GLUT-4myc ectopic expression in L6 myoblasts generates a GLUT-4-specific pool conferring insulin sensitivity

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GLUT-4myc ectopic expression in L6 myoblasts generates a GLUT-4-specific pool conferring insulin sensitivity. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E572–E578, 1999.—Insulin stimulates glucose uptake into muscle and fat cells via recruitment of the glucose transporter 4 (GLUT-4) from intracellular store(s) to the cell surface. Robust stimulation of glucose uptake by insulin coincides with the expression of GLUT-4 during differentiation of muscle and fat cells, but it is not known if GLUT-4 expression suffices to confer insulin sensitivity to glucose uptake. We have therefore examined the effect of expression of a myc epitope-tagged GLUT-4 (GLUT-4myc) into L6 myoblasts, which do not express endogenous GLUT-4 until differentiated into myotubes. Ectopic expression of GLUT-4myc markedly improved insulin sensitivity of glucose uptake in L6 myoblasts. The GLUT-4myc protein distributed equally to the cell surface and intracellular compartments in myoblasts, and the intracellular fraction of GLUT-4myc further increased in myotubes. In myoblasts, the intracellular GLUT-4myc compartment contained the majority of the insulin-regulatable amino peptidase (IRAP) and GLUT-4myc segregated into a specific compartment in L6 myoblasts and confers insulin sensitivity to these cells. L6-GLUT-4myc myoblasts, which are easily transfectable with various constructs, are a useful resource to study insulin action.

GLUT-4 translocation; GLUT-4 vesicles; insulin action; muscle cells

INSULIN PROMOTES the rapid uptake of glucose from the circulation into muscle and fat. These two tissues are unique in their expression of the glucose transporter 4 (GLUT-4) isofrom of facilitated hexose transporters (2, 3, 6, 15, 16). It is now widely accepted that insulin stimulates glucose uptake into muscle and fat cells via the recruitment of GLUT-4 glucose transporters from an intracellular store to the cell surface (2, 5, 13, 15, 22). The development of muscle and fat cell lines has facilitated our understanding of this process, specifically the rat L6 myogenic line and the mouse 3T3-L1 adipogenic line, which are unique in their expression of the GLUT-4 protein (9, 28). Both of these cell lines undergo differentiation in culture, from myoblasts into myotubes in the case of L6 and from fibroblasts into adipocytes in the case of 3T3-L1. In both models, GLUT-4 expression occurs on and after differentiation into myotubes (27) or adipocytes (7). Similarly, the expression of GLUT-4 has been shown to be developmentally regulated in rodent skeletal muscle (33). Both L6 myoblasts and 3T3-L1 fibroblasts display only a minor stimulation of glucose uptake on an insulin challenge (7, 21, 27). In contrast, both L6 myotubes and 3T3-L1 adipocytes mount a robust stimulation of glucose uptake in response to the hormone. The magnitude of the maximum stimulation of glucose uptake by insulin relative to the basal-state uptake is smaller for L6 myotubes (2-fold) than for 3T3-L1 adipocytes (~10-fold). This correlates with the much smaller response to the hormone displayed by isolated skeletal muscles (2- to 5-fold) (12, 31, 42, 45) relative to isolated adipocytes (~20-fold; Refs. 17, 34, 35, 40). Differences in species also contribute to the range of insulin responsiveness, rodent tissues being more responsive than their human counterparts.

The stimulation of glucose uptake coincides with the expression of GLUT-4 in muscle and fat cells, leading to the suggestion that GLUT-4 may be a determinant of the maximum insulin response. However, this scenario has been challenged by the observation that ectopic expression of GLUT-4 into 3T3-L1 fibroblasts or C2C12 myoblasts and myotubes did not elevate the responsiveness of glucose uptake to insulin (11, 14, 23). Therefore, it has been hypothesized that other factors that appear during the differentiation of these cells contribute to conferring insulin responsiveness to the newly expressed GLUT-4 protein.

In the present study, we examined the effect of ectopic expression of a myc epitope-tagged GLUT-4
(GLUT-4myc) into L6 myoblasts on insulin action. We report that the expression of this protein alone confers insulin sensitivity to glucose uptake, of a similar magnitude to that attained during normal differentiation of the cells into myotubes. We further demonstrate that the magnitudes of insulin stimulation of the lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) and of the serine/threonine kinase protein kinase B-α (PKB-α/Akt1) are similar in myoblasts as in myotubes. Furthermore, the ectopically expressed GLUT-4myc protein is targeted to both an intracellular compartment and the plasma membrane in myoblasts, and the intracellular localization of GLUT-4myc is further accentuated in myotubes. The intracellular GLUT-4myc compartment of myoblasts contains all of the intracellular insulin-regulatable aminopeptidase (IRAP) but excludes, in part, the endogenous glucose transporter GLUT-1. Finally, insulin causes a twofold increase in the amount of GLUT-4myc at the myoblast cell surface, matching the extent of stimulation of glucose uptake. We conclude that L6-GLUT-4myc myoblasts are a useful cell system to study insulin regulation of GLUT-4 traffic.

METHODS

Cell culture and glucose uptake. L6 myoblasts expressing a GLUT-4 protein with an exofacial myc epitope tag (L6-GLUT-4myc) were constructed with a clone of L6 myoblasts selected for high fusion (27), as described by Kishi et al. (20). L6-GLUT-4myc or parental L6 myoblasts were grown in α-MEM containing 2% FBS. Myoblasts were used as they reached confluence before any evidence of differentiation (cell fusion). For all experiments, L6-GLUT-4myc or parental L6 cells were serum depleted for 5 h in α-MEM culture medium before insulin stimulation. Hexose uptake into L6-GLUT-4myc or parental L6 myoblasts and myotubes was measured as previously described (19).

Cell surface detection of GLUT-4myc. Cell surface detection of GLUT-4myc was carried out as previously described (43) with slight modifications. Briefly, after incubations with or without insulin at 37°C as indicated, cells were washed twice with PBS at 4°C, incubated with 3% BSA for 10 min, and then reacted with the anti-myc antibody 9E10 (Santa Cruz, CA) in 10% goat serum + 3% BSA in PBS for 1 h at 4°C. After being washed four times with ice-cold PBS, cells were fixed with 3% paraformaldehyde for 3 min at 4°C. After the fixation, cells were incubated with 0.1 N glycine in PBS for 10 min at 4°C, washed with PBS, and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Jackson Laboratories) for 30 min at 4°C. The cells were extensively washed with PBS, and then 1 ml/well of 0.4 mg/ml o-phenylenediamine dihydrochloride reagent (Sigma), prepared according to the instructions of the manufacturer, was added for 30 min at room temperature. The reaction was stopped by addition of 0.25 ml of 3 N HCl, the supernatant was collected, and the optical absorbance was measured at 492 nm. To measure total cellular GLUT-4myc, serum-starved cells were washed twice with PBS and fixed with 3% paraformaldehyde for 3 min at 4°C and then incubated with 0.1 N glycine in PBS for 10 min, followed by a 15-min incubation with 0.1% (vol/vol) Triton X-100 at 4°C. After being washed with PBS, the cells were blocked with 10% goat serum and 3% BSA for 30 min and then incubated with the anti-myc antibody (9E10) for 1 h at 4°C. After being washed with PBS, the cells were incubated with HRP-conjugated donkey anti-mouse IgG as above, followed by reaction with o-phenylenediamine dihydrochloride reagent as described previously.

PI 3-kinase and PKB-α/Akt1 activity assays. The in vitro activity of PI 3-kinase associated with IRS-1 immunoprecipitates, and of PKB-α/Akt1 in PKB-α/Akt1 immunoprecipitates, was assayed exactly as described previously (19, 36).

Characterization of GLUT-4- or IRAP-containing vesicles. Immunoisolation of GLUT-4- or IRAP-containing compartments ("vesicles") from the light-density microsome fraction of L6-GLUT-4myc myoblasts was prepared as previously described for parental L6 cells (37). Briefly, L6-GLUT-4-myc myoblasts were fractionated to remove nuclei, mitochondria, and plasma membranes (30). The supernatant containing low-density microsomes was adjusted to 100 mM potassium phosphate, pH 7.4, and the protein concentration was measured with the bicinchoninic acid reagent (Pierce Chemical, Rockford, IL) according to the instructions of the manufacturer. An aliquot of 50 µg protein of the microsome sample was incubated with 28 µl of anti-IRAP monoclonal antibody precoupled to M-500 magnetic Dynabeads (Lake Success, NY) or 30 µl of the sheep anti-myc IgG (Santa Cruz, CA) for 1 h at 4°C, and an aliquot of 200 µg protein of the microsome sample was incubated with 2 µg of anti-GLUT-4 monoclonal antibody 1F8 (Biogenesis, Sandown, NH) precoupled to M-500 magnetic Dynabeads coated with sheep anti-mouse IgG. Vesicles attached to the beads (termed Pt for immunocomplex pellet) were concentrated via a magnet and washed three times with PBS. The supernatants were subjected to centrifugation at 200,000 g for 60 min, and the sediments were washed three times with SN for immunocomplex supernatant) were resuspended in Laemmli's sample buffer (24) and analyzed by SDS-PAGE. The gelatinolytic activity of PI 3-kinase associated with IRS-1 immunoprecipitates, and of PKB-α/Akt1 in PKB-α/Akt1 immunoprecipitates, was assayed exactly as described previously (19, 36).

RESULTS AND DISCUSSION

We have previously reported that, in contrast to L6 myotubes, L6 myoblasts do not respond significantly with an increase in glucose uptake to an acute exposure to insulin (21). The low responsiveness and sensitivity to insulin of parental L6 myoblasts suggest that the endogenous GLUT-1 and GLUT-3 transporters present in these cells (1, 27) are largely unresponsive to the hormone at this cellular stage. The insulin-stimulated glucose uptake dose-response curves of the parental L6 myoblasts and myotubes are illustrated in Fig. 1A. The results are expressed as hexose uptake per unit time per well of confluent myoblasts or myotubes to compare uptake into the same number of cells. Parental L6 myoblasts only began to display an insulin response when exposed to 100 nM concentrations of the hormone, whereas the parental myotubes showed a statistically significant response beginning at 1 nM insulin. These parental L6 myotubes showed a maximal response of twofold at 100 nM insulin, a concentration
typically used in studies of insulin action in L6 myotubes and 3T3-L1 adipocytes. The rate of glucose uptake in response to 100 nM insulin was statistically different in parental myoblasts and in myotubes (P < 0.05).

In contrast to the lack of response of L6 parental myoblasts to hexose uptake, L6-GLUT-4myc myoblasts displayed higher insulin-responsive glucose transport (Fig. 1B). L6-GLUT-4myc myoblasts showed statistically significant increases in glucose uptake on exposure to 1 nM insulin, and at 100 nM insulin showed a gain in glucose uptake of 103% above the basal value (i.e., 2-fold stimulation). The sensitivity to insulin displayed by L6-GLUT-4myc myoblasts was similar to that of L6-GLUT-4myc myotubes (Fig. 1B). Considering the highest stimulation values shown in Fig. 1 as maxima, the concentrations of insulin yielding half-maximal stimulation of glucose uptake were different for parental L6 myoblasts (80 nM) than for any of the other cells (18 nM for parental L6 myotubes, 13 nM for L6-GLUT-4myc myoblasts, and 20 nM for L6-GLUT-4myc myotubes).

The results in Fig. 1 are expressed per well of confluent myoblasts or myotubes in an attempt to compare the same number of cells in each state. When expressed per unit protein, the myotubes appear to have a lower rate of transport given that they have a higher amount of protein than the myoblasts (see next paragraphs). Reported per well, the responsiveness of the L6-GLUT-4myc myotubes was somewhat higher than that of the parental L6 myotubes, possibly due to the contribution of both the endogenous and transfected versions of the glucose transporter. Indeed, we have recently reported that roughly similar levels of protein expression of the endogenous and the transfected GLUT-4 are attained in the L6-GLUT-4myc myotubes (43). These results also indicate that the level of expression of GLUT-4myc in the myoblasts resembles the endogenous level of GLUT-4 in the myotubes, minimizing the risk of saturating the elements involved in GLUT-4 sorting.

The exofacial location of the myc epitope on GLUT-4myc allowed us to estimate the proportion of GLUT-4myc exposed at the cell surface or the total cellular content of GLUT-4myc by analyzing either intact or permeabilized cells, respectively. The amount of myc epitope exposed at the surface of nonpermeabilized cells was determined by a quantitative assay based on the colorimetric detection of anti-myc antibody bound to a monolayer of L6 cells. The amount of myc epitope present in the entire cells was determined by permeabilization of the cells with 0.1% Triton X-100 before immunolabeling with anti-myc antibody. The intracellular content was then estimated by subtracting the amount present on the cell surface from the amount in the entire cell. In the myoblast stage, 55 ± 6% (n = 3) of the total GLUT-4myc content was found to be exposed at the cell surface, and the remainder 44 ± 6% (n = 3) was intracellularly located. At the myotube stage, the amount of GLUT-4myc present in total membranes was 70% higher than in myoblasts, expressed per unit of protein (data not shown). Because the total protein content of myotubes was about twice that of the same number of myoblasts, the amount of GLUT-4myc protein may rise by as much as three- to fourfold in myotubes. We speculate that the gain in
GLUT-4myc expression might be caused by translational and/or posttranslational mechanisms (e.g., ribosomal transit and/or protein stabilization), given the fact that the transcription of the GLUT-4myc cDNA is determined by the cytomegalovirus promoter, which would presumably have the same activity in myoblasts and myotubes. The amount of GLUT-4myc expressed at the surface of L6-GLUT-4myc myotubes was very similar to that present at the surface of L6-GLUT-4myc myoblasts (1.14 ± 0.03 in myotubes relative to a value of 1.00 ascribed to myoblasts). This result suggests that the power of retention mechanisms, probably in the form of proteins interacting with GLUT-4, increases on cellular differentiation. The GLUT-4 polypeptide expresses retention-endocytosis motifs in both its NH₂-terminal and its COOH-terminal sequences (4, 8, 10, 25, 29, 41). Based on the results of GLUT-4myc distribution discussed above, the functionality of these motifs in myotubes does not appear to be markedly compromised by the insertion of the myc epitope.

In an attempt to understand if the signals emanating from the insulin receptor thought to lead to the mobilization of GLUT-4 also mature during myogenesis, the activation by insulin of the lipid kinase PI 3-kinase and the serine/threonine kinase PKB-α/Akt1 was measured in both myoblasts and myotubes. We have previously shown that PI 3-kinase activation by insulin is a prerequisite for insulin-dependent translocation of GLUT-4 to the cell surface in parental L6 myotubes (38, 39). Recently, we have shown that activation of PKB-α/Akt1 by the hormone appears to be required for the translocation of GLUT-4myc (44) detected by a single-cell immunofluorescence approach. Therefore, we compared the activation of PI 3-kinase and of PKB-α/Akt1 in L6-GLUT-4myc myoblasts and myotubes. Table 1 shows that IRS-1-associated PI 3-kinase activity was stimulated by approximately sevenfold upon rapid exposure to insulin of both GLUT-4myc myoblasts and myotubes. Similarly, the extent of the response of PKB-α/Akt1 activity to insulin was comparable (~6-fold) in L6-GLUT-4myc myoblasts and myotubes (Table 1). This stimulation is equivalent to that reported earlier for parental L6 myotubes (19). Moreover, the phosphorylation of Ser 473 in PKB-α/Akt1 in response to insulin was virtually identical in the parental as in the L6-GLUT-4myc myoblasts (results not shown). The equivalent responsiveness of L6 myoblasts relative to L6 myotubes is in contrast to the poorer response of 3T3-L1 fibroblasts relative to adipocytes (7).

The comparable insulin-dependent stimulation of PI 3-kinase, PKB-α/Akt1, and glucose uptake observed in L6-GLUT-4myc myoblasts and myotubes led us to compare the extent of GLUT-4myc translocation to the cell surface at the two stages of differentiation. Table 1 shows that the hormone doubled the exposure of GLUT-4myc at the surface of both myoblasts and myotubes, matching the stimulation of glucose uptake caused under the same conditions. These observations raise the possibility that L6 myoblasts have the cellular machinery and signaling pathways required for GLUT-4 translocation, although they do not naturally express this transporter isoform.

### Table 1. Insulin-dependent signals, glucose uptake, and translocation of GLUT-4myc in L6-GLUT-4myc myoblasts and myotubes

<table>
<thead>
<tr>
<th>Insulin/Basal</th>
<th>Myoblasts</th>
<th>Myotubes</th>
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<tbody>
<tr>
<td>IRS-1-associated PI 3-kinase activity</td>
<td>7.85 ± 1.44</td>
<td>6.43 ± 0.79</td>
</tr>
<tr>
<td>PKB-α/Akt1 kinase activity</td>
<td>6.19 ± 1.04</td>
<td>6.22 ± 2.09</td>
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<tr>
<td>Glucose uptake</td>
<td>1.79 ± 0.18</td>
<td>2.07 ± 0.16</td>
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<tr>
<td>GLUT-4myc translocation</td>
<td>2.18 ± 0.21</td>
<td>1.98 ± 0.04</td>
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Values are means ± SE of at least 3 experiments (for all assays) and are multiples of the increase of stimulation caused by insulin relative to basal values. L6 myc epitope-tagged GLUT-4 (GLUT-4myc) myoblasts and myotubes were serum depleted for 5 h before treatment with 100 nM insulin for 5 min [phosphatidylinositol 3-kinase (PI 3-kinase)], 10 min [serine/threonine kinase protein kinase B-α (PKB-α/Akt)] or 30 min (glucose uptake and GLUT-4myc translocation assays) as indicated. Insulin receptor substrate-1 (IRS-1)-associated PI 3-kinase activity, PKB-α/Akt1 kinase activity, glucose uptake, and GLUT-4myc translocation were measured as described in METHODS.
transfection of untagged GLUT-4 has been reported to increase insulin-dependent glucose uptake in parental L6 myoblasts (32). This appears to be a property of rat L6 cells, because GLUT-4 transfection into mouse C2c12 muscle cells, which do not express endogenous GLUT-4, did not improve their low insulin response of glucose uptake even when differentiated into myotubes (23). It may be that C2c12 cells do not have the requisite machinery to mobilize GLUT-4 even in the myotube stage. In contrast, transfection of GLUT-4myc has conferred an insulin response to glucose uptake in Chinese hamster ovary cells and increased the response of 3T3-L1 adipocytes, but the response of GLUT-4myc-transfected 3T3-L1 fibroblasts was not examined (18). Neither was the extent of insulin signaling nor the GLUT-4myc compartment identified in that study.

The ability of GLUT-4myc to lodge in an intracellular location and to respond to insulin prompted us to characterize this compartment. Membranes containing GLUT-4myc were immunopurified out of the light-density microsomes of L6-GLUT-4myc myoblasts with the monoclonal antibody 1F8 directed to the COOH-terminal domain of GLUT-4. The immunoisolated vesicles contained the vast majority of the intracellular GLUT-4myc (Fig. 2). In contrast, they only contained 40 ± 2% (n = 3) of the intracellular GLUT-1. These results suggest that GLUT-4 is located in a compartment that in part excludes GLUT-1. This segregation of the two transporters is reminiscent of their distribution in 3T3-L1 adipocytes, in which the oxidative ablation of the endosomal pool obliterated the immunoreactivity of the majority of the GLUT-1 but of only half of the GLUT-4 complements (26). Substantial exclusion of GLUT-1 from the endogenous GLUT-4 pool was also observed in parental L6 myotubes (37). A similar segregation of GLUT-1 and GLUT-4 was reported for cardiac myocytes with graded immunopurification of the GLUT-4 compartment (46). Figure 2 also shows that the immunoisolated vesicles containing GLUT-4myc also contain the vast majority (96 ± 2%) of the intracellular IRAP. This suggests that structural motifs present in both GLUT-4myc and IRAP localize these proteins to the same intracellular compartment. We have previously shown that IRAP expression is lower in parental L6 myoblasts compared with L6 myotubes (37). In L6-GLUT-4myc myoblasts, IRAP expression increased 3.5 ± 0.2-fold on differentiation into myotubes (data not shown).

The complete recovery of IRAP in the GLUT-4 intracellular pool suggests that there is no IRAP in other membranes but does not prove that all GLUT-4-containing membrane structures also contain IRAP. To examine this possibility, intracellular membranes containing IRAP were immunopurified with anti-IRAP specific antibodies. Figure 3 shows that the purification of IRAP-containing vesicles was complete, as there was no IRAP left in the supernatant. It also shows that virtually all of the intracellular GLUT-4 copurified with the IRAP compartment(s). In contrast, the IRAP compartment contained only 42% of the GLUT-1, the remainder being recovered in the supernatant. These results corroborate the overlap of the compartment(s) containing IRAP and GLUT-4 and their segregation from that containing GLUT-1.

In conclusion, ectopic expression of GLUT-4myc markedly improved insulin sensitivity and responsiveness of glucose uptake in L6 myoblasts. The GLUT-4myc protein distributed equally to the cell surface and intracellular compartments in myoblasts. On differentiation into myotubes, GLUT-4myc content increased, as did its retention in the intracellular compartment. In myoblasts, this compartment contained the majority of the IRAP protein but less than half of the GLUT-1 protein, suggesting segregation of GLUT-4 and IRAP to a specific cellular locus. Similar insulin-dependent stimulation of PI 3-kinase or PKB-α/Akt1 activities was observed in myoblasts and myotubes. In both myoblasts and myotubes, GLUT-4myc responded to insulin by translocating to the cell surface. These cells therefore constitute a cellular system in which GLUT-4 translocation can be measured in intact cells via detection of the exofacial myc epitope. Moreover, the cells retain the ability to differentiate into myotubes, allowing comparisons of GLUT-4myc translocation in both stages. Collectively, these results suggest that L6 muscle cells expressing GLUT-4myc are a useful system for studies of insulin action on glucose transport and that the myoblast stage, being easily transfectable with various constructs, may be a useful tool in the analysis of insulin action. In this regard, we have recently used L6-GLUT-4myc myoblasts to transiently express dominant-negative versions of Akt to assess the role of this kinase in GLUT-4myc translocation (44).

We thank Dr. Philip J. Bilan for helpful discussions and input throughout this study and Dr. Morrie Birnbaum (University of Pennsylvania) for the kind gift of the anti-IRAP monoclonal antibody. A. Ueyama is a visiting research fellow from Otsuka Pharmaceutical, 463-10 Kagasuno Kawachi-chou, Tokushima 771-0192, Japan. K. Yaworsky was supported by a Hospital for Sick Children Foundation Scholarship at the University of Toronto. O. M. Rosen was supported by a Canadian Diabetes Association to A. Klip.

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