Activation of Syntaxin 1C, an Alternative Splice Variant of HPC-1/ Syntaxin 1A, by Phorbol 12-Myristate 13-Acetate (PMA) Suppresses Glucose Transport into Astroglioma Cells via the Glucose Transporter-1 (GLUT-1)*

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Syntaxin 1C is an alternative splice variant lacking the transmembrane domain of HPC-1/syntaxin 1A. We found previously that syntaxin 1C is expressed as a soluble protein in human astroglioma (T98G) cells, and syntaxin 1C expression is enhanced by stimulation with phorbol 12-myristate 13-acetate (PMA). However, the physiological function of syntaxin 1C is not known. In this study, we examined the relationship between syntaxin 1C and glucose transport. First, we discovered that glucose transporter-1 (GLUT-1) was the primary isoform in T98G cells. Second, we demonstrated that glucose uptake in T98G cells was suppressed following an increase in endogenous syntaxin 1C after stimulation with PMA, which did not alter the expression levels of other plasma membrane syntaxins. We further examined glucose uptake and intracellular localization of GLUT-1 in cells that overexpressed exogenous syntaxin 1C; glucose uptake via GLUT-1 was inhibited without affecting sodium-dependent glucose transport. The value of $V_{\text{max}}$ for the dose-dependent uptake of glucose was reduced in syntaxin 1C-expressing cells, whereas there was no change in $K_m$. Immunofluorescence studies revealed a reduction in the amount of GLUT-1 in the plasma membrane in cells that expressed syntaxin 1C. Based on these results, we postulate that syntaxin 1C regulates glucose transport in astroglia cells by changing the intracellular trafficking of GLUT-1. This is the first report to indicate that a syntaxin isoform that lacks a transmembrane domain can regulate the intracellular transport of a plasma membrane protein.

The protein machinery that regulates intracellular transport and vesicle formation, docking, and fusion has been the focus of intense research over the last few years. The SNARE hypothesis (soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor) constitutes a widely accepted model in which dynamic interactions among proteins within the acceptor (t-SNARE: syntaxin and SNAP-25) and donor (v-SNARE: VAMP) compartments control exocytosis (1, 2). Recent studies have revealed that syntaxins function in a wide variety of cells and tissues, including neurons, endocrine glands, amphibian ectodermal cells, epithelial cells, cells of the immune system, platelets, and yeast (3). Consequently, a unified role for the SNARE complex in the docking and fusion of vesicles during intracellular trafficking, as well as in nerve terminals, has been proposed.

To date, 15 members of the mammalian syntaxin family have been identified, all of which localize to specific membrane compartments via a transmembrane domain at the C terminus. In contrast to the localization of syntaxins 5–18 to different intracellular compartments, such as the Golgi and post-Golgi apparatus (4), syntaxins 1–4 are restricted predominantly to the plasma membrane, where they mediate constitutive and regulated vesicle trafficking to the cell surface (4). All syntaxins have a coiled-coil helix domain (called H3 in syntaxin 1A) next to the transmembrane domain at the C terminus. The H3 domain is a highly conserved region that interacts with several different SNAP proteins, including SNAP-25, VAMP, and α-SNAP, and to some extent, nSec-1/Munc-18 (4).

Syntaxin 1C is an alternative splice variant of HPC-1/syntaxin 1A. Syntaxin 1A is involved in the docking of synaptic vesicles at active zones in neurons (5, 6), and is deleted hemizygously in patients with the neurodevelopmental disorder, Williams syndrome (7, 8). In a previous study, we demonstrated that syntaxin 1C is expressed as a soluble protein in astroglia cells (9). While the N-terminal domain of syntaxin 1C is the same as that of syntaxin 1A, the functionality of the H3 and transmembrane domain has been lost, caused by the generation of a novel 34-residue C-terminal domain by the insertion of a 91-bp splicing region. Several other isoforms of syntaxin that lack a transmembrane domain by alternative
Facilitative glucose transporters (GLUTs) are proteins that regulate the entry of glucose into cells and maintain cell metabolism and homeostasis throughout the periphery and brain (10). There are at least six different GLUT genes with differential tissue distributions, subcellular localizations, and kinetics for glucose uptake (14). In the brain, there are two GLUT isoforms, namely GLUT-1 and GLUT-3. GLUT-1 appears during early embryogenesis and is required for cell metabolism and homeostasis in glial cells (13, 15). GLUT-3 is found primarily in neurons (15). GLUT-4 is expressed only in muscle and fat cells, where it resides in an intracellular compartment under basal conditions and is translocated to the cell surface after stimulation with insulin (15). Recently, it became clear that syntaxin 4 and several SNARE-related molecules participate in the translocation of GLUT-4 to the plasma membrane (16, 17). In recent studies, however, it was revealed that glucose transport is regulated through several signal transduction pathways, including those that involve mitogen-activated protein kinase, phosphatiidylinositol 3-kinase, and protein kinase C (PKC) (18–20). We showed previously that astroglial cells express syntaxin 1C but not syntaxin 1A, and that the expression of syntaxin 1C protein is up-regulated via a PKC signaling pathway by stimulating cells with phorbol 12-myristate 13-acetate (PMA) (9).

In the present study, we used a human astroglial cell line, which expresses syntaxin 1C to determine whether syntaxin 1C is involved in glucose transport. We found that the induction of endogenous syntaxin 1C expression by PMA caused a reduction in GLUT-1 in the plasma membrane and suppressed glucose uptake. Expression of exogenous syntaxin 1C in T98G cells had the same effects. These results suggest that the physiological function of syntaxin 1C in astroglial cells is the regulation of intracellular trafficking of GLUT-1.

EXPERIMENTAL PROCEDURES

Reagents—All tissue culture reagents were purchased from Invitrogen, Life Technologies (Carlsbad, CA), with the exception of fetal calf serum (FCS), which was purchased from Sigma. Human insulin was purchased from Roche Applied Science (Basel, Switzerland). Acrylamide/bis-acrylamide was obtained from WAKO Chemical (Osaka, Japan). Penicillin (100 U/ml) and streptomycin (100 mg/ml) for drug stimulation, cells were treated for 3–4 h with 1–10 μM PMA (Sigma), 10 μM 4n-PMA (Sigma), or 10 μM forskolin (RBI).

Northern Blot Analysis—Northern blot analysis was carried out, according to the method of Nagamatsu et al. (22). Total RNA (20 μg) isolated from native and transfected (see below) T98G cells was separated by electrophoresis in 1% formaldehyde-agarose denaturing gels. The EcoRIdigested 600-bp fragments of the GLUT-1 and GLUT-3 cDNAs were labeled with 35P by random priming. The GLUT-1 and GLUT-3 cDNAs were a kind gift from Dr. Shinya Nagamatsu (Kyorin University, Japan). The intensity of the autoradiographic signals was measured directly from digital images (Bas 2000, Fuji, Tokyo, Japan).

Cell Culture and Transfection—Two human astroglia cell lines, T98G and U87MG, were provided by Dr. Hiroki Sawaya (Kyorin University, Japan). Cells were grown on 90-mm in diameter plastic dishes in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml). For drug stimulation, cells were treated for 3–4 h with 1–10 μM PMA (Sigma), 10 μM 4n-PMA (Sigma), or 10 μM forskolin (RBI).

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FIG. 1. Identification of GLUT isoforms in the human glioma cell line, T98G. A, dose-dependent 2-DG uptake in T98G cells. Uptake of 2-DG was measured for 13 min. Values are the mean ± S.E. of three independent experiments. \( R^2 = 0.999 \). The value of \( K_m \) (2.42 ± 0.44 mM) suggested that a low affinity glucose transporter, GLUT-2, did not participate in glucose uptake. Eadie-Hofstee plots from A are shown in the upper panel. B, PCR primers and products for GLUT-1, GLUT-2, GLUT-3, and GLUT-4. C, RT-PCR analysis of GLUT-1 and GLUT-3 mRNA in T98G, NB-1, and 3T3-L1 cells. D, glucose uptake in T98G cells treated with 25 mM glucose and 4.9 mg/ml insulin compared to control.
Syntaxin 1C Regulates Glucose Transport via GLUT-1

**Experimental Procedures.** The values are the mean ± S.E. for three independent experiments. Glucose uptake in response to 10 μM PMA, 10 μM 4α-PMA, and 10 μM forskolin was, 8.84 ± 0.62 (black bar), 10.49 ± 0.31 (gray bar), and 11.13 ± 1.16 (striped bar) nmol/10 min/mg, respectively, versus the control (Cont., 10.87 ± 0.24; 10.1% Me2SO; white bar). GLUT uptake was significantly reduced by treatment with PMA. ***, p < 0.01 compared with control. C, time course of syntaxin 1C expression in T98G cells treated with 10 μM PMA. Cells were treated with 10 μM PMA for 12, 24, or 48 h. Expression of syntaxin 1C was quantified as for A. The values are the mean ± S.E. for three independent experiments. Expression of syntaxin 1C increased 4.53 ± 0.51-, 7.34 ± 1.31-, and 8.06 ± 1.52-fold after treatment with PMA for 12, 24, and 48 h, respectively. D, time course of 2-DG uptake in T98G cells treated with 10 μM PMA. Cells were treated with 10 μM PMA for 12, 24, or 48 h. Glucose uptake was quantified as for B. The values are the mean ± S.E. for three independent experiments. Glucose uptake decreased 0.94 ± 0.02-, 0.86 ± 0.05-, and 0.79 ± 0.05-fold after treatment with PMA for 12, 24, and 48 h, respectively.

**Fig. 2.** Relationship between 2-DG uptake via GLUT-1 and expression of syntaxin 1C in T98G astrogliaoma cells treated with PMA. A, expression of syntaxin 1C in PMA-treated T98G cells. The amount of syntaxin 1C expressed in T98G cells that were treated for 24 h with 10 μM PMA, 10 μM forskolin, or 10 μM 4α-PMA was quantified using 12% SDS-PAGE. Trichloroacetic acid lysates (25 μg) were immunoblotted with monoclonal antibody 14D8 (35 kDa). Immunoblotted membranes were reprobed with anti-tubulin IgG (55 kDa). Densitometric analysis was performed using NIH Image. Expression of syntaxin 1C in response to treatment with 10 μM PMA increased 6.82 ± 0.52-fold, compared with the control (Cont., 0.1% Me2SO). B, 2-DG uptake in PMA-treated T98G cells. Cells were treated for 24 h with 10 μM PMA, 10 μM forskolin, or 10 μM 4α-PMA. Glucose uptake via GLUTs was measured as described under “Experimental Proce-dures.” The values are the mean ± S.E. for three independent experiments. Glucose uptake in response to 10 μM PMA, 10 μM 4α-PMA, and 10 μM forskolin was, 8.84 ± 0.62 (black bar), 10.49 ± 0.31 (gray bar), and 11.13 ± 1.16 (striped bar) nmol/10 min/mg, respectively, versus the control (Cont., 10.87 ± 0.24; 10.1% Me2SO; white bar). GLUT uptake was significantly reduced by treatment with PMA. ***, p < 0.01 compared with control. C, time course of syntaxin 1C expression in T98G cells treated with 10 μM PMA. Cells were treated with 10 μM PMA for 12, 24, or 48 h. Expression of syntaxin 1C was quantified as for A. The values are the mean ± S.E. for three independent experiments. Expression of syntaxin 1C increased 4.53 ± 0.51-, 7.34 ± 1.31-, and 8.06 ± 1.52-fold after treatment with PMA for 12, 24, and 48 h, respectively. D, time course of 2-DG uptake in T98G cells treated with 10 μM PMA. Cells were treated with 10 μM PMA for 12, 24, or 48 h. Glucose uptake was quantified as for B. The values are the mean ± S.E. for three independent experiments. Glucose uptake decreased 0.94 ± 0.02-, 0.86 ± 0.05-, and 0.79 ± 0.05-fold after treatment with PMA for 12, 24, and 48 h, respectively.

Inset. B, PCR analysis of GLUT isoforms in T98G cells. Schematic representation of the GLUT isoforms is shown in the upper panel. Open boxes indicate the coding region. The bars indicate the 3'-untranslated regions. Arrowheads indicate the position of the primer pairs (1 + 2). PCR was carried out using the primers indicated in the upper panel. First strand cDNA templates from T98G astrocytoma cells (human glial cell line), NB-1 cells (human neurobrastoma cell line), and 3T3L1 cells (mouse adipocytes cell line) were analyzed on a 2.5% agarose gel. Semiquantitative RT-PCR was carried out for 30 cycles using 5 ng of each cDNA template. Relative expression of GLUT-3 versus GLUT-1 was 0.355 ± 0.076. GLUT-3 and GLUT-3 mRNA in T98G cells. Northern blot analysis of GLUT-1 and GLUT-3 mRNA expression revealed a 2.8-kb fragment that corresponded to GLUT-1 mRNA and a small amount of a GLUT-3 transcript. The amount of GLUT-1 transcript was normalized to the intensity of the 28 S tRNA band. Densitometric analysis revealed that the amount of GLUT-1 mRNA was ~9-fold (949 ± 79%; p < 0.0001) greater than that of GLUT-3. D, functional characterization of GLUT isoforms in T98G cells. Measurement of 2-DG uptake in T98G cells, cultured with low and high concentrations of insulin, is shown in the white and black bar on the right, respectively. Glucose uptake before 2-DG uptake measurement was 12.27 ± 0.75 and 12.77 ± 0.55 nmol/10 min/mg for stimulation with 0 (white bar) and 4 μg/ml (black bar) insulin, respectively. Insulin did not affect 2-DG uptake in T98G cells, which suggested that there is no insulin-responsive GLUT-4 in T98G cells. The data in A-D support the conclusion that GLUT-1 is the main isoform in T98G cells.
The cell cycle analysis was carried out as reported previously (25). Growth of T98G cells that had been plated at a density of 2 × 10⁵ cells per 90-mm in diameter culture dish was arrested by removing serum for 24 h. The cells were restimulated for 40 h with medium containing 10% serum. Thereafter, the cells (1 × 10⁶ cells/ml) treated with 0.5 mg/ml tritiated [3H]-glucose (2-DG) were immunoblotted with anti-GLUT-1 polyclonal antibody. The level of GLUT-1 protein expression (47 kDa) was measured using the DC protein assay from Bio-Rad. The amount of GLUT-1 and GLUT-3 mRNA was quantified by Northern blot. The amount of GLUT-1 and GLUT-3 mRNA was calculated using NIH Image. There was no significant change in the amount of GLUT-1 and GLUT-3 mRNA in T98G cells that were treated with PMA. GLUT-1 protein expression. Cells were treated for 24 h with 10 μM PMA, 10 μM forskolin, or 10 μM 4α-PMA. Membrane fractions (35 μg) were immunoblotted with anti-GLUT-1 polyclonal antibody. The level of GLUT-1 protein expression (47 kDa) was not affected by PMA.

Measurement of Glucose Uptake

Glucose transport was assayed by measuring the uptake of 2-deoxy-[1,2-3H(N)]glucose (2-DG), essentially as described previously (26). Briefly, confluent cultures of T98G cells were incubated with either 5.5 or 25.0 mM d-glucose for 7 days. The culture medium was changed daily to maintain a relatively constant concentration of glucose.

Measurement of 2-DG Uptake via GLUTs and SGLTs in T98G Cells—To study basal glucose uptake via sodium-dependent glucose transporters (SGLTs), cells were treated with Na+-free HBSS buffer containing 0.03 g/100 ml bovine serum albumin, 138 mM N-methyl-D-glucamine (NMDG), 5.6 mM KC1, 0.34 mM NaHPO₄, 0.44 mM KH₂PO₄, 1.27 mM CaCl₂, and 20 mM HEPES (pH 7.4), incubated on 12-well multiplates at 37°C, for 30 min. Glucose uptake was initiated by adding 0.05 μCi of 2-deoxy-[1,2-3H(N)]glucose (2-[3H]DG, PerkinElmer Life Sciences) to 0.5 ml of HBSS buffer, in the presence of 0.1 mM 2-deoxy-D-glucose, in 35-mm in diameter wells. After 15 min at room temperature, uptake was terminated by rapid washing with 1 ml of ice-cold PBS. The uptake of 2-DG was linear between 0 and 20 min of incubation (data not shown). For the kinetic analysis, we used 0.1–100.0 mM 2-DG (0.0064–6.4 μM 2-[3H]DG). The cells were solubilized in 1% SDS, and the amount of radioactivity was measured. Protein content was measured using the DC protein assay from Bio-Rad. The difference in the amount of uptake in the presence and absence of 0.5 mM cytochalasin B (a transport inhibitor) was calculated; this represented glucose transporter-dependent activity. In each experiment, glucose uptake was assayed in triplicate.

We studied the kinetics of glucose uptake using different concentrations of d-glucose, as described previously (26). Briefly, confluent cultures of T98G cells were incubated with either 5.5 or 25.0 mM d-glucose for 7 days. The culture medium was changed daily to maintain a relatively constant concentration of glucose.

RESULTS

Measurement of 2-DG Uptake via GLUTs and SGLTs in T98G Cells—To examine the relationship between syntaxin 1C expression and glucose transport, we first determined whether there was glucose uptake via GLUT in T98G cells. The amount of 2-DG uptake via GLUTs and SGLTs was measured in the
presence of cytocharasin B (an antagonist of GLUTs) and in Na\textsuperscript+}-free medium. GLUT activity accounted for \( \sim 80\% \) of total 2-DG uptake in T98G cells. By contrast, SGLT activity accounted for less than 20\% of total 2-DG uptake (data not shown). Uptake of 2-DG was linear for up to 20 min of incubation (data not shown). These observations indicate that GLUTs are expressed in T98G cells.

Identification of GLUT Isoform in T98G Cells—There are several reports that the GLUT isoforms GLUT-1 and GLUT-3 are the main components of several types of glioma (22, 27, 28). However, whether GLUTs are expressed in T98G cells has not been determined. To determine which GLUT isoform(s) is expressed in T98G cells, we investigated the kinetics of 2-DG uptake. As shown in Fig. 1A, the \( K_m \) of 2-DG uptake in T98G cells was 2.42 \( \pm \) 0.12 mm. GLUT-1, GLUT-3, and GLUT-4 are high affinity glucose transporters, whereas GLUT-2 is a low affinity transporter.

Fig. 4. Localization of GLUT-1 and expression of syntaxin 1C in T98G cells treated with PMA, 4\alpha\textsubscript{PMA}, or forskolin. Cells were treated for 24 h with 10 \( \mu \)M PMA (B and F), 10 \( \mu \)M 4\alpha\textsubscript{PMA} (C and G), or 10 \( \mu \)M forskolin (D and H). Cells were fixed and permeabilized with acetone/methanol (1:1) and double-stained with anti-GLUT-1 polyclonal antibody and monoclonal antibody 14D8, as described under “Experimental Procedures.” The figures are confocal scanning laser microscopic images of GLUT-1 (A–D) and 14D8 (E–H) immunofluorescence. Treatment of cells with PMA increased syn1C and decreased GLUT-1 expression in the plasma membrane.
affinity transporter (29). Because the value of $K_m$ in the present study is far smaller than that of GLUT-2 ($K_m$: 20–40 mM), it is likely that the GLUT isofrom functioning in T98G cells is not GLUT-2, but rather a high affinity transporter, e.g. GLUT-1, GLUT-3, or GLUT-4.

As shown in Fig. 1B, semiquantitative RT-PCR revealed that GLUT-1 and GLUT-3 were expressed in T98G cells; GLUT-2 and GLUT-4 expression was undetectable except under saturated PCR conditions (data not shown). To examine the expression levels of endogenous GLUT-1 and GLUT-3, we studied expression of the mRNA of these GLUT isoforms in T98G cells. GLUT-1 mRNA was quantified by normalizing the band intensity to that of the 28 S tRNA band. The amounts of 2.8-kb GLUT-1 transcript in 20 μg of total RNA were similar in T98G cells that were transfected with either the expression vector alone (T98G-Mock), syntaxin 1A (T98G-Syn1A), syntaxin 1C (T98G-Syn1C), or syntaxin 4 (T98G-Syn4). The membrane fraction (35 μg) of each of the transfected T98G cell lines was immunoblotted with anti-GLUT-1 polyclonal antibody. Expression of GLUT-1 protein (47 kDa) was not affected by the overexpression of syntaxin 1A, syntaxin 1C, or syntaxin 4. D, glucose uptake via GLUTs (black bars) or SGLTs (gray bars) was measured, as described under "Experimental Procedures." The values are the mean ± S.E. for three independent experiments. Glucose uptake via GLUTs in T98G cells that were transfected with either the expression vector alone (T98G-Mock), syntaxin 1A (T98G-Syn1A), syntaxin 1C (T98G-Syn1C), or syntaxin 4 (T98G-Syn4) was 11.39 ± 0.47, 7.19 ± 0.50, 6.80 ± 0.41, and 10.85 ± 0.96 nmol/min/mg, respectively. Glucose uptake via SGLT in T98G cells that were transfected with either the expression vector alone (T98G-Mock), syntaxin 1A (T98G-Syn1A), syntaxin 1C (T98G-Syn1C), or syntaxin 4 (T98G-Syn4) was 1.66 ± 0.51, 1.57 ± 0.42, 1.57 ± 0.56, and 1.37 ± 0.43 nmol/min/mg, respectively. ***, $p < 0.001$, compared with control (T98G-Mock).

GLUT-1 expression can be activated by PMA (9). In the present study, we investigated whether a change in the level of syntaxin 1C expression might affect glucose transport. In Fig. 2, A and B shows the change in syntaxin 1C expression and 2-DG uptake in T98G cells that were treated with either PMA, forskolin, or 4α-PMA (a nonfunctional analog of PMA). Uptake of 2-DG in PMA-treated cells was reduced by ~85%, compared with control cells (Fig. 2B), whereas 2-DG uptake was not affected by either forskolin or 4α-PMA (Fig. 2B). None of the aforementioned treatments had any effect on the uptake of 2-DG via SGLTs (data not shown). In addition, an analysis of the time course of glucose uptake (Fig. 2, C and D) revealed that 2-DG uptake in T98G cells was reduced as the PMA-induced level of syntaxin 1C expression increased. Because syntaxin 1A mRNA is not found in astrogloma cells, irrespective of whether cells are treated with PMA (9), the observed change in glucose uptake in PMA-treated T98G cells was not caused by the actions of syntaxin 1A. To determine whether the

Fig. 5. GLUT-1 mRNA and protein expression and glucose uptake via GLUT-1 in T98G cells transfected with syntaxin 1A, syntaxin 1C, or syntaxin 4. A, expression of HA-tagged syntaxins. Trichloroacetic acid lysates were immunoblotted with an anti-HA monoclonal antibody (3F10). The level of expression of the transfected syntaxins was similar for each type of syntaxin. T98G-Syn1A, T98G-Syn1C, and T98G-Syn4 indicate T98G cells that were transfected with syntaxin 1A, syntaxin 1C, and syntaxin 4, respectively. B, expression of GLUT-1 mRNA. A representative autoradiograph of a Northern blot is shown. The positions of 28 and 18 S rRNA are indicated on the right. The amount of GLUT-1 mRNA was quantified by normalizing the band intensity to that of the 28 S RNA band. The amounts of 2.8-kb GLUT-1 transcript in 20 μg of total RNA were similar in T98G cells that were transfected with either the expression vector alone (T98G-Mock), syntaxin 1A (T98G-Syn1A), syntaxin 1C (T98G-Syn1C), or syntaxin 4 (T98G-Syn4). C, immunoblot for GLUT-1. The membrane fraction (35 μg) of each of the transfected T98G cell lines was immunoblotted with anti-GLUT-1 polyclonal antibody. Expression of GLUT-1 protein (47 kDa) was not affected by the overexpression of syntaxin 1A, syntaxin 1C, or syntaxin 4.

Fig. 2, C and D shows the change in GLUT-1 protein expression and glucose uptake via GLUT-1 in T98G cells that were transfected with syntaxin 1A, syntaxin 1C, or syntaxin 4. GLUT-1 protein expression was more abundant than that of GLUT-3. We also confirmed expression of GLUT-1 protein by immunoblotting (see Fig. 3C) and localization in plasma membrane in T98G cells by cell surface biotinylation assay (data not shown).

It has been reported that glucose uptake via GLUT-1 in cells cultured in low glucose medium is higher than in the presence of high concentrations of glucose (26). We investigated glucose uptake in T98G cells that were cultured with different concentrations of glucose. As expected, 2-DG uptake was ~1.5 times greater in low-glucose medium (5.5 mM glucose), compared with high glucose medium (25 mM glucose) (Fig. 1D). Another property of GLUT-4 is that it translocates to the plasma membrane in cells that have been stimulated with insulin, which results in an increase in glucose uptake (16). Consequently, we tested whether the amount of 2-DG uptake in T98G cells would increase after stimulation with insulin; this was not the case (Fig. 1D). The results shown in Fig. 1, B and D suggest that there is no functional GLUT-4 in T98G cells. The aforementioned results demonstrate that the major isofrom of GLUT in T98G cells is GLUT-1.

Activation of Endogenous Syntaxin 1C by PMA Suppresses Translocation of GLUT-1 to the Plasma Membrane in T98G Cells—Our previous study revealed that T98G astrogloma cells express syntaxin 1C, but not syntaxin 1A, and that syntaxin 1C expression can be activated by PMA (9). In the present study, we investigated whether a change in the level of syntaxin 1C expression might affect glucose transport. In Fig. 2, A and B shows the change in syntaxin 1C expression and 2-DG uptake in T98G cells that were treated with either PMA, forskolin, or 4α-PMA (a nonfunctional analog of PMA). Uptake of 2-DG in PMA-treated cells was reduced by ~85%, compared with control cells (Fig. 2B), whereas 2-DG uptake was not affected by either forskolin or 4α-PMA (Fig. 2B). None of the aforementioned treatments had any effect on the uptake of 2-DG via SGLTs (data not shown). In addition, an analysis of the time course of glucose uptake (Fig. 2, C and D) revealed that 2-DG uptake in T98G cells was reduced as the PMA-induced level of syntaxin 1C expression increased. Because syntaxin 1A mRNA is not found in astrogloma cells, irrespective of whether cells are treated with PMA (9), the observed change in glucose uptake in PMA-treated T98G cells was not caused by the actions of syntaxin 1A. To determine whether the
reduction in glucose uptake was caused by the presence of syntaxin 1C, we examined the expression of syntaxin 2, syntaxin 3, and syntaxin 4 in the plasma membrane. In contrast to the expression of syntaxin 1C, which is increased in a dose-dependent manner by PMA treatment (7.37 ± 1.34-fold increase in response to 10 μM PMA), the expression of syntaxin 2, syntaxin 3, and syntaxin 4 was not affected by PMA (Fig. 3A).

Furthermore, PMA had no effect on the expression of GLUT-1 and GLUT-3 mRNA and protein in T98G cells (Fig. 3B and C). These results suggest that the treatment of T98G cells with PMA did not alter the level of expression of GLUT-1, GLUT-3, or syntaxins other than syntaxin 1C.
We also analyzed the effect of PMA using immunofluorescence and found that in cells in which the expression of endogenous syntaxin 1C had been enhanced by PMA, GLUT-1 expression in the plasma membrane decreased, whereas expression in the intracellular fraction increased (Fig. 4, B and F). By contrast, neither forskolin (Fig. 4, D and H) nor 4α-PMA (Fig. 4, C and G) had any effect on GLUT-1 expression.

Glucose Uptake via GLUT-1 in T98G Cells Transfected with Syntaxin 1A, Syntaxin 1C, or Syntaxin 4—To determine whether syntaxin 1C expression affects glucose transport in astrogliaoma cells, we introduced exogenous syntaxin 1A, syntaxin 1C, or syntaxin 4 tagged with HA into at least three lines of T98G cells for each syntaxin. Western blot analysis revealed that the level of expression of each type of transfected syntaxin was similar (Fig. 5A). There was also no difference among the cell lines with respect to the level of GLUT-1 mRNA and protein expression (Fig. 5, B and C). These observations indicate that the overexpression of exogenous syntaxin did not affect GLUT-1 expression in T98G cells.

Next, we studied 2-DG uptake via GLUTs in each type of syntaxin-transfected cell line. The results indicated that 2-DG uptake was reduced to ~60% in the syntaxin 1C-transfected cell line (T98G-Syn1C; 6.80 ± 0.41 nmol/13 min/mg), compared with cells that were transfected with the expression vector alone (Fig. 5D, T98G-Mock; 11.39 ± 0.47). Similar results were obtained for syntaxin 1A-transfected cell lines (Fig. 5D, T98G-Syn1A; 7.19 ± 0.50). By contrast, there was no change in 2-DG uptake in syntaxin 4-transfected cells (Fig. 5D, T98G-Syn4; 10.85 ± 0.96). Uptake of 2-DG via SGLTs was not altered by overexpression of exogenous syntaxin (Fig. 5D). Similar observations were obtained for cell lines in which other syntaxins were expressed, and in cells that were transfected with exogenous syntaxin without an HA tag (data not shown), suggesting that the suppression of 2-DG uptake was not caused by either clonal variation or the presence of the HA tag motif. Finally, we obtained similar results with another astrogliaoma cell line, namely U87MG (data not shown).

Dose-dependent Glucose Uptake in Untransfected T98G Cells and T98G Cells Transfected with Syntaxin 1C—To determine whether glucose uptake in syntaxin 1C-transfected T98G cells was dose-dependent, we studied kinetic analysis of 2-DG uptake (Fig. 6). The value of $V_{\text{max}}$ (6.041 ± 0.72 mm) was reduced in the HA-tagged syntaxin 1C-expressing cell line (T98G-Syn1C), whereas the value of $K_m$ (2.708 ± 0.29 mm) was unchanged relative to the untransfected cells (T98G (Native cell): $K_m = 2.009 ± 0.46$ mm, $V_{\text{max}} = 10.002 ± 0.86$ mm) and cells that were transfected with the expression vector alone (T98G-Mock: $K_m = 2.657 ± 0.31$ mm, $V_{\text{max}} = 10.001 ± 0.90$ mm) (Fig. 6). These results suggest that the decrease in glucose uptake that is associated with the expression of syntaxin 1C might be caused by a decrease in amount of GLUT-1 in the plasma membrane, whereas the rate of glucose transport by individual GLUT-1 remains the same.

Overexpression of Syntaxin Did Not Affect the Cell Growth or Mitogenesis—It has been reported that glucose transport is associated closely with mitogenic properties of glioma cells, such as cell growth (22). Therefore, we investigated whether syntaxin expression might affect cell proliferation. We measured the growth rate of cells during the logarithmic growth phase (48–96 h). As shown in Table I, the growth rate of cells transfected with syntaxin was not significantly different to that of cells that were transfected with the expression vector alone. To examine cells in each phase of the cell cycle, cell growth was arrested by withdrawing serum for 24 h to synchronize the cell cycle; cells were then restimulated for 40 h with medium containing 10% serum. As shown in Table II, there was no difference after restimulation between the cell cycles of cells that had been transfected with syntaxin, compared with cells that were transfected with the expression vector alone. These
results indicate that the overexpression of syntaxin did not affect the mitogenic properties of T98G cells. Thus, the suppression of glucose uptake by syntaxin 1C was not caused by an alteration in mitogenesis.

**Intracellular Localization of GLUT-1 in T98G Cells Transfected with Syntaxin 1C**—We examined the intracellular localization of endogenous GLUT-1. Immunofluorescence images of the various syntaxin-transfected cell lines are presented in Fig. 7. There was little GLUT-1 expression in the plasma membrane of T98G cells that had been transfected with syntaxin 1C (T98G-Syn1C), and most GLUT-1 appeared to be localized to the intracellular compartment of these cells (Fig. 7). A similar result was obtained in the case of syntaxin 1A-transfected cells (T98G-Syn1A) (Fig. 7). By contrast, in cells that had been transfected with either syntaxin 4 (T98G-Syn4) or the expression vector alone (T98G-Mock), almost all GLUT-1 was present in the plasma membrane, and there was little or no GLUT-1 within the cell (Fig. 7). The same result was obtained for cells that were transfected with HA-tagged syntaxins (data not shown). These observations are consistent with the results of the analysis of glucose and 2-DG uptake (see Figs. 5 and 6, respectively).

We also examined immunostaining in T98G cells that had been transiently transfected with HA-tagged syntaxins (Fig. 8). Only cells that expressed HA-tagged syntaxin 1A (Fig. 8, A, D, and G) and HA-tagged syntaxin 1C (Fig. 8, B, E, and H) exhibited a reduction in GLUT-1 expression in the plasma membrane and an increase in GLUT-1 within intracellular compartments. However, there was no change in localization of GLUT-1 in cells that had been transfected with HA-tagged syntaxin 4 (T98G-Syn4) or the expression vector alone (T98G-Mock).
syntaxin 4 (Fig. 8, C, F, and I). Syntaxin 1A and syntaxin 4, both of which are membrane-bound, were not localized to the plasma membrane in astrogliaoma cells. This might be caused by the absence of the regulatory molecules (e.g. nSec-1/Munc18) that was necessary for localization of plasma membrane syntaxin (30, 31). These findings suggest that soluble syntaxin 1C might prevent the localization of GLUT-1 to the plasma membrane, which would ultimately reduce glucose transport.

**DISCUSSION**

Recent studies have revealed that several signal transduction mechanisms participate in the glucose metabolism, the regulation of GLUT expression and localization, and glucose transport (18–20). We demonstrated previously that syntaxin 1C expression was up-regulated by PMA via PKC signaling in astrogliaoma cells (9). In this report, we demonstrated that glucose transport and the amount of GLUT-1 in the plasma membrane were suppressed in astrogliaoma cells by stimulation with PMA. It is likely that the suppression of glucose transport by PMA was caused by a decrease in the number of GLUT-1 molecules that is present in the plasma membrane, and that this was caused by an increase in the syntaxin 1C expression because: 1) PMA increased the endogenous expression of syntaxin 1C without changing the total expression of syntaxin 2–4, or GLUT-1 in astrogliaoma cells and 2) overexpression of exogenous syntaxin 1C caused the similar phenomenon to that by PMA. These suggest that the PKC signaling, which affects the syntaxin 1C expression, may (at least partly) regulate the glucose metabolism through changing the GLUT-1 expression in the plasma membrane.

However, it was not clear whether the reduced expression of GLUT-1 in the plasma membrane was caused by a suppression of translocation of GLUT-1 to the plasma membrane or an increase in the amount of GLUT-1 that was internalized from the plasma membrane, because most GLUT-1 in the plasma membrane is recycled constitutively between the plasma membrane and intracellular vesicles. Recent studies of GLUT translocation suggest two possibilities. First, syntaxin 1C may play a role in membrane fusion. Although the intracellular transport pathway of GLUT-1 is unknown, studies of GLUT-4 vesicular translocation suggest that the machinery that is used for the intracellular trafficking of GLUT is similar to that in neuroendocrine systems (16). In this model, GLUT-4-containing vesicles are primed to the plasma membrane where fusion is driven by the formation of a stable heterotrimeric complex of syntaxin 4, SNAP23, and VAMP-2. In addition, it has been proposed that the formation of the SNARE complex is regulated by several suppressive molecules, including Munc18c, synip, pantophysin, and Rab4 (17). Syntaxin 1C is distinct from syntaxin 1A in that the C-terminal of syntaxin 1C is converted by alternative splicing to a novel proline-rich region of 35 residues; this results in an absence of both the transmembrane domain and the latter half of the H3 domain (residues 191–267 in syntaxin 1A). Therefore, the C-terminal of syntaxin 1C does not have the capacity to bind most SNAREs or accessory molecules, except nSec-1/Munc18 and SNAP-25. Therefore, unlike syntaxin 4, syntaxin 1C may not be able to form a SNARE core complex. We showed previously that syntaxin 1C is found only in the soluble fraction of astrogliaoma cells (9), which indicates that there is no binding with membrane-bound SNAREs. However, since syntaxin 1C is able to bind Munc18b in vitro (7), it is possible that syntaxin 1C may be in competition with the factors including Munc18b that are able to bind its N-terminal, thereby changing the constitutive intracellular transport of GLUT-1 vesicles to the plasma membrane. Second, syntaxin 1C may act on the cytoskeleton. It was demonstrated recently that components of the cytoskeleton, such as microtubules and actin filaments, are necessary for the translocation of GLUT-1 and GLUT-4 to the plasma membrane (32–34). From this viewpoint, it is interesting that syntaxin 1C possesses a tubulin binding motif in the N-terminal region. An analysis of microtubule reassembly showed that a peptide for the tubulin-binding motif that is found in both syntaxin 1A and syntaxin 1C can directly bind tubulin subunits, and the N-terminal peptide involving this motif could decrease tubulin polymerization in vitro (35, 36), suggesting that syntaxin 1C might affect the local structure of microtubules. The aforementioned observations suggest that the N-terminal region of syntaxin 1C might play an important role in regulating the local structure of the cytoskeleton in astrogliaoma cells. This might explain why the overexpression of syntaxin 1A (which binds the plasma membrane) produced similar effects on glucose transport as did the overexpression of syntaxin 1C.

In the CNS, energy metabolism via GLUT-1 plays a central role in the function of astroglia, which modulates the distribution and metabolism of glucose in the brain (13). The regulation of glucose transport in the CNS is particularly important in the hippocampus and frontal cortex, because these regions are integration centers that are crucial to learning, memory, and personality traits. Several studies have suggested that disruption of glucose metabolism in the CNS is associated with neuronal dysfunction. For example, neuronal activity (particularly cognitive function) may be adversely affected in metabolic disorders, such as diabetes mellitus, in which glucose delivery or utilization in the CNS is disrupted (37–39). Impaired cognitive function can be ameliorated to an extent by administering glucose and insulin. We reported previously that in patients with Williams syndrome, which is characterized by cognitive malfunction that produces hyperactivity, poor attention, relatively intact linguistic function, and visual spatial deficits, the syntaxin 1C gene is located within a region that is deleted hemizygously (8, 40). An examination of glucose metabolism in the CNS of patients with Williams syndrome would be a potentially fruitful avenue of investigation.

In conclusion, we have shown in the present study that syntaxin 1C, a nonmembrane-bound syntaxin, can affect the intracellular transport of a plasma membrane protein. Further studies will enable us to better understand the mechanism of uptake and membrane transport of glucose.

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Activation of Syntaxin 1C, an Alternative Splice Variant of HPC-1/Syntaxin 1A, by Phorbol 12-Myristate 13-Acetate (PMA) Suppresses Glucose Transport into Astrogloma Cells via the Glucose Transporter-1 (GLUT-1)

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