Overexpression of Ha-ras Selectively in Adipose Tissue of Transgenic Mice

EVIDENCE FOR ENHANCED SENSITIVITY TO INSULIN*

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To determine the role of Ras-dependent signaling pathways in adipocyte function, we created transgenic mice that overexpress Ha-ras in adipocytes using the aP2 fatty acid-binding protein promoter/enhancer ligated to the human genomic ras sequence. ras mRNA was increased 8–17-fold and Ras protein 4–5-fold in white and brown fat, with no overexpression in other tissues. The subcellular distribution of overexpressed Ras paralleled that of endogenous Ras. [U-14C]Glucose uptake into isolated adipocytes was increased ~2-fold in the absence of insulin, and the ED$_{50}$ for insulin was reduced 70%, with minimal effect on maximally stimulated glucose transport. Expression of Glut4 protein was unaltered in transgenic adipocytes, but photolabeling of transporters in intact cells with [3H]2-(1-azi-Z,Z,Z-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine revealed 1.7–2.6-fold more cell-surface Glut4 in the absence of insulin and at half-maximal insulin concentration (0.3 nm) compared with nontransgenic adipocytes. With maximal insulin concentration (80 nm), cell-surface Glut4 in nontransgenic and transgenic adipocytes was similar. Glut1 expression and basal cell-surface Glut1 were increased 2–2.9-fold in adipocytes of transgenic mice. However, Glut1 was much less abundant than Glut4, making its contribution to transport negligible. These in vitro changes were accompanied by in vivo alterations including increased glucose tolerance, decreased plasma insulin levels, and decreased adipose mass. We conclude that Ras overexpression in adipocytes leads to a partial translocation of Glut4 in the absence of insulin and enhanced Glut4 translocation at physiological insulin concentration, but no effect with maximally stimulating insulin concentrations.

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Insulin regulates a myriad of cellular events including mitogenesis, gene expression, and various metabolic pathways such as glucose transport, glycogen synthesis, and triacylglyceride synthesis. Insulin stimulates glucose uptake into target tissues primarily via the translocation of Glut4 from intracellular vesicles to the plasma membrane (1–3). Although major strides have been made in elucidating signaling pathways involved in insulin’s regulation of gene expression and mitogenesis, the molecular basis for insulin-stimulated glucose transporter translocation remains elusive. Recent data indicate that activation of PI3-kinase is involved, but PI3-kinase activation alone is not sufficient to achieve insulin-stimulated glucose transport (4–10).

The binding of insulin to its receptor initiates a cascade of signaling events involving activation of the receptor’s intrinsic tyrosine kinase activity, receptor autophosphorylation, and phosphorylation of cellular substrates such as insulin receptor substrate-1 and -2 and Shc (11). These proteins, in turn, act as docking modules for other proteins via SH2 domains, thereby forming multiple signaling complexes. One such signaling complex involves the interaction of insulin receptor substrate-1 and/or Shc with Grb2 and SOS, leading to the activation of the low molecular weight GTP-binding protein Ras. Insulin binding to its receptor causes an increase in cellular concentrations of GDP-bound Ras (12–14), and signaling pathways emanating from Ras have been implicated in mediating at least a portion of insulin’s pleiotropic effects on cell growth and metabolism (15). However, the role of Ras signaling pathways in the regulation of insulin-stimulated glucose transport remains controversial.

Van den Berghe et al. (16) compared the ability of insulin, epidermal growth factor, and thrombin to stimulate the Ras/mitogen-activated protein kinase cascade in 3T3-L1 adipocytes and found that Ras activation alone is not sufficient for insulin-stimulated glucose transport. Osterop et al. (12) and Draznin et al. (17) also reported that enhanced Ras signaling in Rat-1 cells had no effect on insulin-stimulated glucose transport. In contrast, Kozma et al. (18) reported that transfection of 3T3-L1 adipocytes with constitutively activated Ras resulted in enhanced translocation of Glut4 to the plasma membrane in the absence of insulin, and insulin did not further stimulate glucose uptake or Glut4 translocation. Interpretation of the latter study is tempered by the fact that Ras overexpression resulted in an ~95% down-regulation of Glut4 expression. Recently, transient expression of constitutively active Ras in primary rat adipocytes led to increased levels of cotransfected epitope-
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tagged Glut4 at the cell surface (10). Further support for this phenomenon comes from studies in which microinjection of activated Ras into cardiac myocytes caused a significant increase in glucose transport (19). In contrast, microinjection of constitutively active Ras (20) or downstream effectors such as activated Raf (21) into 3T3-L1 adipocytes had no effect on the translocation of Glut4. Additionally, injection of neutralizing antibodies directed against Ras resulted in a 50% inhibition of insulin-stimulated glucose transport in cardiac myocytes (19), but had no effect in 3T3-L1 adipocytes (20). The latter observation is supported by the fact that transfection of rat adipocytes with dominant-negative Ras did not affect translocation of epitope-tagged Glut4 (10). Recently Reich et al. (22) found that lovastatin, which inhibits farnesylation of Ras (and thus appropriate targeting to the plasma membrane), attenuated insulin effects on thymidine incorporation and glucose incorporation into glycogen, but not glucose transport. However, the effects of lovastatin may not be specific to Ras since it also inhibits the rate-limiting step of cholesterol biosynthesis (23), geranylgeranylation of proteins (23), insulin stimulation of PI 3-kinase activity (24), and cell transformation by Raf (25).

We have addressed the question of Ras involvement in insulin-stimulated glucose transport using an in vivo approach, developing transgenic mice that overexpress Ha-Ras exclusively in brown and white adipose tissue. We show that wild-type Ras overexpression leads to a partial translocation of Glut4 and Glut1 in the absence of insulin as well as increased sensitivity to the effects of insulin on Glut4 translocation. However, Ras overexpression does not alter maximally insulin-stimulated glucose transport, suggesting that at maximal insulin concentration, Ras is not rate-limiting for glucose transport in adipocytes.

EXPERIMENTAL PROCEDURES

Transgenic Animals—The adipose-specific Ha-ras transgene (see Fig. 1) was constructed using the fast-specific promoter/enhancer from the fatty acid-binding protein gene, aP2 (gift of Dr. B. M. Spiegelman) and the genomic sequence of human Ha-ras (gift of Dr. G. Cooper). The 5.4-kilobase aP2 DNA fragment contains a consensus transcription initiation site and has been shown to promote high levels of expression of the reporter gene chloramphenicol acetyltransferase (26) and the glucose transporter GLUT4 (27) selectively in adipose tissue. The aP2-ras construct contains a 6.4-kilobase fragment of genomic ras sequence including 61 base pairs of 5'-untranslated sequence. The boundary of the fusion gene was confirmed by sequencing. The transgene was re-ligated into the SpeI fragment of the aP2 promoter blotting to identify offspring carrying the transgene. Mice were housed at 21°C with a 12-h light/dark cycle and were fed Purina mouse chow (Lifescan, Inc., St. Louis, MO).

Determination of Blood Glucose and Insulin Concentrations—Blood was collected from the tail vein prior to injection (time 0) and at 10, 20, 30, 60, 120, and 180 min post-injection. Northern Blotting—Total RNA was extracted from brain, liver, cardiac and skeletal muscle, brown and white adipose tissue, or isolated white fat, perigonadal adipose tissue, and cardiac and hind limb skeletal muscle, and subcellular membrane fractions were prepared from isolated white adipocytes (see method below). Protein concentration was assessed by the BCA technique (Pierce). Proteins were separated via SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose, and immunoblotted for Ras using a monoclonal antibody to human Ha-Ras (Oncogene Science Inc, Cambridge, MA). Briefly, blots were blocked (3% skim milk in Tris-buffered saline with 0.1% Tween 20) for 2 h at room temperature, incubated with antibody (150 μl of solution) for 3 h at room temperature, washed with Tris-buffered saline and 0.1% Tween 20, and incubated with secondary antibody conjugated to horseradish peroxidase. Bands were visualized with ECL and quantified by densitometry. Membranes were also blotted with an antisem specific to the COOH terminus of Glut4 (gift of H. Haspel) and Glut1 (gift of B. Thorens) as described previously (29).

Adipocyte Glucose Transport—Adipocytes were isolated from perigonadal fat pads by collagenase digestion (1 mg/ml; lot CLS1, Worthington) (30). Cells were incubated at 37°C with constant shaking (100 rpm) in a 2% suspension by volume, in Krebs-Ringer-bicarbonate-Hepes (30 mM Hepes) buffer (pH 7.4) supplemented with 2.5% bovine serum albumin (Fraction V, Sigma) and 200 μM adenosine in the presence (insulin-stimulated) or absence (basal) of various concentrations of porcine insulin (gift of Dr. R. Chance, Lilly) for 30 min. [U-14C]Glucose (3 μM final concentration) uptake was performed as described previously (31, 32). This glucose transport assay was chosen because it can be performed on a dilute suspension, which minimizes breakage of large and fragile mouse adipocytes. Furthermore, it requires few cells. Under the conditions utilized, the uptake of [14C]glucose has been shown to directly reflect glucose transport, and results parallel those with 3-O-methylglucose transport (31, 32).

Glucose Transport into Muscle—Intact soleus and extensor digitorum longus skeletal muscles were excised and incubated in vitro, and 2-deoxyglucose uptake was measured as described previously for the rat epididymal fat (33). 2-Deoxyglucose uptake was assessed at conditions in which transport is rate-limiting for glucose utilization (34).

Subcellular Fractionation of Adipocytes—Adipocytes were isolated from gonadal fat pads from 15–30 mice (total fat pad weight of 8–10 g) and incubated for 30 min in the absence or presence of 80 nM insulin as described above. A small aliquot (4 × 10^6 μl) of both basal and insulin-stimulated cells was taken for determination of total cell protein (31). The remaining cells were washed twice with Trizma (Tris base)/EDTA/sucrose buffer (pH 7.4, 18°C) and homogenized, and plasma membranes, low density microsomes, and high density microsomes were prepared by differential centrifugation (35). Relative purity and recovery of the fractions were assessed by measurement of cytochrome c reductase activity (marker of the endoplasmic reticulum) (36) and immunoblots of the subcellular fractions with an anti-Ras antibody (Oncogene Science Inc) since p21H ras is found primarily in the plasma membrane.

Photobinding of Cell-surface Glucose Transporters—Photobinding of Glut4 and Glut1 was carried out essentially by the method of Holman et al. (37) for rat adipocytes. Isolated adipocytes were prepared from 3- to 4-week-old Sprague-Dawley rats. Fragmentation gel purification of the transgene was injected into the pronucleus of fertilized zygotes from FVB mice and transferred to pseudopregnant females. Genomic DNA from tail clippings was digested with EcoRI and analyzed by Southern blotting to identify offspring carrying the transgene. Mice were housed at 21°C with a 12-h light/dark cycle and were fed Purina mouse chow (5008 ad libitum. All studies were conducted using heterozygous mice.

Determination of Blood Glucose and Insulin Concentrations—Blood glucose levels were measured using a One Touch 11 glucose meter (LifeScan, Inc.). Plasma insulin concentrations were determined with a radioimmunoassay kit using rat insulin standards (Linco Research, Inc., St. Louis, MO).

Glucose Tolerance Tests—Subsequent to a 14-h fast, conscious mice were injected with d-glucose (1 mg/g of body weight) intraperitoneally. Blood was collected from the tail vein prior to injection (time 0) and at 10, 20, 30, 60, 120, and 180 min post-injection.

Northern Blotting—Total RNA was extracted from brain, liver, cardiac and skeletal muscle, brown and white adipose tissue, or isolated white fat, perigonadal adipose tissue, and cardiac and hind limb skeletal muscle, and subcellular membrane fractions were prepared from isolated white adipocytes (see method below). Protein concentration was assessed by the BCA technique (Pierce). Proteins were separated via SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose, and immunoblotted for Ras using a monoclonal antibody to human Ha-Ras (Oncogene Science Inc, Cambridge, MA). Briefly, blots were blocked (3% skim milk in Tris-buffered saline with 0.1% Tween 20) for 2 h at room temperature, incubated with antibody (150 μl of solution) for 3 h at room temperature, washed with Tris-buffered saline and 0.1% Tween 20, and incubated with secondary antibody conjugated to horseradish peroxidase. Bands were visualized with ECL and quantified by densitometry. Membranes were also blotted with an antisem specific to the COOH terminus of Glut4 (gift of H. Haspel) and Glut1 (gift of B. Thorens) as described previously (29).
Expression of the Transgene—Two independent lines of transgenic mice that overexpress Ha-ras were generated using the transgene shown in Fig. 1. Lines were designated as moderate and high level overexpressing. Fig. 2 A shows Northern blotting of RNA from brown adipose tissue. For Fig. 2 (A and B), equivalent loading of lanes was verified by ethidium bromide staining of the gels. ras mRNA in brown adipose tissue was increased ~8-fold in the moderate level overexpressing line and ~16-fold in the high level overexpressing line compared with nontransgenic litter mates. Fig. 2B shows that ras overexpression was limited to brown and white adipose tissue in the moderate overexpressing line, with no overexpression in heart, skeletal muscle, brain, or liver. Although in this particular Northern blot the size of ras mRNA appears slightly larger in nontransgenic white adipose tissue compared with all other tissues, this was not observed in other samples of nontransgenic white adipose tissue analyzed on other Northern blots. In the high level overexpressing line, increased levels of ras mRNA were also observed in several other tissues in addition to fat (data not shown). Thus, in vivo studies of glucose homeostasis were not pursued in the high level overexpressing line.

Fig. 3A (upper panel) shows that the overexpression of Ras at the protein level in white adipose tissue was also severalfold higher in the high level overexpressing line compared with the moderate line. The exposure shown was chosen to illustrate the difference in the levels of Ras protein in the two transgenic lines and thus is too light to see Ras protein in nontransgenic fat. However, Ras protein could be observed in nontransgenic lanes with longer exposure. Fig. 3A (lower panel) shows a representative blot of Ras protein in post-nuclear membranes prepared from isolated adipocytes from nontransgenic and moderate level Ras-overexpressing mice. The mean of multiple blots shows that Ras protein is increased 3-4-fold in the moderate level overexpressing mice compared with nontransgenic mice. No adjustments need to be made to compare the amount of Ras expression per cell since the recovery of total membrane protein was not different in isolated adipocytes from nontransgenic and transgenic mice (nontransgenic, 447 ± 99 pg/cell; transgenic, 595 ± 66 pg/cell; n = 4, p = not significant). Levels of Ras protein could not be assessed in isolated adipocytes from high level overexpressing mice due to the difficulty in harvesting large amounts of isolated adipocytes from this line. However, the quantitative information in the moderate overexpressing line is most important since that is the line in which we pursued both in vivo and in vitro studies of insulin action.

Subcellular Distribution of Ras—Fig. 3B shows the subcellular distribution of Ras. In nontransgenic adipocytes (upper panel), Ras resides primarily in the plasma membrane and high density microsomes and does not translocate in response to insulin. A very small amount of Ras can be seen in the low density microsomes also. In transgenic adipocytes (lower panel), Ras distribution parallels that in nontransgenic adipocytes, with the highest amounts in the plasma membrane. Ras is clearly detectable in the high density microsomes as well. Because this fraction is enriched in the endoplasmic reticulum, Ras in this fraction most likely represents newly synthesized protein. Alternatively, it may be due to contamination from plasma membranes, which is always seen in the high density microsome fraction (35). A similar amount of contamination of the high density microsomes with plasma membranes in nontransgenic and transgenic mice would result in much greater levels of Ras in transgenic high density microsomes due to the higher levels of Ras in the plasma membranes of transgenic cells. Due to the high level of Ras overexpression in adipocytes from transgenic mice, Ras is also detectable in the low density microsomes. However, the amount of Ras in this fraction is less than that in the homogenate. There is an enrichment only in the plasma membranes and high density microsomes, which is similar to the distribution in nontransgenic adipocytes. Ras protein was not detectable in cytosol from either nontransgenic or transgenic adipocytes (data not shown). Thus, the overall pattern of Ras distribution between nontransgenic and transgenic cells is similar. However, since Ras is increased in all membrane fractions of transgenic adipocytes, we cannot rule out the possibility that some of the effects observed are due to increased levels of Ras protein in internal membranes.

This similarity in the relative distribution of Ras between nontransgenic and transgenic cells is one indication that the membranes from the nontransgenic and transgenic cells are

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y = response \text{ variable (glucose transport)}, \quad \delta = \text{asymptotic value of } Y \text{ at } X = 0 \text{ (basal)}, \quad X = \text{concentration of insulin}, \quad Y = \text{asymptotic value of } Y \text{ at } X = \infty, \quad \mu = \text{concentration of insulin that produces } \frac{\delta}{2}(Y - \delta) + \delta, \quad \beta = \text{standardized slope parameter where } \frac{\beta Y}{4\mu} \text{ is the rate of change in the response at } X = \mu \text{. Curve fitting was performed with the Prophet program (National Institutes of Health). Parameter estimates were obtained for each individual dose-response experiment, and the results from multiple experiments were meaned.}
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fractionating in a similar manner. As an independent assessment of this, we measured cytochrome c reductase activity, a marker for the endoplasmic reticulum. High density microsomes from both nontransgenic and transgenic cells were enriched ~3-fold in cytochrome c reductase activity, whereas low density microsomes and plasma membranes showed no enrichment in both genotypes. Recoveries of cytochrome c reductase activity were similar for both genotypes. Adequate membrane protein was not available for additional marker enzyme assays.

Body Weight, Fat Pad Weight, and Adipocyte Size—To determine whether the increased Ras expression in adipocytes would alter adipose tissue development, we measured body weight, gonadal fat pad weight, and adipocyte size in sex- and age-matched nontransgenic and transgenic litter mates (Table I). We initially compared mice at each week of age. In male mice, there was no difference in body weight between nontransgenic and moderate level Ras-overexpressing mice at any age from 12 to 25 weeks. For each of these genotypes, there was less than a 2-g increase in mean weight over this time period, so the body weight data for the combined ages are shown on the first line of Table I. To illustrate that the same conclusion is reached when comparing mice of exactly the same age, the weight comparison for male mice at 17 weeks is shown on the second line. High level Ras-overexpressing male mice were several grams lighter than nontransgenic mice. In females, body weights for both moderate level and high level Ras-overexpressing mice were 1.6 g lighter than nontransgenic mice when combining ages from 12 to 19 weeks. When a single age was examined, the difference in weight between the nontransgenic and transgenic mice ranged from 1.7 to 3.0 g. The 15-week data for female mice are also included separately in Table I to illustrate the greatest difference that was seen at any age.

Gonadal fat pad weight was reduced with both moderate and high level Ras overexpression in both male and female mice compared with nontransgenic mice. Because expression of the transgene had marked effects on gonadal fat pad weight and cell size at all ages and the effects were of similar magnitude from 10 to 24 weeks in males and from 12 to 20 weeks in females, the data for the combined ages are shown in Table I. In male mice, there was a “dose-dependent” effect of the transgene, so an even greater reduction in fat pad weight was seen with high level Ras overexpression compared with moderate overexpression. Adipocyte size was reduced in both male and female transgenic mice compared with nontransgenic mice (p < 0.05). High level overexpression tended to have a greater effect on adipocyte size than moderate level overexpression (p < 0.05 in females, but not significant in males). Grossly, fat pad morphology was normal with moderate level Ras overexpression, but strikingly different with high level Ras overexpression where increased vascularity was observed. This, coupled with the very small adipocyte size, made floatation and isolation of adipocytes from the high level overexpressing line very difficult. Thus, only limited studies could be carried out using the high level overexpressing line.

In Vivo Glucose Homeostasis—To determine whether Ras overexpression in isolated adipocytes could alter insulin action and glucose homeostasis in vivo, we assessed the effects of moderate level Ras overexpression on blood glucose and plasma insulin concentrations and on glucose tolerance. Blood glucose concentrations in fasted (14 h) or fed animals were unaffected by Ras overexpression (Table II), although fed levels tended to be slightly lower in male transgenic mice compared with nontransgenic males (p < 0.07). Even when the data were analyzed for each week of age individually, there were no statistically significant differences between nontransgenic and transgenic mice. Plasma insulin concentrations in the fed state were significantly lower in transgenic mice compared with nontransgenic litter mates (p < 0.05). Glucose tolerance tests (Fig. 4) revealed that the normal increase in blood glucose following intraperitoneal glucose injection was attenuated in transgenic mice, indicating significant enhancement in net glucose disposal in transgenic mice compared with nontransgenic litter mates (area under curve; p < 0.01).

Glucose Transport into Muscle in Vitro—We wanted to determine whether the improved glucose tolerance in transgenic mice could be due to enhanced uptake of glucose into muscle since muscle accounts for at least 80% of insulin-stimulated glucose uptake in normal animals and humans (40). We measured 2-deoxyglucose uptake in soleus (slow twitch) and extensor digitorum longus (primarily fast twitch) muscles from nontransgenic and transgenic mice. Insulin stimulated
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Effects of Ras overexpression on body weight, fat pad weight and adipose cell size

Values are means ± S.E. Numbers of mice are indicated in parentheses.

| Age | Nontransgenic | Ras transgenic |
|-----|---------------|----------------|
|     |               | Moderate overexpression | High overexpression |
| Male |               |                         |                    |
| Body weight (g) | 12–25 | 33.8 ± 0.5 (26) | 32.6 ± 0.6 (35) |
|         | 17    | 33.4 ± 0.9 (11) | 33.0 ± 1.5 (10) |
| Gonadal fat pad weight (g) | 10–24 | 0.87 ± 0.07 (19) | 0.43 ± 0.05 (26) |
|       | 15    | 28.3 ± 0.9 (11) | 25.3 ± 0.6 (13) |
| Adipocyte size (μg lipid/cell) | 10–24 | 0.399 ± 0.026 (17) | 0.178 ± 0.016 (16) |
|       | 15    | 0.61 ± 0.07 (18) | 0.29 ± 0.02 (27) |
| Female |       | 0.309 ± 0.03 (8) | 0.115 ± 0.02 (5) |

Different from nontransgenic mice at p < 0.05.  
Different from moderate level ras-overexpressing mice at p < 0.05.  
Some determinations of adipocyte size were performed on cells pooled from 10 to 20 mice, so each mean reflects over 50 mice.

Blood glucose and plasma insulin levels in nontransgenic and transgenic mice

Values are means ± S.E. Numbers of mice are indicated in parentheses. Fasting values were obtained following an overnight fast in mice that were 10–12 weeks of age. Fed values were obtained between 8:00 and 10:00 a.m. in mice that were 12–18 weeks of age.

| Fasting glucose | Fed glucose | Fed insulin |
|-----------------|-------------|-------------|
| Nontransgenic   | Transgenic  | Nontransgenic | Transgenic  | Nontransgenic | Transgenic  |
| Male | 116 ± 3 (4) | 117 ± 5 (5) | 154 ± 5 (9) | 141 ± 5 (10) | ND | ND |
| Female | 105 ± 3 (25) | 107 ± 5 (30) | 126 ± 3 (4) | 125 ± 4 (8) | 5.8 ± 1.1 (5) | 1.9 ± 0.8 (5) |

p < 0.05 compared with nontransgenic mice of same sex.  
ND, not determined.  
p < 0.05 compared with nontransgenic mice of same sex.

depicted in Fig. 5. In the left panel, transport rates are shown as nmol/cell/min for a representative experiment. When results from seven nontransgenic and six transgenic experiments are combined (data not shown), basal transport rates are elevated 1.8–2.5-fold in transgenic mice compared with nontransgenic litter mates (p < 0.01). Maximal insulin-stimulated transport is not increased in transgenic adipocytes. However, a shift to the left in the dose-response curve indicates increased insulin sensitivity. The right panel shows the data after curve fitting using the rising logistic equation given under “Experimental Procedures,” which takes into account the difference in basal transport. Curve fitting data from seven nontransgenic and six transgenic experiments gave the parameters shown in Table III. The mean ED₅₀ for the transgenic mice is reduced 80% (as shown in the inset in the right panel; p < 0.04), which indicates increased insulin sensitivity. To confirm these results in an independent line, we examined the effects of high level Ras overexpression on glucose transport in adipocytes (Fig. 6). The left panel shows that basal transport was elevated 3.3–4.2-fold, and maximally insulin-stimulated transport was increased 60% (p < 0.05). The right panel shows the results after curve fitting and thus correcting for the difference in basal transport. The curve fitting parameters are shown in Table III. In this separate transgenic line, there is also a shift to the left in the dose-response curve, and the ED₅₀ (inset in the right panel) is reduced 48% compared with nontransgenic litter mates (p < 0.05), again indicating increased sensitivity to insulin.

The difference in the ED₅₀ values for the controls in Figs. 5 and 6 is due to the fact that different sexes were studied. Normal female mice are more sensitive to insulin than males, both in vivo and in adipocytes in vitro (27). By studying both sexes, we demonstrate that the increased insulin sensitivity is present in adipocytes from Ras-overexpressing mice of both sexes (Figs. 5 and 6 and Table III).

Glut1 and Glut4 Protein Levels in Adipose Tissue—Western blotting of post-nuclear membranes from gonadal adipose tissue revealed no difference in Glut4 protein levels in transgenic
was increased 4-fold (data not shown). Glut4 expression was not increased, and Glut1 expression with nontransgenic litter mates (Fig. 7).

Insulin dose-response curves for glucose transport into isolated adipocytes from nontransgenic and moderate level Ras-overexpressing mice. Adipocytes were isolated and incubated with varying concentrations of insulin as described under "Experimental Procedures." Left panel, glucose transport expressed as amol/cell/min. Right panel, data after curve fitting, which corrects for differences in basal rates of transport. Data are means ± S.E. of quadruplicate samples for a single experiment using male nontransgenic (open squares) and moderate level Ras-overexpressing transgenic (closed squares) mice that were 14 weeks of age. Curves are representative of seven experiments for nontransgenic and six experiments for transgenic male mice that were 12–14 weeks of age (see Table III). Right panel inset, ED₅₀ values calculated by curve fitting. Values for ED₅₀ are means ± S.E. for seven nontransgenic (NTG) and six transgenic (TG) male mice that were 12–14 weeks of age. *, different from nontransgenic mice at p < 0.04.

| Table III | Curve fitting parameters for insulin dose-response curves for glucose transport |
|-----------|---------------------------------|
|           | Nontransgenic | Transgenic moderate overexpression | Transgenic high overexpression |
| R²        | 0.993 ± 0.001 | 0.994 ± 0.002 | 0.997 ± 0.001 |
| β (basal) | 4.4 ± 0.6    | 7.6 ± 1.7*    | 7.7 ± 1.2    |
| Y (Rmax)  | 23.7 ± 6.0   | 31.1 ± 3.6    | 42.9 ± 4.9   |
| µ (ED₅₀)  | 0.09 ± 0.31  | 0.20 ± 0.06*  | 0.33 ± 0.06  |
| β         | 2.50 ± 0.64  | 1.20 ± 0.26   | 1.62 ± 0.22  |

* Significant difference from nontransgenic mice of the same sex at p < 0.05.

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mice with moderate levels of Ras overexpression compared with nontransgenic litter mates (Fig. 7A). In contrast, Glut1 expression was increased 2-fold in gonadal adipose tissue from transgenic mice (p < 0.01). With high level Ras overexpression, Glut4 expression was also increased, and Glut1 expression was increased 4-fold (data not shown).

Photolabeling of Glut1 and Glut4 in Intact Adipocytes—Because of the increase in Glut1 expression in fat cells from transgenic mice, we photolabeled cell-surface Glut1 and Glut4 in intact adipocytes to determine whether the overexpression of Glut1 in transgenic adipocytes could contribute significantly to the increase in basal glucose transport in adipocytes from transgenic mice. These studies were carried out in the moderate level overexpressing line for the reasons stated above. Efficiencies of photolabeling with the bismannose label and of immunoprecipitation are similar for the two transporters (37, 41). Thus, the labeling results for the two transporters can be directly compared. In cells from nontransgenic mice in the basal state, Glut1 is not detectable above background levels, whereas Glut4 is clearly detectable (data not shown). In the maximally insulin-stimulated state, Glut1 is slightly above background levels, and Glut4 is ~8 times more abundant than Glut1 in nontransgenic fat cells. In adipocytes from moderate level Ras-overexpressing transgenic mice in the basal state, Glut1 levels are very low compared with Glut4 (Fig. 7B, left panel), even though Glut1 expression is increased 2-fold. In the insulin-stimulated state, Glut1 is less than one-sixth as abundant as Glut4 in Ras overexpressing transgenic adipocytes (Fig. 7B, right panel).

Fig. 8 shows a representative experiment in which photolabeling of plasma membrane Glut4 in the basal state was 2-fold greater in adipocytes from moderate level Ras-overexpressing mice compared with nontransgenic mice. This enhanced translocation of Glut4 in the absence of insulin was mirrored by a 2.8-fold increase in basal glucose transport in transgenic adipocytes. When data from three experiments were meaned (cells pooled from a total of n = 20 nontransgenic mice and n = 26 transgenic mice), we observed a 2.4 ± 0.4-fold increase in photolabeling and a 2.2 ± 0.3-fold increase in glucose transport in transgenic adipocytes compared with nontransgenic adipocytes (p < 0.05). Similar results were observed when Glut4 was measured by immunoblotting plasma membranes prepared from isolated adipocytes (data not shown).

To determine if the enhanced sensitivity of glucose transport to insulin (Fig. 5) was due to enhanced translocation of Glut4, photoaffinity labeling with the bismannose photolabel was conducted after adipocytes were incubated with half-maximal insulin concentration (0.3 nm) (Fig. 8). Half-maximally insulin-stimulated glucose transport and photolabeled Glut4 were approximately 2-fold higher than in nontransgenic cells.
DISCUSSION

The cellular mechanism(s) that regulate insulin-stimulated Glut4 translocation remain largely unknown. Insulin stimu-
lates the activation of p21ras, and the Ras/mitogen-activated protein kinase pathway has been shown to regulate many of the effects of insulin on mitogenesis and protein synthesis (15). However, the role of Ras in insulin-stimulated glucose trans-
port remains controversial. Data are conflicting as to whether pathways emanating from Ras are important or even involved in insulin stimulation of Glut4 translocation. Using a trans-
gen ic approach, we examined the role of p21ras in the regula-
tion of Glut4 translocation in adipose tissue. Mice that overex-
press wild-type Ha-Ras exclusively in adipose tissue have higher rates of basal glucose transport and increased insulin-
sensitive glucose transport in adipocytes. The mechanism ap-
ppears to be insulin "mimicking," with enhanced translocation of Glut4 to the plasma membrane in the absence of insulin and in
response to submaximal, but not maximal, concentrations of insulin.

The fact that wild-type Ras (which is not structurally modified to be constitutively active) has this insulin-like effect is somewhat unexpected since Ras activation is generally thought to require ligand binding to a receptor. The effects we observe suggest that Ras can be activated to some degree in the absence of ligand binding. Support for this possibility comes from the observation that p21ras colocalizes with cell-surface receptors (42) and may facilitate oligomerization, leading to receptor activation independent of ligand by cross-phosphorylation (43). Furthermore, our results indicate that the amount of Ras protein is rate-limiting for activation of signaling for GLUT4 translocation in the absence of insulin and at submaximal insulin concentrations, but not at maximally stimulating insulin concentrations.

Our results are entirely consistent with reports of the effects of activated Ras either transfected into rat adipocytes (10) or microinjected into cardiac myocytes (19). In the former study, cell-surface GLUT4 was increased in the absence of insulin, and in the latter, basal glucose transport increased with no change in GLUT4 gene expression. Finally, chronic overexpression of constitutively active Ras in 3T3-L1 adipocytes (18) also increased cell-surface GLUT4 in the absence of insulin; however, interpretation of these data is compromised by the 95% reduction in GLUT4 expression in these cells.

Others have shown that the Ras/mitogen-activated protein kinase pathway is not sufficient or necessary for insulin-stimulated glucose transport or GLUT4 translocation by overexpressing activated Ras (20), dominant-negative Ras (20), or activated Raf (21) in 3T3-L1 adipocytes or by treating these cells with a specific mitogen-activated protein kinase kinase inhibitor (44). We also have data, in another transgenic mouse model using Asn17 dominant-negative Ras, that signaling via Ras is not essential for maximally insulin-stimulated glucose transport (45). In contrast, PI 3-kinase-mediated signaling has recently been shown to be necessary for insulin-stimulated glucose transport, albeit not sufficient (4–10). The requirement for PI 3-kinase signaling in the regulation of GLUT4 translocation does not necessarily preclude a role for Ras since the Ras and PI 3-kinase pathways have recently been shown to potentially converge (46–48). Taken together with the data in the literature, our current study indicates that more than one pathway may be capable of eliciting GLUT4 translocation in the absence of insulin and at submaximal insulin concentrations. Additional evidence for more than one pathway comes from the fact that GLUT4 translocation stimulated by exercise and hypoxia does not appear to involve PI 3-kinase as it is not sensitive to the PI 3-kinase inhibitor, wortmannin (49).

Transgenic mice overexpressing wild-type Ras have reduced adipocyte size, and one might question whether this could affect glucose transport rates. The changes in glucose transport in the adipocytes from the Ras-overexpressing mice are unlikely to be accounted for by smaller cell size since studies in rodent models have shown that smaller adipocytes have lower basal glucose transport rates and no change or lower transport in response to submaximal and maximal insulin stimulation (50, 51). In this study, we observe higher basal and submaximally insulin-stimulated transport rates in the smaller cells from transgenic mice. In addition, our own data on nontransgenic FVB mice, the strain used for this study, show no correlation between cell size and either the ED50 for insulin-stimulated glucose transport or the maximal insulin-stimulated glucose transport rate (data not shown). Thus, the increase in basal and submaximally insulin-stimulated glucose transport in adipocytes from Ras transgenic mice cannot be explained by differences in cell size.

The changes in glucose transport also cannot be attributed to effects on GLUT1 expression in adipocytes. Insulin is known to regulate the expression of GLUT1 (20, 52), and this appears to be true even in the p21rasmitogen-activated protein kinase pathway (20). In our study, moderate level overexpression of wild-type Ras in fat of transgenic mice results in a 2-fold overexpression of GLUT1 in adipose tissue. However, this increase in GLUT1 gene expression does not explain the enhanced glucose transport in adipocytes from transgenic mice since GLUT1 is much less abundant than GLUT4 (37, 53), and our photolabeling data show that this is true even in Ras-overexpressing adipocytes. Furthermore, GLUT1 has a 3-fold lower turnover number (mol of glucose transported per transporter/time) than GLUT4 (54). Thus, GLUT1 appears to play a very minor role in glucose transport into the adipocyte in both nontransgenic and transgenic mice.

The effects of Ras overexpression at submaximal insulin concentrations are physiologically relevant since these concentrations fall in the normal range for circulating insulin levels. Thus, the effects of Ras overexpression on glucose transport in adipocytes may explain, at least in part, the apparent increased glucose tolerance (Fig. 4) and enhanced sensitivity to insulin (Table II) in the Ras overexpressing transgenic mice in vivo. Although it may be surprising that increased glucose uptake selectively into adipose tissue can affect whole body insulin action, this is supported by recent studies showing enhanced glucose tolerance and increased insulin sensitivity in transgenic mice overexpressing GLUT4 only in adipose tissue (27). The direct uptake of glucose into fat may account for only a part of the enhanced glucose disposal in Ras-overexpressing mice. However, the fact that glucose transport into skeletal muscles from these mice is not altered in vitro makes it unlikely that chronic alterations in muscle account for the enhanced insulin sensitivity in vivo. Although leanness is often associated with increased insulin sensitivity and obesity is associated with insulin resistance, these changes usually persist in skeletal muscle in vitro (55). In our model, enhanced insulin sensitivity may be present in muscle in vivo due to changes in the hormonal/metabolic milieu. Thus, future studies will determine whether the effects on insulin action in vivo in the Ras overexpressing transgenic mice could also be due to secondary changes in circulating levels of substrates (i.e. free fatty acids) that are known to affect glucose uptake or peptides (tumor necrosis factor and leptin) whose synthesis or release may be affected by altering Ras expression.

These studies also clearly establish a role for Ras in adipocyte development and maintenance in vivo. Previous evidence indicated that Ras might be important for adipocyte differentiation. Transfection of a dominant-negative mutant form of Ras into 3T3-L1 preadipocytes resulted in inhibition of differentiation to adipocytes (13). Thus, one might expect that overexpression of Ras in adipocytes of transgenic mice would foster adipocyte differentiation, and the enhancement of glucose transport would increase lipogenesis, resulting in increased adipose mass. However, we observe reduced gonadal fat pad weight and reduced adipocyte size in transgenic mice with moderate levels of Ras overexpression, and this effect is enhanced with high levels of Ras overexpression. The possibility that Ras plays an important role in the regulation of adipose mass is strengthened by our preliminary observation of increased adipose mass in transgenic mice overexpressing dominant-negative Ras in adipocytes (45). Future investigations will determine whether these results from direct effects of Ras in adipocytes or indirect effects due to changes in the levels of circulating substrates and hormones that affect metabolism.
and eating behavior. For example, lower circulating insulin levels (Table II) may result in increased lipolysis and decreased lipogenesis in vivo. The possibility that these changes in adipose mass could be mediated by leptin, the protein product of the newly cloned ob gene (56), is currently under investigation. In conclusion, while signaling emanating from Ras is not essential for maximally insulin-stimulated glucose transport, overexpression of wild-type Ras in adipocytes of transgenic mice results in increased basal and submaximally insulin-stimulated glucose transport. The mechanism involves increased cell-surface Glut4. The in vivo consequences of increased wild-type Ras expression include reduced adipose tissue mass and increased insulin sensitivity.

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REFERENCES

1. Simpson, I. A., and Cushman, S. W. (1989) Annu. Rev. Biochem. 58, 1059–1089.
2. Mueckler, M., (1990) Diabetes 39, 6–11.
3. Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukuimoto, H., and Seino, S. (1990) Diabetes Care 13, 198–208.
4. Cheatham, B., Vlahos, C. H., and Blenis, J. (1994) Mol. Cell. Biol. 14, 4902–4911.
5. Clarke, J., Young, P., Yonezawa, K., Kasuga, M., and Holman, G. (1994) Biochem. J. 300, 631–635.
6. Gould, G. J., et al., Andrews, G., Herbst, J., Plevin, R., and Gibbs, E. (1994) J. Biol. Chem. 269, 26622–26625.
7. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3568–3573.
8. Sanchez-Margalef, V., Goldfine, I. D., Vlahos, C. J., and Sung, C. K. (1994) Biochem. Biophys. Res. Commun. 204, 446–452.
9. Wiese, R. J., Mastick, C. C., Lazar, D. F., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 3442–3446.
10. Quon, M. J., Chen, H., Ing, B. L., Liu, M. L., Zarnowski, M. J., Yonezawa, K., Kasuga, M., Cushman, S. W., and Taylor, S. I. (1995) Mol. Cell. Biol. 15, 5403–5411.
11. Cheatham, B., and Kahn, R. C. (1995) Endocr. Rev. 16, 117–142.
12. Otero, A., Medema, R., Bos, J., Zon, G. V. D., Moller, D., Flier, J., Moliver, W., and Maassen, J. (1992) J. Biol. Chem. 267, 14647–14653.
13. Porras, A., Nebreda, A. R., Benito, M., and Santos, E. (1992) J. Biol. Chem. 267, 21124–21131.
14. Burgering, B. M., Medema, R. H., Maassen, J. A., Van de Wetering, M. L., Van der Beugel, A. J., McCormick, F., and Bos, J. L. (1991) EMBO J. 10, 1103–1109.
15. White, M., and Kahn, C. (1994) J. Biol. Chem. 269, 1–4.
16. Van den Berge, N., Ouwens, D. M., Maassen, J. A., Van Makenbeel, M. G. H., Sips, B. C. M., and Krans, H. M. J. (1994) Mol. Cell. Biol. 14, 2372–2377.
17. Draznin, B., Chang, L., Leitner, J., Takata, Y., and Olefsky, J. (1993) J. Biol. Chem. 268, 19998–20001.
18. Kauma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E., and Czech, M. P. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4460–4464.
19. Manchester, J., Kong, X., Lowry, O. H., and Lawrence, J. C., Jr. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4644–4648.
20. Hausdorff, S. F., Frangi, J. V., and Birnbaum, M. J. (1994) J. Biol. Chem. 269, 21391–21394.
21. Fingar, D. C., and Birnbaum, M. J. (1994) J. Biol. Chem. 269, 10127–10132.

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