, Dulbecco's modified eagle medium; EC₅₀, half effective concentration; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

14647
**Experimental Procedures**

**Materials**—All chemicals used were analytical grade. Tissue culture media, sera, G418, and metotrexate were from Gibco. Phosphate-free DMEM and bovine insulin were from Sigma. Bovine serum albumin (RIA grade, insulin free) was from United States Biochemical Corp. Control monoclonal antibody KT3 (anti-SV40 large T), rabbit polyclonal antibody anti-rat IgG, and the anti-p21ras monoclonal antibody, Y13-259, were kindly provided by Dr. A. Zanetta (this department). The anti-insulin receptor monoclonal antibody MA-5, [32P]orthophosphate (carrier free) and [3H]2-deoxyglucose were from Amersham. Polyethyleneimine-cellulose F plates were purchased from Merck and Protein G-Sepharose 4 fast flow beads were from Pharmacia.

**Cell Lines**—Clonal cell lines expressing different numbers of insulin receptors were made by transfecting NIH3T3 and CH09 cells with a plasmid containing the human wild type insulin receptor cDNA (18) under control of the SV40 early promoter. The vector contained also a neomycin resistance genes for selection and a dihydrofolate reductase gene for amplification. By stepwise amplification, a panel of CHO-cell lines (IR25-IR800) was constructed expressing increasing insulin receptor numbers. Transfection of NIH3T3 cells yielded the A14 cell line. The cell lines were maintained in DMEM, 9% FCS (dialyzed against phosphate-buffered saline), 25 mM metotrexate. CH09 cell lines IR25, and CL-1 expressing approximately 1.10^6 wild type or mutant insulin receptors, and control cell line C-Neo, transfected with only the neomycin resistance gene, were prepared as previously described (19). These cells were cultured in Ham’s F-10, 9% FCS, 600 μg/ml G418.

The characteristics of the various cell lines are listed in Table 1. Quantitation of insulin-binding sites in cell lines was by Scatchard analysis of insulin binding data.

**Rate-1-derivived cell lines H9 and H13 overexpress wild type H-ras protein and RR2, RR3, and RR7 express mutant H-ras (16). They were cultured in DMEM, 9% FCS.

**Cell Labeling, Receptor Stimulation, and Phase Separation**—The procedures were essentially as described (16). Experiments were performed at least twice, two replicates/experiment. Cells were deprived of serum for 16 h, subsequently labeled for 3 h with [3H]orthophosphate (0.1-0.2 mCi/ml), and stimulated for 3 or 15 min with insulin or with monoclonal antibody MA-5 (10^-11 M) for 30 min. The cell cultures were rapidly washed and lysed in buffer containing 500 mM, and the detergent phase was used to determine p21ras-bound GTP.

**Serum-starved cells were labeled with [32P]orthophosphate (0.1-0.2 mCi/ml), and stimulated for 3 or 15 min with insulin or with monoclonal antibody MA-5 (10^-11 M) for 30 min.**

**TABLE 1**

| Parental cell line | Insulin receptor no. | Nature of insulin receptor |
|--------------------|----------------------|-----------------------------|
| A14                | NIH3T3               | WT                          |
| CH09               | NA                   | 5.10^-6                      |
| IR25               | CHO                  | 8.10^-10                     |
| IR100              | CHO                  | 1.10^-10                     |
| IR100A             | CHO                  | 1.20.10^-10                  |
| IR200              | CHO                  | 7.10^-9                      |
| IR800              | CHO                  | 1.6.10^-10                   |
| C-Neo              | CHO                  | 5.10^-6                      |
| A5                 | CHO                  | 1.3.10^-10                   |
| B5                 | CHO                  | 1.10^-10                     |
| CL-1               | CHO                  | 1.10^-10                     |

* Endogenous receptors.
* Plasmid with only the G418 resistance marker is transfected.
* Insulin receptor numbering is according to Ebina *et al.* (20).

The aqueous phase was made 1% in Triton X-100 and 500 mM in NaCl and used for receptor isolation.

**Analysis of Guanine Nucleotides Bound to p21ras**—The diluted Triton X-114 detergent phase was preclreated for 15 min with Protein G-Sepharose beads coupled to rabbit anti-rat IgG and further processed according to Burge et al. (16). In short, the supernatant was incubated for 1 h with the anti-p21ras monoclonal antibody Y13-259 or the control monoclonal antibody KT3 coupled to Protein G-Sepharose beads. The immunoprecipitates were collected and washed. GTP and GDP were eluted and separated on polyethyleneimine-cellulose F plates. GTP and GDP spots were visualized by autoradiography, and the radioactivity in GTP and GDP was quantitated by liquid scintillation counting. The values were corrected by substraction of background values obtained with the control antibody KT3 (16).

**Analysis of Insulin Receptor Autophosphorylation**—After phase separation, the aqueous phase was incubated for 16 h with an antiphosphotyrosine polyclonal antibody coupled to Protein G-Sepharose. The immunoprecipitates were collected and washed four times with 50 mM HEPES buffer, pH 7.4, 500 mM NaCl, 5 mM MgCl2, 10 mM NaF, 1 mM NaVO3, 0.1% Triton X-100, 0.005% SDS. Proteins were analyzed by reducing SDS-PAGE and autoradiography. Incorporation of [32P] into the insulin receptor β-chain was quantitated by liquid scintillation counting of corresponding gel slices. The ratio Tyr-P, Ser-P, and Thr-P in the insulin receptor β-chain after insulin stimulation was determined by phosphoamino acid analysis according to Cooper et al. (22).

**2-Deoxyglucose Uptake**—Uptake of 2-DOG was measured as described (23). In short, A14 cells were grown in 6-cm dishes to confluence. 24 h before assay 2-DOG uptake, fresh medium was added to the cells. To stimulate hexose uptake, the cells were incubated for 1 h with 0.1 μM insulin or MAb 259 coupled to Protein A at 37°C. Subsequently, [3H]2-DOG was added up to a concentration of 20 μM, and the incubation was continued for 10 min at 37°C. Free 2-DOG was washed away, and the cells were lysed in 1 ml of 0.1 M NaOH. The [3H] content was quantitated by liquid scintillation counting.

**RESULTS**

The Relation between Ras-GTP Formation and Insulin Receptor Number in CHO Cells—Clonal CHO cell lines, expressing varying numbers of insulin receptors/cell were used to determine the relationship between insulin-stimulated Ras-GTP formation and the number of insulin receptors/cell. Serum-starved cells were labeled with [32P]orthophosphate for 3 h and stimulated for 3 min with excess of insulin (4 μM). Cells were lysed and the amount of GTP and GDP bound to p21ras was determined by precipitating the p21ras-guanine nucleotide complexes with the antisera Y13-259 followed by separation of the nucleotides by thin layer chromatography. The radioactivity in the GTP and GDP spots was quantitated and used to calculate the percentage of Ras-GTP formation in the p21ras-bound guanine nucleotide pool, i.e. GTP divided by GTP+GDP. The relation between the increment in the percentage of Ras-GTP and the number of insulin receptors is given in Fig. 1.

Already at low numbers of insulin receptor, as in parental CH09 cells (5.10^6 insulin receptors/cell), Ras-GTP formation is stimulated from a basal 20% Ras-GTP to 30%. When the insulin receptor number rises a steep increase in Ras-GTP formation is reached. At very high receptor numbers (7.10^6 and 1.6.10^6), the plateau value of a maximum in Ras-GTP formation is reached.

Most of the insulin receptors in IR800 cells becomes autophosphorylated on tyrosine after 3 min of incubation with 4 μM insulin as inferred from anti-phosphotyrosine antibody immunoprecipitations followed by precipitating the remaining supernatant with anti-insulin receptor serum and subsequent analysis of the immunoprecipitates on Western blots using...
Phosphorylation in IR800 Cells—In the same experiment, the insulin concentration was determined. After stimulation with 0.4 µM insulin, the radioactivity in p21ras-bound GTP and GDP was determined, and the percentage of GTP was calculated. The GTP percentage in the absence of insulin (20%) is subtracted. The insulin-induced increase in the percentage of GTP is plotted against the number of insulin receptors/cell. Averaged data, of two independently performed experiments consisting of two replicates/experiment, are shown, and the standard deviation is less than 10% of the averaged values.

The Relation between Insulin Receptor Number, Ras-GTP Formation, and Insulin-stimulated Incorporation of Thymidine into DNA—A panel of CHO-derived cells expressing different numbers of insulin receptor, as listed in Table I, was serum starved and subsequently labeled with [3P]orthophosphate. Cells were incubated for 3 min with or without 4 µM insulin. The radioactivity in p21ras-bound GTP and GDP was determined, and the percentage of GTP was calculated. The GTP percentage in the absence of insulin (20%) is subtracted. The insulin-induced increase in the percentage of GTP is plotted against the number of insulin receptors/cell. Averaged data, of two independently performed experiments consisting of two replicates/experiment, are shown, and the standard deviation is less than 10% of the averaged values.

Remarkably, both basal- and insulin-stimulated incorporation decreases strongly whereas FCS-induced levels remain largely unchanged. When insulin stimulation is expressed as fold stimulation of unstimulated [3H]thymidine incorporation the pattern shown in the inset of Fig. 2 is observed. Initially, upon increasing the number of insulin receptors, insulin becomes more potent in increasing basal uptake whereas at higher receptor numbers, the stimulation decreases. These data indicate that the effect of insulin on [3H]thymidine incorporation is not simply related to the Ras-GTP levels in these cells after insulin stimulation.

The Relation between Insulin Concentration and Ras-GTP Formation in CHO Cells Overexpressing Insulin Receptors—The IR800 cell line, a CHO cell line expressing approximately 1.6 x 10^6 insulin receptors/cell, was serum starved and labeled with [3P]orthophosphate. Cells were stimulated for 3 min with indicated concentrations of insulin. Radioactivity in GTP bound to p21ras was determined and plotted against the insulin concentration. The experiment was performed in duplicate, two replicates/experiment (S.D. < 10%).

Serum-starved cells were 32P-labeled and incubated for 3 min with different concentrations of insulin. The amount of radioactivity in GTP and GDP bound to p21ras was determined and was plotted against the insulin concentrations. A typical dose-response curve was obtained with an EC_{50} at 6.10^{-9} M insulin (Fig. 3).

The Relation between Insulin Concentration and Receptor Phosphorylation in IR800 Cells—In the same experiment, the relation between the insulin receptor β-chain phosphorylation and insulin concentration was determined. After stimulation of IR800 cells for 3 min with increasing insulin concentrations, the phosphorylated receptor was isolated using anti-phosphotyrosine polyclonal antibody. The immunoprecipitated proteins were separated by SDS-PAGE (Fig. 4, inset), and radioactivity in the β-chain of the insulin receptor was determined. When this value was plotted against the insulin concentration, the relation shown in Fig. 4 was obtained. The EC_{50} being at 4.10^{-8} M insulin.

It should be noted that this relatively high EC_{50} is due to...
The mean value of the radioactivity was plotted against the insulin concentration. The inset shows the autoradiographs of the insulin receptor β-chain phosphorylation of cells stimulated with the following concentrations of insulin:

- lane 1: 0 M
- lane 2: 1.10^-8 M
- lane 3: 1.10^-8 M
- lane 4: 3.10^-8 M
- lane 5: 5.10^-8 M
- lane 6: 1.10^-7 M

The short (3 min) incubation time with insulin, when the incubation time is 15 min, the EC50 shifts to 1.5x10^-9 M.

Ras-GTP Does Not Mediate the Insulin-stimulated Hexose Uptake and Glycogen Synthesis—As p21ras is known to be a mitogenic signaling intermediate, we investigated whether it may also play a role in the activation of insulin-stimulated metabolic effects. Three different approaches were followed. First a panel of CHO cells (CL-1, B25, A5, Table I) with similar expression levels of wild type, mutant Ser1200, and mutant Thr134 insulin receptors, respectively, was examined for insulin stimulation of Ras-GTP formation. The Ser1200 insulin receptor has been shown to be capable of mediating metabolic signals including the activation of glucose transport but is inactive in mitogenic signaling (19). In response to insulin, no detectable tyrosyl autophosphorylation of the β-chain is apparent, although some increase in serine-threonine phosphorylation may occur as indicated by an increase in β-chain labeling in anti-insulin receptor immune precipitates which is not observed when anti-phosphotyrosine serum is used. The Thr134 mutation appears to completely inactivate insulin receptor signaling (19). Additionally, no insulin-stimulatable autophosphorylation is seen. As a control for these experiments, cells expressing only the neomycin resistance plasmid without insulin receptor cDNA (C-Neo) were used.

Upon insulin stimulation some Ras-GTP formation was detected in the mutant cell lines A5 and B25 (Fig. 7). The
observed increment in Ras-GTP formation was lower than seen in the C-Neo cells and much lower than in the CHO cell line CL-1 which expresses a similar number of wild type receptors as the cell lines A5 and B25. As the Ser'200 mutant insulin receptor in the B25 cell line can mediate metabolic signaling, the results suggest that metabolic signaling does not require Ras-GTP formation.

These findings were strengthened by the results of the second approach. The monoclonal antibody MA-5 directed against an extracellular epitope of the insulin receptor has been shown to activate metabolic signaling via the insulin receptor β-chains (24). We have tested the effect of this antibody on 2-deoxyglucose uptake, receptor tyrosine autophosphorylation, and Ras-GTP formation in A14 cells.

The antibody was active in stimulating 2-DOG uptake. However, no detectable stimulation of receptor tyrosyl autophosphorylation or Ras-GTP formation was detected in these experiments (Table II). The antibody had no effect on [3H]thymidine incorporation in CHO9 cells (results not shown).

In the third approach, we used cell lines expressing either normal levels of p21ras (i.e. the Rat-1 cell line), cell lines overexpressing H-ras (H9 and H13 cells), and cell lines expressing mutant H-ras which remains permanently in the active GTP-bound state (RR2, RR3, and RR7 cells).

Insulin stimulated 2-DOG uptake in these cell lines to a similar extent (Table III), whether there was a constitutively high expression of GTP-bound p21ras or not, again arguing against the requirement of Ras-GTP formation for stimulation of hexose uptake.

It should be noted that the basal hexose uptake in the RR2, RR3, and RR7 cells is elevated compared to the other cells (Table III) which is probably due to the increased expression of the Glut-1 glucose transporter in these cells (25).

**DISCUSSION**

We showed previously that insulin stimulation of NIH3T3-A14 cells, a cell line expressing ~7.10^6 insulin receptors/cell, results in a rapid conversion of Ras-GDP into Ras-GTP and that this step is linked to the induction of the immediate early genes c-fos, c-jun, and p53 (16). In this paper we have examined in more quantitative terms the relation between insulin receptor autophosphorylation and conversion of p21ras into the GTP-bound state.

The experiments in which CHO cell lines, expressing varying numbers of insulin receptors were used, indicate that already at physiologically relevant receptor numbers Ras-GTP formation is observed and that the number of insulin receptors determines the level of Ras-GTP formed. Even in parental CHO9 cells, insulin increases the amount of Ras-GTP from 20 to 30%. At high receptor numbers (above 7.10^6 receptors/cell), the system is saturated at 60% Ras-GTP.

When NIH3T3-A14 cells, overexpressing the insulin receptor, were stimulated with different concentrations of insulin, initially a linear relationship between Ras-GTP levels and receptor autophosphorylation was observed, with a slope of 0.4, until approximately 60% of p21ras was converted into the GTP form. Taking into account that tyrosine phosphorylation represents only 40% of the insulin-stimulated increase in radioactivity in the insulin receptor β-chain, the slope is approximately 1 when only the tyrosine phosphorylation is considered. The 32P isotopic content of the β- and γ-phosphates in nucleotide triphosphates are in rapid equilibrium by the enzymes nucleoside diphosphate kinase and adenylate kinase. The isotopic content of the α-phosphate in nucleotides is more slowly equilibrated, so the specific activity of the α-phosphate in GTP will be between zero and the values for β- and γ-phosphate (26). Insulin stimulation results in a rapid phosphorylation of 3–6 tyrosine residues/insulin receptor α-β subunit (27). Therefore, when corrected for the fraction that is tyrosyl phosphorylated, a given level of 32P in insulin receptors and GTP reflects approximately similar numbers of molecules. Thus, a slope of 1 in the relation between Ras-GTP formation and receptor tyrosyl autophosphorylation suggests that approximately 1 mol of p21ras is converted into

**Table II**

| Cell line | Increment in 2-DOG uptake |
|-----------|---------------------------|
| Rat-1     | 0.13 ± 0.02               |
| H9        | 0.18 ± 0.02               |
| H13       | 0.18 ± 0.03               |
| RR2       | 0.12 ± 0.04               |
| RR3       | 0.11 ± 0.03               |
| RR7       | 0.18 ± 0.05               |

* Rat-1, parental cell line; H9 and H13 cell lines overexpressing normal p21ras; RR2, RR3, and RR7 cell lines transformed by mutant p21ras.

**Table III**

| Control | Insulin MA-5 |
|---------|--------------|
| cpm in Ras-GTP | 57±69 | 193±194 |
| cpm in β-chain  | 294±354 | 3420±554 |
| Relative 2-DOG uptake | 1.00 (0.06) | 1.35 (0.09) |

* The increment in 2-DOG uptake by 1 μM insulin is expressed as nmol/10^6 cells and is an average of five determinations. Standard deviations are indicated. Basal uptake was approximately 0.3 nmol, except in the mutant p21ras-transformed cell lines where basal uptake was 0.6 in RR2, 0.8 in RR3, and 1.7 in RR7.
the GTP form/mol of insulin receptor, as α-β subunit. Of course, in these experiments we only determine the actual Ras-GTP level, the turnover of Ras-GTP and the phosphorylated insulin receptor is not taken into account. So the ratio of 1:1 can only be seen as an estimation.

A similar relation between receptor activation and Ras-GTP formation, as in NIH3T3-A14 cells, is seen in the CHO-IR800 cell line, although in this cell line the yield of Ras-GTP is somewhat lower, being approximately 0.2 mol of Ras-GTP/mol of autophosphorylated receptor, as α-β subunit. These findings indicate that CH09- and NIH3T3-derived cell lines may utilize non-amyli phying signaling pathways that link the insulin receptor and Ras. In addition, we have measured the relation between the insulin-stimulated receptor auto-phosphorylation and Ras-GTP formation in A14 cells at two time points, i.e. 3 and 15 min. The EC₅₀ for receptor auto-phosphorylation and Ras-GTP formation showed a parallel shift to the left when stimulated for 15 min. The yield of Ras-GTP formed per tyrosyl-autophosphorylated receptor did not change. These data indicate that hormone binding or receptor auto-phosphorylation is a rate-limiting step and not Ras-GTP generation.

The mechanism by which p21ras is converted into the GTP form is not known. The level of Ras-GTP in cells is determined by the rate of formation, via GDP-GTP exchange on Ras-GDP, and the rate of degradation of Ras-GTP, i.e. the stimulation of the intrinsic GTPase activity of the Ras protein by GAP and/or NF-1 (28-32). The tyrosine-kinase activity of the insulin receptor may regulate the activity of some of these components via phosphorylation. In this situation, we might expect an amplification of the signal as the activated receptor is likely to phosphorylate several target molecules. Another potential mechanism by which receptors with tyrosine-kinase activity may function is by autophosphorylation, followed by complex formation with proteins having SH2 domains. In such a situation, the process should depend strictly on the amount of autophosphorylated receptor and is expected to depend less on the time during which the receptor is activated.

As we observe no amplification in Ras-GTP formation, we judge that these data are more consistent with a model where Ras-GTP is stabilized within a complex rather than a model in which the activated receptor modulates the activity of a target by direct phosphorylation. One can only speculate about the nature of the components involved in such a complex formation. GAP, having SH2 domains, is an attractive candidate because it can interact with both the autophosphorylated receptor and p21ras. We have recently obtained evidence that the insulin receptor is able to form complexes with GAP under certain conditions.

By the use of CHO cell lines expressing similar levels of wild type, and mutant insulin receptors, the involvement of p21ras in insulin-stimulated hexose uptake and glycogen synthesis was investigated. The mutant insulin receptors Ser¹²⁰⁰ and Thr¹⁷⁵⁴ demonstrate markedly impaired insulin-stimulated tyrosyl autophosphorylation although some phosphorylation on serine and threonine residues occurs after insulin stimulation of the Ser¹²⁰⁰ mutant. When overexpressed in CHO cells, the Ser¹²⁰⁰ mutant receptor shows discordant signaling and is capable of mediating insulin-induced stimulation of glucose transport and glycogen synthesis but is inactive in mitogenic signaling. The Thr¹⁷⁵⁴ mutant is inactive in both metabolic and mitogenic signaling. Both mutants show no stimulation of Ras-GTP formation above the level which is generated which is sufficient for maximal stimulation of hexose uptake if Ras-GTP is in this signaling pathway and thereby preventing any additional upstream stimulation by insulin. However, insulin induces a similar increase in 2-DOG uptake in cells expressing wild type or oncogenic p21ras.

These conclusions are corroborated by the observation that the monoclonal antibody MA-5, which does activate glucose uptake and glycogen synthesis, also has no effect on the level of Ras-GTP. This antibody enhances Ser-Thr phosphorylation and may only minimally activate the tyrosyl autophosphorylation activity of the insulin receptor β-subunit (33).

We judge that these results suggest that insulin signaling bifurcates early at the post-receptor level. One way proceeds via Ras-GTP and requires receptor autophosphorylation on tyrosine, whereas in other signaling pathways, like glucose uptake, Ras-GTP is not involved.

When [³H]thymidine incorporation in CHO cell lines is investigated it is observed that the level after insulin stimulation decreases when cells express increasing numbers of insulin receptors. The fetal calf serum-induced response remains largely unchanged indicating that no large changes in cellular properties have occurred. Also, the high basal [³H]thymidine incorporation, which is characteristic of parental unstimulated CHO cells, decreases when insulin receptor number increases. These findings show that the Ras-GTP level as such is a poor predictor for indicating the magnitude of the [³H]thymidine incorporation. Studies by Cai et al. (34) have shown that ras proteins seem involved in at least two parallel mitogenic pathways, one of which depends on protein kinase C activation whereas the other seems independent. In contrast to several other receptors with a tyrosine kinase activity, the activated insulin receptor seems a poor activator of phospholipase C-γ and protein kinase C (35). Thus, in case of insulin-induced Ras-GTP formation the coactivation of PKC or other additional factors by insulin may be insufficient for obtaining a full mitogenic response.

Similar observations that Ras-GTP levels are a poor predictor for mitogenic activity of cells have been described previously, as with PC12 and REF52 cells where Ras-GTP induces differentiation and growth arrest, respectively (36, 37).

REFERENCES

1. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, L. M., Dall, T. J., Gray, A., Coussens, L., Luo, Y.-C., Tsubokawa, M., Masen, A., Seeburg, P. H., Gronfeld, C., Rosen, O. M., and Ramachandran, J. (1985) Nature 313, 756-761
2. Castled, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kappeller, R., and Soltoff, S. (1991) Cell 64, 281-292
3. Lawrence, J. C., and Lerner, J. (1979) J. Biol. Chem. 253, 2104-2113
4. Miller, D. S. (1989) J. Biol. Chem. 264, 10438-10446

7 G. J. Pronk, manuscript in preparation.
