Insulin-dependent Phosphorylation of the Insulin Receptor-Protein Kinase and Activation of Glucose Transport in 3T3-L1 Adipocytes

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Insulin stimulates hexose transport and phosphorylation of the insulin receptor in monolayer cultures of intact 3T3-L1 adipocytes. To assess the phosphorylation state of the receptor in situ, cells were equilibrated with [32P]orthophosphate and then disrupted under denaturing conditions which preserved the phosphorylation state of the receptor, as determined in the cell. The insulin receptor, isolated by lectin adsorption and two-dimensional nonreducing/reducing polyacrylamide gel electrophoresis, occurred as a single oligomeric species with an apparent $\alpha_{12}$ $\beta_{12}$ subunit composition. This oligomeric structure was not altered by treating cells with insulin. Only the $\beta$-subunit of the receptor was phosphorylated; [32P]phosphoserine and [32P]phosphotyrosine were both identified in the $\beta$-subunit from cells in the unstimulated state, but only [32P]phosphotyrosine increased in cells stimulated with insulin. Neither insulin-like growth factors I nor II stimulated insulin receptor $\beta$-subunit phosphorylation, although both activated hexose transport. Upon the addition of insulin, [32P]orthophosphate incorporated into the $\beta$-subunit increased 4.5-fold (7-fold with respect to [32P]phosphotyrosine) and was complete within 1 min ($t_{1/2} = 8$ s). Following the removal of insulin from the monolayers, [32P]subunit fell to the basal level ($t_{1/2} = 2.5$ min); there was no lag phase before either transition. The tyrosine protein kinase activity, measured in vitro with a model substrate, was higher with immunoaffinity-purified insulin receptor from insulin-stimulated cells than from cells in the basal state.

Hexose transport rate, measured using 3-O-[methyl-$^{14}$C]glucose, was half-maximally stimulated at 2 nM insulin. A 1-min latency period followed insulin addition, after which a 7-fold increase in the steady-state rate of hexose uptake was achieved within 5 min. Upon the removal of insulin, hexose transport continued at the stimulated steady-state rate for 2.5 min and then declined to the basal rate with a half-time of 8 min. These kinetic experiments in situ and protein kinase activity measurements in vitro support the hypothesis that $\beta$-subunit phosphorylation is an intermediate step linking insulin binding to the increased glucose transport rate.

Insulin stimulates a variety of cellular responses by interaction with the insulin receptor. A general characteristic of these responses is their sensitivity to insulin at concentrations well below half-maximal saturation of hormone binding. A well-documented example is the activation of glucose transport in rat adipocytes (1). In the case of cultured mouse 3T3-L1 adipocytes (2), however, the insulin dependence for acute stimulation of glucose uptake is closer to that for hormone binding, yet the magnitude of the response is comparable to that found with rat adipocytes. This mouse cell line is, therefore, a good choice to study the biochemical events that link the insulin receptor to this biological effect.

The receptor itself is an intrinsic membrane glycoprotein containing two each of the insulin-binding $\alpha$-subunit and the protein kinase $\beta$-subunit. There is evidence from other laboratories that the insulin receptor in rat adipocytes (4), IM-9 lymphocytes, or 1135 rat hepatoma cells (5, 6) occurs in multiple oligomeric forms. It was suggested that these forms could have biological significance. However, the only oligomeric form from 3T3-L1 adipocytes purified by insulin affinity chromatography was this $\alpha_{12}$ $\beta_{12}$ heterotetramer (3). Therefore, it is necessary to determine if oligomers other than the $\alpha_{12}$ $\beta_{12}$ form occur in the 3T3-L1 adipocytes.

Insulin stimulates the tyrosine-specific protein kinase activity of the $\beta$-subunit (7-12), both for autophosphorylation and substrate phosphorylation. It now appears that the former process is responsible for increased catalytic activity toward exogenous substrates (13-15). With the original observation that insulin also elevated $\beta$-subunit phosphorylation in intact cells (16, 17), it became evident that this enzymatic activity might participate in signal transduction. To justify further research into this exciting hypothesis, it is necessary to show that the presumed regulatory event occurs before the biological response. Toward this end, kinetic studies show that an insulin-dependent change in $\beta$-subunit phosphorylation precedes the changes in glucose transport rate. It is also demonstrated that the in situ phosphorylation state of the $\beta$-subunit affects its kinase activity.

**Experimental Procedures**

Cell Culture and Labeling with [32P]Orthophosphate—3T3-L1 cell monolayers were cultured and differentiated as described previously (2). Briefly, 3T3-L1 fibroblasts were grown to confluence, and 2 days later the conversion to adipocytes was induced by addition of methyl isobutylxanthine, dexamethasone, and insulin to Dulbecco's modified Eagle's medium (DME)1 containing 10% fetal bovine serum. The culture medium was supplemented with insulin for 2 more days.

1 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; EGTA, [ethylenebis(oxycarbonynitrit)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA-Sepharose, wheat germ agglutinin-Sepharose CL-4B; IGF, insulin-like growth factor.

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followed by 4–6 days in DMEM supplemented only with 10% fetal bovine serum.

For the determination of 32P-labeled phosphoproteins, monolayers in 60-mm culture dishes were washed four times with 3 ml of DMEM prepared without phosphate-containing salts (DMEM minus Pi) and were then incubated with 2 ml of DMEM minus Pi. After a 5 min exposition to insulin or the insulinase, the monolayer was washed four times with 3 ml each of Ca2+ and Mg2+-free DMEM containing 32P, was added. Equilibration of the adenine nucleotide pool was complete within 2 h. Insulin at various concentrations was added for fixed periods of time, as indicated in the figure legends. The additions of 32P, and of insulin were made so that the total incubation time of each treatment was never more than 2 h. The 32P-incubation with 32P, was constant. Thus, at the time of each monolayer was extracted, the radioactivity in [32P]ATP was constant (see "Results").

To measure time-dependent changes in 32P-labeled β-subunit, duplicate 60-mm cell monolayers were used for each time point. Although phosphorylation and dephosphorylation reactions were shown to occur only in the intact cell by harvesting cells under denaturing conditions and in the presence of metal chelators (see below), further precaution was taken to exclude any time-dependent artifacts arising after the cells were lysed in SDS. Specifically, the time periods for stimulation by insulin or for the "relaxation" after insulin removal were arranged in random order in the experimental protocol.

To measure decreases in 32P content of phosphoproteins following the withdrawal of insulin, the above protocol was used with the following changes. Unbound insulin was removed from the monolayer with 32P, by washing twice with 2 ml each of CO2-equilibrated DMEM minus Pi, over a 30-s period. The medium was then replaced with 2 ml of DMEM minus Pi, preconcentrated for 2 h on monolayers in the presence of 32P, at the same concentration used for the stimulation phase of the experiment; insulin was not present at this time. Following this "washout" and medium replacement, cells were returned to the incubator for fixed time periods. This procedure is not a pulse-chase protocol but measures changes in phosphorylation states due to de novo phosphorylations and/or dephosphorylations (see "Results"). The total time of exposure of cells to 32P, was 2 h 40 min. Since the addition of CO2 to the monolayer was for 10 min and the relaxation periods varied from 1 to 30 min, the insulin stimulation was initiated between 2 and 2.5 h. Therefore, controls for the insulin-stimulated steady state were done to bracket this interval, terminating the incubations after 2 h 10 min, 2 h 25 min, and 2 h 40 min, where the final 10 min were in the presence of insulin.

Isolation of the 32P-Labeled Insulin Receptor β-Subunit—After the labeling period (described above), cell monolayers were washed 4 times with 3 ml of ice-cold phosphate-buffered saline during a 6-s period, followed immediately by lysis by the cells in 2 mL of 25 mM Tris, 192 mM glycine, 100 mM EDTA, 5 mM EGTA, pH 8.3 (TGE buffer containing 0.1% SDS). The homogenate was washed twice with TGE containing 1.7% Triton X-100 (v/v), and the combined lysates were hand homogenized in a Potter-Elvehjem homogenizer. Dilution of SDS with Triton X-100 was described by Mostow and Blobel (18) for efficient immunoprecipitation. The homogenates were centrifuged for 45 min at 40,000 rpm in a Beckman 70.1 Ti rotor at 4°C. Four ml of the infranatant below the lipid layer was taken for adsorption to 50 μl of WGA-Sepharose (3.5 mg of lectin coupled per ml of Sepharose CL-4B, prepared as described previously (31)). Adsorption was carried out for 90 min at room temperature. The WGA-Sepharose was then washed 5 times with 1 ml of TGE containing 0.4% SDS and 1% Triton X-100. Prior to loading the samples onto the first dimension SDS-PAGE tube gel, the affinity matrix was incubated for 30 min with Lasemml sample buffer containing calcium and 1.5 mM N-acetylgallosucrose (final concentrations). Two-dimensional nonreducing/reducing SDS-PAGE was then performed as previously described (3), but using a 5% T (2.5% C) separating gel in the first dimension (6% T; 5% w/v) total acrylamide; 2.5% C (2.5% w/v) of the total acrylamide was bisacrylamide). The proteins were visualized by silver staining in some experiments. More frequently, the second dimension gel was dried on Whatman 3MM paper (50 μg of 32P-labeled supernatant [3]20,000 g of 25 μM HEPES (pH 7.2), 25 mM MgCl2, 1 mM glucose, and adding 300 μl of 2 μg (2 units of enzyme in an ammonium sulfate suspension obtained from Sigma); the final pH was 7. After 60 min at room temperature, an aliquot was removed and analyzed by thin layer chromatography (20). In Vitro Phosphorylation Using Immunoprecipitated Insulin Receptor and [32P]ATP—Insulin receptor isolated by the above immunoaffinity procedure was derived from cells that were either not exposed to insulin or exposed to 1 μM insulin for 10 min prior to harvesting. These cells had been equilibrated with 32P, for 2 h as described above. Autophosphorylation was examined at 20 μM (y-32P)ATP, 5 mM Mn(CH3CO2)2, 50 mM HEPES, 0.1% Triton X-100, pH 6.5. The presence or absence of the C-terminal phosphoacceptor of insulin receptor was measured using reduced-and-carboxamidomethylated lysozyme (15). Phosphoproteins were resolved by SDS-PAGE after heating the Protein A-Sepharose-adSORbed material for 2 min at 10°C in the presence of 2% SDS and 20 mM diithiothreitol in order to release the phosphorylated β-subunit.

[32P]Phosphoamino Acid Analysis—32P-Labeled proteins were localized by autoradiography with wet unfixed gels, and the appropriate segments of the gels were excised after alignment with the autoradiograms. Each segment was soaked for 2 days in 12 ml of water (4× washes) to denature the protein and to lyophilize. The nmol of inorganic phosphate was equal to the 32P in the y position of ATP, since no activity was by two-dimensional thin layer high voltage electrophoresis (19). Phosphoamino acid standards were visualized with ninhydrin, and the [32P]phosphoamino acids were identified by autoradiography and subsequent alignment.

Analysis of Cellular 32P-Labeled Adenine Nucleotides—The distribution of 32P, among cellular ATP, ADP, and AMP was measured to assess equilibration of the adenine nucleotide pool in cells incubated with 32P. These measurements were first made at half-hour intervals during the incubation of cells with 32P, this established that 2 h were required to reach a steady-state distribution of 32P, among adenine nucleotides. The relative activity of 32P, incorporated into the γ position of ATP following the labeling of cells was also determined. Cells were incubated with 32P, then stimulated or not for 10 min with 1 μM insulin, and then extracted as described above. Aliquots of 300 μl each were taken from the extract and the protein precipitated by addition of 75 μl of 50% trichloroacetic acid. The precipitates were discarded, and each supernatant was washed 4 times with 5 ml of ether to extract the trichloroacetic acid. An aliquot of each aqueous phase was mixed with adenine nucleotide standards and analysed by two-dimensional thin layer chromatography using LiCl and sodium formate buffers (20). The standards were visualized under ultraviolet light and the labeled compounds by autoradiography. Quantification was done by liquid scintillation counting. The activity at the γ position of [32P]ATP was measured by mixing a 25-μl aliquot of the ethanolic supernatant [3]30,000 g of 30 mM HEPES (pH 9.0), 3 mM MgCl2, 1 mM glucose, and adding 300 μl of 2 μg (2 units of enzyme in an ammonium sulfate suspension obtained from Sigma); the final pH was 7. After 60 min at room temperature, an aliquot was removed and analyzed by thin layer chromatography (20).

In Vitro Phosphorylation Using Immunoprecipitated Insulin Receptor and [32P]ATP—Insulin receptor isolated by the above immunoaffinity procedure was derived from cells that were either not exposed to insulin or exposed to 1 μM insulin for 10 min prior to harvesting. These cells had been equilibrated with 32P, for 2 h as described above. Autophosphorylation was examined at 20 μM (y-32P)ATP, 5 mM Mn(CH3CO2)2, 50 mM HEPES, 0.1% Triton X-100, pH 6.5. The presence or absence of the C-terminal phosphoacceptor of insulin receptor was measured using reduced-and-carboxamidomethylated lysozyme (15). Phosphoproteins were resolved by SDS-PAGE after heating the Protein A-Sepharose-adSORbed material for 2 min at 10°C in the presence of 2% SDS and 20 mM diithiothreitol in order to release the phosphorylated β-subunit.

The labeled ATP was quantitatively converted to glucose 6-phosphate by reaction with glucose in the presence of hexokinase and led to a stoichiometric increase in the 32P present in ADP and glucose 6-phosphate on the chromatogram. The increase in [32P]glucose 6-phosphate was equal to the 32P in the γ position of ATP, since no...
changes in radioactivity were observed in other positions of the chromatograms. This change was equal to one-third of the total cpm in the original ATP spot and one-half the increase in [32P]ADP, indicating complete equilibration of the adenine nucleotide pool.

3-O-[methyl-14C]Glucose Transport—The transport assay was essentially that described earlier (2). For this purpose 3T3-L1 cells were grown and differentiated on 1.5-cm plastic coverslips (Thermanox, Miles Scientific) set into Lindbro multiwell culture dishes of similar well diameter. Coverslips were loaded into Lucite dippers designed according to Norton and Munck (21), and 3-O-[methyl-14C]glucose uptake was monitored by a dipping technique described in detail earlier (2). Briefly, cell monolayers which had been preincubated in the presence or absence of 1 μM insulin for 15 min at 37 °C in Krebs-Ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.25 mM MgSO4, and 5 mM NaHPO4 at pH 7.4) were incubated with the radiolabeled sugar (50 μM; 4.8 mCi/mmol) for 10 or 20 s. Uptake was terminated by dipping the cell monolayers into a series of three phosphate-buffered saline washes containing 0.3 mM phloretin. For relaxation experiments, cells were exposed to 5 mM insulin for 15 min and then dipped through a series of five washes of Krebs-Ringer phosphate buffer containing 5 mM glucose and then incubated in that same buffer for the required time. Just before measuring labeled 3-O-methylglucose uptake, the cells were dipped through a series of five more washes of Krebs-Ringer phosphate buffer alone to remove the glucose. Nonspecific uptake was determined in the presence of 20 μM cytochalasin B. Radioactivity was determined by liquid scintillation after lysing cells in 0.1% SDS.

Materials—[32P]Orthophosphate, [γ-32P]ATP, and L-[35S]methionine were obtained from Amersham Corp.; 3-O-[methyl-14C]glucose and 2-deoxy-[14C]glucose were from New England Nuclear; culture media and sera were from Gibco; wheat germ agglutinin was from Calbiochem; protein A-Sepharose was from Pharmacia; trypsin (tosylphenylalanyl chloromethyl ketone treated) was from Worthington; anti-insulin receptor antibody, used in earlier studies from this laboratory (22), was derived from the IgG fraction from the serum of a diabetic patient, B-10, with acanthosis nigricans and was supplied by Dr. Phillip Gordon of the National Institutes of Health; insulin-like growth factors I and I1 were the gift of Dr. Goesta Enberg, The Karolinska Institute, Stockholm, Sweden. All other chemicals were reagent or electrophoresis grade.

RESULTS

Understanding the mechanism of signal transduction by the insulin receptor presents a complex biochemical problem. The results described below address two hypotheses of insulin action on its receptor and thus upon the biological response to insulin. The first hypothesis is that insulin causes a change in the distribution of insulin receptor subunits among cellular pools of free subunits and various oligomeric states, as proposed by others (5, 6, 23). The different forms may be responsible for different biological effects. The key fact in devising an experimental test of this hypothesis is that the receptor’s subunits are disulfide bond linked (24), which permits analysis by nonreducing/reducing two-dimensional SDS-PAGE. The observation of a single oligomeric species of insulin receptor purified from 3T3-L1 adipocytes (3) further suggests employment of this analytical method in testing the second hypothesis.

The insulin receptor is both a phosphoprotein and protein kinase. The potential regulatory significance of these facts in signal transduction was recognized at the time of their discovery (7–12, 16, 17, 25). One aspect of the hypothetical link between insulin-stimulated insulin receptor phosphorylation and insulin-stimulated glucose uptake is a clear demonstration that changes in phosphorylation precede changes in transport. With this criterion satisfied, the question of concomitantly altered protein kinase activity could be addressed.

Analysis of Insulin Receptor Subunit Structure—Two-dimensional nonreducing/reducing SDS-PAGE was used to analyze the oligomeric state of the insulin receptor isolated from cells labeled with [35S]methionine (Fig. 1A). The pulse-chase labeling conditions were selected based upon earlier work (26) to enhance detection of the mature form of the α- and β-subunits. Essentially identical results were obtained from a 60-min pulse and 11-h chase, where virtually all of the metabolically labeled receptor was present at the cell surface (results not shown). The autoradiogram shows the presence of both α- and β-subunits “inside” the diagonal, indicating that the lower migration in the first dimension was due to intersubunit disulfide bonds. This figure shows that there are no other oligomeric forms of the insulin receptor in 3T3-L1 adipocytes, by the absence of α- and/or β-subunits at other coordinates inside the diagonal of the two-dimensional gel. The presence or absence of free subunits cannot be established by this procedure alone because of the background of labeled proteins along the diagonal. However, the identity of these proteins as insulin receptor subunits as well as the absence of free subunits was verified by quantitative analysis of [35S] methionine-labeled receptor, obtained by the above protocol and by immunoprecipitation with anti-insulin receptor antibodies.

The standard protocol was adsorption of detergent-ex-

![Fig. 1. Identification of insulin receptor subunits in the glycoprotein fraction from cell extracts. 3T3-L1 adipocytes in monolayer culture were metabolically labeled with [35S]methionine (Panel A) or [32P]orthophosphate (Panels B and C). Proteins in the detergent extract adsorbed to WGA-Sepharose were resolved by nonreducing/reducing two-dimensional SDS-PAGE; reduction of disulfide bonds with 20 mM dithiothreitol was carried out after electrophoresis in the first dimension. Radioiodinated proteins were visualized by autoradiography for 16 h at −70 °C, as described under "Experimental Procedures." Panel A, [35S]methionine-labeled glycoproteins after a 60-min pulse with [35S]methionine and 6-h chase (cf. Ref. 22). The positions of the α-subunit (<α) and the β-subunit (<β) of the insulin receptor were assigned on the basis of the same coordinates observed with the purified insulin receptor from 3T3-L1 adipocytes (Fig. 10 in Ref. 18). Additional proof of the identities of these insulin receptor subunits is given in the text. Panels B and C, [32P]-labeled glycoproteins from cells not stimulated by insulin (B) or following a 10-min exposure to 1 μM insulin (C). The ratio of 32P in the β-subunits (<β) in the presence versus the absence of insulin was 4.2. The arrow indicates a 240-kDa glycoprotein whose phosphorylation on serine was increased 8-fold by insulin stimulation.](image)
tracted glycoproteins onto WGA-Sepharose, followed by two-
dimensional SDS-PAGE analysis. Typical results are shown in
Fig. 1A. When the WGA-Sepharose eluate was immuno-
precipitated with anti-insulin receptor antibodies and protein
A-Sepharose, the polypeptides designated α- and β-subunits in
Fig. 1A were quantitatively recovered in the immunoprecip-
itate, which was analyzed in a one-dimensional reducing SDS-PAGE system (not shown); these polypeptides were
absent from autoradiograms of the supernatant fraction after
immunoprecipitation. Therefore, the α- and β-polypeptides in
Fig. 1A are immunologically identified as insulin receptor subunits. In complementary experiments, one-half of the deter-
genium-extracted protein was analyzed by the WGA-Sepha-
rose adsorption and the two-dimensional SDS-PAGE system and the other half by immunoprecipitation and reducing SDS-
PAGE. There were 5800 cpm of [35S]methionine-labeled re-
ceptor subunits (α + β) in the former case and 5780 cpm in
the latter case. These data show that all immunologically
identifiable receptor is accounted for by the single oligomeric
species found by two-dimensional SDS-PAGE. Furthermore, α-
and β-subunits were not present in the protein fraction not
adsorbed to WGA-Sepharose in the standard protocol. This
proved that all α- and β-subunits in the initial detergent
extract were bound to WGA-Sepharose. This result was
confirmed by both analytical procedures that the ratio of
[35S]methionine-labeled subunits (α/β = 0.63 ± 0.08 in 8 experiments). This is the same ratio of label incorporated into the subunits of insulin affinity-
purified receptor (3) which was α/β = 0.67. These facts also
corroborate the identity of the insulin receptor subunits.
Finally, the total [35S]methionine-labeled insulin receptor at
these coordinates (cf. Fig. 1A) was unchanged by exposing
cells to 1 μM insulin (α + β = 2365 ± 83 without insulin
versus 2287 ± 292 with insulin); the same result was observed
by immunoprecipitation and one-dimensional SDS-PAGE
analysis. Thus, insulin did not cause a redistribution of mass
among putative receptor subpopulations, and signal transduc-
tion by the insulin receptor of 3T3-L1 adipocytes proceeds
through the intact holoreceptor.

The above results demonstrate that the protocol involving
adsorption of detergent-extracted proteins to WGA-Sepha-
rose and analysis by two-dimensional nonreducing/reducing
SDS-PAGE detects all the insulin receptor protein kinase of
3T3-L1 adipocytes. This protocol was, therefore, used to
measure insulin-dependent changes in the incorporation of
[32P] into the β-subunit of the insulin receptor in intact cells. To
conclude that the 32P content of the isolated β-subunit truly
represented its state in the intact cell (in situ), it was
necessary to show that changes in its 32P content did not
occur in vitro during the isolation protocol. Results of the
salient control studies, as outlined by Garrison (27), are
described first to provide a firm experimental foundation for
the subsequent results and analyses.

**Cellular Uptake of [32P], and Equilibration among Adenine Nucleotides**—The level of [32P], incorporated into phosphoprotein in intact cells is governed by a balance between the rates of phosphorylation by protein kinases and dephosphorylation by phosphoprotein phosphatases. These activities are measured accurately only when the adenine nucleotide pool has reached a steady-state level of 32P content (27) since ATP is the primary phosphate donor for most protein kinases, including the insulin receptor-protein kinase. Incubation of 3T3-L1 adipocyte monolayers in phosphate-free DMEM, compared to complete medium, had no detectable effect on the cellular ATP concentration, measured as described by Frost and Lane (2). The activity of cellular ([32P]ATP was determined after cells were incubated with 32P, for fixed time periods, to establish that 2 h were required to reach a steady state. The cellular [32P]adenine nucleotides were separated chromatographically from trichloroacetic acid-soluble components, as described under "Experimental Procedures." The cellular [32P]ATP changed from 8,600 ± 750 cpm after 30 min of uptake of 32P, from the medium to 18,400 ± 2,000 cpm after 60 min, 26,300 ± 2,400 cpm after 120 min, and 24,000 ± 2,200 cpm after 180 min. Therefore, a steady-state level of 32P incorporation into cellular ([32P]ATP was reached after 120 min. It was also established that the distribution of cellular
32P, among ATP, ADP, and AMP was not altered by insulin
after 32P label in this pool reached a steady-state level. Insulin
added to the cells had no effect on the radioactivity recovered
from cellular [32P]ATP (49,400 versus 50,400 cpm), [32P]ADP
(12,600 versus 13,800 cpm), or [32P]AMP (1,100 versus 1,200
cpm), without or with 1 μM insulin, respectively. Moreover,
there was no change due to insulin in 32P activity at the γ
position of cellular ATP, as measured in vitro by the hexoki-
glucose 6-phosphate, as described under "Experimental Pro-
cedures." Therefore, increased 32P in phosphoproteins, fol-
lowing stimulation of the cells by insulin, was due only to
increased protein kinase and/or decreased phosphoprotein phosphatase activities.

**Quenching of Phosphorylation and Dephosphorylation**—To investigate insulin-dependent changes in 32P labeling of the
insulin receptor in intact cells, it was necessary to establish
that neither phosphorylation nor dephosphorylation occurred
during the isolation of phosphoproteins prior to analysis by
SDS-PAGE. In experiments described below, insulin-dependent
changes in 32P-labeled proteins in situ were determined after
cells had been equilibrated for 2–2.5 h in phosphate-free
medium to which traer amounts of [32P] had been added. How-
over, to determine whether phosphorylation occurred
during the isolation procedure, cells were maintained in
phosphate-free medium for 2.3 h, but in the absence of 32P.
This mimicked the equilibration protocol without introducing
the radiolabel in culture. Cells were then extracted in 2 ml of
TGE buffer with 0.1% SDS and containing 0.1 mCi of
carrier-free [γ-32P]ATP to test for subsequent incorporation
into protein as a test for protein kinase activity. The glyco-
protein fraction obtained by adsorption to and elution from
WGA-Sepharose contained >95% of the insulin receptor (see
below). Analysis by nonreducing/reducing SDS-PAGE and
autoradiography showed that 32P was not incorporated from
[γ-32P]ATP even after an 84-h exposure of the dried gel
(compare with the exposure times for Fig. 1, B and C); insulin,
added to the cells or during the extraction, had no effect on
this result. Furthermore, the quantities and distributions of
proteins visualized by silver staining, including detection of
the α- and β-subunits of the insulin receptor, were the same
from experiments where the 32P label was introduced in
culture (as [32P]orthophosphate) or during the extraction (as
[γ-32P]ATP). Therefore, the extraction and isolation condi-
tions precluded effects of all protein kinase activity.

Dephosphorylation during the extraction and isolation pro-
cedures was also ruled out by the following experiment. Cells
were equilibrated with 32P, for 2.5 h to permit cellular phos-
phoproteins to become 32P labeled, stimulated or not with 1
μM insulin, and then extracted in the TGE buffer containing
0.1% SDS, as described under "Experimental Procedures." Duplicate aliquots were removed; trichloroacetic acid precipi-
tation was done immediately or after 4 h, which was the time
required to prepare samples for SDS-PAGE. No difference in
trichloroacetic acid-insoluble radiolabel was found between
samples precipitated immediately after extraction or just prior to electrophoresis. Thus, dephosphorylation was not observed during the isolation procedure. Furthermore, when the time between extraction of the $[^32P]$-labeled cells and the initiation of adsorption to WGA-Sepharose was varied from 1.3 to 3.5 h (see "Experimental Procedures"), the $[^32P]$ content of the $\beta$-subunit was unchanged. These findings prove that the results to be described below represent phosphorylation states in the intact 3T3-L1 adipocytes. When 100 mM NaF and 10 mM sodium pyrophosphate were included in the extraction buffer, the following was observed; as in the standard extraction protocol, the $\alpha$-subunit was not $[^32P]$-labeled. Slightly more $[^32P]$ was found in the $\beta$-subunit, but the difference in cpm of $[^32P]$ between the basal and insulin-stimulated states was not altered. There was no qualitative effect on the conclusions drawn from phosphoamino acid analyses of the $[^32P]\beta$-subunit described below.

$[^32P]$Phosphoamino Acid Composition of the $\beta$-Subunit—Fig. 1, B and C, shows the 4-fold difference in $[^32P]\beta$-subunit of the insulin receptor resulting from a 10-min exposure of $[^32P]$-equilibrated cells to 1 $\mu$M insulin or without insulin, respectively. Two important findings from this experiment were: 1) the $\alpha$-subunit was not phosphorylated under either circumstance, and 2) increased in situ phosphorylation due to insulin was not a general phenomenon but showed specificity for certain proteins, one of which has been identified as the insulin receptor's $\beta$-subunit.

Phosphoamino acid analyses of the $[^32P]\beta$-subunit derived from cells in the basal and insulin-stimulated states are shown in Fig. 2. $[^32P]$Phosphoserine and $[^32P]$phosphothreonine were detected under both circumstances, but only $[^32P]$phosphothreonine increased after cells were exposed to insulin. Although there was a 4-fold overall elevation of $[^32P]$, incorporated into the $\beta$-subunit, there was approximately a 7-fold increased incorporation into $[^32P]$phosphothreonine.

The conditions for extraction by proteolysis of phosphopeptides from the acrylamide gels were chosen after trial experiments with several proteinases and different $[^32P]$tyrosine-labeled proteins because of high yield and low levels of free $[^32P]$. The conditions used for acid hydrolysis were established in preliminary experiments and maximized the yield of $[^32P]$phosphothreonine. Because longer hydrolysis times are required for the release of $[^32P]$phosphoserine, these analyses are only qualitatively accurate for the relative levels of each phosphoamino acid (e.g. phosphothreonine $versus$ phosphoserine). They are, however, quantitatively reproducible for changes in $[^32P]$phosphothreonine and phosphoserine comparing the basal and insulin-stimulated state of phosphorylation of the $[^32P]\beta$-subunit. Finally, the apparent differences in migration of $[^32P]$-labeled components is less at pH 1.9 in Fig. 2A versus Fig. 2B; this is an artifact of the electrophoresis of multiple samples on a single thin-layer plate in the electrophoretic apparatus used and does not indicate different phosphoamino acid compositions of the residual $[^32P]$-labeled phosphopeptides.

Insulin Dependence of $\beta$-Subunit Phosphorylation and 3-O-[methyl-$^1^4$C]Glucose Transport Rate—Increased phosphorylation of the $\beta$-subunit and enhanced 3-O-[methyl-$^1^4$C]glucose transport rate were saturable with increasing concentrations of insulin administered to the cells, as shown in Fig. 3A. The half-maximally effective concentrations of insulin were 8 and 2 nM for $[^32P]\beta$-subunit phosphorylation and for hexose transport, respectively. A 4-fold maximal stimulation of $[^32P]\beta$-subunit was observed. For hexose transport, a 7-fold stimulation was observed.

Fig. 3A also shows the hormonal specificity for insulin; neither 67 nM IGF-1 nor 64 nM IGF-2 had any detectable effect on the phosphorylation state of the insulin receptor's $\beta$-subunit. These concentrations of IGF-1 and IGF-2 based upon their reported molecular weights (28, 29) were sufficient to give 86--96% and 90--100% of the maximal stimulation of hexose transport, respectively (Fig. 3B). The dose-response curves in Fig. 3B show similar characteristics in the relative sensitivities to insulin $versus$ the IGFs, and the maximal stimulation of transport was 16-fold above basal with IGF-1, IGF-2, or insulin (legend, Fig. 3B). The dose-response curves for transport measured by the uptake of 3-O-[methyl-$^1^4$C]glucose (Fig. 3A) versus 2-[methyl-$^1^4$C]deoxyglucose (Fig. 3B) indicate only slightly different half-maximally effective concentrations of insulin, and the relative shapes of both curves are nearly the same. Thus, uptake measurements using either glucose analog reflect the same coupling mechanism, as suggested previously by Ciaraldi and Olefsky (30). The mechanism by which insulin activates hexose transport may differ from the mechanism by which the IGFs activate hexose transport in these cells.

Time Course of Stimulation by Insulin—If the insulin-dependent $\beta$-subunit phosphorylation state serves a regulatory function with respect to glucose uptake, then changes in the former must occur first. Fig. 4 shows the time course for stimulation of $[^32P]\beta$-subunit phosphorylation and hexose transport following the addition of 1 $\mu$M insulin to 3T3-L1 adipocytes. The increased $\beta$-subunit phosphorylation reached a new steady state within 1 min after the addition of insulin. The first time point at 8 s showed 50% of the final transition, which is the approximate half-time for this process. Glucose transport rate, however, displayed a 1-min lag during which transport remained at its basal rate, followed by a linear rate increase with a "half-time" of 100 s. The observation of a 1-min lag phase is not due to the time required to measure the transport rate, which was 20 s. The same lag time was found using 10-s uptake assays (not shown). The new steady state
The basal state) and 4-fold for "P incorporation into the subunit of concentrations of insulin for 10 min prior to measuring the rate of 3-
cyles. The amounts of [32P]subunit following a 10-min exposure of
phosphorylation and hexose transport rate in 3T3-L1 adipocytes.
The basal transport rate was 87 units: insulin, 1220; IGF-1, 1330; IGF-2, 1280.
The basal state to the basal state after the withdrawal of insulin (Fig.
should also occur first in the transition from the stimulated steady-state, 100
and hexose transport rate, 87 ± 8 pmol/10^6 cells/min. The hormonally activated rates (maxima) were in the same units: insulin, 1220 ± 86; IGF-1, 1330 ± 20; IGF-2, 1280 ± 20.
was reached at 5 min. Both phenomena remained at their respective elevated steady-state levels until insulin was withdrawn.
The insulin-stimulated levels of [32P]β-subunit and glucose uptake rate are both maintained in the continuous presence of insulin (Fig. 4). If the state of phosphorylation of the β-subunit has a regulatory function, changes in this parameter should also occur first in the transition from the stimulated state to the basal state after the withdrawal of insulin (Fig. 5). To follow the "relaxation" of the phosphorylation state of the [32P]β-subunit, 20 nM insulin was employed, providing a sufficient difference between the elevated and basal states of phosphorylation to gather data during the transition periods following insulin addition or withdrawal. At 20 nM insulin, the elevated steady-state level of [32P]β-subunit was achieved at 5 min. Removal of insulin after 10 min resulted in a decline from the stimulated steady state. The decrease in [32P]β-subunit level did not exhibit a lag phase and appeared to coincide with the removal of insulin, having a halftime of 2.5 min for the transition (Fig. 5, right panel). At 5 nM insulin, the levels of [32P]β-subunit and glucose uptake were also measured.

FIG. 3. Hormone dependence of insulin receptor β-subunit phosphorylation and hexose transport rate in 3T3-L1 adipocytes. Panel A, 3T3-L1 adipocytes were incubated with the indicated concentrations of insulin for 10 min prior to measuring the rate of 3-O-[methyl-14C]glucose uptake (O; 20-s assay time) or for 5 min to determine the increase in [32P]β-subunit (C). Maximal stimulation above the basal state was 7-fold for 3-O-[methyl-14C]glucose uptake rate (10.6 ± 1.0 pmol of hexose transported per min per 10^6 cells in the basal state) and 4-fold for [32P] incorporation into the β-subunit of the insulin receptor (120 ± 11 cpm of [32P] incorporated per 4 × 10^6 cells). The amounts of [32P]β-subunit following a 10-min exposure of cells to 0.5 μg/ml each of IGF-1 (●) or IGF-2 (▲) is also shown. Panel B, hexose transport, stimulated by a 15-min preincubation with insulin (○), IGF-1 (●), or IGF-2 (▲), was measured by the uptake of 2-[14C]deoxyglucose over a 2.5-min assay period, as described by Frost and Lane (2). The basal transport rate was 87 ± 8 pmol/10^6 cells/min. The hormonally activated rates (maxima) were in the same units: insulin, 1220 ± 86; IGF-1, 1330 ± 20; IGF-2, 1280 ± 20.

FIG. 4. Time course of insulin-stimulated β-subunit phosphorylation and hexose transport rate. 3T3-L1 adipocytes were exposed to 1 μM insulin for the time periods indicated. The uptake of 3-O-[methyl-14C]glucose (C, average from 4 monolayers) was measured for a 20-s period after the indicated periods of time following the addition of insulin. To determine the rate of increase of [32P]β-subunit (C), from individual monolayers, cells were exposed to insulin for the times indicated, but the total time of exposure to [32P] was constant at 150 min. Further details are given under "Experimental Procedures."
hexose transport rate reached an elevated steady-state level at 12 min. Withdrawal of insulin after 15 min produced a decline from the stimulated steady state. An apparent first-order decay in hexose transport rate was observed with a \( t_{1/2} \) of 8 min, following the initial 2.5-min delay which preceded the decline (Fig. 5, right panel). These subsaturating concentrations of insulin were selected because they produced approximately the same relative stimulation of each process (Fig. 3A and Fig. 5, legend). The significance of these lag periods for the activation and relaxation of hexose transport will be discussed below.

In Vitro Autophosphorylation of Receptor from Basal-state and Insulin-stimulated Cells—The extent of insulin receptor autophosphorylation in vitro, especially that activated by insulin, should be inversely related to the degree of tyrosyl phosphorylation that occurred on the receptor in the intact cell (in situ). This prediction assumes one of two possible situations: either the autophosphorylation sites in situ and in vitro are the same or, if different, they are mutually exclusive.

To test this prediction, two 6-cm dishes each containing 5 x 10^5 3T3-L1 adipocytes were equilibrated with 4.5 mCi of ^32P; one cell monolayer was exposed to 1 \( \mu \text{M} \) insulin for 10 min to elevate \(^{32}\text{P}\)β-subunit to the stimulated steady-state level of phosphorylation (cf. Fig. 4), and the other cell monolayer did not receive insulin. Cells were lysed in TEGE buffer containing Triton X-100 but without SDS, and the extracts were treated as described above for the isolation of inactive receptor. Glycoproteins adsorbed to WGA-Sepharose were eluted in TEGE buffer containing 0.1% Triton X-100 and 0.3 M \( N \)-acetylglucosamine, and the insulin receptor was "purified" by immunoprecipitation as described under "Experimental Procedures." The washed immunoprecipitates were divided into fractions representing 9 x 10^6 cells each and were handled in the following manner: 1) treated with sample buffer for SDS-PAGE to determine the endogenous level of \(^{32}\text{P}\)β-subunit (Fig. 6, A and B); 2) autophosphorylated with 20 \( \mu \text{M} \) \([γ^{32}\text{P}]\text{ATP} \) (Fig. 6 C(−) and D(−)); 3) autophosphorylated with 20 \( \mu \text{M} \) \([γ^{32}\text{P}]\text{ATP} \) in the presence of 1 \( \mu \text{M} \) insulin (Fig. 6, C(+) and D(+)). \[^{32}\text{P}\]β-Subunit was measured by one-dimensional SDS-PAGE; there was no detectable \[^{32}\text{P}\]labeled insulin receptor precursor following either in situ or in vitro phosphorylation reactions (not shown).

For these labeling conditions and based upon results given above for the isolation of inactive receptor, 40 and 160 cpm were expected from the \(^{32}\text{P}\)β-subunit labeled in situ without

or with insulin stimulation, respectively, and 30 and 150 cpm were found. Thus, within experimental error, the endogenous levels of \(^{32}\text{P}\)β-subunit were maintained during the isolation from cells in the basal state (Fig. 6A) and in the stimulated state (Fig. 6B). Insulin receptor derived from cells in the basal state (Fig. 6A) was sensitive in vitro to insulin, as shown by the 4-fold stimulated autophosphorylation, comparing Fig. 6, C(−) to C(+). Insulin receptor derived from cells in the insulin-stimulated steady state (Fig. 6B) was capable of auto-phosphorylation (Fig. 6D(−)) but did not undergo significantly increased autophosphorylation when insulin was added in vitro (Fig. 6D(+)). The possibility that the insensitivity to insulin in vitro shown in Fig. 6D was due to residual insulin was considered in the following experiment. Adding 1 \( \mu \text{M} \) insulin to detergent extracts from basal-state cells and then obtaining immunoprecipitated receptor, as described above, resulted in levels of autophosphorylation comparable to 6C(+), but without substantial enhancement by insulin added to the final immune complex (not shown).

In Vitro Substrate Phosphorylation Catalyzed by Receptor from Basal-state and Insulin-stimulated Cells—When autophosphorylation of the insulin receptor is stimulated in vitro by insulin, an increased rate of exogenous substrate phosphorylation is observed (13–15). The analogous experiment was conducted to determine the effect of phosphorylation in situ on subsequent substrate phosphorylation in vitro. Reduced- and-carboxamidomethylated lysozyme was used as the substrate (15) with immunoprecipitated insulin receptor that was prepared exactly as described above. In addition to receptor isolated from cells that were not exposed to insulin or that were stimulated for 10 min with 1 \( \mu \text{M} \) insulin, receptor was also prepared from cells stimulated with 20 nM insulin for 10 min and from which insulin was then withdrawn and relaxation permitted to occur for 20 min, also giving a basal phosphorylation state (see Fig. 5). Fig. 7 shows that a higher rate of substrate phosphorylation was achieved by receptor iso-
DISCUSSION

Two hypotheses, put forward by several laboratories, place different emphasis on the roles of certain structural and enzymatic properties of the insulin receptor in the mechanism of signal initiation by insulin. One hypothesis states that the diversity of biological responses initiated by insulin in a given cell type arises from multiple oligomeric forms of the receptor, wherein the distribution of cellular receptor among these forms is governed by insulin (5, 6). The second hypothesis specifically concerns the phosphorylation state and catalytic activity of the insulin receptor’s β-subunit, which is a protein kinase (7–15, 31). It is envisioned that regulation of the biological response is controlled through insulin-dependent receptor autophosphorylation, which in turn alters the rate of substrate phosphorylation (13–15). Because this receptor’s kinase is specific for tyrosyl groups and most insulin-stimulated phosphorylations in cells result in phosphorylated seryl and threonyl residues, the receptor would be part of a cascade of protein kinase/phosphatase activities. Such a system has the advantage of signal amplification (32, 33). In a combination of these hypotheses, it would be biologically pertinent to study the relative enzymatic activities of the various possible oligomeric states of the receptor (34–36). With respect to these hypotheses, evidence presented in this paper supports a biological role for the insulin receptor’s β-subunit phosphorylation state and/or protein kinase activity, but is not consistent with more than one functionally significant oligomeric form of the receptor.

The 3T3-L1 adipocyte insulin receptor has been purified as a single oligomeric species (3). To determine the native oligomeric state of the insulin receptor in the intact cell, it was first necessary to concentrate the detergent-extracted receptor. In most laboratories, this has been accomplished with WGA-Sepharose, generally as a step preliminary to immunoprecipitation. In this glycoprotein fraction, there are few enough disulfide-linked proteins (cf. Fig. 1A) to permit the unequivocal identification of oligomers of the 135-kDa α- and β-subunits in a two-dimensional nonreducing/reducing SDS-PAGE system. Often immunoprecipitation with anti-insulin receptor antibodies is performed, giving the additional element of specificity before the last analytical step (5, 6). However, preliminary experiments indicated that the [35S]methionine-labeled insulin receptor could not be eluted efficiently from the immunoprecipitate during electrophoresis except under reducing conditions and/or with prior boiling of the sample. As much as 20% of the labeled proteins remained associated with the immune complex after electrophoresis, whereas direct electrophoresis from WGA-Sepharose left <0.02% of the protein associated with the affinity matrix; there were no receptor subunits in the residual fraction. It was also found that heating the sample without reducing agent, whether in solution or bound to an affinity matrix, resulted in the formation of lower-order oligomers of this receptor. It was concluded that these oligomers were an artifact of sample preparation because they were quantitatively equal to the single species observed without prior heating of the sample. With this understanding, the occurrence of a single endogenous oligomer of the insulin receptor, apparently an αβ2 heterotetramer, was established. The absence of free subunits was also confirmed by complementary experiments involving immunoprecipitation of [35S]methionine-labeled insulin receptor, as described under "Results." Crettaz et al. (6) reported a complex array of insulin receptor oligomers and free subunits in both IM-9 lymphocyte and rat hepatoma cells. Massague et al. (4) demonstrated 3 oligomeric forms in rat adipocytes, and the receptor purified by Fujita-Yamaguchi (37) from human placenta also occurs in a multiplicity of oligomeric states. All of these investigators boiled the samples prior to electrophoresis. It is, therefore, not clear that the native insulin receptor occurs only in the αβ2 form in all cell types, but it is certain that in 3T3-L1 adipocytes this is its native state, which is unperturbed by insulin.

The study by Crettaz et al. (6) addressed the long-term effect of insulin upon the down-regulation of its receptor. Our investigations are focused on the short-term action of this hormone in stimulating glucose transport, a process that attains the fully activated steady state 5 min after the addition of 1 μM insulin to the cell monolayer (Fig. 4). The absence of an effect of insulin on the oligomeric structure of the receptor, comparing the basal and insulin-stimulated states 10 min after the cells were exposed to 1 μM insulin, requires that the signal transduction occur through the αβ2 form of the receptor. Thus, the hypothesis involving alteration of the β-subunit’s phosphorylation state was examined using the unique oligomeric and disulfide-linked properties of the receptor to isolate the β-subunit. Ultimately, this approach yielded an extremely low and reproducible background of radioactivity in the gels, 4–5-fold lower than could be obtained with 32P-labeled proteins when receptor was immunoprecipitated directly from the detergent extract and one-dimensional gels were used in the analysis (data not shown).

When applied to intact cells, insulin stimulated phosphorylation of the receptor's β-subunit (Fig. 1B versus 1C). This increase was not due to changes in the specific activity of cellular [γ-32P]ATP, since insulin had no effect on this parameter (see "Results"). The increase of [32P]β-subunit was due to increased [32P]phosphotyrosine, which was present, but at a much lower level, in the basal state (Fig. 2). The *in vitro* autophosphorylation of insulin receptor occurs on tyrosyl groups even in the absence of insulin (15, 38). Therefore, it was necessary to show no *in vitro* autophosphorylation occurred during the extraction and isolation procedures. As described under "Experimental Procedures," with or without insulin, there was no incorporation into proteins of [32P], from [γ-32P]ATP presented in the TGGE buffer at the time of cell lysis and extraction. This is compelling evidence that the [32P] phosphotyrosine found in the [32P]β-subunit represents the *in situ* phosphorylation state of the insulin receptor. This is the first reported occurrence of [32P]phosphotyrosine in the β-subunit from unstimulated cells, and it is not necessarily a general occurrence. Other laboratories have reported increases in [32P]phosphoserine (16, 39-42), which was not the case here. Failure to find higher levels of [32P]phosphoserine may be due to the duration of exposure of these cells to insulin, i.e. exposure of insulin for longer than 10 min might be required to demonstrate increased [32P]phosphoserine. This might be consistent with the apparently slower increase of [32P]phosphotyrosine reported by Pang et al. (42) in Fao hepatoma cells. However, from the point of view of insulin action, especially in the stimulation of glucose transport, the kinetics of insulin-activated β-subunit phosphorylation and glucose uptake (Fig. 4) together with the amino acid specificity (Fig. 2) support a potential mechanistic regulatory role in this activation process for the changing level of phosphotyrosine and not phosphoserine in the β-subunit. This does not preclude all significance of phosphoserine in the biological or enzymatic activity of the β-subunit. The data merely argue against its participation in the process leading to accelerated glucose uptake, because the phosphoserine content did not change when the glucose transport activity increased.

With 3T3-L1 adipocytes, there is only a small difference between the insulin concentration dependencies of β-subunit phosphorylation and glucose uptake rate (Fig. 9). The extent of β-subunit phosphorylation appears to be closely related to insulin binding, since half-maximal insulin-binding occurs at 7 nM insulin (data not shown), and the time course of hormone binding (Fig. 7 in Ref. 2) is comparable to that for the increase in [32P]β-subunit (Fig. 5). This suggests a difference in the coupling between insulin-receptor interaction and the activation of glucose transport in this adipocyte cell system versus the rat adipocyte system, where low apparent receptor occupancy gives a maximal response (1). Although no direct comparison has yet been made, the basis for this difference could be due to nonequivalent catalytic efficiencies of the insulin receptor-protein kinase from each cellular source. Alternatively, the difference may be at some point in the regulatory pathway distal to the receptor itself. In line with this possibility, it is noteworthy that neither IGF-1 nor IGF-2 affected insulin receptor β-subunit phosphorylation (Fig. 3A) although each was as effective as insulin in stimulating hexose transport (legend, Fig. 3B). It is, therefore, probable that these growth factors act through their respective receptors. Jacobs et al. (43) have demonstrated specific *in situ* IGF-1-stimulated phosphorylation of the IGF-1 receptor in IM-9 lymphocytes. Sasaki et al. (44), using BRL-3A2 rat liver cells, showed stimulated phosphorylation of the IGF-1 receptor β-subunit both by IGF-1 and by insulin, but a ~3-fold higher concentration of insulin was required to achieve the same effect. With 3T3-L1 adipocytes, IGF-1 was ineffective in promoting insulin receptor β-subunit phosphorylation at a concentration that did stimulate glucose uptake (Fig. 3); thus, there is hormonal specificity of the phosphorylation observed here, in addition to the evidence of electrophoretic mobility and immunological specificity described above. Preliminary studies have detected only low levels of IGF-1 receptor in this cell line. It may be that the action of IGF-1 in this system is analogous to that of insulin in rat adipocytes, *vis à vis* an undetectable or extremely low extent of functionally significant phosphorylation of its cognate receptor (45).

The receptors for insulin (7-12, 31) and for IGF-1 (46) are both protein kinases, so that a common regulatory pathway could be envisioned *a priori*. The IGF-2 receptor apparently lacks this activity. Glucose transport, however, is sensitive to a variety of agents such as hydrogen peroxide (2) that do not appear to act through specific receptors. The most simple interpretation would be that there are multiple entry points into the pathway controlling the activation of transport, not all involving phosphorylation among the earliest events.

To discern if there are kinetic grounds permitting a link between insulin-stimulated β-subunit phosphorylation and acceleration of the glucose transport rate, the time dependencies of both processes were measured (Figs. 4 and 5). It is a prerequisite of any functional association that the presumed regulatory event occurs before, or is coincident with, the process under its control. Data satisfying this requirement are presented not only for the stimulation of each process at a saturating insulin concentration (Fig. 4), but also for the relaxation of each process following the removal of subsaturating insulin from the cell monolayers (Fig. 5). Under both circumstances, the change in [32P]β-subunit phosphorylation state coincided with the addition or removal of insulin. The rate of glucose uptake, however, was unaltered for 1 min or for 2.5 min following the addition or removal of insulin, respectively. At 5 nM insulin, the lag time between insulin addition and the first increase in the glucose transport rate was nearly 2 min (Fig. 5, *left*). Even at 20 nM insulin, there was no detectable lag in the increase or decline of [32P]β-subunit level (Fig. 5, *left*). The occurrence of a latency period between presentation of the stimulus and the onset of the response, as well as lengthening of this lag time with lower concentrations of the hormone, is indicative of the accumulation of intermediates effector in the response system. Similar lag phases between presentation of insulin and activation of glucose uptake (30) or rubidium transport (47) have been reported in rat adipocytes.

The additional value of the data in Figs. 4 and 5 is that the time frame for the accumulation of intermediates is defined. Since we are testing the hypothesis that involves β-subunit phosphorylation in the mechanism of insulin action, the possibility that these intermediates are phosphoproteins is being tested. However, as noted above, *in vitro* studies suggest that increased β-subunit autophosphorylation enhances this protein kinases' catalytic activity (13-15). The increased [32P] phosphotyrosine content observed here (Fig. 2) suggested that this might be true *in situ*. Thus, to support circumstantially a biological role for the protein kinase activity of this receptor, the [32P]β-subunit phosphorylation state generated *in situ* was shown to affect substrate phosphorylation as measured *in vitro*. The immunoprecipitated receptor, isolated and characterized as described under "Results," was shown to catalyze substrate phosphorylation at a higher rate when the receptor was isolated from insulin-stimulated cells than from cells in the basal state (Fig. 7). The kinase activity of receptor isolated
after the relaxation protocol was identical to that of receptor from unstimulated cells. This shows that the in situ fall in the level of [32P]β-subunit is accompanied by a corresponding decrease in catalytic activity. The difference in rates of substrate phosphorylation in Fig. 7 is not sufficiently large to permit accurate determination of changes in the kinase activity during transition between stimulated and basal states. These results were obtained after insulin was applied to the cell monolayers. To test for a stimulatory effect of residual insulin, i.e. insulin still bound to receptor in the immunoprecipitate, 1 μM insulin was added in vitro before initiating substrate phosphorylation. There was no stimulation by insulin of the rates of substrate phosphorylation (not shown).

The absence of a stimulatory effect by insulin present at the start of substrate phosphorylation is due to substrate inhibition of autophosphorylation (15). Therefore, the results reflect differences in the [32P]β-subunit generated in situ and are not due to residual insulin in the immunoprecipitate. Thus, it is concluded that the in situ increase in [32P]β-subunit caused by insulin is functionally analogous to that observed in vitro in terms of elevating the protein kinase activity of the insulin receptor. This finding of increased catalytic capability as well as the recent report of White et al. (49) encourages us to search for one or more [32P]phosphotyrosyl-labeled proteins as possible intermediates of signal transduction in the insulin-dependent acceleration of glucose transport.

The approximately 2-fold difference in catalytic activity, as the recent report of White et al. (49) demonstrated a 4.4-fold lower in vitro phosphorylation in the basal state receptor and identifying, fewer sites would remain available in vitro and less 32P would be incorporated into the receptor from the 1 μM insulin-stimulated cells, supporting the latter proposition that most of the receptor’s autophosphorylation sites were indeed phosphorylated in situ. Since the immunoprecipitation protocol employed does not distinguish surface from intracellular receptors, it is impossible to comment on the subcellular location of the phosphorylated receptor.

In 3T3-L1 adipocytes, it is now established that insulin-dependent changes in the β-subunit phosphorylation state are both rapid and extensive and do not involve oligomeric reorganization of the native receptor. Further investigations are being directed toward understanding the significance of phosphotyrosine in the basal state receptor and identifying, through the use of kinase and transport inhibitors, intermediate steps in the activation of glucose transport.

REFERENCES