

c-Fos Activates Glucosylceramide Synthase and Glycolipid Synthesis in PC12 Cells*

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It has been demonstrated that c-Fos has, in addition to its well recognized AP-1 transcription factor activity, the capacity to associate to the endoplasmic reticulum and activate key enzymes involved in the synthesis of phospholipids required for membrane biogenesis during cell growth and neurite formation. Because membrane genesis requires the coordinated supply of all its integral membrane components, the question emerges as to whether c-Fos also activates the synthesis of glycolipids, another ubiquitous membrane component. We show that c-Fos activates the metabolic labeling of glycolipids in differentiating PC12 cells. Specifically, c-Fos activates the enzyme glucosylceramide synthase (GlcCerS), the product of which, GlcCer, is the first glycosylated intermediate in the pathway of synthesis of glycolipids. By contrast, the activities of GlcCer galactosyltransferase 1 and lactosylceramide sialyltransferase 1 are essentially unaffected by c-Fos. Co-immunoprecipitation experiments in cells co-transfected with c-Fos and a V5-tagged version of GlcCerS evidenced that both proteins participate in a physical association. c-Fos expression is tightly regulated by specific environmental cues. This strict regulation assures that lipid metabolism activation will occur as a response to cell requirements thus pointing to c-Fos as an important regulator of key membrane metabolisms in membrane biogenesis-demanding processes.

Membrane biogenesis is a complex process that couples nuclear responses to growing environmental cues with appropriate morphological and functional changes of the cell. The proteins and lipids that are required for cell membrane expansion, *i.e.* during cell proliferation, neuritogenesis, tumorigenesis, etc. are provided by a complex endomembrane system

whose major constituents are the endoplasmic reticulum (ER)⁵ and the Golgi complex. Phospholipids, together with cholesterol and integral membrane proteins, are synthesized in the ER and incorporated into preexisting membrane. Nascent membranes bud at ER exit sites and move by vesicular transport toward the plasma membrane passing through the Golgi complex where a series of post-translational modifications on cargo and membrane-bound proteins occur. The lipid composition of membranes is also adjusted in the Golgi complex by the addition of glycolipids and sphingomyelin. Finally, at the most trans region of the Golgi, vesicles are targeted to their final destination: the plasma membrane, endosomes, lysosomes, among others.

Although the molecular and cellular basis of intracellular vesicle transport has been described in detail (reviewed in Ref. 1), less is known about the molecular mechanisms that enable the endomembrane system to adapt to fluctuations in the cell's demands of the membrane according to its diverse functional states. It can be anticipated that, in cells that are actively involved in proliferation or in plasma membrane extension processes that demand massive membrane biogenesis, the organellar homeostasis must be different to that of cells that are neither dividing nor actively growing. However, the nature of the regulatory events underlying such processes are yet unknown.

The proto-oncogene *c-fos* is known to be rapidly and transiently induced in many cell types as part of the nuclear response to a plethora of stimuli such as growth factors, sensorial stimulation, and neurotransmitter release (2–6). In the last years it has been demonstrated that its protein product c-Fos has, in addition to its transcription factor activity, the capacity to act as a cytoplasmic activator of the biosynthesis of phospholipids (7–10). In PC12 cells induced to undergo differentiation by feeding of cells with nerve growth factor (NGF) (11), *c-fos* transcription is rapidly

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⁵ The abbreviations used are: ER, endoplasmic reticulum; NGF, nerve growth factor; ASO, c-Fos mRNA antisense oligonucleotide; SO, corresponding sense oligonucleotide; GlcCerS, ceramide glucosyltransferase; GalT1, glucosylceramide galactosyltransferase; SialT1, lactosylceramide sialyltransferase; C, chloroform; M, methanol; C₆-NBD-Cer, N-6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-4-D-erythro-sphingosine; BD, basic domain (amino acids 139–159) of c-Fos; NA, deletion mutant of c-Fos containing amino acids 1–139; NB, deletion mutant of c-Fos containing amino acids 1–159; LZC, c-Fos deletion mutant of containing amino acids 165–380; LacCer, lactosylceramide; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HA, hemagglutinin.

induced (12, 13), c-Fos associates to the ER and activates the synthesis of phospholipids necessary for membrane biogenesis associated to cell growth and neurite formation (10). Only ER-associated c-Fos is capable of activating phospholipid synthesis (14). c-Fos/ER association/dissociation and consequently its capacity to activate phospholipid synthesis is regulated by the phosphorylation state of c-Fos tyrosine residues: quiescent cells contain small amounts of c-Fos, which is tyrosine-phosphorylated and dissociated from the ER membranes. Upon induction of cells to re-enter growth, concomitant with the induction of c-Fos expression, pre-existing c-Fos is dephosphorylated, it associates to the ER, and it activates phospholipid synthesis (14).

Because membrane biogenesis requires the coordinated supply of its various integral membrane components, in this work we address the emerging question of whether c-Fos also activates the synthesis of glycolipids, another ubiquitous membrane component. It is shown that c-Fos activates the metabolic labeling of glycolipids in differentiating PC12 cells. Enzyme determinations show that c-Fos activates glucosylceramide synthase (GlcCerS), indicating that the c-Fos effect is due to stimulation of formation of GlcCer, the first glycosylated intermediate in the pathway of glycolipid synthesis. This, in its turn, results in the subsequent increase in glycolipid metabolic labeling in these differentiating cells. Results of co-immunoprecipitation experiments are compatible with GlcCerS and c-Fos participating of a physical association.

EXPERIMENTAL PROCEDURES

Cell Cultures—PC12 cells were grown at 37 °C in 5% CO₂ in DMEM (Sigma-Aldrich) supplemented with 0.04 mM glutamine and 5% fetal bovine serum (Invitrogen) plus 5% horse serum (Invitrogen). For establishing quiescence, cells grown for 72 h in DMEM supplemented with 5% horse serum plus 5% fetal bovine serum had their medium changed to DMEM supplemented with 1% horse serum for an additional 36 h. After this time in culture, cells had depleted the medium of serum growth factors and were growth-arrested (15). To induce cells to differentiate, fresh medium containing 100 ng/ml NGF (Invitrogen) replaced medium in each well. Control cultures received the same volume of fresh medium without NGF.

In experiments in which cellular effectors such as NGF, oligonucleotides (5'-G₅A ACA TCA TGG TCG ₅T-3' c-fos mRNA antisense oligonucleotide (ASO), or the corresponding sense oligonucleotide (SO), Biosynthesis, Lewisville, TX) were used, these were added to quiescent cell cultures in 5 μl of medium as indicated for each experiment. Control cells received 5 μl of medium alone. Medium was replaced with fresh medium containing the corresponding cellular effectors as indicated for each experiment at 48 and 72 h after initiation of cell treatment.

For transfection of PC12 cells, cells were seeded onto acid-washed coverslips coated with polylysine (1 mg/ml) in 24-well multiwell plates containing 1 ml of DMEM, and attached cells were transiently transfected with 1 μg of total DNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. cDNAs of human GlcCerS cloned into

pcDNA3.1/V5-His-TOPO (Invitrogen) (a generous gift from Y. Hirabayashi, Institute of Physical and Chemical Research, RIKEN, Saitama, Japan) were co-transfected with Iip33-pEYFP (16) or with SialT2-pEYFP (17). For transfection of c-Fos or of its deletion mutants, the pcDNA 3.1 eukaryotic expression vector containing the coding sequence of the corresponding deletion polypeptide obtained from the full-length c-Fos cDNA (kindly supplied by J. Blenis, Harvard Medical School, Boston, MA) inserted in the EcoRI site of the polylinker sequence of the vector was used as described previously (10).

Expression and Purification of c-Fos—c-Fos was synthesized as a His-tagged protein in *Escherichia coli* and purified from cell lysates by nickel affinity chromatography by running through HisBin resin (Novagen, Madison, WI) as described previously (18).

Endogenous Acceptor and Metabolic Labeling of Glycolipids—For the determination of the *in vitro* endogenous glycolipid acceptor capacity, PC12 cells were suspended in 10 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose, washed twice in the same solution, and homogenized. Homogenates were incubated for 2 h in an incubation system that contained, in a final volume of 20 μl: 70 μg of homogenate protein, 45 mM MnCl₂, 25 mM HEPES-KOH (pH 7.0), 25 mM KCl, 2.5 mM magnesium acetate, and 50 μM UDP-[³H]Glc (10.9 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) (19). When indicated, 1.35 μl of recombinant c-Fos was added to a final concentration of 1 ng of c-Fos/μg of homogenate protein. Control incubates received 1.35 μl of vehicle. Reactions were stopped by addition of trichloroacetic acid-phosphotungstic acid (5% to 0.5% w/v, respectively, final concentration). For metabolic labeling of glycolipids, cells cultured during 4 days under the indicated experimental conditions (3 × 10⁵ cells/35-mm dish) were metabolically labeled with 1.5 μCi/ml of D-[U-¹⁴C]galactose ([¹⁴C]Gal, 303 mCi/mmol, Amersham Biosciences) during the last 8 h prior to harvesting.

Radioactivity Determinations and Lipid Chromatography—Radioactivity incorporated into endogenous glycolipid acceptors was determined in total lipids extracted from trichloroacetic acid-phosphotungstic acid precipitates with chloroform:methanol (C:M) 2:1 (v/v) (20). For chromatographic analysis of glycolipids, after metabolic labeling of cells *in culture* with [¹⁴C]Gal, cells were washed with cold PBS, scrapped from the plate, and pelleted by centrifugation. Lipids were extracted from the pellet with C:M (2:1 v/v) and freed from water-soluble contaminants by passing through a Sephadex G-25 column. Lipid extracts were used for radioactivity quantification (a 10% aliquot evaporated in a vial) or for chromatographic analysis. For this, the extract was supplemented with appropriate amounts of standard lipids and chromatographed on HPTLC plates (Merck, Germany) using C:M:0.2% CaCl₂ (60:36:8 v/v) as solvent. Standard lipids were visualized by exposure of the plate to iodine vapors. Routinely 2000–3000 cpm were spotted on each lane. Radioactive lipids were visualized by fluorography after dipping the plate in 0.4% melted 2,5-diphenyloxazole in 2-methylnaphthalene and exposure to a radiographic film at –70 °C, usually during 4–6 days (19).

Determination of Glycolipid Glycosyltransferase Activities—Transferase activities were determined in PC12 cell homoge-

nates as follows: GlcCer galactosyltransferase (GalT1), was determined in an incubation system that contained, in a final volume of 30 μ l, 100 μ M GlcCer, 500 μ M UDP-[3 H]Gal (450,000 cpm, 30 Ci/mmol, PerkinElmer Life Sciences), 20 mM cytidine 5'-diphosphocholine (as competitor of pyrophosphatases), 10 mM MnCl₂, 10 mM MgCl₂, 100 μ g of Triton X-100, 64 mM sodium cacodylate-HCl buffer (pH 7.2), and cell homogenate (100 μ g of protein). LacCer sialyltransferase (SialT1) was determined in an incubation system that contained, in a final volume of 30 μ l, 400 μ M LacCer, 100 μ M CMP-[3 H]NeuAc (250,000 cpm, 32 Ci/mmol, PerkinElmer Life Sciences), 20 mM MnCl₂, 1 mM MgCl₂, 20 μ g of Triton CF54/Tween 80 (2:1 w/w), 100 mM sodium cacodylate-HCl buffer (pH 6.5) and cell homogenate (40 μ g of protein). Incubations were performed at 37 °C for 90 min. Samples without exogenous acceptor were used to correct the incorporation into endogenous acceptors (21). Reactions were stopped by the addition of trichloroacetic acid-phosphotungstic acid (5% to 0.5% w/v, final concentration), and the radioactivity incorporated into lipids was determined as described above.

GlcCerS activity was assayed according to Veldman *et al.* (22) with slight modifications. The incubation system contained, in a final volume of 125 μ l, 20 μ M *N*-6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-4-*D*-erythro-sphingosine (C₆-NBD-ceramide, Molecular Probes, Carlsbad, CA) complexed with bovine serum albumin in a 1:1 molar ratio, 400 μ M 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 400 μ M UDP-glucose, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM EDTA in 50 mM HEPES (pH 7.2) and cell homogenate (100 μ g of protein). After the incubation period at 37 °C stated in each case, reactions were stopped by the addition of 625 μ l of C:M 2:1, v/v to extract C₆-NBD lipids in the lower phase. After centrifugation at 1000 \times *g* for 5 min, the lower phase was evaporated under nitrogen and subjected to HPTLC by using C:M: H₂O (65:25:4, v/v) as solvent. C₆-NBD lipids present in the chromatograms were visualized by UV illumination of the HPTLC. When indicated, 1.35 μ l of recombinant c-Fos in 8 M urea was added to the corresponding enzymatic assay to a final concentration of 1 ng of c-Fos/ μ g of homogenate protein. Control incubates received 1.35 μ l of vehicle.

Cell Immunofluorescence Analysis—Cells grown on round, acid-washed coverslips were fixed, blocked, and immunolabeled as described previously (10). Briefly, rinsed cells were fixed at 37 °C for 10 min in 3% paraformaldehyde, 4% sucrose in 10 mM PBS, washed twice, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at 37 °C. Washed coverslips were blocked with 1% bovine serum albumin/0.1% Tween 20 (v/v) in 10 mM PBS (blocking buffer) for 2 h and incubated overnight at 4 °C in blocking buffer containing rabbit anti-c-Fos 4 antibody (Santa Cruz Biotechnology, dilution 1/300), mouse anti-TGN-38 (BD Biosciences, dilution 1/100), goat anti-calnexin (Santa Cruz Biotechnology, dilution 1/300), or mouse anti V5 tag (Serotec Ltd., , Oxford, UK, dilution 1/80) antibodies. Washed cells were incubated with anti-rabbit Alexa 546- or anti-mouse Alexa 488-conjugated antibodies (Molecular Probes and Invitrogen) each diluted 1/1000 in blocking solution, washed, mounted in ProLong Antifade (Molecular Probes and Invitrogen) and visualized on a confocal laser scanning microscope LSM 5 (Carl Zeiss, Germany).

Immunoprecipitation Experiments—Immunoprecipitation in PC12 cells between c-Fos and the indicated enzymes was performed as described in Current Protocols in Cell Biology (Unit 7.2) under nondenaturing conditions. Briefly, cells arrested during 48 h in DMEM were co-transfected with the following plasmids: c-Fos cloned into pEYFPN1 for rat c-Fos; pcDNA3.1/V5-His-TOPO for human V5-tagged GlcCerS; pHAN1 for mouse His-tagged GalT1 and pCINeo for mouse HA-tagged SialT1; 24 h later cells were lysed in nondenaturing lysis buffer (1% w/v Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA), centrifuged 15 min at 16,000 \times *g*, and the supernatant was then precleared for 30 min at 4 °C using Protein G-Sepharose beads (Amersham Biosciences). The cleared lysates were incubated with Protein-Sepharose G beads conjugated to a mouse anti c-Fos (Santa Cruz Biotechnology), anti-V5 (Serotec Ltd.), anti-His (Roche Diagnostics), or anti-HA (Sigma-Aldrich) mouse monoclonal antibodies, and immunoprecipitates were washed three times with 0.1% w/v Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA and analyzed by Western blot as described.

Western Blot Analysis—Ten micrograms of protein were subjected to SDS-PAGE on 12% poly acrylamide gels and transferred to nitrocellulose membrane as described previously (10). Blocked membranes were incubated with rabbit c-Fos antibody (Santa Cruz Biotechnology, dilution 1/5000) or mouse DM1A raised against α -tubulin (Sigma-Aldrich, dilution 1/5000), washed twice for 15 min in PBS-Tween and then incubated with a secondary antibody biotin-conjugated (Vector Laboratories Inc., Burlingame, CA) raised against each corresponding primary antibody for immunodetection. Samples were then incubated with streptavidin peroxidase-conjugated (Amersham Biosciences), and immunodetection was performed using ECL plus (Amersham Biosciences). For co-immunoprecipitation experiments, an anti-V5 mouse antibody (Serotec Ltd.) was used.

RESULTS

NGF-treated PC12 Cells Show c-Fos-dependent Glycolipid Synthesis Activation—It has been previously shown that phospholipid synthesis is activated in PC12 cells induced to grow and differentiate by the addition of NGF to the culture medium (10). To evaluate if glycolipid synthesis is also activated under these experimental conditions, the synthesis of these lipids was determined in PC12 cells cultured for 4 days in the presence or the absence of NGF and metabolically labeled with [14 C]Gal during the last 8 h prior to harvesting. The incorporation of radioactivity into total glycolipids was ~60% higher in +NGF cells as compared with control cells (Fig. 1A). To examine the dependence of glycolipid synthesis activation on c-Fos expression, metabolic labeling experiments were carried out in +NGF cells cultured in the presence of a c-Fos mRNA ASO, which specifically blocks c-Fos expression. Under these conditions, the activation of total glycolipid labeling induced by NGF was abolished highlighting the role of c-Fos in the activation of glycolipid synthesis. The corresponding c-Fos SO had no effect on NGF activation of total glycolipid labeling (Fig. 1A). For each experimental condition, the expression level of c-Fos was mon-

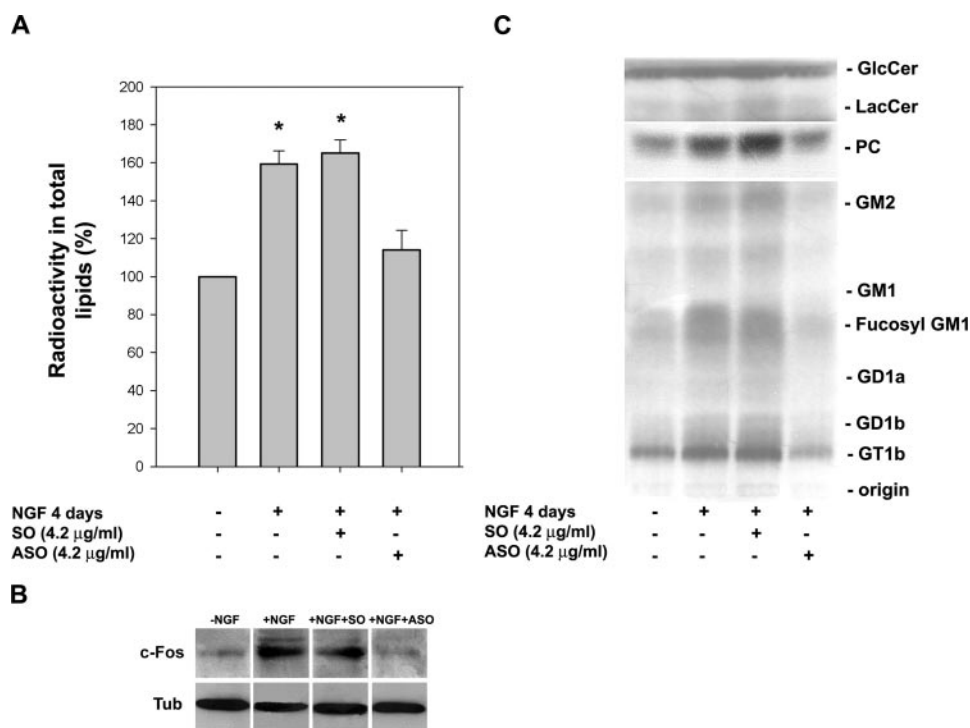


FIGURE 1. Metabolic labeling of glycolipids. Cells cultured 4 days with or without NGF and in the presence of oligonucleotides sense (SO) or antisense (ASO) to c-Fos mRNA, as indicated, were metabolically labeled with [14 C]Gal 8 h before harvesting. Lipids were extracted, purified, and analyzed as described under "Experimental Procedures." **A**, radioactivity (~ 2000 – 3000 cpm per lane) of total lipid extracts. Results are the mean \pm S.D. of three independent experiments performed in triplicate. *, $p < 0.005$ with respect to $-$ NGF-treated cells as determined by Student's t test. Note that c-Fos ASO abrogates the increase of glycolipid labeling observed in cells induced to differentiate by feeding of NGF. **B**, Western blot determinations of c-Fos in cells cultured as in **A**. Nitrocellulose membranes immunostained for c-Fos were stripped and stained for α -tubulin labeling as a gel loading control. **C**, lipid extracts from cells cultured as in **A** were purified, chromatographed, and visualized as indicated under "Experimental Procedures." The positions of co-chromatographed glycolipid standards are indicated. To avoid overexposure of the phosphatidylcholine bands, film exposure time of the middle part of the chromatogram was shorter than that for the rest.

itored by Western blot. Abundant c-Fos was found in the $+NGF$ and the $+NGF + SO$ cells, contrasting with that found in the control ($-NGF$) and the $+NGF + ASO$ cells, which were at the limit of detection (Fig. 1B), reinforcing the dependence of the NGF-activated synthesis of glycolipids on c-Fos. As found previously (10), oligonucleotide incorporation into cells showed an efficiency of $\sim 80\%$.

A TLC analysis of total radioactive glycolipids was carried out to determine if particular species of these lipids were specifically activated and, if so, responsible for the observed increase in the total labeling shown in Fig. 1A. As reported (23–25), the pattern of glycolipids of PC12 cells is dominated by complex species (Fig. 1C). However, the autoradiograms failed to evidence any species whose individual labeling could have accounted for the $\sim 60\%$ increase found in the labeling of total glycolipids (Fig. 1C). Rather, a global stimulation of the glycolipid-synthesizing machinery in the c-Fos-mediated response of PC12 cells to NGF occurred. Phosphatidylcholine is also labeled with [14 C]Gal and, as expected from previous results with other radioactive precursors of phospholipids (9, 10), its labeling also increased in a c-Fos-dependent manner (Fig. 1C).

c-Fos Activates the Labeling of Endogenous GlcCer in Vitro—To go further in the analysis of the activating effect of c-Fos on glycolipid formation, homogenates prepared from cells treated

with effectors as in Fig. 1A were assayed to determine their capacity to incorporate [3 H]Glc from UDP-[3 H]Glc into endogenous glycolipid acceptors. Homogenates prepared from $+NGF$ cells showed a ~ 2 -fold increase in [3 H]Glc incorporation into endogenous glycolipid acceptors with respect to those from control cells (Fig. 2), indicating that the activated condition of the glycolipid-synthesizing machinery was maintained in the isolated membranes and was not attained in membranes from cells in which c-Fos expression was precluded by ASO. Because the donor nucleotide in the *in vitro* reaction is UDP-[3 H]Glc, this result indicates that an increased amount of ceramide is converted to GlcCer by the GlcCerS when c-Fos is present in the cell homogenate.

The Basic Domain of c-Fos Activates the Labeling of Endogenous Acceptors in Vitro—It has been previously shown that the c-Fos deletion mutants that contain the basic domain (BD) of c-Fos (amino acids 139–159) are capable of activating phospholipid synthesis *in vitro*, whereas those lacking this domain are not (10). To evaluate if the BD of c-Fos is also relevant for

the activation of glycolipid synthesis, PC12 cells were mock transfected (Control) or transfected to express c-Fos or its deletion mutants NA (amino acids 1–139, which lacks BD), NB (amino acids 1–159, which contains BD), or LZC (amino acids 165–380, which lacks BD) (Fig. 3). Transfected cells were cultured for 2 days in the presence of NGF ($+NGF$) to prime cells to differentiate and extend neurites, and then culture medium was replaced by fresh medium without NGF and cultures continued to complete 4 days. Under these experimental conditions, the neurites extended during the first 2 days in culture stop growing and retract unless cells had been transfected to constitutively express c-Fos or the BD-containing deletion mutant NB (10). To evaluate the glycolipid-labeling capacity of membranes from these cell preparations, the *in vitro* incorporation of [3 H]Glc into endogenous glycolipid acceptors was determined. Homogenates obtained from transfected cells grown 2 days with NGF followed by 2 days without NGF were incubated for 2 h with UDP-[3 H]Glc and the incorporation of [3 H]Glc into endogenous glycolipids determined as indicated for Fig. 2. Homogenates from cells expressing c-Fos or the BD-containing deletion mutant (NB) showed a ~ 4 -fold activation of [3 H]Glc incorporation into endogenous glycolipid acceptors with respect to homogenates from mock transfected cells (Control) (Fig. 3, right panel). On the other hand, those from

cells expressing the deletion mutants NA or LZC showed labeling values comparable to those of the mock transfected cells. In experiments not shown, it was found that labeling values of homogenates from cells grown 4 days without NGF were essentially the same as those of the mock transfected cells grown 2 days +NGF followed by 2 days –NGF and that protein expression of c-Fos or its deletion mutants was essentially the same in all transfected cells. These results underscore the importance of the BD domain of c-Fos to

attain glycolipid synthesis activation. They also rule out that glycolipid synthesis activation is reflecting an increase in glycolipid glycosyltransferase mRNA induced by c-Fos-AP-1 dimers or NGF stimulation, because transfected cells cultured 2 days with NGF followed by 2 days without NGF showed control levels of [3 H]Glc incorporation unless cells were transfected to express a BD-containing protein (full-length c-Fos or NB deletion mutant).

Glucosyl-, but Not Sialyl- or Galactosyltransferases Are Activated by c-Fos—To examine if all or only particular enzymes of the initial steps in the pathway of synthesis of glycolipids (GlcCer \rightarrow LacCer \rightarrow Sialyl-LacCer (GM3)) are activated by c-Fos, the activities of GlcCerS, glucosylceramide galactosyltransferase (GalT1), and LacCer sialyltransferase (SialT1) were determined *in vitro* in the presence of exogenous acceptors, in conditions of linearity with the incubation time and protein concentration, with or without the addition of recombinant c-Fos to the incubates.

For the determination of GlcCerS activity, the conversion of C₆-NBD-ceramide (C₆-NBD-Cer) into C₆-NBD-glucosylceramide (C₆-NBD-GlcCer) was measured. Fig. 4A shows a representative experiment of the formation of C₆-NBD-GlcCer at different incubation times, in the absence and the presence of c-Fos. Quantification of the fluorescent C₆-NBD-GlcCer formed is shown in Fig. 4B (results are the mean of four independent experiments). It is clear that, although in the incubates performed in the absence of c-Fos a low activity was detected, which practically reached plateau at 15 min of incubation, in the +c-Fos incubates GlcCerS activity increased up to 60 min and reached a plateau value ~4-fold higher than that obtained in the absence of c-Fos. Three additional experiments were performed under the same experimental conditions while incubating the samples for 30 min. The mean arbitrary densitometric units \pm S.D. of the HPTLC scan of C₆-NBD-GlcCer formation was: –c-Fos 209 \pm 36; +c-Fos: 665 \pm 150; $p < 0.005$ as determined by Student's *t* test. By contrast, neither SialT1 nor GalT1 activities were found stimulated by c-Fos (Table 1). These results strongly suggest that c-Fos is capable of activating the bulk of glycolipid synthesis by activating GlcCerS.

c-Fos Increases V_{max} of GlcCerS without Modifying K_m Values for Either UDP-Glc or C₆-NBD-Cer—To gain information about the mechanism by which c-Fos activates GlcCerS, the kinetic parameters for the conversion of C₆-NBD-Cer into C₆-NBD-GlcCer were determined in the presence and absence of c-Fos. Increasing the concentration of UDP-Glc while keeping NBD-Cer constant and at saturating concentration allows the calculation of K_m values for UDP-Glc of 6.9 and 7.9 μ M in the absence and presence of c-Fos, respectively. Under the same conditions, calculated V_{max} values were 178 and 385 densitometric units of NBD-GlcCer formed.h^{–1}.mg protein^{–1} in the presence and the

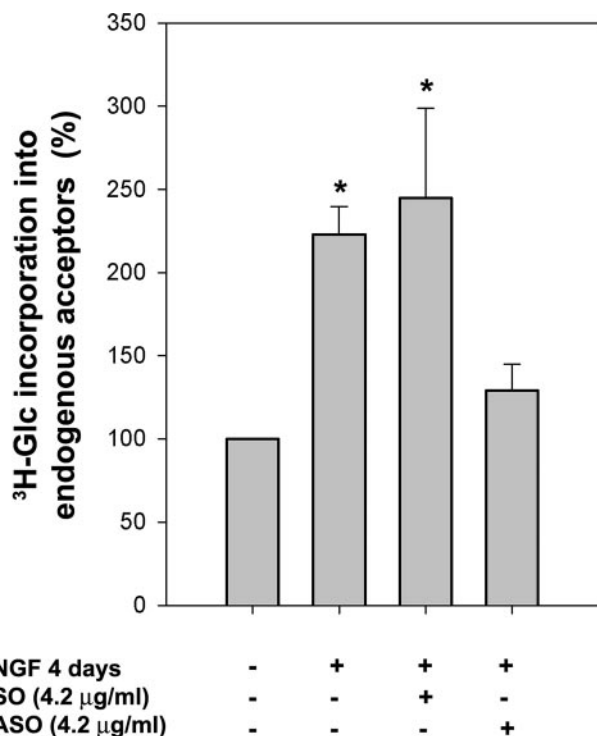


FIGURE 2. c-Fos-containing homogenates show activated labeling of endogenous glycolipid acceptors *in vitro*. Homogenates prepared from PC12 cells cultured 4 days under the indicated experimental conditions were incubated *in vitro* for 2 h with UDP-[3 H]Glc. After incubation, labeled glycolipids were purified, and lipid-bound radioactivity was quantified. Results are the mean \pm S.D. of three independent experiments performed in triplicate. *, $p < 0.005$ with respect to –NGF-treated cells as determined by Student's *t* test.

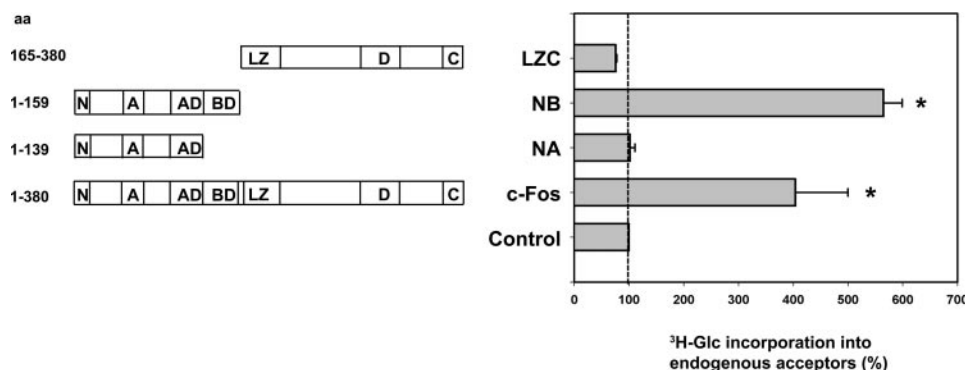


FIGURE 3. *In vitro* glycolipid labeling activation by c-Fos or c-Fos deletion mutants. Cells mock transfected (control) or transfected to express c-Fos or the indicated c-Fos deletion mutants (depicted on the left) were cultured for 2 days with NGF, medium was replaced by fresh medium without NGF, and cultures were continued for an additional 2 days. Homogenates prepared from these cells were incubated for 2 h with UDP-[3 H]Glc, and glycolipid labeling was determined as in Fig. 2. Results are the mean \pm S.D. of three independent experiments performed in triplicate. *, $p < 0.0001$ with respect to control, mock transfected cells as determined by Student's *t* test.

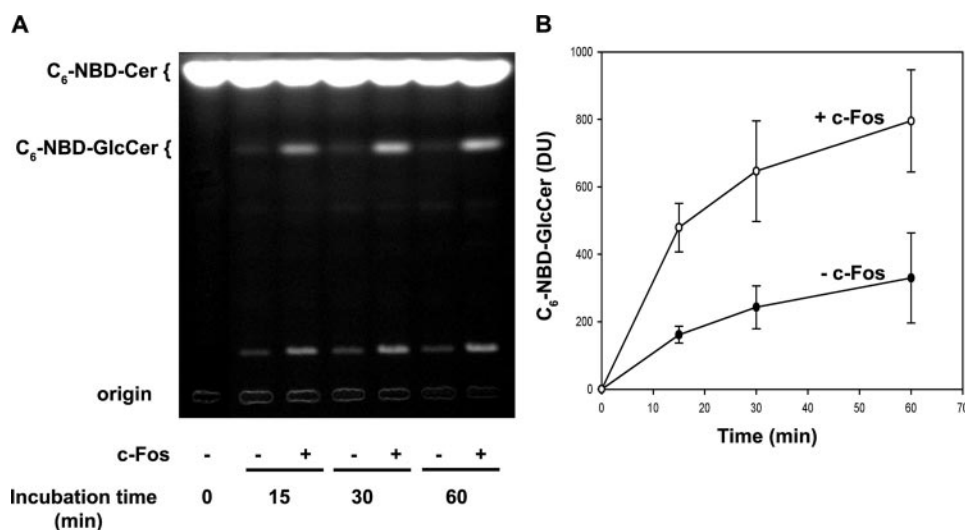


FIGURE 4. GlcCerS activity in homogenates determined in the presence or the absence of c-Fos. The *in vitro* conversion of C_6 -NBD-Cer to C_6 -NBD-GlcCer was assayed in homogenates obtained from cells cultured in the absence of NGF for 4 days with or without the addition of recombinant c-Fos. *A*, formation of C_6 -NBD-GlcCer with and without addition of c-Fos (1 ng/ μ g of homogenate protein) to the incubates as determined at the indicated incubation times after HPTLC separation and UV visualization. *B*, quantification (expressed as arbitrary densitometric units) of C_6 -NBD-GlcCer formation in the absence of c-Fos (closed circles) or the presence of c-Fos (open circles). Results are the mean \pm S.D. of four independent experiments.

TABLE 1

GlcCerS, GalT1, and SialT1 activities in PC12 cells, in the presence or absence of c-Fos

Homogenates obtained from PC12 cells cultured in the absence of NGF were used as enzyme source for the determination of GlcCerS, GalT1, and SialT1 activities, in the presence of recombinant c-Fos (1 ng/ μ g of homogenate protein) or of vehicle (Control). Samples without exogenous glycolipid acceptor were used to correct the incorporation into endogenous acceptors. Values of GlcCerS activity obtained at 60 min of incubation are expressed in arbitrary densitometric units (Fig. 4A). Values for GalT1 and SialT1 activities are expressed as picomoles of sugar transferred/mg protein/h. Results of GalT1 and SialT1 are the mean \pm S.D. of two experiments performed in duplicate.

	GlcCerS	GalT1	SialT1
Control	178.2	159.7 \pm 2.8	152.2 \pm 2.8
+ c-Fos	872.0	160.2 \pm 24.2	163.6 \pm 5.5

absence of c-Fos, respectively (Fig. 5A). Increasing the concentration of NBD-Cer while keeping UDP-Glc constant and at saturating concentration resulted in comparable K_m values for NBD-GlcCer in the absence or presence of c-Fos (13 μ M and 20 μ M, respectively) but >3-fold (240 versus 787) densitometric units of NBD-Glc-Cer formed. h^{-1} .mg protein $^{-1}$ in the absence and the presence of c-Fos, respectively. These results indicate that the catalytic efficiency, rather than the affinity for the substrates, is increased in the presence of added recombinant c-Fos.

c-Fos and GlcCerS Participate in a Physical Association— The experiments of Fig. 4 raise the question of whether c-Fos and GlcCerS can form physical associations to attain the c-Fos-dependent activated state of GlcCerS. To examine this possibility, co-immunoprecipitation assays were performed with homogenates from PC12 cells co-transfected to express c-Fos and V5-tagged GlcCerS. Previously, the subcellular distribution of the V5-tagged GlcCerS was controlled by immunofluorescence. PC12 cells were co-transfected with V5-tagged GlcCerS and YFP-tagged SialT2 as a Golgi marker or YFP-tagged lip33 as an ER marker. GlcCerS co-localized with the ER marker (Fig. 6A, lower row) and with the Golgi marker (Fig. 6A, upper row) as already shown (26). Endogenous c-Fos was found clearly co-

localizing with GlcCerS (Fig. 6B, upper row) and with the ER marker calnexin (Fig. 6B, lower row) but less clearly with the Golgi marker TGN-38 (Fig. 6B, middle row). Physical association between c-Fos and GlcCerS was evidenced with co-immunoprecipitation assays carried out with lysates of cells co-transfected to express c-Fos and V5-tagged GlcCerS. Fig. 7A shows that immunoprecipitation of c-Fos or of GlcCerS was capable of mutually co-immunoprecipitate the other protein, supporting a physical association between them. To monitor the specificity of the association, membranes from c-Fos and His-tagged GalT1 or c-Fos and HA-tagged SialT1 co-transfected cells were also subjected to co-immunoprecipitation assays. Immunoprecipitates using V5, His, or HA antibodies

were Western blotted with c-Fos antibody, as indicated. Controls of mock transfected cells were run in parallel (not shown). No c-Fos was observed co-immunoprecipitating either with GalT1-His or with SialT1-HA or in lysates from cells expressing only c-Fos and immunoprecipitated with anti V5 antibody (Fig. 7B), indicating that c-Fos physically associates only with specific membrane components.

DISCUSSION

PC12 cells are a good model to investigate the diverse events leading to neural cell differentiation (27, 28). These events can be broadly divided in two: first are the genomic events that trigger the differentiation process, which are followed by the activation of the metabolic machinery that provides all the components the differentiating neuron requires to grow. Whereas much progress has been made in deciphering the nuclear events leading to neuronal differentiation, including the participation of nuclear c-Fos as an AP-1 transcription factor (reviewed in Ref. 29), much less is known about the accompanying metabolic events. However, the possibility of having a few proteins whose expression is very tightly regulated, in charge of commanding both aspects of the differentiation process at different subcellular levels, nuclear and cytoplasmic, seems a reasonable mechanism to facilitate the coordination of the diverse events of this growth process.

Glycolipids are present in nearly all animal cells but the membranes from the nervous system are particularly concentrated in these lipids. The level of expression of glycolipids can be controlled by regulating the activity of the glycosyltransferases that participate in their biosynthesis. In this sense, different levels of regulation have been reported, *i.e.* transcriptional, translational, post-translational (30), and organellar topology (17, 31). c-Fos, as an AP-1 transcription factor, could have been a good candidate to regulate GlcCerS transcription. However, two lines of evidence seem to rule out this possibility:

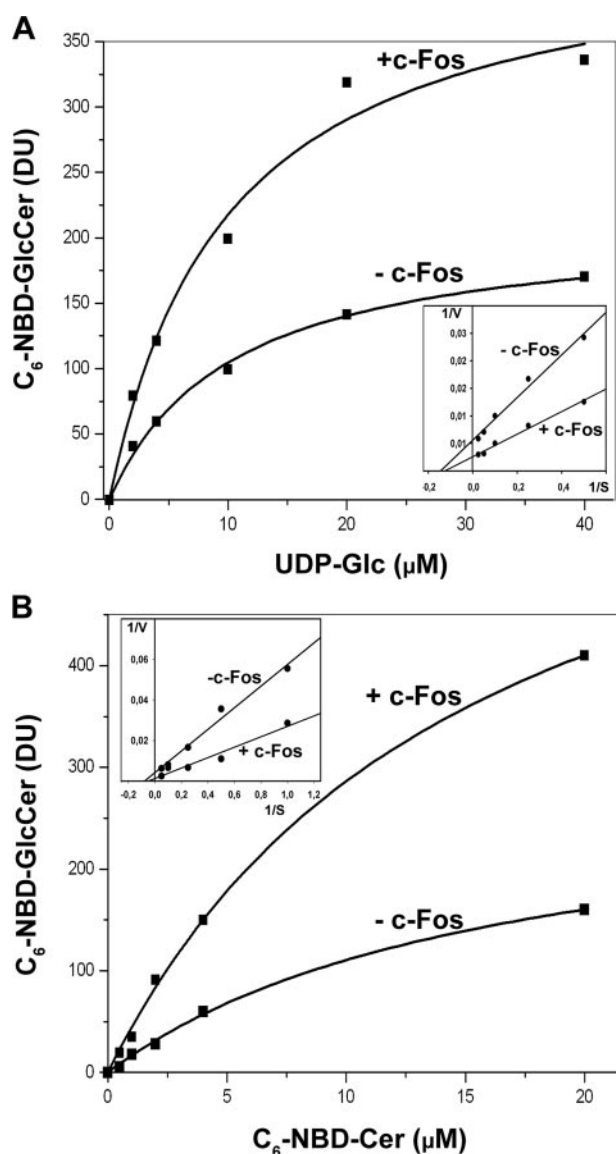


FIGURE 5. GlcCerS kinetic parameters in the presence or the absence of c-Fos. Enzyme activity was determined at 15 min of incubation as indicated in Fig. 4 at the indicated increasing concentrations of UDP-Glc and constant saturating concentration of C₆-NBD-Cer (20 μM) (A) or at increasing concentration of C₆-NBD-Cer and constant saturating concentration of UDP-Glc (400 μM) (B) with or without the addition of c-Fos (1 ng/μg of homogenate protein) as indicated. Insets show the Lineweaver-Burk plots for K_m and V_{max} calculations.

the first is that a computerized search (Genomatix, Transfac, and AliBaba2.1 programs) for putative transcription factor binding sites in the 5'-upstream region from the ATG codon of rat GlcCerS gene revealed sites for SP1/GC, GATA-1, E2F, and ETS1, among others, but no AP-1 sequence was detected as had been reported for the promoter of mouse GlcCerS gene (32). The second is the observation that, in culture, cells transfected to express NB attain a similar glycolipid synthesis activation to those treated with NGF despite NB lacking the leucine zipper domain required for the heterodimerization of c-Fos to form AP-1 transcription factors.

A key step in glycolipid synthesis is the formation of GlcCer, the first glycosylated intermediate. This synthesis is catalyzed by GlcCerS, an ER and Golgi (26, 33, 34) or Golgi membrane-

concentrated enzyme (35, 36). Inhibition of GlcCerS with the specific inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol results in glycosphingolipid depletion and reduced growth of neuroblastoma (37) and PC12 cells (38) and axonal growth of hippocampus (39) and cerebral cortical neurons (40) in culture. Taking into consideration the results presented herein, an additional level of control of glycosphingolipid synthesis, commanded by cellular effectors directly acting on key steps of their biosynthetic machinery should be considered. Even if c-Fos is the first protein identified with the capacity to activate phospholipid and glycolipid synthesis, it can be foreseen that other effectors with similar regulatory activities but acting at different levels of the biosynthetic pathway will emerge as we progress in our understanding of the differentiation and growth processes. It should be underscored that c-Fos is not a constitutive component of cells; rather, its expression is very tightly regulated, responding to specific environmental cues (2, 41; reviewed in Refs. 6, 42). This strict regulation of c-Fos expression assures that the activation of glycolipid and phospholipid metabolisms will occur at the moment required.

For the c-Fos-dependent activation of phospholipid synthesis (9, 10), activation of all the enzymes involved in the corresponding biosynthetic pathway was not a requirement to attain an overall lipid activated state; rather, only specific enzymes, *i.e.* phosphatidic acid phosphatase and acyl transferase were activated and yet a global activation was obtained (43). For the case of glycolipids, activation of GlcCerS, which provides the precursor for higher order glycolipids, seems to be sufficient for global activation of the synthesis; GalT1 and SialT1 were essentially unaffected by c-Fos. GlcCerS is unique among glycolipid glycosyltransferases in the sense that its catalytic site is oriented toward the cytosolic face of Golgi (35) and pre-Golgi membranes (33, 34), whereas the subsequent transferases are type II membrane proteins with the catalytic site oriented toward the lumen of the Golgi.

Concerning the molecular mechanism by which c-Fos activates the synthesis of these lipids, two mechanisms seem at first sight feasible: one is that c-Fos directly interacts with the enzymes of both lipid pathways that it activates, and the other is that c-Fos interacts with other components and modifies the intermolecular organization of the membrane in such a way that the modification of the microenvironment activates specific enzymes that sense these changes. In fact both possibilities have experimental data that support them. With respect to the first possibility of a direct interaction between c-Fos and the activated enzymes, preliminary results obtained by co-immunoprecipitation and fluorescence resonance energy transfer studies between specific integral-membrane enzymes of the pathway of phospholipid synthesis and c-Fos are evidencing that this is indeed the case.⁶ As for the enzyme GlcCerS, the sidedness of this type I transmembrane enzyme makes this possibility attractive. It has been recently reported that GlcCer formed on the cytoplasmic face of proximal Golgi is conveyed by FAPP2 to the distal Golgi (44) where it is translocated to the lumen for

⁶ A. Alfonso Pecchio and B. L. Caputto, unpublished results.

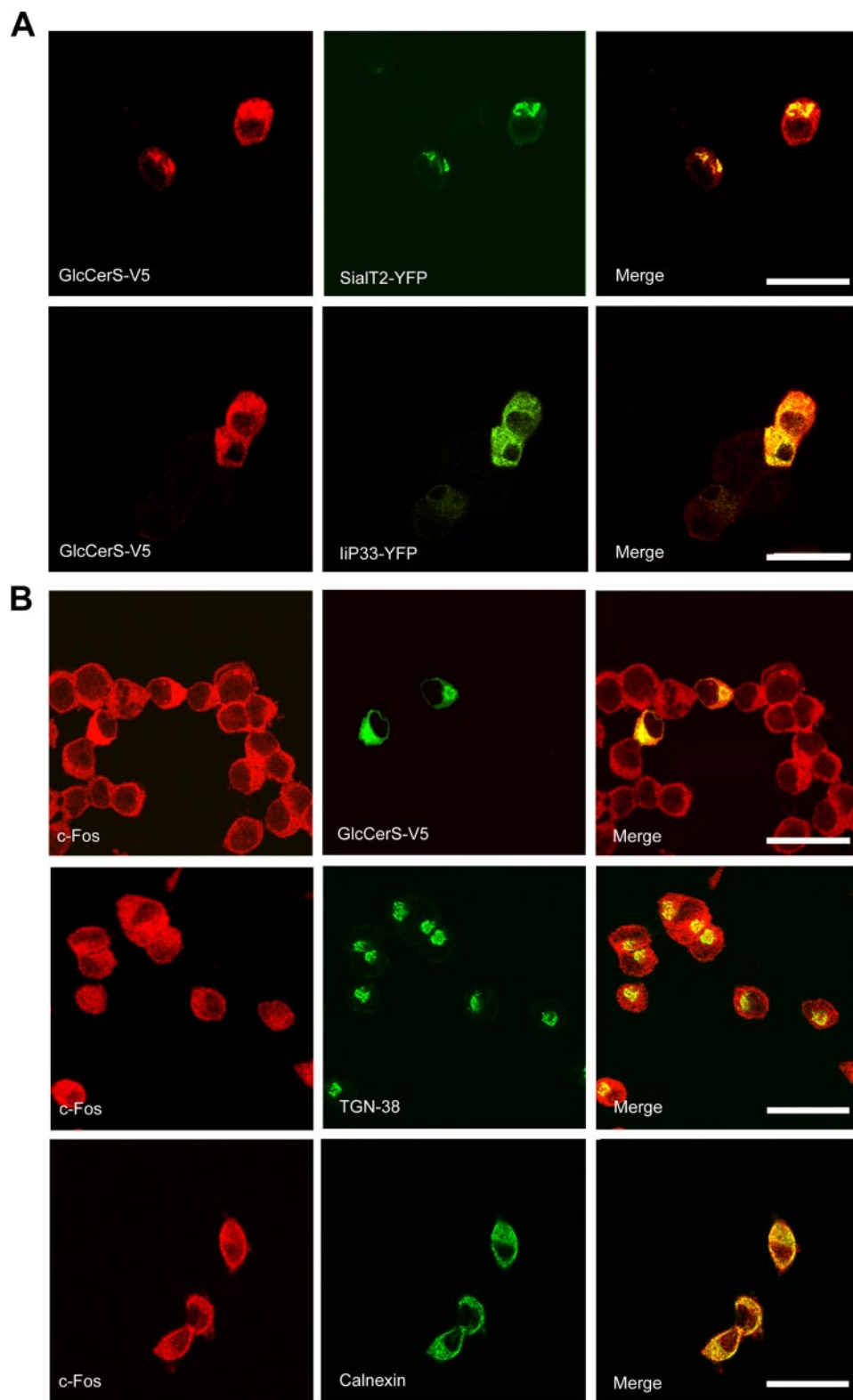


FIGURE 6. Immunofluorescence examination shows GlcCerS and c-Fos co-localizing at the ER. *A*, subcellular localization of GlcCerS was examined in PC12 cells co-transfected to express V5-tagged-GlcCerS and the Golgi-resident YFP-tagged SialT2 (upper row) or the ER-resident, YFP-tagged lip33 (lower row). GlcCerS was evidenced with V5 and Alexa 488 antibodies as described under "Experimental Procedures." The YFP-tagged markers were detected by intrinsic fluorescence. Note the ER and Golgi distribution of GlcCerS. *B*, subcellular localization of c-Fos examined in PC12 cells cultured +NGF for 7.5 min that were fixed and immunostained for c-Fos and the GlcCerS tag V5 (top panel) or the Golgi marker TGN-38 (middle panel) or the ER marker calnexin (bottom panel) as described under "Experimental Procedures." Note co-localization of c-Fos/V5-GlcCerS and of c-Fos/ER. Bar: 10 μ m.

synthesis of more complex glycolipids. Simultaneously, it was reported that FAPP2 is also capable of transporting GlcCer from the Golgi to the ER, where it translocates to the lumen and reaches the Golgi for further glycosylation by vesicular transport (36). The results reported herein strongly support that activation of GlcCerS by cytoplasmic c-Fos involves a physical association between them, raising the possibility that the activated complex provides FAPP2 with extra amounts of GlcCer for transport to the translocation sites. The second possibility is sustained by strong biophysical evidence: c-Fos is an amphitropic, highly surface active protein that differentially interacts with phospholipid monolayers with a selective dependence on the lipid polar head group and the lateral surface pressure. As a consequence of this interaction on the monolayers, c-Fos can modulate the activity of phospholipases at the interfacial level (18, 45, 46). To add complexity to the interpretation of this regulatory mechanism, the fact that tyrosine phosphorylation/dephosphorylation events on c-Fos influence its ER association (14) should also be considered as plausible for glycolipid synthesis activation. The finding that c-Fos increases GlcCerS V_{max} values for both substrates without substantially modifying the K_m values does not rule out either of these possibilities. Even if further studies will be required to determine if one, both, or some other molecular mechanism is participating in the activation phenomenon, c-Fos is emerging as an important regulator of key membrane metabolism during such complex processes demanding membrane biogenesis as is neuronal differentiation. It should be kept in mind that, to achieve the differentiation process, not only the metabolism of lipids but also that of all other cell components required for growth must be activated. If the expression of c-Fos reflects what occurs with various yet

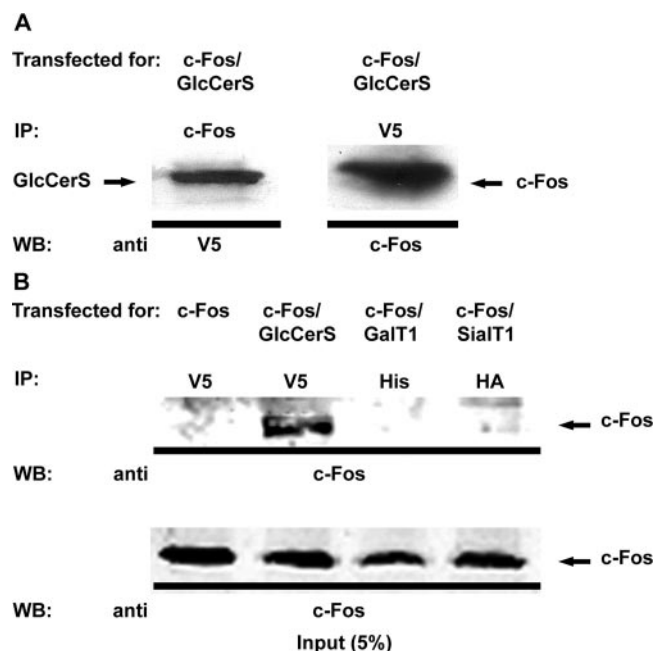


FIGURE 7. GlcCerS but not GalT1 or SialT1, physically associates with c-Fos. A, lysates from quiescent cells co-transfected to express c-Fos and V5 tagged-GlcCerS were immunoprecipitated using anti-c-Fos or V5 antibodies as indicated. Immunocomplexes were analyzed by SDS-PAGE followed by Western blotting anti-V5 or c-Fos antibodies as indicated. B, top panel, lysates from quiescent cells transfected to express c-Fos or co-transfected to express c-Fos and V5-tagged-GlcCerS or c-Fos and His-tagged GalT1 or c-Fos and HA-tagged SialT1 were immunoprecipitated using, from left to right, V5 (first two lanes) or His or HA antibodies as indicated. Immunocomplexes were analyzed by SDS-PAGE followed by Western blotting with anti-c-Fos antibody. The lower panel shows the Western blots of c-Fos input (5%) of the corresponding cell lysates of the upper panel.

unknown regulatory proteins or if c-Fos will result in a key regulator of other metabolisms (*i.e.* proteins) will no doubt be important aspects to focus on in future work.

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