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Mechanism of insulin action on membrane protein recycling: A selective decrease in the phosphorylation state of insulin-like growth factor II receptors in the cell surface membrane

(protein sorting/endocytosis/anti-insulin-like growth factor II receptor antibody/immunoblotting/^{32}P-labeled adipocytes)

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ABSTRACT **Insulin action in adipocytes leads to an increase in the steady-state number of cell surface glucose transporters and insulin-like growth factor II (IGF-II) receptors that appear to cycle continuously between the plasma membrane and a low-density membrane fraction. The IGF-II receptor could be labeled to constant specific activity by incubating adipocytes with [^{32}P]phosphate for 2 hr. The extent of phosphorylation of IGF-II receptors in plasma membranes and in low-density microsomes was compared using ^{125}I-labeled IGF-II binding and immunoblotting to quantitate the receptors present in each fraction. Receptors in the plasma membrane fraction of control cells incorporated approximately 1 molecule of phosphate per IGF-II binding site or 2 to 3 times more phosphate than was incorporated into IGF-II receptors in the low-density microsomes. Addition of insulin to labeled adipocytes did not change the specific activity of the γ-phosphate of ATP but produced a specific and sharp decrease in the ^{32}P-phosphate content of IGF-II receptors in the plasma membrane. No change due to insulin in the phosphorylation of receptors derived from low-density microsomes was observed. The insulin-mediated decrease in the [^{32}P]phosphate content of IGF-II receptors from the plasma membrane was rapid in onset, paralleled the increase in the number of IGF-II receptors on the cell surface, and persisted for at least 30 min in the presence of insulin. Furthermore, when the effect of insulin to increase the number of IGF-II receptors in the cell surface was prevented by cooling cells to 5°C, the decrease in phosphorylation of plasma membrane receptors could still be observed, indicating that this latter effect is not secondary to receptor redistribution. These data indicate that insulin inhibits one or more IGF-II receptor kinases or increases phosphatase activity, or both. Decreased phosphorylation of such insulin-sensitive plasma membrane components as IGF-II receptors may play a role in increasing their steady-state cell surface concentration, perhaps by delaying their internalization.**

One of the most important and well-studied effects of insulin is the rapid stimulation of hexose transport activity in muscle and fat cells. Cushman and Wardzala (1) as well as Suzuki and Kono (2) demonstrated that this increase in glucose transport is accompanied by a redistribution of hexose transporters from a low-density microsome fraction to the plasma membrane fraction of disrupted cells. These workers proposed that insulin action leads to the recruitment of intracellular hexose transporters to the cell surface membrane. This hypothesis has been confirmed by the direct demonstration that insulin causes increased expression of transporters on the cell surface of intact adipocytes (3). We recently showed that another membrane component, the type II insulin-like growth factor (IGF) receptor, responds to insulin in a manner remarkably similar to the glucose transporter (4). Thus, increased numbers of IGF-II receptors were found to reside in the plasma membrane fraction of insulin-treated adipocytes, whereas a concomitant decrease in low-density microsome receptors due to insulin action was apparent (4). These data underscore the likelihood that insulin may be modulating hexose transporters and IGF-II receptors through a similar mechanism.

Recent studies on the transferrin receptor have suggested that increased phosphorylation of such membrane components might be associated with the process of internalization (5, 6). These findings raise the possibility that this type of covalent modification may be a general mechanism through which the cycling of membrane components could be regulated. The aim of the present study was to determine whether a reversible phosphorylation of the IGF-II receptor may be involved in the pathway of its internalization and recycling to the plasma membrane and whether such phosphorylation might be a focal point for insulin action. The results presented in this paper show that the effect of insulin to increase the number of receptors on the cell surface is accompanied by a sharp and stable decrease in the specific activity of ^{32}P in these receptors in the plasma membrane.

MATERIALS AND METHODS

Cell Isolation and Labeling. Male rats weighing 100–150 g were used (SD strain, Taconic Farms, Germantown, NY). Fat cells were isolated from the epididymal fat pads as described by Rodbell (7) in phosphate-free Hepes-buffered Krebs–Ringer solution, pH 7.4. For ^{32}P-labeling experiments, 5 ml of the cell suspension (0.5–1 × 10^6 cells per ml) was incubated at 37°C in 25-cm^2 culture bottles with carrier-free [^{32}P]orthophosphate (1 mCi/ml; 1 Ci = 37 GBq; New England Nuclear). Routinely, 10 nM insulin (porcine, Lilly) was added to each culture bottle after a 2-hour labeling period for the times indicated. In experiments designed to evaluate the influence of glucose metabolism on the effects of insulin, 5 mM glucose was present in the buffer throughout.

Plasma Membrane and Low-Density Microsome Preparations. Plasma membranes and low-density microsomes were prepared in 0.25 M sucrose/10 mM Tris·HCl/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.1 mM Na_3VO_5/50 mM NaF/50 mM $Na_2H_2P_2O_7$, pH 7.4, at 4°C by differential centrifugation as described by McKeel and Jarett (8), with some modifications (9). Membrane protein determinations were carried out by the Bradford procedure (10) (Bio-Rad protein assay) using egg albumin (Sigma) as standard. These plasma membranes and low-density microsome fractions have previously been characterized in detail (1, 4).

Immunoprecipitation. To separate ^{32}P-labeled IGF-II receptor from other phosphoproteins, plasma membranes and

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Abbreviation: IGF-II, insulin-like growth factor II.

low density microsomes were solubilized in 500 μl of 25 mM Hepes/1.5% Triton X-100/1% sodium deoxycholate/1% bovine serum albumin/0.1% $NaDodSO_4$/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.1 mM Na_3VO_5/50 mM NaF/50 mM $Na_2H_2P_2O_7$/0.5 M NaCl (lysis buffer) and immunoprecipitated in a final volume of 1 ml with 15 μg of polyvalent antibody against the IGF-II receptor (9, 11) previously immobilized on Affi-Gel 10 (Bio-Rad). After incubating with constant mixing overnight the resin was extensively washed with lysis buffer. After a final wash with 25 mM Hepes/0.1% $NaDodSO_4$/0.1% Triton X-100, the resin was boiled for 1 min in 50 mM Tris, pH 6.8/1% $NaDodSO_4$/20% glycerol/0.005% bromophenol blue. The solubilized samples were electrophoresed on 6% polyacrylamide slab gels as described by Laemmli (12).

Immunoblotting. Immediately after electrophoresis, proteins were transferred from the gels onto nitrocellulose paper (Schleicher & Schuell), essentially as described by Burnette (13), with several modifications. The buffer used was 20 mM Tris base/150 mM glycine/20% methanol/0.1% $NaDodSO_4$. The transfer was conducted at constant current (200 mA) for 10–12 hr (≈10 V/cm). The nitrocellulose was then incubated for 1 hr at 22°C in 15 mM Tris/150 mM NaCl/2% bovine serum albumin, pH 7.4, and then for 8–12 hr with the polyvalent antibody (20 μg/ml) against the IGF-II receptor in the same buffer (TNB buffer). The immunoglobulin solution was removed and the paper was washed, incubated with 2×10^6 cpm of ^{125}I-labeled protein A in 5 ml of TNB buffer for 40 min at room temperature, and washed again. The paper was then exposed to Kodak X-Omat film at −70°C in the presence of a DuPont Lightning Plus intensifying screen for 2–5 hr. The receptor bands were then localized and excised, and ^{125}I was determined in a Packard gamma counter and ^{32}P, by Cerenkov counting in a Packard Tri-Carb liquid scintillation counter. Background radioactivity was determined for each lane by excising a similar-sized region of the paper immediately below each band.

ATP Specific Activity. The ^{32}P specific activity of the γ-phosphate of ATP was measured by the method of England and Walsh (14) using cells labeled as described above.

RESULTS

Immunoblotting of IGF-II Receptors. In preliminary experiments, a linear relationship was found between the amount of membrane protein extracted for immunoprecipitation and the amount of ^{125}I-labeled protein A associated with the immunoblotted IGF-II receptor band. The amount of IGF-II receptor in 50–300 μg of low-density microsome protein is equivalent to that in 500–3000 μg of plasma membrane protein (results not shown). Thus, the redistribution of IGF-II receptors between low-density microsomes and plasma membrane that occurs on addition of insulin to rat adipocytes could be quantitatively analyzed by immunoblotting of the different subcellular fractions.

Fig. 1 (*Insets*) shows autoradiographs of immunoblots derived from plasma membrane (*A*) and low-density microsomes (*B*) obtained from control cells and cells treated with 10 nM insulin. Under control conditions, the amount of receptor per μg of membrane protein is 8- to 10-fold higher in low-density microsomes than in plasma membrane. Insulin action produces a marked concentration-dependent increase in the number of IGF-II receptors found in the plasma membrane fraction and a concomitant decrease of receptors in the low-density microsomal fraction. The dose–response relationship for the increase in plasma membrane IGF-II receptors and for the decrease in low-density microsome receptors indicates a half-maximal effect at 0.1 nM insulin. A maximal effect of insulin on IGF-II receptor concentration in both membrane fractions was obtained at about 1 nM insulin.

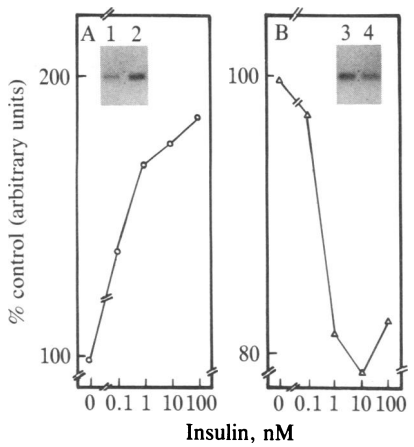

FIG. 1. Dose dependence of the insulin-mediated increase in plasma membrane (*A*) and decrease in low-density microsomal (*B*) IGF-II receptors quantitated by immunoblotting. Immediately after isolation, fat cells were incubated for 15 min in the presence or absence of insulin at the concentrations indicated. The cells were fractionated and 100 μg of plasma membrane protein and 50 μg of low-density microsomal protein were electrophoresed and immunoblotted. The nitrocellulose paper was exposed for 3 hr and autoradiograms were analyzed by densitometric scanning. The value in arbitrary units of each sample was expressed as the percentage of the control (not insulin treated) samples. Plotted are the averaged results from two different experiments. (*Insets*) Autoradiographs obtained from the immunoblots. Lanes: 1, plasma membranes from control cells; 2, plasma membranes from cells treated with 10 nM insulin; 3, low-density microsomes from control cells; 4, low-density microsomes from cells treated with 10 nM insulin.

It is likely that the insulin concentrations that result in this biological response are overestimates because of the extensive insulin degradation rates that are expected under our incubation conditions with concentrated cell suspensions.

Incorporation of ^{32}P into IGF-II Receptors. Because immunoblotting appeared to give a precise estimate of IGF-II receptor concentrations in membrane samples, it was possible to analyze quantitatively the relative amount of ^{32}P incorporated into this receptor under different conditions. Incubation of fat cells with 1 mCi of ^{32}P/ml results in the rapid incorporation of radiolabel into IGF-II receptors (Fig. 2). The ratio of ^{125}I-labeled protein A associated with the immunoprecipitated IGF-II receptor after electrophoresis and immunoblotting to the amount of ^{32}P present in this receptor band reflects the specific activities of ^{32}P in the IGF-II receptors derived from different membrane fractions. In IGF-II receptors of both membrane fractions the incorporation of ^{32}P increases steadily for approximately 60 min and is essentially unchanged thereafter. A striking finding in these experiments is that the plasma membrane IGF-II receptors exhibit a higher steady-state incorporation of ^{32}P than those in the low-density microsomal fraction (Fig. 2). Thus, incubation of the cells with ^{32}P for up to 4 hr results in a 2- to 3-fold higher apparent specific activity of the plasma membrane receptors as compared to those in the low-density microsomes.

Effect of Insulin on IGF-II Receptor Phosphorylation. The results presented in Figs. 1 and 2 raised the possibility that the phosphorylation state of the IGF-II receptor is functionally related to the steady-state distribution of this receptor between the cell-surface membrane and intracellular low-density membrane vesicles. If such were the case, the effect of insulin to produce a redistribution of IGF-II receptors between these two cellular compartments might be accompanied by a change in the phosphorylation state of the receptors. From experiments designed to evaluate such effects of insulin, it can be seen (^{125}I-labeled protein A values,

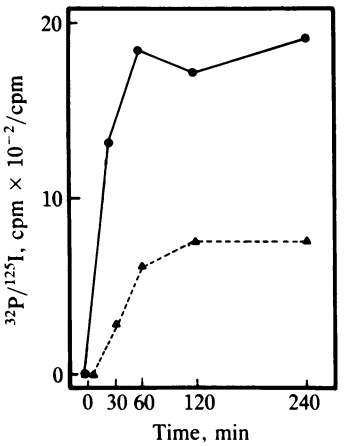

FIG. 2. Time course of incorporation of ^{32}P into IGF-II receptors in adipocyte membrane fractions. Cells were incubated in the presence of 1 mCi of [^{32}P]phosphate/ml for the times indicated, and plasma membranes (●) and low-density microsomes (▲) were prepared and used for immunoprecipitation, electrophoresis, and immunoblotting. Receptor bands were excised and ^{32}P incorporated was quantitated by Cerenkov counting. The ^{125}I-labeled protein A associated to the same bands was quantitated by gamma radiation counting. The ratio of cpm of ^{32}P to cpm of ^{125}I (minus background) was obtained and plotted. The ^{125}I radioactivity in plasma membrane and low-density microsomes at 120 min was 841 cpm and 1107 cpm, respectively, and the ^{32}P radioactivity was 152 cpm and 75 cpm, respectively. Results shown are from a representative experiment, which was repeated two times with similar results.

Table 1) that insulin produces an increase in the amount of IGF-II receptors that fractionate with the plasma membranes and a reciprocal decrease in receptors that are contained in the low-density microsomes, as previously noted.

Significantly, the increase in the number of IGF-II receptors in the plasma membrane is not accompanied by a proportional increase in the ^{32}P found in these receptors (Table 1). Thus, insulin causes a sharp decrease in the phosphorylation state of IGF-II receptors in the plasma membrane fraction. The ($^{32}P/^{125}I$) × 100 value declines from 44 ± 11 to 19 ± 7 because of the action of insulin. In contrast, the insulin-mediated decrease in the number of low-density microsome IGF-II receptors is paralleled by a similar decrease in the ^{32}P found in the receptor bands. Therefore, no change in the phosphorylation of IGF-II receptors in this fraction in response to insulin is observed [($^{32}P/^{125}I$) × 100 = 12 ± 3 versus 11 ± 3].

An estimate of the total number of IGF-II receptors present in insulin-treated vs. control cells, obtained by addition of the IGF-II receptors (^{125}I-labeled protein A) in the plasma membranes plus those in the whole low-density microsomes recovered from a suspension of 3×10^6 cells, shows that the total number of IGF-II receptors in these combined cell fractions is unchanged by insulin treatment (3700 ± 650 vs. 3850 ± 720 total cpm of ^{125}I in IGF-II receptors from 3×10^6 control and insulin-treated cells, respectively). In contrast, the amount of ^{32}P associated with total IGF-II receptors is decreased by insulin treatment (637 ± 200 vs. 456 ± 180 total cpm of ^{32}P in receptors from control and insulin treated cells, respectively). The overall ($^{32}P/^{125}I$) × 100 values of the IGF-II receptors from control and insulin-treated cells are estimated to be 15.3 ± 3 and 11.2 ± 3, respectively. The paired difference analysis of these values in each experiment shows that this difference is statistically significant ($P < 0.05$).

The possibility that the effect of insulin to decrease the incorporation of [^{32}P]phosphate into IGF-II receptors was a reflection of an insulin-mediated decrease in the specific activity of the γ-phosphate of ATP rather than of a true decrease in the phosphorylation state of the receptor was considered. Therefore, the specific activity of the γ-phosphate of ATP in adipocytes was measured under each of the experimental conditions tested. The results presented in Table 2 show that the ATP pool reached constant specific activity after ≈60 min of incubation. The incorporation of ^{32}P into the IGF-II receptor closely parallels the labeling of the γ-phosphate of ATP. This evidence indicates that the phosphate groups of the IGF-II receptor turn over very rapidly. When the values for specific activity of ATP (Table 2) and for receptor concentration calculated by Scatchard analysis of ^{125}I-labeled IGF-II binding (0.4 ± 0.2 pmol/mg of protein) are used, the calculated stoichiometry for the phosphorylation of plasma membrane IGF-II receptors is between 0.75 and 1.5 molecules of phosphate per IGF-II binding site. Addition of 10 nM insulin after a 120-min–labeling period did not alter the specific activity of the γ-phosphate of ATP to any detectable extent. Thus, the data indicate that the decrease in the

Table 1. Effect of insulin on number and phosphorylation state of IGF-II receptors

Cell locus and condition	^{125}I-protein A associated with receptor/μg of membrane protein, cpm/μg	^{32}P in receptor/μg of membrane protein, cpm/μg	$^{32}P/^{125}I$, cpm × 10^{-2}/cpm
Plasma membranes			
Control	0.96 ± 0.20	0.41 ± 0.10	44 ± 11
Insulin	1.93 ± 0.50*	0.36 ± 0.10‡	19 ± 7*
Low-density microsomes			
Control	8.30 ± 2.0	1.07 ± 0.40	12 ± 3
Insulin	6.40 ± 2.0†	0.75 ± 0.31†	11 ± 3‡

Isolated adipocytes were incubated for 2 hr in the presence of 1 mCi/ml [^{32}P]orthophosphate. Insulin (10 nM) was added and incubations were continued for another 15 min. Plasma membranes and low-density microsomes were isolated and detergent solubilized. Approximately 80 μg of low-density microsomal protein and 400 μg of plasma membrane protein were used for immunoprecipitation, which was followed by electrophoresis and immunoblotting. The IGF-II receptor bands were cut out and analyzed for ^{125}I and ^{32}P. cpm of ^{125}I or ^{32}P obtained are expressed as functions of the amount of protein initially used for immunoprecipitation. $^{32}P/^{125}I$ ratios obtained for each band provided estimates of the specific activity of ^{32}P in IGF-II receptors. Values represent mean ± SEM of five different cell preparations, two of which were made in the absence and three in the presence of 5 mM glucose. The data were pooled because the results were similar. Differences between control versus insulin-treated cells were calculated by Student's t test of the paired differences between conditions; *, $P < 0.025$; †, $P < 0.05$; ‡, not significant.

Table 2. Effect of insulin in the presence and absence of glucose on the incorporation of [^{32}P]phosphate into the IGF-II receptor and into the γ-phosphate of ATP

Time of ^{32}P exposure, min	Glucose (5 mM)	Insulin (10 nM)	Specific radioactivity in γ-phosphate, cpm/pmol	^{32}P/^{125}I, cpm × 10^{-2}/cpm	
				PM	LDM
30	−	−	162	13	3
60	−	−	209	18	7
120	−	−	222	20	10
120	−	+	239	11	9
120	+	−	ND	19	6
120	+	+	ND	8	9

Cells were incubated for the times indicated in the presence or absence of glucose. Where shown, insulin was present during the last 10 min of incubation. Cells were processed for the measurement of specific radioactivity of the γ-phosphate of ATP or the phosphorylation state of the IGF-II receptor. Data presented are from one experiment. The average values from five experiments for the specific activity of the γ-phosphate of ATP after 2 hr of incubation with ^{32}P were 292 ± 74 and 300 ± 83 cpm/pmol in control and insulin-treated cells, respectively. ND, not determined; PM, plasma membrane; LDM, low-density microsomes.

amount of [^{32}P]phosphate in the IGF-II receptor as a consequence of insulin action is due to a decrease in the phosphate content of the receptor. Recently, it has been shown that some of the effects of insulin on the phosphorylation state of glycogen synthase are secondary to the effects of the hormone on glucose metabolism (15). In view of these data, experiments were performed to determine whether the decrease in the phosphorylation state of the IGF-II receptor was dependent on the stimulation of glucose metabolism induced by insulin. In the presence or absence of glucose, insulin caused a marked decrease in the phosphorylation state of the IGF-II receptor in the plasma membrane (Table 2).

The insulin-mediated decrease in the phosphorylation state of the plasma membrane IGF-II receptor coincides with the enrichment of this fraction with receptors from the low-density microsomal fraction. This raises the possibility that the decrease in phosphorylation of the IGF-II receptor in the plasma membrane fraction may be a consequence of dilution with less-phosphorylated receptors from the low-density microsomes. Two different experiments were performed to evaluate this possibility. First, the time course of the insulin effect was studied. If the possibility were correct, it would be predicted that the poorly phosphorylated receptors from the low-density microsomes upon being inserted into the plasma membrane would rapidly become as heavily phosphorylated as those receptors initially present in the plasma membrane. Thus, the ^{32}P/^{125}I ratio would quickly return to the control values observed before addition of insulin. It was found that the increase in the number of IGF-II receptors in the plasma membrane occurs relatively quickly after the addition of insulin and is completed within 5 min of incubation with the hormone (Fig. 3*A*). The decrease in the specific activity of ^{32}P in IGF-II receptors of the plasma membrane is equally fast. However, the insulin-mediated decrease in the phosphorylation state of the plasma membrane IGF-II receptors is stable and remains virtually unchanged even after 30 min of incubation with the hormone. No change in the phosphorylation of IGF-II receptors in the low-density microsomes occurs throughout this time period (Fig. 3*B*).

In the second experiment, use was made of the fact that, at low temperature, most events involving endocytosis or exocytosis are inhibited, while insulin binding to and activation of its receptor kinase are not (16). It can be seen in Fig. 4 that the insulin-mediated increase in the number of plasma membrane IGF-II receptors, which is readily apparent at

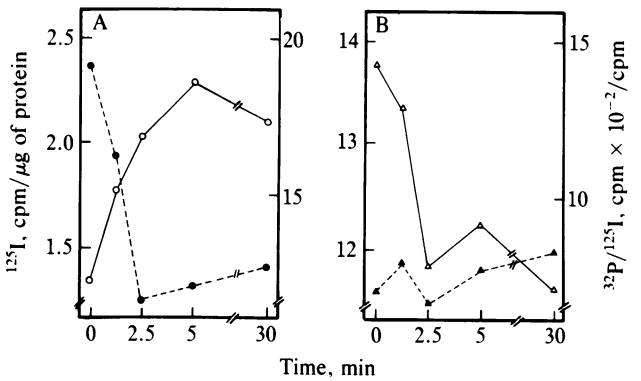

FIG. 3. Time course of effect of insulin to produce redistribution of IGF-II receptors and decrease the phosphorylation state of the receptor in the plasma membrane. Cells were incubated for 2 hr with ^{32}P at 1 mCi/ml. Insulin (10 nM) was added to each bottle and incubations were continued for the times indicated. The incubation medium was removed as quickly as possible and the cells were homogenized. The earliest time point that could be recorded, ≈1.2 min, was the shortest period of time between the addition of insulin and the homogenization of the cells. Plasma membranes (*A*) and low-density microsomes (*B*) were solubilized and the IGF-II receptor was immunoprecipitated, electrophoresed, and immunoblotted. ^{125}I and ^{32}P radioactivities in the receptor and the ^{32}P/^{125}I ratios were quantitated as described in the legend to Fig. 2. ^{125}I radioactivity is expressed as a function of the amount of protein initially used for immunoprecipitation. ^{125}I radioactivity per μg of protein (○ and △) and ^{32}P/^{125}I ratios (● and ▲) for the IGF-II receptor are plotted.

37°C (*A*), is virtually abolished when the temperature is decreased to 5°C (*B*). However, the insulin-mediated decrease in the phosphorylation of the IGF-II receptor in the plasma membrane is equally apparent at 5°C as at 37°C. Thus, insulin is capable of reducing the phosphorylation state of IGF-II receptors in the plasma membrane even under conditions in which the insertion of receptors from the low-density microsomes is prevented.

DISCUSSION

The results presented in this study show that the IGF-II receptor is phosphorylated in intact cells. The incorporation of [^{32}P]phosphate into the receptor closely parallels the labeling of the γ-phosphate of ATP and both reach constant

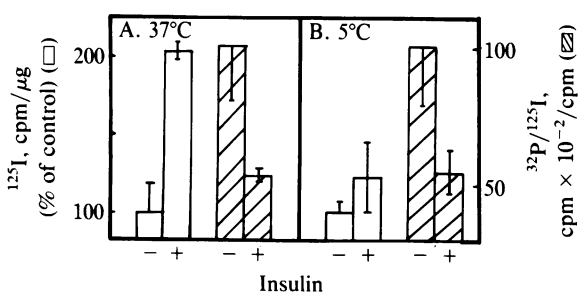

FIG. 4. Effect of insulin on number and phosphorylation state of the plasma membrane IGF-II receptor at 37°C and 5°C. Adipocytes were incubated with ^{32}P at 1 mCi/ml at 37°C for 2 hr. Half the cells were left at 37°C and half were transferred to an ice-cold water bath. Five minutes after transfer of the cells, 10 nM (*A*) or 1 μM (*B*) insulin was added to the cell suspensions. After a further 10-min incubation, cells were homogenized. The rest of the procedure was as described in Table 1. Results are presented as percentage of the values obtained in the absence of insulin, which were 5.1 ± 0.2 and 5.2 ± 0.4 cpm of ^{125}I/μg of protein at 37°C and 5°C, respectively, and 1750 ± 400 and 1550 ± 400 cpm of ^{32}P/cpm of ^{125}I at 37°C and 5°C, respectively. Data are mean ± SEM of three experiments.

specific activity after ≈90 min incubation of the cells with [^{32}P]phosphate. This indicates that the phosphate groups in the receptor turn over very rapidly. Furthermore, the stoichiometry of receptor phosphorylation is approximately one molecule of phosphate per IGF-II-binding site for the receptors in the plasma membrane fraction. Thus, both the kinetics and the extent of phosphate incorporation into the IGF-II receptor support a role of phosphorylation in regulating the function or distribution of this receptor within the cell. This latter possibility is highlighted by the finding in this study that in isolated adipocytes under basal conditions the degree of steady-state IGF-II receptor phosphorylation varies depending on the location of the receptor within the cell (Fig. 2). Our data show that the amount of phosphate in IGF-II receptors in the plasma membrane is 2–3 times higher than that in receptors from the low-density microsomal pool.

The difference in the phosphorylation state of IGF-II receptors between plasma membrane and low-density microsomes combined with the fact that IGF-II receptors are rapidly cycling between these membrane compartments (17) suggest that IGF-II receptors, newly inserted into the plasma membrane, undergo rapid phosphorylation prior to their internalization. Once internalized, the highly phosphorylated IGF-II receptors must be dephosphorylated. These concepts lead further to the hypothesis that one or more phosphorylation sites on plasma membrane IGF-II receptors may serve a functional role in directing receptor internalization. Recent data showing that phorbol diester-directed phosphorylation of the transferrin receptor correlates with receptor internalization (5, 6) has led to similar conjectures.

The insulin-mediated redistribution of IGF-II receptors between cellular membrane fractions is accompanied by a marked and specific decrease in the phosphorylation state of this receptor in the plasma membranes (Fig. 4 and Table 1). Three of our present findings strongly support the view that this effect of insulin is a primary event rather than solely a consequence of receptor redistribution. The first is that a net decrease in the amount of ^{32}P incorporated into the total pool of IGF-II receptors can be observed in response to insulin treatment. This overall effect is relatively small because most of the cellular IGF-II receptors reside in the low-density microsomes and the phosphorylation state of these receptors is not affected by insulin. Nevertheless, the insulin effect to decrease the phosphorylation state of total IGF-II receptors is consistent and statistically significant.

Second, the insulin-mediated decrease in the phosphorylation state of IGF-II receptors in the plasma membrane is stable with time. It is clear that the labeling of IGF-II receptors occurs rapidly during the first minutes of incubation with ^{32}P (Fig. 2). However, even after 30 min of insulin exposure equivalent to at least two rounds of IGF-II receptor recycling (17), plasma membrane IGF-II receptors are still less phosphorylated compared to those in control cells (Fig. 3). Taken together, these data suggest that the presence of insulin markedly retards the phosphorylation of IGF-II receptors in the plasma membrane.

Third, the effect of insulin to decrease the phosphorylation state of IGF-II receptors in the plasma membrane persists under conditions in which receptor redistribution is blocked (Fig. 4). This evidence argues strongly against the possibility that the decrease in the phosphorylation of plasma membrane IGF-II receptors is only a reflection of the enrichment of this fraction with receptors from the low-density microsomes. Rather, the insulin-mediated inhibition of phosphorylation of plasma membrane IGF-II receptors could be a primary effect of the hormone.

We cannot distinguish from our data whether the decreased phosphorylation state of plasma membrane IGF-II receptors in response to insulin is due to an increase in phosphatase activity, a decrease in kinase activity, or both. However, it is known that the phosphorylation processes involved in the regulation of glycogen synthesis and pyruvate dehydrogenase activity are affected by insulin in a manner that implicates the stimulation of phosphatase activity (15, 18–20). Regardless of the potential mechanism, it is clear that the effect of insulin to alter the dynamics of plasma membrane IGF-II receptor phosphorylation is closely associated with the redistribution of receptor that occurs on exposure of fat cells to the hormone.

The data presented here lead us to a working hypothesis on the mechanism by which insulin increases the steady-state number of cell surface IGF-II receptors. It is proposed that the inhibition of plasma membrane IGF-II receptor phosphorylation by insulin action delays the process whereby these receptors are directed to be internalized. According to this view, insulin action decreases the phosphorylation rate of one or more key sites on the IGF-II receptor involved in targeting the receptor toward an endocytotic pathway. Thus, by inhibiting an IGF-II receptor kinase or stimulating a phosphatase, insulin would shift the kinetics of receptor recycling such that an increased steady-state number of receptors would reside in the cell surface membrane. This hypothetical mechanism could operate in the absence of a direct effect of insulin on the cellular exocytotic or endocytotic processes. However, such effects of insulin on general endocytosis or exocytosis cannot be excluded (21). In any case, studies directed toward characterizing the kinases and phosphatases that regulate the phosphorylation state of the IGF-II receptor and their roles as targets for insulin action will be of great interest.

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