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Modulation of the synthesis and glycosylation of the glucose transporter protein by transforming growth factor-β1 in Swiss 3T3 fibroblasts

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Transforming growth factor-β1 (TGF-β1) stimulated growth and glucose uptake in Swiss mouse fibroblasts. DNA synthesis was increased 2–3-fold after 48 h incubation of growing 3T3 cells with TGF-β1 in calf serum-containing medium. Glucose transport activity in the cells was increased within 3 h after addition of TGF-β1 and this stimulation continued during incubation for 48 h. TGF-β1 also increased the levels of a brain type-glucose transporter (GLUT1) mRNA and the GLUT1 protein (55 kDa) in the membranes, consistent with the increase in glucose uptake. Furthermore, a longer exposure of TGF-β1 for 24–48 h induced a marked increase in the 65 kDa GLUT1 in 3T3 cell membranes. Other growth factors such as epidermal growth factor, fibroblast growth factor, transforming growth factor-α, and insulin did not elevate glucose uptake and the levels of 55 and 65 kDa GLUT1 proteins. Adding tunicamycin or deoxymannojirimycin to the TGF-β1-treated and untreated cells caused these 55 and 65 kDa glucose transporters to migrate as one band at 40–43 kDa. In addition, treating membrane proteins with glycopeptidase F, which removes N-linked oligosaccharides, also generated a glucose transporter of 40 kDa, suggesting that the 55 and 65 kDa GLUT1 proteins have a similar or identical core polypeptide but with different N-linked oligosaccharides. These results indicate that TGF-β1 modulates the synthesis of GLUT1 protein as well as its glycosylation in Swiss 3T3 cells, and that these changes may contribute to the control of cell proliferation by TGF-β1.

Introduction

TGF-β1, an active polypeptide homodimer of 25 kDa present in blood platelets, is a potent modulator of growth and differentiation in a number of diverse systems (for reviews, see Refs. 1, 2). TGF-β1 can confer to normal fibroblasts properties associated with transformation, such as growth in soft agar, loss of contact inhibition, and increased DNA synthesis and cell growth [1–3]. TGF-β1 also acts as a potent inhibitor of cell proliferation in many types of cells, including epithelial cells and lymphocytes [3,4]. Although it has been reported that TGF-β1 modulates the expression of variety of proteins and genes including fibronectin, collagen, fibronectin receptor, proteinase inhibitors and junB in several cell types [5–9], the molecular mechanism by which TGF-β1 regulates signal transduction and cell growth remains largely unknown.

An early event in the transformation of cultured cells by tumor viruses or chemical carcinogens is the increased rate of hexose transport [10–12]. In a previous study, we demonstrated that growth factors such as serum, PDGF and FGF stimulated glucose uptake, accompanied by an increase in the expression of a brain-type glucose transporter (GLUT1) mRNA in quiescent Swiss 3T3 fibroblasts [13]. The increased expression of GLUT1 mRNA by growth factors was regulated at the transcriptional level [14,15]. Several glucose transporter isoforms are present in mammalian cells [16]. The glucose transporter denoted GLUT1 is prevalent in the brain, erythrocytes and several fibroblasts

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Abbreviations: TGF-β1, transforming growth factor-β1; TGF-α, transforming growth factor-α; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; GLUT1, brain-type glucose transporter; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; 2-DG, 2-deoxyglucose; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; hRBC, human erythrocytes; kb, kilobases; Con A, concanavalin A; WGA, wheat germ agglutinin; PHA-L4, phytohemagglutinin-L4; DSA, datura stramonium agglutinin.

[17–19]. GLUT1 protein in cultured cell lines is a 55 kDa glycoprotein which posesses a complex N-linked oligosaccharide at Asn-45, and the form of GLUT1 lacking this oligosaccharide is a polypeptide of 38–41 kDa [20–23].

We previously observed that the stimulated glucose uptake and the expression of GLUT1 mRNA in quiescent 3T3 cells by growth factors was enhanced synergistically in the presence of TGF-β1 [24]. In the present study, we further demonstrated that TGF-β1 modulates the synthesis and glycosylation of GLUT1 protein in the membranes of growing Swiss 3T3 cells. Possible roles of these changes in glucose transporter and glucose transport activity in TGF-β1-dependent growth stimulation are also discussed.

Materials and Methods

Materials. Human platelet TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Bovine brain bFGF and mouse submaxillary grand EGF were obtained from Toyobo (Osaka, Japan). Bovine insulin and PMA were from Sigma (St. Louis, MO) and LC Service (Boston, MA), respectively. Human TGF-α was purchased from Biomedical Technologies (Stoughton, MA). 2-Deoxy[1-^{3}H]glucose (17 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol) and [^{125}I]protein A (30 mCi/mg) were purchased from Amersham, UK. Rat brain GLUT1 cDNA [17], and antibodies against human erythrocyte GLUT1 [25] or C-terminal peptide 480–492 of the GLUT1 [26] were gifts from Dr. Ora M. Rosen (Sloan-Kettering Cancer Center, NY) and Dr. M. Kasahara (Teikyo University, Tokyo), respectively. Tunicamycin, deoxymannojirimycin and glycopeptidase F (*Flavobacterium meningosepticum*) were purchased from Sigma, Toronto Research Chemicals (Downsview, Ontario) and Boehringer-Mannheim, Yamanouchi (Tokyo), respectively. Biotynyl lectins (Con A, WGA, PHA-L4 and DSA) were obtained from Seikagaku (Tokyo).

Cell culture. Swiss 3T3 cells were grown in DMEM (Flow Laboratories, Boston) containing 10% FCS (Flow Laboratories), penicillin (100 units/ml) and streptomycim (100 μg/ml) in a humidified 5% CO_2/95% air at 37°C as described previously [13,24].

DNA synthesis. Swiss 3T3 cells were plated in 35 mm plastic dishes in 1 ml of DMEM containing 10% FCS at a density of $6 \cdot 10^4$ cells per dish. At 18–24 h after plating, TGF-β1 was added. At the indicated time, [^{3}H]thymidine (0.5 μCi/ml, 1 μM) was then added and the cells were incubated for another 2 h. The radioactivity incorporated into 5% TCA-insoluble materials was determined, as described previously [13].

Measurement of 2-deoxyglucose uptake. At 18–24 h after plating of Swiss 3T3 cells as described above, TGF-β1 or growth factors were added to the cells and incubated at 37°C for the indicated periods. The treated cells were washed with PBS and 2-DG uptake was then determined by incubation with 2-[^{3}H]DG (0.5 μCi/ml, 1 μM) at 37°C for 15 min in glucose-free DMEM, as described previously [13,24]. During this period, incorporation of the radioactivity into the cells was shown to be linear [13]. The radioactivity incorporated into the cells was extracted with 5% TCA and counted.

RNA blot hybridization. Total cellular RNA was isolated from Swiss 3T3 cells with guanidine isothiocyanate and electrophoresed in 1.5% agarose (20 μg/lane) containing 2.2 M formaldehyde using the procedure described by Maniatis et al. [27], then transferred to a nylon membrane (Hybond-N, Amersham). The membrane was hybridized with a ^{32}P-labeled GLUT1 cDNA probe [17] at 42°C for 18–24 h then washed at room temperature in 2 × SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0) with 0.1% SDS, and again in 0.2 × SSC with 0.1% SDS at 45°C, as described previously [13, 24]. The hybridized blot was exposed to Fuji X-ray film at −70°C for 1–2 days.

Membrane isolation. Mouse 3T3 cells grown in 10 cm dishes were washed twice with cold PBS (pH 7.4) and scraped into iced sonication buffer (SB) containing 5 mM EDTA, 250 mM sucrose, 25 mM benzamidine, 50 mM NaF, 1 mM PMSF and 100 mM NaP_i (pH 7.4) as described [20]. The cells were lysed by sonication (3 × 30 s at 100 W on ice) and the lysate was centrifuged at 550 × *g* for 5 min at 4°C. These membranes were suspended in membrane buffer (MB) (1 mM EDTA, 250 mM sucrose, 1 mM PMSF, 5 mM NaP_i (pH 7.4)) and stored at −20°C until used.

Immunoblot and lectin blot analyses. Suspensions of membrane protein (30–50 μg) were diluted 1:1 with 2 × sample buffer (4% SDS, 40% glycerol, 100 mM dithiothreitol, 125 mM Tris-HCl, pH 6.8) and incubated for 30 min at 37°C, followed by separation on SDS-PAGE (10% acrylamide; Ref. 28). The proteins in the gels were electrophoretically transferred to durapore filters (Millipore), as described [29]. The filters were then incubated for 2 h at 37°C in TBS-Tween (500 mM NaCl, 20 mM Tris, pH 7.5 plus 0.1% Tween 20) containing 5% skim milk, and incubated further with either the anti-human GLUT1 antibody or a biotinyl lectin (Con A, WGA, PHA-L4, DSA) for 2 h at room temperature. For GLUT1 blotting, the filters were then washed twice with TBS-Tween for 10 min, incubated for 2 h with 1 μCi/ml of ^{125}I-labeled protein A, followed by extensive washes with TBS-Tween. The filters were then dried and autoradiographed. For lectin blotting, the biotinyl lectin-treated filters were incubated with streptoavidin-peroxidase conjugate for 15 min at 37°C, followed by washes with TBS-Tween and visualized with tetramethylbenzidine, as described [30].

Treatment of 3T3 cells with glycosylation inhibitors. Growing 3T3 cells cultured with DMEM and 10% FCS

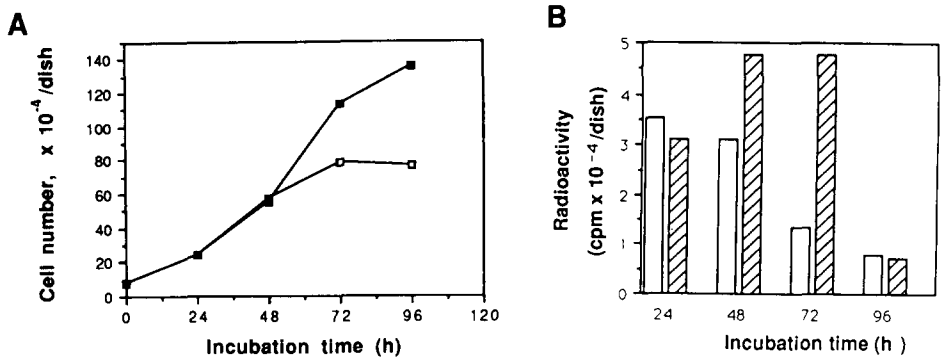

Fig. 1. (A) Effect of TGF-β1 on proliferation of 3T3 cells. Swiss 3T3 cells, $6 \cdot 10^4$, were plated in 35 mm plastic dishes containing DMEM plus 10% FCS and 100 pM TGF-β1 was added on the following day. At the indicated time thereafter, the numbers of viable cells from control (□) and TGF-β1-treated cultures (■) were counted. (B) Kinetics of [^{3}H]thymidine incorporation into growing 3T3 cells. Swiss 3T3 cells were incubated with (hatched column) or without (open column) 100 pM TGF-β1 as described in (A) and the incorporation of [^{3}H]thymidine (0.5 μCi/ml, 1 μM) into TCA-insoluble materials for 2 h was determined at the indicated time, that was the mid-point of each labeling period. The indicated data are the average results from duplicate samples, and these data are representative of two or three separate experiments.

in 10 cm dishes were incubated for 24 h with either tunicamycin (0.1–2 μg/ml) or deoxymannojirimycin (0.1–0.2 mg/ml) in the presence or absence of 50 pM TGF-β1, followed by membrane isolation and immunoblot analysis for GLUT1 protein.

Glycopeptidase F digestion of the membrane proteins. Isolated 3T3 cell membranes (30 μg protein suspended in 20 μl MB at pH 7.4) were mixed with 30 μl reaction medium containing 21.7 mM EDTA, 1.25% NP-40, 16.7 mM β-mercaptoethanol, 2 mM NaP_i (pH 7.2) and 0.6 units of glycopeptidase F. The reaction mixture was then incubated for 18 h at 37°C in a total volume of 50 μl. After this digestion, the reaction mixture was mixed with the SDS-sample buffer, followed by electrophoresis and immunoblot analysis for changes in the expressed GLUT1 protein.

Protein determination. Protein concentration was measured by the method of Bradford [31] or Lowry [32] with bovine serum albumin as the standard.

Results

Effect of TGF-β1 on the proliferation of Swiss 3T3 cells

The effect of TGF-β1 on the growth of Swiss 3T3 cells was examined. A 2-fold increase in [^{3}H]thymidine incorporation compared with the untreated cells was observed at 48–72 h after adding 100 pM TGF-β1 to the medium containing 10% serum (Fig. 1B). This represented about a 50% increase in cell density (Fig. 1A). The 3T3 cells were a flat confluent monolayer in the serum-containing medium. In the presence of TGF-β1, however, there appeared to be morphological

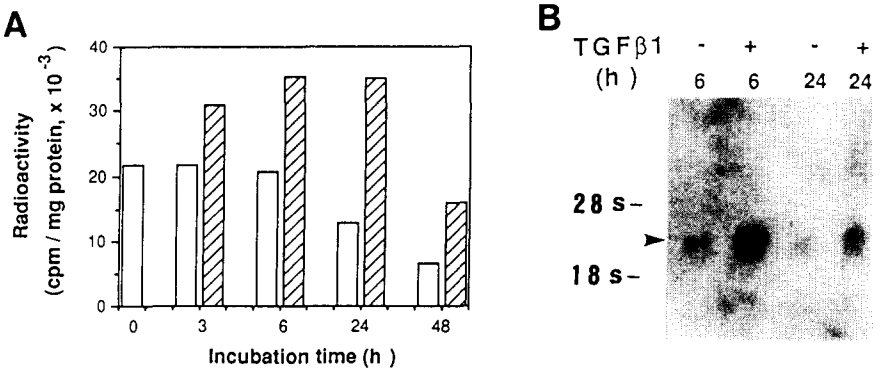

Fig. 2. Effects of TGF-β1 on 2-deoxyglucose uptake and the expression of GLUT1 mRNA in growing 3T3 cells. (A) 3T3 cells were cultured with 10% FCS-containing DMEM in the presence (hatched bars) or absence (open bars) of 100 pM TGF-β1 for the indicaded periods. After the incubation, 2-deoxy[^{3}H]glucose uptake (0.5 μCi/ml, 1 μM) for 15 min was determined as described under Materials and Methods. The data are average of duplicate samples, and the results are representative of three separate experiments. (B) 3T3 cells were incubated with 10% serum-containing DMEM in the presence (+) or absence (−) of 100 pM TGF-β1 for either 6 or 24 h. The total RNA was then extracted from the treated cells and the GLUT1 mRNA level was determined by RNA blot analysis as described under Materials and Methods. The RNA sizes were estimated by acridine orange staining (0.33 mg/ml) of the agarose gel, the 28S and 18S rRNA being used as markers. The arrow indicates the 2.8 kb GLUT1 mRNA.

changes such as a disorganized criss-crossed cell arrangement (data not shown).

TGF-β1 stimulates glucose uptake and the GLUT1 mRNA expression in growing 3T3 cells

The addition of TGF-β1 to growing 3T3 cells caused an increase in 2-[^{3}H]DG uptake within 3 h and led to a 3-fold increase in transport activity compared with the control cells at 24 h (Fig. 2A). 2-DG uptake by 3T3 cells decreased with increasing cell density, but the elevated levels in the TGF-β1-treated cells remained during incubation for 48 h. Dose response studies demonstrated that a measurable increase in glucose uptake occurred with 20 pM TGF-β1 and maximal stimulation of the uptake was obtained with 50–100 pM TGF-β1 at 24 h after treatment (data not shown).

The effect of TGF-β1 on the changes in GLUT1 mRNA levels in growing 3T3 cells was determined by RNA blot analysis. Compared with untreated cells, adding TGF-β1 to growing 3T3 cells for 6 h induced a 3-fold increase in 2.8 kb GLUT1 mRNA expression, that was still evident 24 h after the addition (Fig. 2B).

Modulation of the GLUT1 protein expression by TGF-β1

The level of membrane-associated GLUT1 protein was determined by immunoblotting isolated 3T3 cell membranes (Fig. 3). A 55 kDa GLUT1 protein was observed in membranes from untreated 3T3 cells (Fig. 3A). A broad band of about 55 kDa for GLUT1 in the membranes of human red blood cells (hRBC) was also detected under the same conditions, as described previously [20,21,25]. Consistent with the results from glucose uptake and the expression of GLUT1 mRNA, exposure of 3T3 cells to TGF-β1 for 6 h increased the amount of the 55 kDa GLUT1 by 2-fold (Fig. 3A). In cells treated with 100 pM TGF-β1 for 24–48 h, a marked increase in a 65 kDa protein as well as the 55 kDa GLUT1, both of which were recognized by anti-hRBC GLUT1 antibody, was observed. As shown in Fig. 3B, TGF-β1-treatment of 3T3 cells for 24 h resulted in a dose-dependent increase in the 55 and 65 kDa proteins. The maximal increase in the 55 and 65 kDa GLUT1 expression was obtained with 20–100 pM TGF-β1. This newly detected 65 kDa protein was also GLUT1, since it, as well as the 55 kDa GLUT1, was recognized by an antibody against C-terminal oligopeptide specific for GLUT1 (Fig. 3C; Refs. 21, 25).

Effect of various growth factors on glucose uptake and the GLUT1 protein expression

The specificity of TGF-β1 for modulation of glucose uptake and the GLUT1 protein expression was determined by comparing with other growth factors. As shown in Fig. 4A, little or no stimulation of glucose uptake was observed in 3T3 cells incubated for 24 h with various growth factors (EGF, FGF, PMA, TGF-α or insulin) in serum-containing medium. These growth factors also did not change the level of the 55 and 65

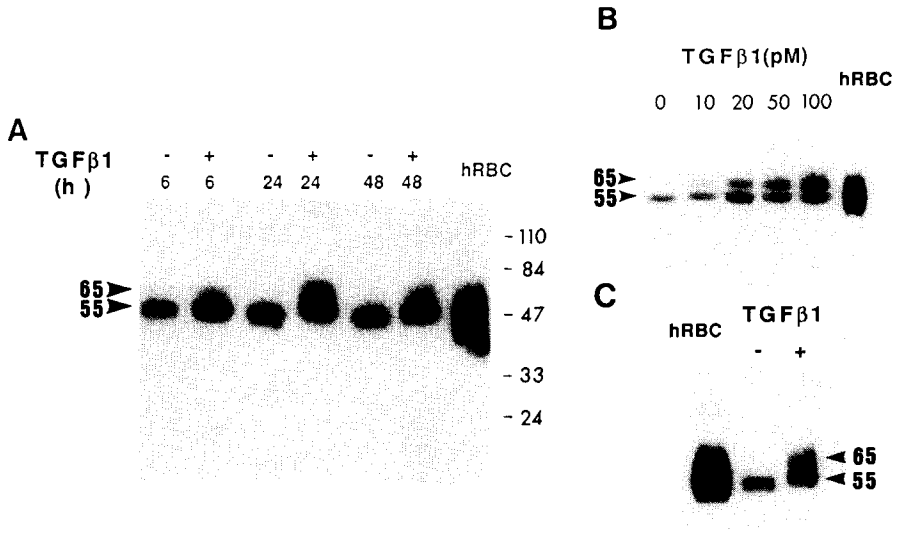

Fig. 3. Immunoblot analysis of GLUT1 in the membranes from control and TGF-β1-treated 3T3 cells. (A) The growing 3T3 cells were incubated with (+) or without (−) 100 pM TGF-β1 for the indicated periods and membranes were prepared as described under Materials and Methods. Membrane proteins (50 μg/lane) were separated by SDS-PAGE, followed by immunoblotting with anti-hRBC GLUT1 antibody. Membrane proteins (0.1 μg) from human RBC (hRBC) were also determined simultaneously as positive controls. The numbers on the right are the molecular masses of markers in kDa. (B) Growing 3T3 cells were treated with various concentrations of TGF-β1 for 24 h and the expression of GLUT1 protein in the membranes was also determined as described in (A). (C) Similar immunoblotting experiments were performed by using antibody against C-terminal oligopeptides of GLUT1 in the membranes isolated from 100 pM TGF-β1-treated or untreated 3T3 cells. Arrows indicate molecular mass in kDa.

kDa GLUT1 proteins (Fig. 4B), suggesting a specific effect of TGF-β1 on the stimulation of glucose uptake and GLUT1 expression in growing 3T3 cells.

Stimulation of GLUT1 expression by TGF-β1 in quiescent 3T3 cells

We have recently shown that TGF-β1 synergistically stimulates glucose transport and the expression of GLUT1 mRNA with serum or growth factors in quiescent 3T3 cells [24]. As expected, the expression of the 55 kDa GLUT1 was increased by the treatment with serum and TGF-β1 in accordance with the increased glucose uptake (Fig. 5). The 65 kDa GLUT1 protein was also induced in quiescent 3T3 cells incubated with TGF-β1 and serum for 24 h, but the extent was much less than that in the case of growing 3T3 cells described in Figs. 3 and 4.

Effect of tunicamycin and deoxymannojirimycin on the synthesis of GLUT1

To examine whether the formation of 65 kDa GLUT1 in the TGF-β1-treated growing cells was due to an increase in glycosylation or whether it was due to

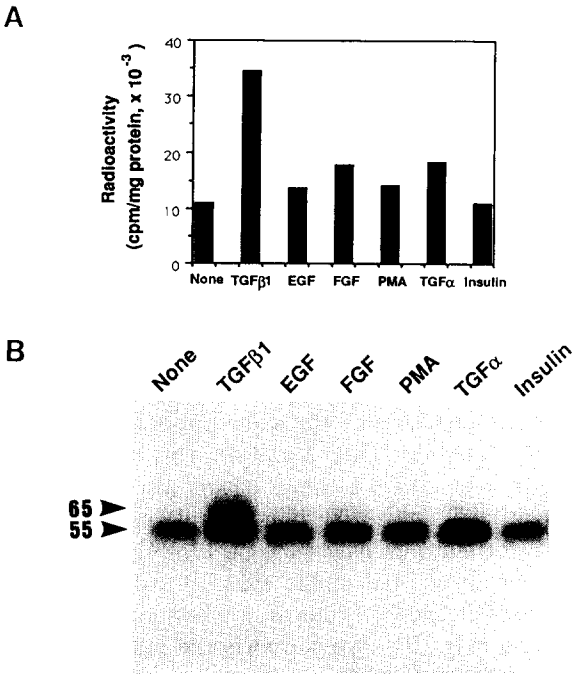

Fig. 4. Effects of various growth factors on 2-deoxyglucose uptake and the expression of GLUT1 in growing 3T3 cells. Growing 3T3 cells were exposed to various growth factors (50 pM TGF-β1, 50 ng/ml EGF, 25 ng/ml PMA, 50 ng/ml TGF-α and 1 μg/ml insulin) as indicated at 37°C for 24 h in DMEM containing 10% FCS. After incubation, 2-deoxyglucose uptake (A) or immunoblot analysis (30 mg membrane proteins/lane) for GLUT1 protein (B) was performed as described in Figs. 2 or 3, respectively. Arrows indicate the molecular mass (kDa) of GLUT1. The data in 2-DG uptake are average of duplicate samples, and these results are representative of two separate experiments.

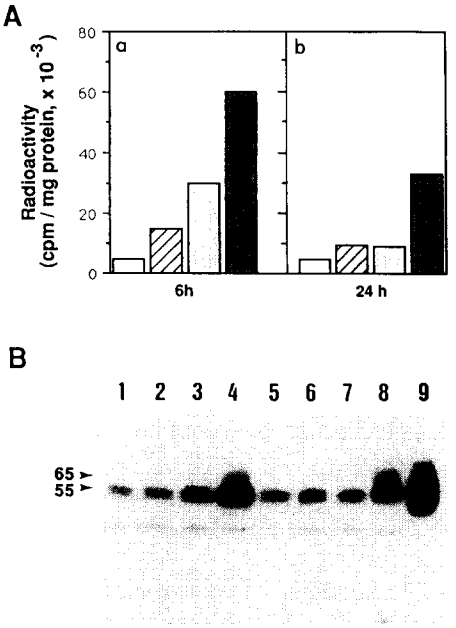

Fig. 5. Stimulation of glucose uptake and the expression of GLUT1 protein by TGF-β1 in quiescent 3T3 cells. (A) Quiescent 3T3 cells prepared as described previously [13] were incubated for 6 (a) or 24 h (b) with DME alone (□), DME+50 pM TGF-β1 (▨), DME+10% FCS (▩), or DME+10% FCS+50 pM TGF-β1 (■), then 2-deoxyglucose uptake for 15 min was determined. The data are average of duplicate samples, and the results are representative of three separate experiments. (B) The quiescent 3T3 cells were incubated for 6 h (lanes 1–4) or 24 h (lanes 5–8) with DME alone (lanes 1 and 5), DME+50 pM TGF-β1 (lanes 2 and 6), DME+10% FCS (lanes 3 and 7), or DME+10% FCS+50 pM TGF-β1 (lanes 4 and 8), and the expression of GLUT1 protein in the isolated membranes was determined by immunoblotting as described in Fig. 3. Human RBC membranes (0.1 μg) were determined at the same time (lane 9). Arrows indicate molecular mass in kDa.

an alteration in the peptide backbone of GLUT1, 3T3 cells were incubated with TGF-β1 for 24 h in the presence of inhibitors of glycosylation processing. Tunicamycin (0.2–2 μg/ml), which blocks the formation of a protein N-glycosydic linkage [33], inhibited the formation of both 55 and 65 kDa GLUT1 in the TGF-β1-treated and untreated cells, and a 40 kDa protein, which corresponds to the unglycosylated GLUT1 [20–23], was detected (Fig. 6A). Deoxymannojirimycin (0.2 mg/ml), an inhibitor of mannosidase I in the Golgi apparatus [33], also inhibited the synthesis of both 55 and 65 kDa GLUT1 and a 43 kDa protein, which may be a GLUT1 precursor containing a N-linked high-mannose oligosaccharide, was recognized by the GLUT1 antibody (Fig. 6B).

It should be noted that the amount of 40 or 43 kDa protein in TGF-β1 and inhibitor-treated 3T3 cells was still increased by about 1.5–2-fold compared to that in the control cells (Fig. 6), suggesting that these glycosylation inhibitors did not affect the stimulated synthesis of GLUT1 at the levels of transcription and translation.

Both 55 and 65 kDa GLUT1 are derived from the same core polypeptide

To confirm further the TGF-β1-induced modulation of the GLUT1 glycosylation, membranes isolated from TGF-β1-treated 3T3 cells for 24 h were directly digested with glycopeptidase F, which cleaves the N-linked oligosaccharides of glycoproteins at the asparagine residue [34]. Both 55 and 65 kDa GLUT1 of the TGF-β1-treated membranes were degraded to 40 kDa after this treatment (Fig. 7). The molecular mass of GLUT1 of untreated 3T3 membranes also shifted from 55 to 40 kDa. These results indicate that these 65 and 55 kDa GLUT1 proteins were derived from an identical core polypeptide but contain different N-linked oligosaccharides.

Effect of TGF-β1 on glycosylation of other membrane proteins

Lectin blot analysis was performed to determine the modulation by TGF-β1 of glycosylation of membrane proteins from growing 3T3 cells. As shown in Fig. 8, TGF-β1 did not affect the molecular masses of Coomassie blue-stained proteins nor those of binding proteins to several lectins such as Con A, WGA, PHA-L4 and DSA. GLUT1 protein was not identified under these conditions.

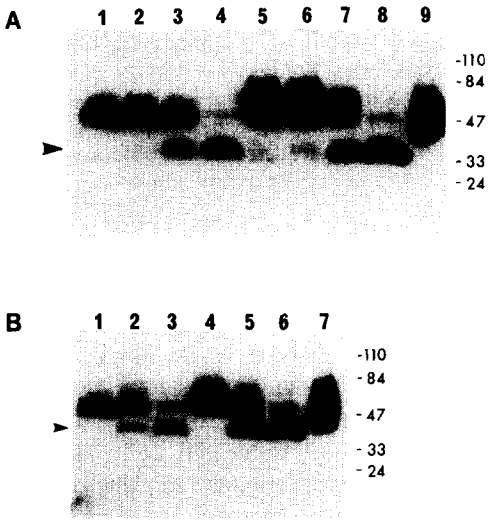

Fig. 6. Effects of tunicamycin and deoxymannojirimycin on the expression of 55 and 65 kDa GLUT1 in TGF-β1-treated 3T3 cells. (A) Growing 3T3 cells were incubated for 24 h with various concentrations of tunicamycin in the absence (lanes 1–4) or presence (lanes 5–8) of 50 pM TGF-β1, and membrane proteins (50 μg/lane) were then immunoblotted as described in Fig. 3. The tunicamycin concentrations used were 0 (lane 1, 5), 0.1 (lane 2, 6), 0.2 (lane 3, 7), and 2 (lane 4, 8) μg/ml. Arrow indicates 40 kDa. In lane 9, hRBC membranes (0.1 μg) were determined. (B) Growing 3T3 cells were similarly incubated with deoxymannojirimycin in the absence (lanes 1–3) or presence (lanes 4–6) of 50 pM TGF-β1 for 24 h, then immunoblotted as described in (A). The deoxymannojirimycin concentrations used were 0 (lane 1, 4), 0.1 (lane 2, 5), and 0.2 (lane 3, 6) mg/ml. Human RBC membranes (0.1 μg) were determined in lane 7. Arrow indicates 43 kDa. The numbers on the right sides of figures are molecular masses of markers in kDa.

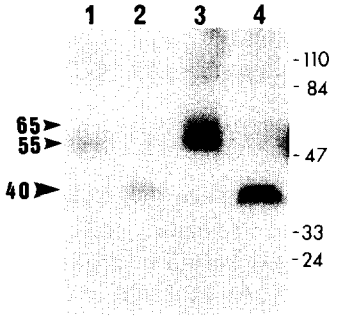

Fig. 7. Glycopeptidase F digestion of GLUT1 proteins in 3T3 membranes. 30 μg of the membrane proteins isolated from growing 3T3 cells (lane 1, 2) or those treated with 100 pM TGF-β1 for 24 h (lane 3, 4) were digested with (lane 2, 4) or without (lane 1, 3) glycopeptidase F (0.6 units) for 18 h at 37°C. These samples were then electrophoresed, followed by immunoblotting to determine GLUT1 levels. Numbers on the right represent molecular masses of markers in kDa and the arrows indicate the position of 65, 55 and 40 kDa GLUT1, respectively.

Discussion

In the present paper, we demonstrated that TGF-β1 stimulated proliferation and glucose uptake of growing Swiss 3T3 fibroblasts cultured in calf serum-containing medium, accompanied by an increase in the expression of the GLUT1 mRNA and a 55 kDa GLUT1 protein. A longer exposure of the cells to TGF-β1 induced the synthesis of 65 kDa GLUT1 protein, whose glycosylation was modulated.

Polyclonal antibodies against the hRBC GLUT1 recognized a GLUT1 protein of 55 kDa in Swiss 3T3 cells (Fig. 3), as reported in other cell systems [17,20–23,25]. Exposure of growing 3T3 cells to TGF-β1 for 24–48 h caused a marked increase in the 65 kDa protein recognized by the same antibody in a dose-dependent manner (Figs. 3 and 4). This newly expressed protein was GLUT1, since it was also recognized by the antibody against an oligopeptide corresponding to the C-terminal domain of GLUT1 (Fig. 3C). The fact that the level of a single GLUT1 mRNA at 2.8 kb increased in the 3T3 cells after treatment with TGF-β1 for 24 h (Fig. 2B) also supports the notion that the 65 kDa protein is GLUT1.

This TGF-β1-dependent increase in the molecular weight of the GLUT1 was due to the altered glycosylation of this protein, since tunicamycin and deoxymannojirimycin, which are inhibitors of glycosylation at different steps [33], converted the 55 and 65 kDa GLUT1 proteins into those of 40 or 43 kDa (Fig. 7), which corresponded to the expected deglycosylation form or the high-mannose one of GLUT1 protein [19,20,35], respectively. Furthermore, the in vitro diges-

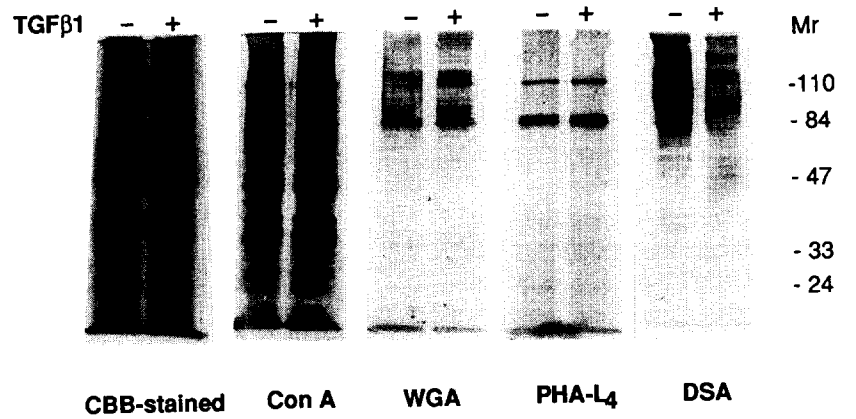

Fig. 8. Lectin blot analysis of 3T3 membrane proteins. Twenty micrograms of the membrane proteins from 3T3 cells treated (+) or untreated (−) with 50 pM TGF-β1 for 24 h were separated by SDS-PAGE. The proteins were then stained with Coomassie brilliant blue (CBB-stained), or blotted onto durapore membranes, followed by incubated with the indicated biotynyl lectins. The lectin binding proteins were then visualized as described in Materials and Methods.

tion of the 3T3 cell membranes by glycopeptidase F also altered the molecular mass of the GLUT1 from 65 and 55 kDa to 40 kDa (Fig. 7). These results clearly indicate that TGF-β1 modulated the processing of the N-linked oligosaccharide structure of the GLUT1 protein in 3T3 cells, and that this modulating activity for the protein glycosylation is specific for TGF-β1 (Fig. 4). In addition, the modulation by TGF-β1 of the glycosylation of GLUT1 seemed to be preferable to actively growing 3T3 cells, since the induction of the 65 kDa GLUT1 was much smaller in the quiescent state (Fig. 5). Although it has been reported that the expression of a variety of glycoproteins such as matrix proteins and cell adhesion receptors is modulated by TGF-β, glycosylation changes have not been observed [5,6,9].

The oligosaccharide structures of many glycoproteins appear to play important roles in cell-cell recognition, protein secretion, protection of proteolytic degradation, and targeting of proteins into cellular organelles [33,34]. It has also been reported that changes in the N-linked oligosaccharides on glycoproteins are associated with malignant transformation, metastasis and differentiation processes of mammalian cells [34]. One of the consistently observed alterations following neoplastic transformation is the expression of larger N-linked oligosaccharides [35–37]. However, most of these investigations occurred with whole glycoproteins in the membranes.

In the present study, we observed for the first time, that TGF-β1 is a powerful modulator of glycosylation of the GLUT1 protein in Swiss 3T3 cells. Although the TGF-β1-induced structural changes in oligosaccharides of GLUT1 are largely unknown, the modulating effect of TGF-β1 on the oligosaccharide structure of glycoprotein might be not a general phenomenon for whole glycoproteins since the binding patterns of several lectins such as Con A, WGA, PHA-L4 and DSA, which have different affinities for carbohydrate moieties, to glycoproteins of 3T3 membranes were not altered significantly by TGF-β1-treatment (Fig. 8). It has been recently reported that hRBC GLUT1 contains a high-mannnose-type oligosaccharide and complex-type oligosaccharides with polylactosamine chains [38]. Based on this information, we are further investigating the altered oligosaccharide structures of the GLUT1 protein in more detail.

A functional relevance of the altered glycosylation in GLUT1 protein to the transport activity remains unknown. The stimulating effect of TGF-β1 on 2-DG uptake in 3T3 cells was still observable when glycosylation inhibitors such as tunicamycin and deoxymannojirimycin were included, although the transport activity was decreased by 30–50% (unpublished data). In accordance with these results, the levels of GLUT1 protein in TGF-β1-treated 3T3 membranes were higher than those in untreated cells even in the presence of the glycosylation inhibitors (Fig. 6). Asano et al. have recently reported that a glycosylated form of GLUT1 protein at Asn-45 had a 2–2.5-fold higher affinity for 2-DG compared to that of unglycosylated GLUT1 in CHO cells [23]. An alteration in glucose transport due to the modified glycosylation of GLUT1 protein in TGF-β1-treated 3T3 cells is further investigated.

Stimulation of DNA synthesis and proliferation by TGF-β1 of growing 3T3 cells was evident after incubation for 48 h (Fig. 1), whereas an increase in 2-DG uptake and the increased levels of the GLUT1 mRNA as well as the protein in the TGF-β1-treated 3T3 cells was observed within 3–6 h of incubation, and these increases were maintained during incubation for up to 48 h (Figs. 2 and 3). These results together with the previous data on quiescent 3T3 cells [13,14,24] suggest that stimulated glucose uptake is a prerequisite for the TGF-β1-induced growth stimulation of 3T3 cells. The stimulation of glucose uptake with the increased

GLUT1 gene expression as well as the altered glycosylation of GLUT1 protein may be partly involved in the transforming activity of TGF-β1 in fibroblastic cells. The increased glucose uptake and GLUT1 mRNA levels are also well characterized in various types of transformed cells [10–12,39,40].

Further experiments are required to evaluate possible roles of the TGF-β1-induced modification of the oligosaccharide structure of GLUT1 protein in regulation of glucose transport activity and cell growth of mamalian cells.

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