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The Ras signaling pathway mimics insulin action on glucose transporter translocation

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ABSTRACT **Recent observations suggest that insulin increases cellular levels of activated, GTP-bound Ras protein. We tested whether the acute actions of insulin on hexose uptake and glucose-transporter redistribution to the cell surface are mimicked by activated Ras. 3T3-L1 fibroblasts expressing an activated mutant (Lys-61) N-Ras protein exhibited a 3-fold increase in 2-deoxyglucose uptake rates compared with nontransfected cells. Insulin stimulated hexose uptake by ≈2-fold in parental fibroblasts but did not stimulate hexose uptake in the N-Ras^{61K}-expressing fibroblasts. Overexpression of N-Ras^{61K} also mimicked the large effect of insulin on 2-deoxyglucose transport in 3T3-L1 adipocytes, and again the effects of the two agents were not additive. Total glucose transporter protein (GLUT) 1 was similar between parental and N-Ras^{61K}-expressing 3T3-L1 fibroblasts or adipocytes, whereas total GLUT-4 protein was actually lower in the N-Ras^{61K}-expressing compared with parental adipocytes. However, expression of N-Ras^{61K} in 3T3-L1 adipocytes markedly elevated both GLUT-1 and GLUT-4 in plasma membranes relative to intracellular membranes, and insulin had no further effect. These modulations of glucose transporters by N-Ras^{61K} expression are not due to upstream regulation of insulin receptors because receptor tyrosine phosphorylation and association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated proteins were unaffected. These results show that activated Ras mimics the actions of insulin on membrane trafficking of glucose transporters, consistent with the concept that Ras proteins function as intermediates in this insulin signaling pathway.**

Insulin is a major physiological regulator of many metabolic pathways, including those directing protein synthesis, lipogenesis, and glucose utilization. These biological actions of insulin are initiated by the activation of a specific, heterotetrameric receptor (1) with intrinsic tyrosine kinase activity (2), leading to rapid autophosphorylation of receptor tyrosine residues (3), as well as the tyrosine phosphorylation of other cellular substrates (4). Early cellular events preceding insulin regulation of target metabolic enzymes include the association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated proteins (5–8), an increase in the amounts of the protooncogene product Ras present in the active, GTP-bound state (9–11), and the activation of numerous protein serine/threonine kinases (12, 13). Some of the insulin-activated protein kinases appear to operate in cascades (14, 15), and recent evidence indicates that phosphatase I modulation by insulin is a result of protein kinase activations (16). It is thought that insulin-regulated protein kinase and phosphatase activities catalyze downstream phosphorylation/dephosphorylation reactions that acutely regulate insulin-sensitive proteins and enzymes. Thus, conversion of the elevated insulin-receptor tyrosine kinase activity to serine/threonine phosphorylation activity is a key element of signal transmission by insulin.

Recent evidence suggests that activation of endogenous Ras proteins in response to stimulation of insulin (9–11) and other growth factor (17–23) receptor tyrosine kinases may be a necessary step for triggering protein serine/threonine kinase cascades. Activated, GTP-bound Ras is thought to interact with GTPase-activating proteins or other effector proteins to catalyze signal transmission (for review, see ref. 24). Other observations implicate Ras function as requisite for insulin action on downstream signaling of bio-responses. However, these reports only describe insulin action on nuclear events, such as control of c-*fos* transcription (25–28) and cell differentiation (29). Indeed, a recent report concluded that Ras signaling was not involved in acute regulation of glucose transport by insulin in a poorly responsive fibroblast line (30). We designed the present studies to test whether activated Ras may mediate such acute actions of insulin in a cell type more similar to primary insulin-responsive tissues. Cultured 3T3-L1 cells were chosen, which when differentiated to an adipocyte phenotype, express the insulin-regulated glucose transporter protein (GLUT) 4 isoform and are highly sensitive to insulin. A major mode by which insulin rapidly increases glucose transport in these cultured cells and in primary muscle and fat cells is to modulate membrane trafficking of GLUTs, increasing their localization to the cell-surface membrane (31). The results reported here show that 3T3-L1 adipocytes expressing a mutant, activated form of Ras indeed exhibit elevated hexose-transport rates and a marked translocation of GLUTs to the plasma membrane. These data implicate Ras proteins as key intermediaries in the signaling pathway whereby insulin modulates glucose transport.

EXPERIMENTAL PROCEDURES

Cell Culture. Wild-type 3T3-L1 cells were seeded at 3.5×10^4 cells per cm^2 in Dulbecco's modified Eagle's medium (DMEM)/10% calf serum/penicillin at 50 units/ml/streptomycin sulfate at 50 μg/ml. 3T3-L1 cells transfected with p21^{Ras61K} were seeded at $\approx 0.5 \times 10^5$ cells per cm^2 in DMEM/penicillin at 50 units/ml/streptomycin sulfate at 50 μg/ml/active Geneticin at 250 μg/ml/10% calf serum that had been treated with activated charcoal and Bio-Rad AG 1-X8 anion-exchange resin. Fibroblasts were used at ≈90% confluence.

Two days after the fibroblasts reached confluence, differentiation into adipocytes was induced by incubating the cells for 3 days in DMEM/10% fetal bovine serum/insulin at 5 μg/ml/1 μM dexamethasone/0.5 mM 3-isobutyl-1-methylxanthine/penicillin at 50 units/ml/streptomycin sulfate at 50 μg/ml. The cells were then incubated for an additional 3 days in the same medium without 3-isobutyl-1-methylxanthine.

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Abbreviation: GLUT, glucose transporter protein.

Thereafter the cells were maintained in DMEM/10% fetal bovine serum/penicillin at 50 units/ml/streptomycin sulfate at 50 μg/ml, with medium changes every 2 or 3 days for an additional 4–10 days. At this time, >95% of the cells expressed the adipocyte phenotype (32).

Measurement of 2-Deoxyglucose Uptake in 3T3-L1 Cells. 2-Deoxyglucose uptake rates were measured in fibroblasts and adipocytes, essentially as described by Frost and Lane (33). Briefly, cells were washed twice with DMEM and incubated for 2 hr at 37°C in DMEM with no supplements. Cells were then washed twice in phosphate-buffered saline (PBS) (137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4/0.68 mM $CaCl_2$/0.49 mM $MgCl_2$, pH 7.4) and incubated in 1 ml of PBS per 6-well tissue culture dish (fibroblasts) or 0.5 ml of PBS per 12-well tissue culture dish (adipocytes) with or without 200 nM insulin for 30 min at 37°C. Cells were then washed once with PBS, and then 1 ml of PBS/0.1 mM 2-deoxyglucose/2-deoxy-D-[2,6-^{3}H]glucose at 1 μCi/ml (1 Ci = 37 GBq) was added to each well for 5 min. Assays were terminated by rapidly washing the cells three times with ice-cold PBS. Cells were solubilized with 0.4 ml of 1.0% SDS, and ^{3}H was detected in 4 ml of scintillant by using a Beckman LS5000TD scintillation counter. Nonspecific deoxyglucose uptake was measured in the presence of 20 μM cytochalasin B and was subtracted from each determination to obtain specific uptake.

Determination of Phosphatidylinositol 3-Kinase Activity in Immunoprecipitates. Lysates were prepared from adipocytes according to the method of Ruderman *et al.* (6). Briefly, cells were serum deprived for 2 hr at 37°C and then stimulated or not for 10 min with 100 nM insulin. The cells were then rinsed with PBS/150 μM sodium vanadate and then scraped in 2 ml of buffer (20 mM Tris, pH 8.1/137 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/10% glycerol/1% Nonidet P-40) containing 150 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin at 10 μg/ml. Lysates were homogenized by drawing through a 25-gauge needle twice and were clarified by centrifugation at 13,000 × *g* for 15 min. Clarified lysates were then immunoprecipitated by using either CT-1, a mouse monoclonal antibody specific for the insulin receptor (34), or 1G2, a mouse monoclonal antibody raised against phosphotyrosine (35), and goat-anti-mouse IgG bound to Sepharose beads (Cappel). The immune complexes were washed according to the established procedure (6), and phosphatidylinositol 3-kinase activity was assayed directly in the immune complex, according to the procedure of Hayashi *et al.* (36). Briefly, the reaction is carried out in a total volume of 50 μl consisting of 20 mM Tris, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA, 100 μM phosphatidylinositol, 100 μM phosphatidylserine, 120 μM adenosine, and [γ-^{32}P]ATP (10 μM ATP; 0.2 μCi/μl). After 10 min at 30°C, the reaction was stopped by adding 200 μl of 1 M HCl and 160 μl of chloroform/methanol, 1:1 (vol/vol). The organic phase was re-extracted with 100 μl of 1 M HCl. The reaction products (15 μl) were separated on Silica gel 60 thin-layer plates by using the chromatography system of Walsh *et al.* (37), which separates phosphatidylinositol 3′-phosphate from phosphatidylinositol 4′-phosphate. The results were quantitated by using a BetaScope from Betagen (Waltham, MA).

Subcellular Membrane Fractionation. Fractionation of subcellular membranes from 3T3-L1 adipocytes was done essentially as described (38). Briefly, cells were grown and differentiated in 150 × 20 mm culture plates and incubated for 2 hr in unsupplemented DMEM; the cells were then incubated for an additional 30 min with or without 200 nM insulin in the same medium. Next, cells were washed once with PBS/1 mM phenylmethylsulfonyl fluoride, warmed to 37°C, and then scraped into 24 ml of ice-cold TES (20 mM Tris/1 mM EDTA/250 mM sucrose, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and immediately homogenized with 15 strokes of a motor-driven Teflon pestle in a 40-ml homogenization vessel (Wheaton Scientific). The homogenate was subjected to centrifugation at 16,000 × g_{max} for 20 min, and the resuspended pellet was applied to a sucrose cushion (1.12 M sucrose in TES), and subjected to centrifugation at 100,000 × g_{max} for 1 hr. Plasma membranes were removed from the top of the sucrose cushion, resuspended in 25 ml of TES, and subjected to centrifugation at 250,000 × g_{max} for 10 min, while the supernatant from the 16,000 × *g* centrifugation was subjected to centrifugation at 250,000 × g_{max} for 1.5 hr to collect the low-density microsome fraction.

GLUT Immunoblot Analysis. The antisera and method have been described (38). Briefly, aliquots of subcellular membrane fractions containing equal amounts of total protein were subjected to SDS/10% PAGE, in duplicate gels, under reducing conditions, as described by Laemmli (39). The resolved proteins were electrophoretically transferred to Schleicher & Schuell nitrocellulose filters (40). Duplicate filters were then incubated in blocking buffer (250 mM NaCl/20 mM Tris/0.5% bovine serum albumin/0.5% gelatin/0.1% Tween 20, pH 7.5) for 2 hr at room temperature. One of the duplicate filters was then incubated for 12 hr at 4°C with a 1:1000 dilution of R-480 antiserum (anti-C-terminal GLUT-1 peptide) (38) prepared in antibody dilution buffer (250 mM NaCl/20 mM Tris/1 mM EDTA/0.1% gelatin/0.05% Tween 20, pH 7.5). The other duplicate filter was incubated for 12 hr at 4°C with a 1:500 dilution of R-1288 antiserum (anti-C-terminal GLUT-4 peptide) (38) prepared in antibody dilution buffer. The filters were washed six times for 5 min each with antibody dilution buffer and then incubated for 1 hr at room temperature with ^{125}I-labeled protein A at 0.05 μCi/ml prepared in antibody dilution buffer. The filters were then washed as before, air dried, wrapped in Saran-Wrap, and subjected to autoradiography with Kodak X-Omat film at −70°C with a DuPont LightningPlus intensifying screen. Quantitation was done by using an LKB densitometer.

RESULTS AND DISCUSSION

Previous studies demonstrated the stable transfection of 3T3-L1 fibroblasts with cDNA encoding mutant N-Ras^{61K} under the control of the dexamethasone-sensitive Moloney murine leukemia virus promoter, and the stable differentiation of these cells to the adipocyte phenotype (32). Overexpression of the activated Ras protein occurs in the absence of dexamethasone in these cells but is further enhanced by the agent (32). As shown in Fig. 1, parental 3T3-L1 fibroblasts responded to 100 nM insulin with a 2-fold increase in 2-deoxy[^{3}H]glucose uptake, consistent with published results (41). Fibroblasts expressing N-Ras^{61K} exhibited a 2.2-fold stimulation of 2-deoxy[^{3}H]glucose uptake compared with the nontransfected cells, and insulin had no significant effect (Fig. 1). As previously observed, differentiated parental 3T3-L1 adipocytes were more responsive to insulin than were fibroblasts (Fig. 2), in parallel with the expression of GLUT-4. 3T3-L1 adipocytes harboring N-ras^{61K} also displayed a marked stimulation of 2-deoxy[^{3}H]glucose transport, similar to the effect of insulin in untransfected cells. Again, insulin had no significant effect over that of N-Ras^{61K} expression in the cultured adipocytes (Fig. 2). Taken together, these data show that overexpression of an activated Ras protein mimics the stimulatory effect of insulin on 2-deoxy[^{3}H]glucose transport in both cultured fibroblasts and adipocytes.

We tested whether the effects of N-Ras^{61K} on 3T3-L1 cells shown in Figs. 1 and 2 might simply be from upstream activation of insulin receptors rather than from a downstream signaling pathway. Immunoblot analysis of lysates prepared from control and insulin-stimulated cultured adipocytes re-

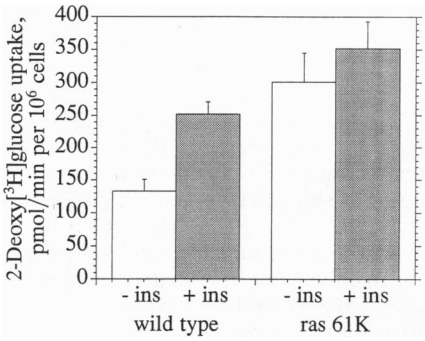

FIG. 1. Effect of insulin on the rate of 2-deoxyglucose uptake in ras^{61K}-transfected and nontransfected 3T3-L1 fibroblasts. Cells were grown to ≈90% confluence, as described, and then incubated in serum-free medium for 2 hr, washed with Dulbecco's PBS and incubated for 30 min in the same buffer with or without 100 nM insulin (ins) at 37°C. 3T3-L1 fibroblasts were assayed for 2-deoxyglucose uptake at 0.1 mM substrate, as described. Values shown are averages ± the SD of six experiments. Assays were done in quadruplicate for each experimental condition. Specific uptake was determined by subtracting appropriate nonspecific control values, as described. Open and shaded bars indicate 2-deoxyglucose uptake rates for control and insulin-stimulated fibroblasts, respectively.

vealed that both parental and N-Ras^{61K}-expressing cell types display approximately the same levels of insulin receptors (data not shown). Both cell types also responded equally well to insulin by autophosphorylating insulin receptors on tyrosine residues (data not shown). An even more sensitive indicator of insulin-receptor activation in intact cells is the rapid elevated association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated proteins (5–8). Parental and N-Ras^{61K}-expressing 3T3-L1 adipocytes were therefore incubated with or without 100 nM insulin for 10 min before preparation of cell lysates with 1% Nonidet P-40. Lysates were immunoprecipitated with the anti-insulin-receptor antibody CT-1 or with an anti-phosphotyrosine antibody, and the precipitates were assayed for phosphatidylinositol 3-kinase activity. Fig. 3 shows that insulin stimulated the coimmunoprecipitation of phosphatidylinositol 3-kinase activity with both insulin receptors (≈6-fold) and tyrosine-phosphorylated proteins (≈9-fold) in parental cultured adipocytes. Importantly, untreated N-ras^{61K}-transfected cells showed no elevation in coimmunoprecipitated phosphatidylinositol 3-kinase activity, and insulin addition exerted the same stimulatory effect as in untransfected cells (Fig. 3). These combined results indicate that the actions of activated Ras to mimic insulin action on glucose-transport activity in

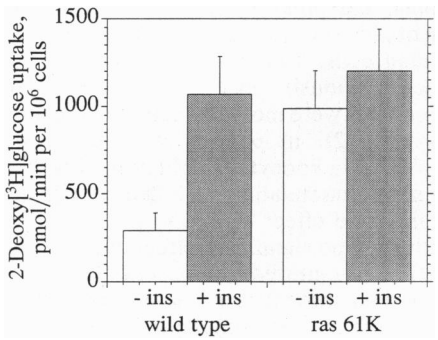

FIG. 2. Effect of insulin (ins) on the rate of 2-deoxyglucose uptake in ras^{61K}-transfected and nontransfected 3T3-L1 adipocytes. Cells were grown and differentiated, as described. 2-Deoxyglucose-uptake assays were done, as described. Values shown are averages ± the SD of 11 experiments. Open and shaded bars indicate 2-deoxyglucose uptake for control and insulin-stimulated adipocytes, respectively.

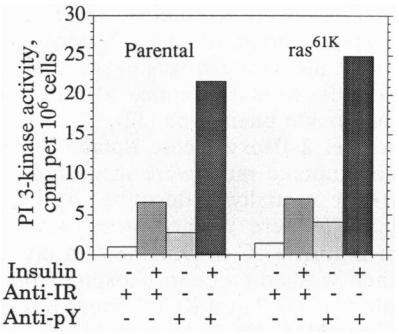

FIG. 3. Effect of insulin on the immunoprecipitation of phosphatidylinositol 3-kinase activity by antiinsulin receptor (Anti-IR) and antityrosine phosphate (Anti-pY) antibodies in ras^{61K}-transfected and nontransfected 3T3-L1 adipocytes. Cells were grown and differentiated, as described, and were then incubated in serum-free medium for 2 hr before the experiment. The cells were then stimulated with or without 100 nM insulin, as indicated, for 10 min before lysis and immunoprecipitation of either the insulin receptor or phosphotyrosine-containing proteins, as described. Phosphatidylinositol (PI) 3-kinase activity was measured in washed immune complexes by incubation with 100 μM phosphatidylinositol and 10 μM [γ-^{32}P]ATP for 10 min at 30°C in a total volume of 50 μl. Phospholipids were extracted and separated by TLC, as described. The radioactivity present in the phosphatidylinositol 3-phosphate spot was quantitated using a Betagen BetaScope and was normalized to cell number. Results of a representative experiment are shown.

3T3-L1 adipocytes is not due to activation of insulin receptors.

A simple mechanism whereby activated Ras might stimulate hexose-transport activity is by increasing the cellular levels of GLUTs. To test this possibility, immunoblot analysis was performed on total membranes prepared from parental and N-ras^{61K}-transfected fibroblasts and adipocytes to examine the amounts of the two isoforms of GLUTs (isoform 1 and 4) known to be expressed in 3T3-L1 cells. Expression of N-Ras^{61K} had no effect on total GLUT-1 protein in either fibroblasts or adipocytes, whereas total GLUT-4 protein was markedly decreased by ≈95% in the N-ras^{61K}-transfected adipocytes compared with controls (data not shown). We next tested whether activated ras affects the membrane distribution of glucose transporters by immunoblot analysis of plasma membranes and intracellular low-density microsomes purified from 3T3-L1 adipocytes. As expected, insulin action caused ≈1.5- and 2-fold increases in the amounts of GLUT-1 and GLUT-4, respectively, recovered in plasma membranes of untransfected cultured adipocytes (Figs. 4 and 5 *Upper*). Corresponding decreases in GLUT-1 and GLUT-4 due to insulin in these cells were seen in the intracellular membranes. Strikingly, the relative distributions of GLUT-1 and GLUT-4 in the plasma membrane versus low-density microsome fractions of untreated N-ras^{61K}-transfected adipocytes were even more markedly increased when compared with the above insulin effect (Figs. 4 and 5 *Bottom*). For GLUT-4, virtually all detectable transporter protein was translocated to the plasma-membrane fraction in adipocytes expressing activated Ras (Fig. 5 *Bottom*). Moreover, insulin treatment of N-ras^{61K}-transfected adipocytes caused no further change in plasma membrane or low-density microsome content of either GLUT-1 or GLUT-4 (Figs. 4 and 5 *Bottom*).

The above data are consistent with the hypothesis that expression of the N-Ras^{61K} protein in 3T3-L1 adipocytes mimics the effect of insulin to translocate GLUTs to the cell-surface membrane. However, differences between the effects of N-Ras^{61K} and insulin in these experiments suggest caution be applied to interpretation about underlying mechanisms. The greater magnitude of the N-Ras^{61K} effect com-

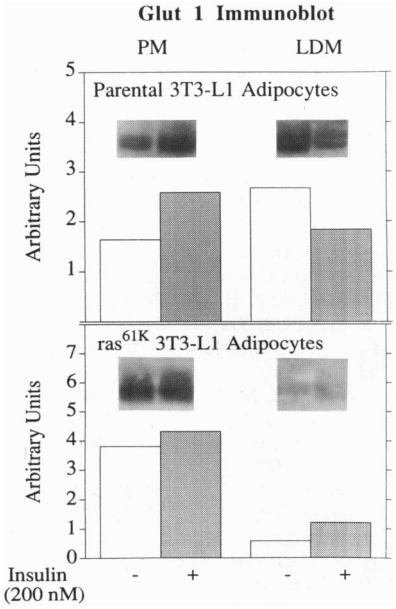

FIG. 4. Effect of insulin and N-Ras^{61K} expression on GLUT-1 levels in membrane fractions prepared from 3T3-L1 adipocytes. Confluent 150-mm tissue culture dishes of N-ras^{61K}-transfected and untransfected 3T3-L1 adipocytes were incubated in serum-free medium for 2 hr and then stimulated or not stimulated with insulin. Monolayers were washed once with PBS, and membrane fractions were prepared, as described. Fifty micrograms of membrane protein was solubilized in electrophoresis sample buffer and resolved by SDS/PAGE on 10% acrylamide gels. After resolution, proteins were transferred electrophoretically to nitrocellulose sheets at 200 mA for 2 hr. Immunoblot analysis was performed by using an anti-GLUT-1-C-terminal peptide rabbit polyclonal antibody, followed by ^{125}I-labeled protein A. Radiolabeled proteins were visualized by autoradiography and were quantitated by densitometry, as described. Results shown are for plasma membrane fractions (PM) and for low-density microsome fractions (LDM) in a representative experiment of three replications.

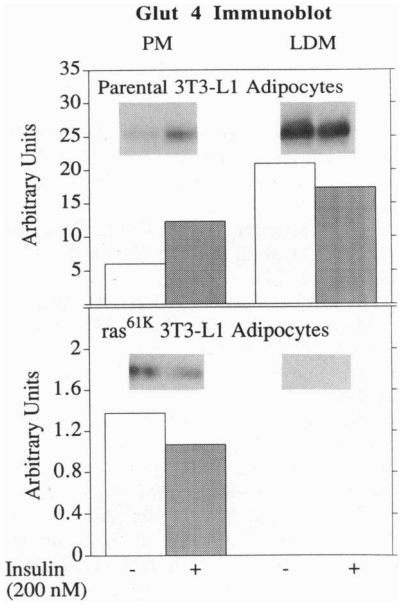

FIG. 5. Effect of insulin and N-Ras^{61K} expression on GLUT-4 levels in membrane fractions prepared from 3T3-L1 cells. Samples were prepared and treated, as described and as described in the legend to Fig. 4, except that a GLUT-4-specific rabbit polyclonal antibody was used for the immunoblot analysis. Results shown are for plasma membrane fractions (PM) and for low-density microsome fractions (LDM) in a representative experiment of three replications.

pared with that of insulin may reflect the higher levels of activated, GTP-bound Ras protein achieved in the transfected cells compared with parental cells treated with insulin. Alternatively, N-Ras^{61K} may act on cellular distribution of GLUTs by a different mechanism than insulin. The fact that so little GLUT-4 is expressed in N-ras^{61K}-transfected cells also complicates comparisons of GLUT-4 distributions in these cells versus parental cultured adipocytes. Further work is required to rigorously evaluate the molecular basis whereby activated Ras modulates glucose transporters.

The results in Figs. 4 and 5 indicate that increases of both GLUT-1 and GLUT-4 at the plasma membrane contribute to the elevated hexose uptake in N-ras^{61K}-transfected adipocytes. However, GLUT-4 transporters probably contribute less to this effect of the activated Ras protein than to insulin action in these experiments due to the much lower amounts of GLUT-4 in the transfected cells. It is important to note that a decreased expression (≈50%) of GLUT-4 is also observed in 3T3-L1 adipocytes chronically treated with insulin over 48 hr (42). In this sense, chronic exposure of 3T3-L1 adipocytes to the N-Ras^{61K} protein or to insulin yields decreased GLUT-4 expression. However, N-Ras^{61K} causes a much greater effect than insulin, and it is not possible to conclude that similar mechanisms are operating. The elevated glucose-transport activity in 3T3-L1 adipocytes treated acutely with insulin is mostly due to GLUT-4 (43). However, in cells treated chronically with insulin, high transport activity is due mostly to increased GLUT-1 protein (44), as is probably the case in N-ras^{61K}-transfected adipocytes (Fig. 2). In any case, the reported effect of insulin to activate endogenous Ras proteins in 3T3-L1 adipocytes (32), coupled with the demonstrated action of the activated Ras protein to cause glucose-transporter redistribution (Figs. 4 and 5), are consistent with the hypothesis that Ras may be an intermediate in insulin action on glucose transport.

The detailed molecular mechanisms whereby insulin causes membrane redistribution of GLUTs are not known. Reported results indicate that membrane-bound GLUT-4 transporters are rapidly recycling between intracellular and cell-surface membranes and that insulin action is associated with both an increased exocytosis rate and decreased endocytosis (45). It is thought that small GTP-binding proteins, such as the Ras-like Rab proteins are involved in membrane trafficking and fusion events in secretory pathways, in general (46, 47), and potentially in GLUT-4 exocytosis (48, 49). One possible mechanism whereby Ras causes glucose-transporter translocation is the modulation of such small GTP-binding proteins. Hypothetically, this could occur through Ras activation of protein serine/threonine kinases or phosphatases or through protein–protein interactions of components that bind Ras and Rab proteins. It is noteworthy with respect to this latter postulate, that Ras GTPase-activating protein binds a 190 kDa protein containing sequences similar to those associated with GTPase-activating proteins specific for another small GTP-binding protein, Rho (50). It will be important to test these hypotheses on the molecular basis of Ras action on glucose-transporter trafficking in future experiments.

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