THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 20, Issue of May 19, pp. 12094–12099, 1995
Printed in U.S.A.

Nutrient Control of GLUT1 Processing and Turnover in 3T3-L1 Adipocytes*

(Received for publication, December 23, 1994, and in revised form, March 6, 1995)

Robert J. McMahon and Susan C. Frost‡

From the Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

Metabolic labeling and immunoprecipitation were used to analyze the glucose-dependent regulation of GLUT1 synthesis, processing, and turnover in a murine adipocyte cell line. Metabolically labeled GLUT1 from control cells migrated as a 46-kDa protein, while GLUT1 from cells deprived of glucose for more than 12 h migrated as a 37-kDa protein. On the basis of tunicamycin sensitivity, both GLUT1 species arose from a common protein migrating at 36 kDa. In addition, the rate of synthesis of GLUT1 in control and glucose-deprived cells was similar. In short pulse-chase experiments, we distinguished two species arising from the core GLUT1 protein in control cells; an intermediate and the mature 46-kDa species. In contrast, only one glycoform, the 37-kDa species, arose from the core protein in glucose-deprived cells, which was not further processed in either the presence or absence of glucose. Although 12–18 h of glucose deprivation were required to affect GLUT1 glycosylation, glucose-deprived cells quickly recovered the ability to correctly glycosylate GLUT1 upon the readdition of glucose ($t_{1/2}$ <1 h). GLUT1 in control adipocytes exhibited a half-life of approximately 14 h, while that in glucose-deprived adipocytes was greater than 50 h. This effect was readily reversed upon the readdition of glucose. In total, these data show that glucose deprivation alters both the processing (glycosylation) and turnover (degradation) of GLUT1. These results are discussed in light of transport function.

The transport of glucose across the plasma membrane is accomplished by a family of integral membrane glycoproteins termed GLUT proteins (1). The regulation of these proteins plays a key role in the metabolism of sugar. In particular, the glucose-dependent regulation of glucose transport has been described in many cell types (for review, see Ref. 2). In each of these cell types, the result of glucose deprivation is an increase in the maximal velocity of glucose transport. However, the mechanism(s) (*i.e.* transcriptional, post-transcriptional, and post-translational) responsible for this stimulation vary according to cell type. Several investigators have postulated that one component might be an alteration of transporter turnover, particularly GLUT1 (3–10). Few studies, however, have investigated the processing and turnover of GLUT1 directly because of the technical difficulties involved (11). Thus our primary goal was to define the kinetics of GLUT1 turnover.

In 3T3-L1 adipocytes, glucose deprivation results in no significant change in the level of GLUT1 protein (as measured by Western blotting) over the period in which transport induction is observed (12). Beyond this time, we and others have reported the accumulation of a lower molecular weight GLUT1 species which results in a 2-fold increase in the total GLUT1 mass by 48 h of glucose deprivation (9, 12, 13). We have previously investigated the role of this second GLUT1 protein in transport stimulation and provided evidence that it is not responsible for the activation of transport during glucose deprivation (12). The origin and characteristics of this lower molecular weight GLUT1 and its relationship to the normal GLUT1 glycoform remain unclear. It is possible that the new GLUT1 glycoform is a normal intermediate in GLUT1 processing that accumulates during glucose deprivation. Alternatively, the new glycoform could contain an aberrant oligosaccharide generated in the absence of glucose. To address these issues, we modified existing methods of GLUT1 immunoprecipitation to improve recovery and reduce nonspecific interactions. This has allowed us to clearly model the processing and turnover of the metabolically labeled transporter in normal and glucose-deprived adipocytes.

EXPERIMENTAL PROCEDURES

Materials—DMEM[1] was obtained from Life Technologies, Inc. Glucose-free DMEM was prepared exactly as commercially available except for the omission of glucose. Calf serum (no. 1100-90) and fetal bovine serum (no. 1020-75) were obtained from Intergen. Glucose-free fetal bovine serum was prepared by dialyzing serum against PBS, pH 7.4, for 48 h at 4 °C, with a molecular mass cutoff of 13,000 daltons. Tran^{35}S-label (1100 Ci/mmol) was obtained from ICN. Polyoxyethylene 9 lauryl ether ($C_{12}E_9$), octanoic acid, and anti-rabbit IgG-conjugated horseradish peroxidase was obtained from Sigma. All other reagents were of the highest quality commercially available.

Cell Culture—3T3-L1 fibroblasts were cultured and differentiated as described previously (14). All other cell culture procedures, including glucose deprivation, have been described previously (12). 3T3-L1 adipocytes cultured in 100-mm plates (12×10^6 cells) were used for all immunoprecipitations.

Antibody Production and Purification—A polyclonal antiserum, designated GT1, was generated to a peptide corresponding to the COOH terminus of GLUT1 (CEELFHPLGADSQV) conjugated to keyhole limpet hemocyanin as described previously (15). Total IgG was purified from rabbit serum as described previously (16) except that the final IgG was dialyzed against PBS. Two mg of the GT1 peptide were coupled to a Sulfolink matrix via the cysteine residue following the manufacturer's instructions (Pierce). Fifteen mg of total IgG were incubated with the peptide column and rotated end-over-end at room temperature for 2 h. Unbound IgG was washed from the column with PBS. Specifically bound IgG was eluted with 0.1 M glycine, pH 3.0, and neutralized with 0.1 ml 1 M Tris base. Eluted IgG fractions were pooled before being dialyzed against PBS for 12 h at 4 °C. The final IgG was stored at −20 °C as a 0.5 mg/ml solution.

Metabolic Radiolabeling of 3T3-L1 Adipocytes—3T3-L1 adipocytes were incubated in 8 ml of methionine- and cysteine-free DMEM without added serum for 1 h to deplete intracellular pools. The depletion medium was then removed, and the cells were incubated in 2 ml of

* This work was supported by National Institutes of Health Grant DK45035 (to S. C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 904-392-3207; Fax: 904-392-2953.

[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; GT1, glucose transporter carboxyl-terminal peptide; $C_{12}E_9$, polyoxyethylene 9 lauryl ether.

methionine- and cysteine-free DMEM containing 200–500 μCi/ml Tran^{35}S-label (as noted in the figure legends) for the specific times indicated (10–180 min). For chase periods, the labeling medium was aspirated and replaced with 8 ml complete DMEM, 10% fetal bovine serum containing 2 mM methionine and 2 mM cysteine. These conditions were established to generate an adequate signal for immunoprecipitation while minimizing methionine and cysteine depletion based on previous reports of alterations in protein turnover with prolonged amino acid depletion (11).

Immunoprecipitation—For GLUT1 immunoprecipitation, we utilized a procedure described previously (17), with the following modifications. Total membrane fractions, prepared as described previously (12), were homogenized with 20 strokes in 1 ml of extraction buffer (PBS containing 1 mM EDTA, 2% $C_{12}E_9$, 0.1% SDS, and protease inhibitor (20 μg/ml each of aprotinin, leupeptin, pepstatin, tosylphenylalanyl chloromethyl ketone, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was sedimented in a microcentrifuge for 5 min at 4 °C. The supernatant was recovered, frozen in liquid nitrogen, and stored at −20 °C. Thawed extracts (1.5–2 mg) were pre-cleared with 5 μl of an unrelated nonimmune antiserum and collected with 25 μl of a 50% suspension of protein A-Sepharose for 1 h at 4 °C. Samples were spun briefly in a microcentrifuge, and pre-cleared supernatants were transferred to new tubes. Extracts were adjusted to equal protein concentration and incubated with 5 μg of anti-GT1 antibody for 3 h at 4 °C with mixing. Twenty-five μl of the protein A-Sepharose suspension was then added for 2 h to collect the immunoprecipitates. The protein A-Sepharose was then washed three times with extraction buffer, followed by four times for 10 min with extraction buffer containing 1 M NaCl. Immunoprecipitates were released by incubation in 0.1 ml of sample dilution buffer containing 6 M urea and 10% β-mercaptoethanol for 15 min at 37 °C. The supernatants were loaded onto an 8% SDS-PAGE gel and run overnight at 50 V. For fluorography, the gels were fixed in 10% trichloroacetic acid, 40% MeOH for 30 min, soaked in water for 30 min, and then soaked in 1 M sodium salicylate for 1 h before drying at 60 °C under vacuum. Dried gels were exposed to Amersham Hyperfilm typically for 4 days.

Endoglycosidase Digestions—Twenty μg of membrane protein from control or glucose-deprived adipocytes and 10 μg from LEC1 CHO cells were denatured in 50 mM β-mercaptoethanol, 0.5% SDS for 20 min at 37 °C. Then, for *N*-glycosidase F digestions, the samples were brought to 150 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, protease inhibitors as above, 1.25 units of *N*-glycosidase F and then incubated at 37 °C for 2 h. For endoglycosidase H digestions, the denatured protein sample was brought to 75 mM sodium citrate, pH 5.5, protease inhibitors as above, and 2.5 milliunits of endoglycosidase H and incubated at 37 °C for 2 h. An equal volume of 2 × sample dilution buffer was added and loaded onto an 8% SDS-PAGE gel for separation.

Electrotransfer and Western Blotting—Transfer of proteins to nitrocellulose and Western analysis were performed as described previously (12).

RESULTS

Specificity and Efficiency of GLUT1 Immunoprecipitation—The GLUT1 immunoprecipitation procedure was assessed using metabolically labeled control or glucose-deprived adipocytes. Fig. 1 shows a fluorograph of the immunoprecipitates. A wide band migrating at 46 kDa (*p46*) was immunoprecipitated from membrane extracts of control cells using purified anti-GT1 antibody (*−GT1*, *+ glucose*). In contrast, a 37-kDa protein was immunoprecipitated from cells deprived of glucose for 36 h (*−GT1*, *−glucose*). The specificity of the immunoprecipitation procedure was verified by immunoprecipitating GLUT1 in the presence of 1 μg of the GT1 peptide. Inclusion of this peptide completely blocked the immunoprecipitation of p46 GLUT1 in control cells, as well as p37 GLUT1 in glucose-deprived cells (*+GT1*, *± glucose*). In a similar competition experiment, a GLUT4 carboxyl-terminal peptide (CSTELEYLGPDEND) did not block the immunoprecipitation of GLUT1 (*+GT4*, *± glucose*). GLUT1 protein of either molecular mass was not observed in membrane extracts immunoprecipitated with protein A-Sepharose alone, with preimmune serum, or with 5 μg of IgG from an unrelated antisera (*−Ab*, *PIS*, *IRR*, *± glucose*). GLUT1 protein was also not observed when extracts were immunoprecipitated with an equal amount of the unbound antibody fraction (flow-through) from peptide purification of the same antisera, indicating that the purification step removed the majority of the GLUT1 specific IgG (*FT*, *± glucose*).

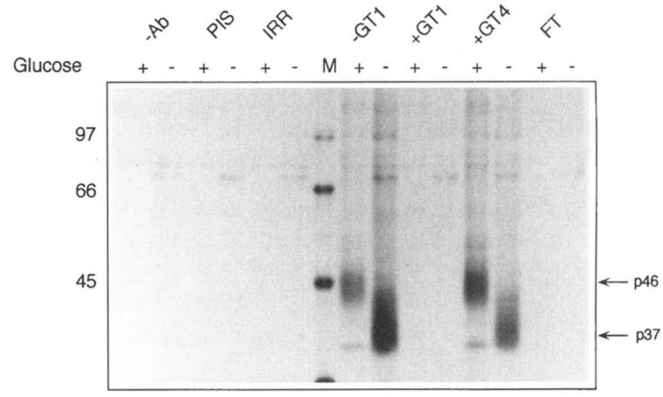

FIG. 1. **Specificity and efficiency of GLUT1 immunoprecipitation.** Cells, cultured with or without glucose for 36 h, were metabolically labeled with 200 μCi/ml Tran^{35}S-label for 1 h as described under "Experimental Procedures." GLUT1 was immunoprecipitated from a total membrane fraction as indicated, separated by SDS-PAGE, and visualized by fluorography. *−Ab*, no antibody; *PIS*, preimmune serum; *IRR*, unrelated serum; *−GT1*, immunoprecipitated in the absence of peptide; *+GT1*, inclusion of 1 μg of GT1 peptide; *+GT4*, inclusion of 1 μg of GT4 peptide; *FT*, unbound antibody fraction from peptide purification.

We determined the efficiency of the immunoprecipitation protocol by sequential rounds of immunoprecipitation with the anti-GT1 antibody. Approximately 80% of GLUT1 was collected during the first round of immunoprecipitation in either control or glucose-deprived cells. The remainder was recovered during a second round of immunoprecipitation (data not shown). In the experiments described herein, only one round of immunoprecipitation was used.

Synthesis and Glycosylation of GLUT1 in Control and Glucose-deprived 3T3-L1 Adipocytes—To determine the role of glucose in regulating the synthetic rate of GLUT1, control and glucose-deprived adipocytes were metabolically labeled for 10–180 min. Fig. 2 shows a fluorograph of the GLUT1 immunoprecipitates. Two GLUT1 species were synthesized in control cells during the first 10 min of labeling, a 36-kDa protein and a glycosylated intermediate migrating at 42 kDa. The 36-kDa protein is very likely the core protein based on the predicted molecular weight of GLUT1 in the absence of oligosaccharide and tunicamycin experiments (see below). With 20 min of labeling, the mature 46-kDa GLUT1 was observed along with p36 and p42. In glucose-deprived cells labeled for 10 min, only the 37-kDa GLUT1 protein was observed. The 36-kDa core GLUT1 protein is likely obscured due to the width of the p37 protein. Lighter exposures provided no further discrimination. No additional GLUT1 species were synthesized during the remainder of labeling period. Although the intensity of total GLUT1 protein in glucose-deprived cells was greater than that of control cells (Fig. 2*B*), this effect was not specific to GLUT1 when taking into account an equal increase in radioactivity of the membrane fraction from glucose-deprived cells (Fig. 2*B*, *inset*). This phenomenon likely represents an increase in the specific activity of the methionine and cysteine pools in glucose-deprived cells. Taken together, these data demonstrate that the synthetic rate of GLUT1 was unaffected by glucose availability despite the differences in glycosylation.

To confirm the hypothesis that p37 GLUT1 arises from post-translational modification with *N*-linked oligosaccharide, control and glucose-deprived adipocytes were incubated in the presence or absence of tunicamycin before labeling. In the absence of tunicamycin, the synthesis of the 46- and 37-kDa GLUT1 species was observed in control and glucose-deprived cells, respectively, as expected (Fig. 3). In contrast, GLUT1

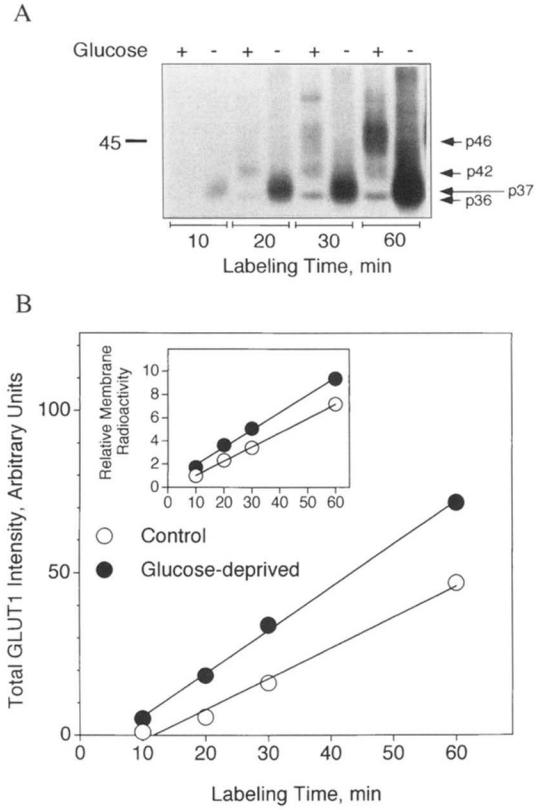

FIG. 2. **GLUT1 synthesis in control and glucose-deprived adipocytes.** *Panel A*, cells, cultured with or without glucose for 36 h, were metabolically labeled with 200 μCi/ml Tran^{35}S-label for the times indicated as described under "Experimental Procedures." GLUT1 was then immunoprecipitated from membrane extracts and analyzed by SDS-PAGE and fluorography. *Panel B*, densitometry of total GLUT1 during labeling. Total GLUT1 intensity was obtained in all experiments by summing the appropriate bands after densitometry of each species separately (*i.e. p36 + p42 + p46*). *Inset*, radioactivity in membrane fraction during labeling relative to 10 min label. This result is representative of at least two independent experiments.

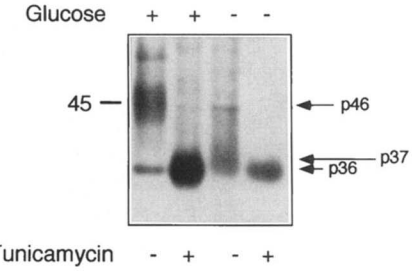

FIG. 3. **Glycosylation of GLUT1 in control and glucose-deprived adipocytes.** Cells, cultured with or without glucose for 36 h, were incubated in the presence (+) or absence (−) of tunicamycin (2.5 μg/ml) for 24 h before being metabolically labeled with 200 μCi/ml Tran^{35}S-label for 1 h as described under "Experimental Procedures." GLUT1 was then immunoprecipitated from membrane extracts and visualized by SDS-PAGE and fluorography. This result is representative of at least two independent experiments.

immunoprecipitated from tunicamycin-treated cells migrated at 36 kDa regardless of metabolic state. This demonstrates that both p46 and p37 arise from the core protein and that both species are glycosylated, albeit to different extents.

Regulation of GLUT1 Processing by Glucose—To examine the processing pathway of GLUT1 in control and glucose-deprived adipocytes, cells were metabolically labeled for 10 min and then chased for a total of 60 min. In control cells, the 36- and 42-kDa GLUT1 precursors synthesized during the pulse disappeared followed by the emergence of the mature GLUT1 at 46 kDa after

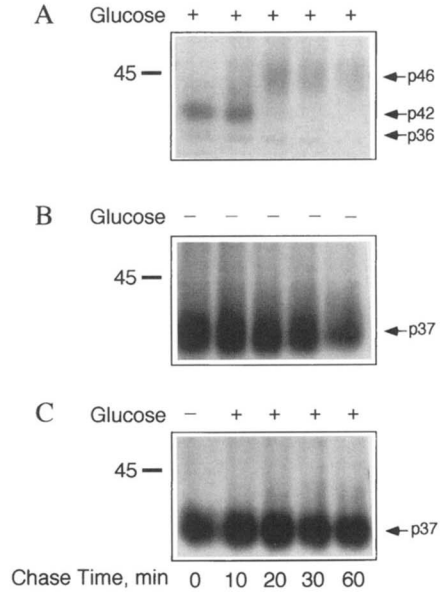

FIG. 4. **Processing of GLUT1 in control and glucose-deprived adipocytes.** Cells incubated with (*+glucose*) or without (*−glucose*) glucose for 36 h were labeled with 500 μCi/ml Tran^{35}S-label for 10 min and then chased for a total of 60 min. GLUT1 was immunoprecipitated from membrane extracts of each and analyzed by SDS-PAGE and fluorography. *Panel A*, control adipocytes chased in the presence of glucose. *Panel B*, glucose-deprived adipocytes chased in the absence of glucose. *Panel C*, glucose-deprived cells chased in the presence of glucose. Shown is the result of a single experiment.

20 min of chase (Fig. 4*A*). In contrast, p37 synthesized in glucose-deprived cells was unchanged when chased in the absence of glucose (Fig. 4*B*). Although this indicated that p37 was not processed in glucose-deprived cells, it did not rule out the possibility that p37 *could* be processed if glucose was present. We therefore tested this hypothesis and found that when glucose-deprived cells were labeled for 10 min and then chased in the presence of glucose, p37 remained unchanged (Fig. 4*C*). These results demonstrate that although 3T3-L1 adipocytes have the capacity to glycosylate GLUT1 to some extent in the absence of glucose, the transporter cannot be further processed due to either the structure of the oligosaccharide or the location of GLUT1 along the processing pathway.

Synthesis of GLUT1 during Glucose Deprivation and Refeeding—To determine the time required for glucose deprivation to affect GLUT1 glycosylation, control cells were placed into glucose-free medium and labeled for 1 h at specific times during the next 36 h. Despite glucose withdrawal, p46 was the only GLUT1 glycoform synthesized during the first 12 h of glucose deprivation (Fig. 5*A*). Beyond 12 h of glucose deprivation, the major GLUT1 glycoform synthesized shifted to p37. Densitometry of the GLUT1 bands indicated that the total amount of GLUT1 in glucose-deprived cells remained nearly constant (Fig. 5*B*). To examine the reversibility of this effect, cells deprived of glucose for 36 h were placed into medium containing glucose and labeled for 1 h during refeeding. As expected, p37 was the only form synthesized in the glucose-deprived cells. Within 6 h of refeeding, however, only p46 was synthesized (Fig. 5*C*). Further, the readdition of glucose resulted in a 3.5-fold increase in GLUT1 despite only a 40% increase in total membrane radioactivity (Fig. 5*D*). To examine this recovery in more detail, we immunoprecipitated GLUT1 from adipocytes labeled for 1 h at each hour during the first 6 h of refeeding. Surprisingly, within 1 h of glucose readdition, only the normal p46 glycoform was synthesized; no metabolically labeled p37 was observed (data not shown).

GLUT1 Half-life in Control and Glucose-deprived Adipo-

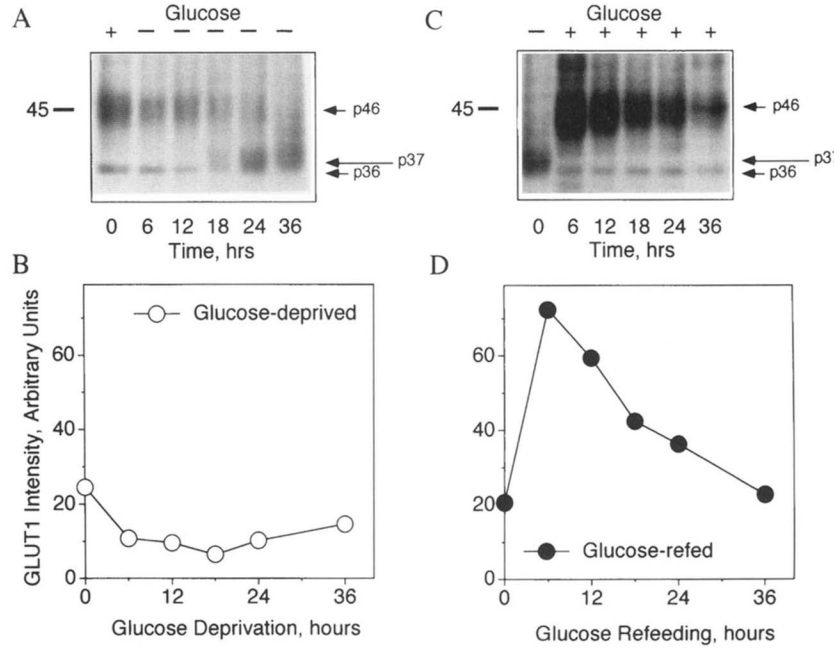

FIG. 5. **Synthesis of GLUT1 during glucose deprivation and refeeding.** *Panel A*, control cells were placed into glucose-free medium and labeled for 1 h with 200 μCi/ml Tran^{35}S-label at the times indicated. GLUT1 was immunoprecipitated from membrane extracts and analyzed by SDS-PAGE and fluorography. *Panel B*, densitometry of total GLUT1 during glucose deprivation. *Panel C*, glucose-deprived (36 h) cells were placed into medium containing glucose and labeled for 1 h with 200 μCi/ml Tran^{35}S-label at times indicated. GLUT1 was then analyzed as in (*A*). *Panel D*, densitometry of total GLUT1 during glucose refeeding. These results are representative of at least three independent experiments.

cytes—To determine the glucose-dependent regulation of GLUT1 turnover in 3T3-L1 adipocytes, control and glucose-deprived cells were metabolically labeled and then chased in either the presence or absence of glucose. GLUT1 (p46) in control adipocytes exhibited a half-life of approximately 14 h (Fig. 6, *A* and *B*). GLUT1 from control adipocytes chased in the absence of glucose exhibited a similar turnover until 12 h of glucose deprivation. After this time, the degradation of GLUT1 was significantly reduced. GLUT1 (p37) synthesized in glucose-deprived cells was not significantly degraded in the absence of glucose (Fig. 6, *C* and *D*). Although not present during the initial labeling (time = 0), a second 44-kDa glycoform appeared during the chase, whose turnover time was identical to that of p37. In glucose-deprived adipocytes chased in medium containing glucose, the turnover of GLUT1 (both p44 and p37 glycoforms) was similar to that of p46 in control adipocytes.

Glycosidase Digestion of GLUT1 Glycoforms—In order to gain insight into the type of oligosaccharide structure attached to p37, we compared its mobility and sensitivity to endoglycosidases with GLUT1 from LEC1 CHO cells, a cell line which lacks *N*-acetylglucosaminyl transferase I activity (18). Thus, total membrane protein from control adipocytes, glucose-deprived adipocytes, and LEC1 CHO cells were treated with or without *N*-glycosidase F, which removes *N*-linked oligosaccharides leaving core protein, or endoglycosidase H, which specifically cleaves oligosaccharides with high mannose structure. As expected, the normal GLUT1 glycoform (p46) which contains a complex oligosaccharide was sensitive to digestion by *N*-glycosidase F but not endoglycosidase H as assessed by Western blotting (Fig. 7). The mobility of GLUT1 from LEC1 CHO cells was significantly faster than p46, a consequence of the inability of these cells to process glycoproteins beyond a high mannose structure. Thus, GLUT1 in these cells was sensitive to both *N*-glycosidase F and endoglycosidase H. In glucose-deprived adipocytes, both p46 and p37 were observed by Western blotting and both were sensitive to *N*-glycosidase F treatment. The migration of p37 GLUT1 was not altered by treatment with endoglycosidase H, indicating that it does not contain an oligosaccharide similar to GLUT1 from LEC1 CHO cells despite their similar migration.

DISCUSSION

In this report, we used immunoprecipitation to analyze the effect of glucose deprivation on the biosynthesis, processing, and turnover of GLUT1 in 3T3-L1 adipocytes. Although several studies have reported the appearance of a low molecular weight form of GLUT1 resulting from the withdrawal of glucose, the relationship, if any, between this form and the normal molecular weight species has not been investigated. Thus we have extended these prior studies in several ways. First, our work demonstrated that p37 GLUT1 is a newly synthesized protein rather than a breakdown product of the normal transporter. Second, we determined that p37 is not a precursor to the normal form of GLUT1 (p46). Finally, we demonstrated that p37 is post-translationally processed in 3T3-L1 adipocytes by a pathway that is unique from that of p46 resulting in an abbreviated and likely aberrant oligosaccharide. These conclusions are based on the following observations. In control cells, we identified a single mature form of GLUT1 that migrated as a broad band at approximately 46 kDa. Two precursor forms were distinguished from the mature GLUT1. These represent the core protein (36 kDa) and an intermediate (42 kDa) in the processing pathway consistent with analysis of GLUT1 processing in a cell-free system (19). When metabolically labeled GLUT1 was immunoprecipitated from glucose-deprived cells, we observed a 37-kDa protein. In the presence of tunicamycin, only one form of GLUT1 (p36) was synthesized in either control or glucose-deprived cells, indicating that the core protein was not altered by glucose deprivation. This also indicates that p37, like the normal GLUT1 glycoform, arose from the 36-kDa core protein by modification with an *N*-linked oligosaccharide. It should be noted that not all cells appear competent to glycosylate GLUT1 in the absence of glucose. The lower molecular mass GLUT1 observed in glucose-deprived rat kidney cells, for example, migrates at the same molecular weight as GLUT1 from these cells incubated with tunicamycin or GLUT1 transporter isolated from these cells treated with endoglycosidase (9). Glycosylation therefore represents another cell type-specific aspect of the regulation of GLUT1 expression by glucose.

The GLUT1 glycoform in glucose-deprived 3T3-L1 adipocytes is interesting from several perspectives. First, it is intriguing that over 12 h are required for its appearance. This indicates that

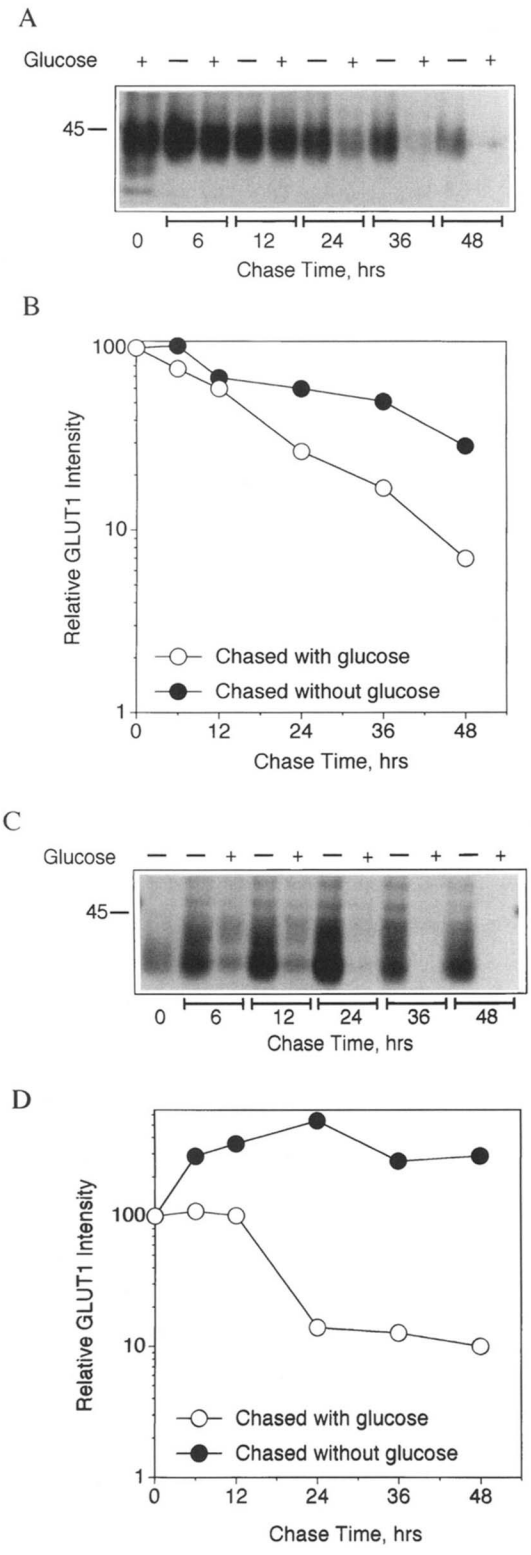

FIG. 6. **Turnover of GLUT1 in control, glucose-deprived, and refed adipocytes.** Control or glucose-deprived cells were labeled with 200 μCi/ml Tran^{35}S-label for 1 h and then chased in the presence (+) or absence (−) of glucose. GLUT1 was immunoprecipitated from membrane extracts and analyzed by SDS-PAGE and fluorography. *Panel A*, GLUT1 immunoprecipitates from control cells chased in the presence or absence of glucose. *Panel B*, Densitometry of total GLUT1 during the chase period from *Panel A*. *Panel C*, GLUT1 immunoprecipitates from glucose deprived cells chased in the presence or absence of glucose. *Panel D*, Densitometry of total GLUT1 during the chase period from *Panel C*. Results shown are from one experiment which is representative of at least four independent experiments.

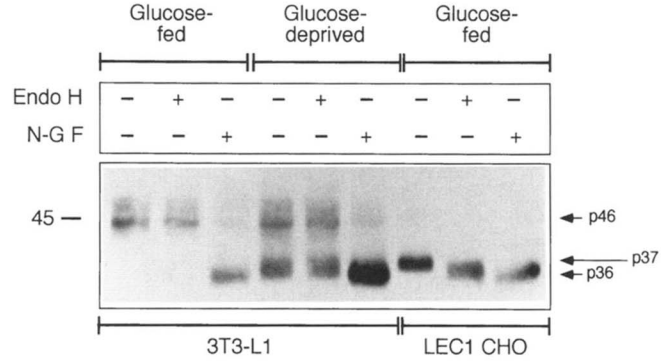

FIG. 7. **Endoglycosidase digestion of GLUT1 glycoforms from 3T3-L1 adipocytes and LEC1 CHO.** Membrane protein from control and glucose-deprived 3T3-L1 adipocytes (20 μg) and from LEC1 CHO cells (10 μg) was denatured and treated with endoglycosidase as described under "Experimental Procedures." Membrane proteins were then separated by SDS-PAGE and transferred to nitrocellulose. GLUT1 was then detected by Western analysis and visualized by enhanced chemiluminesence. Results shown are representative of at least four independent experiments.

sufficient sugar is available to support normal glycosylation for this period of glucose deprivation. Yet, it is unlikely that any "free" sugar would be available to support core oligosaccharide biosynthesis due to the high rate of glucose utilization.[2] However, glycogen breakdown may provide sufficient substrate for this process during the early phase of deprivation. Only when the glycogen pool is depleted would p37 accumulate. Yet, in the face of extended glucose deprivation with no apparent source of glucose for oligosaccharide synthesis, GLUT1 (p37) is still glycosylated, albeit in abbreviated form. It is possible that carbohydrate is scavenged from the degradation of other glycoproteins whose function is not needed under these conditions. Alternatively, the lipid-linked oligosaccharide pool may not be completely depleted between 12 and 36 h of deprivation and thus provides continuous core oligosaccharide.

The oligosaccharide structure on p37 form is currently unknown, although it is evidently not the same as that on GLUT1 from LEC1 cells based on endoglycosidase H sensitivity. However, previous studies provide some clues as to the type of oligosaccharide that might be generated in the absence of glucose (20). In CHO cells deprived of glucose, the synthesis of lipid-linked oligosaccharide shifts from a normal $Glc_3Man_9GlcNAc_2$ to an alternative structure, $Glc_3Man_5GlcNAc_2$. The transfer of this oligosaccharide to protein acceptors occurred normally, although the resulting glycoproteins were endoglycosidase H insensitive. It could therefore be hypothesized that 3T3-L1 adipocytes might also generate a similar oligosaccharide in the absence of glucose, resulting in a GLUT1 protein that was both slightly greater in molecular weight than the core protein and insensitive to endoglycosidase H. Although p37 GLUT1 displays these characteristics, confirmation of this hypothesis would require purification of the transporter in significant quantities and subsequent carbohydrate sequencing.

The function and targeting of p37 remains to be determined. Studies directed toward understanding the role of glycosylation in transport activity have provided ambiguous results. Cells expressing a mutant GLUT1 transporter missing the glycosylation site, Asn^{45}, retained some glucose transport activity (21). However, treatment of GLUT1-containing vesicles with exo- and endoglycosidases resulted in a loss of transport activity (22). Likewise, the role of glycosylation in targeting GLUT1 to

[2] 3T3-L1 adipocytes utilize glucose at a rate of approximately 350 nmol/10^6 cells/h (H. H. Kitzman, R. J. McMahon, P. M. Fadia, and S. C. Frost, submitted for publication).

the functional compartment (*i.e.* plasma membrane) is not clear. The mature GLUT1 transporter in 3T3-L1 adipocytes and other cell types at the cell surface is a complex-type glycoprotein. However, GLUT1 from the LEC1 CHO cell line resides at the cell surface as evidenced by the ability of these cells to transport glucose (23). Clearly, the type of oligosaccharide present or endoglycosidase sensitivity alone cannot be used to predict or define protein localization. The glucose deprivation model, coupled with subcellular fractionation, may provide information regarding the effects of alternative glycosylation on GLUT1 targeting.

Finally two previous reports attempting to measure the half-life of GLUT1 by direct methods have proven contradictory. Haspel *et al.* (11) observed a half-life of 90 min for GLUT1 in 3T3-L1 cells. In contrast, Sargeant and Paquet (24) studied the turnover of GLUT1 in these cells and observed a half-life of approximately 19 h. Our data showing a half-life of 14 h is more consistent with the latter. In neither of these earlier papers was the effect of glucose deprivation measured. Thus our study describes an aspect of GLUT1 regulation not previously identified. Even though glucose deprivation had no specific effect on the rate of GLUT1 synthesis (despite the difference in glycosylation), the effect on GLUT1 turnover was striking. Clearly, the degradation of p37 was significantly inhibited. Interestingly, so was the turnover of the total pool of membrane protein. P46 turnover is affected only after 12 h of glucose deprivation concurrent with the onset of abnormal or deficient glycosylation as indicated by the appearance of p37. Thus, the loss of glucose itself is not the signal for the inhibition of turnover. Rather, the depletion of a specific glycoprotein, required for degradation, may impair turnover. This is supported by that fact that glucose readdition quickly restores normal GLUT1 glycosylation and turnover.

REFERENCES

1. Gould, G. W., and Bell, G. I. (1990) *Trends Biochem. Sci.* **15,** 17–23
2. Klip, A., Tsakiridis, T., Marette, A., and Ortiz, P. (1994) *FASEB J.* **8,** 43–53
3. Kletzien, R. F., and Perdue, J. F. (1975) *J. Biol. Chem.* **250,** 593–600
4. Yamada, K., Tillotson, L. G., and Isselbacher, K. J. (1983) *J. Biol. Chem.* **258,** 9786–9792
5. Germinario, R. J., Rockman, H., Oliveira, M., Manuel, S., and Taylor, M. (1982) *J. Cell. Physiol.* **112,** 367–372
6. Walker, P. S., Ramlal, T., Donovan, J. A., Doering, T. P., Sandra, A., Klip, A., and Pessin, J. E. (1989) *J. Biol. Chem.* **264,** 6587–6595
7. van Putten, J., and Krans, H. M. J. (1985) *J. Biol. Chem.* **260,** 7996–8001
8. Shawver, L. K., Olson, S. A., White, M., and Weber, M. J. (1987) *Mol. Cell. Biol.* **7,** 2112–2118
9. Haspel, H. C., Mynarcik, D. C., Ortiz, P. A., Honkanen, R. A., and Rosenfeld, M. G. (1991) *Mol. Endocrinol.* **5,** 61–71
10. Tordjman, K. M., Leingang, K. A., and Mueckler, M. M. (1990) *Biochem. J.* **271,** 201–207
11. Haspel, H. C., Birnbaum, M. J., Wilk, E. W., and Rosen, O. M. (1985) *J. Biol. Chem.* **260,** 7219–7225
12. Kitzman, H. H., Jr., McMahon, R. J., Williams, M. G., and Frost, S. C. (1993) *J. Biol. Chem.* **268,** 1320–1325
13. Reed, B. C., Shade, D., Alperovich, F., and Vang, M. (1990) *Arch. Biochem. Biophys.* **279,** 261–274
14. Frost, S. C., and Lane, M. D. (1985) *J. Biol. Chem.* **260,** 2646–2652
15. Sigel, M. B., Sinha, Y. N., and VanderLaan, W. P. (1983) *Methods Enzymol.* **93,** 3–14
16. Dankert, J. R., Shiver, J. W., and Esser, A. F. (1985) *Biochemistry* **24,** 2754
17. Kozka, I. J., Clark, A. E., and Holman, G. D. (1991) *J. Biol. Chem.* **266,** 11726–11731
18. Chaney, W., and Stanley, P. (1986) *J. Biol. Chem.* **261,** 10551–10557
19. Mueckler, M., and Lodish, H. F. (1986) *Cell* **44,** 629–637
20. Rearick, J. I., Chapman, A., and Kornfeld, S. (1981) *J. Biol. Chem.* **256,** 6255–6261
21. Asano, T., Katagiri, H., Takata, H., Lin, J. L., Ishihara, H., Inukai, K., Tsukuda, K., Kikuchi, M., Hirano, H., Yazaki, Y., and Oka, Y. (1991) *J. Biol. Chem.* **266,** 24632–24636
22. Feugeas, J. P., Neel, D., Goussault, Y., and Derappe, C. (1991) *Biochim. Biophys. Acta* **1066,** 59–62
23. Haspel, H. C., Revillame, J., and Rosen, O. M. (1988) *J. Cell. Physiol.* **136,** 361–366
24. Sargeant, R. J., and Paquet, M. R. (1993) *Biochem. J.* **290,** 913–919