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Effect of Growth Factors and Steroids on Transglutaminase Activity and Expression in Primary Astroglial Cell Cultures

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Type-2 transglutaminase (TG-2) is a multifunctional enzyme involved in the regulation of cell differentiation and survival that recently has been shown to play an emerging role in astrocytes, where it is involved in both proliferation and differentiation processes. Growth factors (GFs) such as EGF, basic fibroblast growth factor, insulin-like growth factor–I (IGF-I), and insulin (INS) are trophic and mitogenic peptides that participate in neuron–glia interactions and stimulate neuronal and astroglial proliferation and differentiation. Steroid hormones such as glucocorticoids and estrogens also play a pivotal role in neuronal and astroglial proliferation and differentiation and are key hormones in neurodegenerative and neuroprotective processes. We investigated the effects of the interaction of GFs with dexamethasone (DEX) or 17β-estradiol (E_2) on TG-2 activity and their expression in cultured astrocytes. We observed a significant increase in TG-2 activity and expression in astroglial cells treated for 24 hr with IGF-I, EGF, or INS. Priming of the cells with DEX or E_2, for 48 hr also led to an increase in TG-2 levels. When growth factors were present in the last 24 hr of the steroid treatment, a reduction in TG-2 expression and activity and a different subcellular TG-2 distribution were found. Our data indicate that steroid hormone–GF interaction may play an important role in astroglial function. The effect on TG-2 could be part of the regulation of intracellular pathways associated with the astrocyte response observed in physiological conditions and, possibly, also in neuropathological diseases.

Key words: growth factors; estrogens; astrocytes; glucocorticoids

Transglutaminases (TGs) form a family of calcium-dependent crosslinking enzymes that catalyze the formation of intra- and intermolecular bonds between the γ-carboxyamide moiety of glutamine and primary amino groups (Lorand and Graham, 2003). Type-2 transglutaminase (TG-2), which is the most ubiquitous TG isoform, is a multifunctional enzyme involved in the regulation of cell differentiation and survival (Milakovic et al., 2004). TG-2 is induced during apoptotic death and is implicated in a variety of human disorders including central nervous system (CNS) disorders (Fesus and Piacentini, 2002; Mastroberardino et al., 2002). Ca^{2+} ions are key regulators of TG-2 activity. When intracellular Ca^{2+} is low, TG-2 behaves like a G protein, coupling different receptors to phospholipase C (Nakaoka et al., 1994).

The multiplicity of TG-2 function also depends on its intracellular location. TG-2 has been found in the cytosol, in mitochondria (Piacentini et al., 2002; Jeitner et al., 2005) and in the nucleus (Campisi et al., 2003), where it is presumably involved in the regulation of gene expression.

Astrocyte activity may be modulated by steroid hormones and growth factors. Astrocytes express estrogen

Abbreviations used: bFGF, basic fibroblast growth factor; DC, monodansylcadaverine; CLSM, confocal laser scanning microscopy; DEX, dexamethasone; EGF, epidermal growth factor; E_2, estradiol; IGF- I, insulin-like growth factor–I; INS, insulin; GFAP, glial fibrillary acidic protein; GFs, growth factors; MAPK, mitogen-activated protein kinase; TG-2, tissue transglutaminase; TGase, transglutaminase.

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receptors (ERs) and glucocorticoid receptors (Avola et al., 1988b; Chou et al., 1991; Jung-Testas et al., 1992; Sato et al., 2003; Chaban et al., 2004; Pawlak et al., 2005) in both the nucleus and the cell membrane, as well as receptors for growth factors, such as epidermal growth factor (EGF), insulin-like growth factor–I (IGF-I), and insulin (INS) receptors (Mazzoni and Kenigsberg, 1994; Jafferali et al., 2000; Aberg et al., 2003; Morita et al., 2005). Steroids regulate the interaction between glia and neurons and glial gene expression and promote the production of astrocytic growth factors, which then may act in both an autocrine and a paracrine fashion (Azcoitia et al., 2001). Glucocorticoids as well as estrogens are involved in neuronal and astroglial cell differentiation and in neurodegeneration/neuroprotection processes in the CNS. Growth factors (GFs) such as basic fibroblast growth factor (bFGF), EGF, IGF-I, and INS act as mitogenic peptides and participate in neuron–glia cross talk, stimulating cultured nerve cell proliferation and differentiation (Avola et al., 1988a, 1988b; Marletta et al., 2001; Spina et al., 2002). In particular, GFs play a pivotal role in the dynamic flow of signaling molecules between neurons and astroglia (Avola et al., 2000).

We previously showed that "competence," or "progression," GFs and estrogens have an effect on DNA labeling and cytoskeletal protein expression during astroglial cell proliferation and differentiation in 17β-estradiol (E_2)– and bFGF-treated cultures (Avola et al., 2002).

Recently, GF–estradiol interaction in DNA labeling and cytoskeletal protein [glial fibrillary acidic protein (GFAP) and vimentin] expression in cultured rat astrocytes was also investigated (Avola et al., 2004). Interestingly, vimentin is a substrate of TG-2, which is expressed in cultured neurons and astrocytes (Ientile et al., 2002; Campisi et al., 2003, 2004). TG-2 expression/activity may be modulated by GFs, as hepatocytes treated with EGF show a significant increase in TG-2 activity concomitant with an increase in *TG-2* mRNA (Katoh et al., 1996). Little is known about how growth-promoting or differentiating agents regulate the expression of TG-2 and the overall TG-2 activity in astrocytes.

In the present study we investigated the interaction between GCs or E_2 and GFs and the effects of this interaction on TG-2 activity and expression in 15 DIV astrocyte cultures starved for 24 hours and then treated with dexamethasone (DEX) or E_2 either alone (48 hr) or in combination with some GFs added in the last 24 hr. We can report that IGF-I, EGF, and INS markedly increased TG-2 expression and activity and that this effect was reduced when glial cultures were primed with both dexamethasone and estradiol, indicating that regulation of TG-2 in astrocytes is signal- and hormone dependent.

MATERIALS AND METHODS

Materials

Cell culture medium and sera were from Invitrogen (Milan, Italy). Monodansyl-cadaverine (DC) and other chemicals of analytical grade were from Sigma (Milan, Italy). TG-2 antibody CUB 7402 was from NeoMarkers (Fremont, CA). Texas red–conjugated anti-IgG, horseradish peroxidase–conjugated antibody, and the enhanced chemiluminescence (ECL) developing system for immunoblots were purchased from Amersham Pharmacia Biotech (Milan, Italy).

Astroglial Cell Cultures

Primary cultures of astrocytes were prepared from newborn albino rat brains (from 1- to 2-day-old Wistar strain rats) as previously described (Avola et al., 1988a, 1988b; Bramanti et al., 2007). In particular, cerebral tissues, after dissection and careful removal of the meninges, were mechanically dissociated through 82-mm-pore sterile mesh (Nitex). Isolated cells were suspended in Dulbecco's Modified Eagle's Medium, supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (50 mg/mL), and penicillin (50 U/mL) and plated at a density of 3×10^6 cells/100-mm dish and 0.5×10^5 cells/chamber in Lab-Tek II multichambered slides. The low initial plating density of dissociated cells was meant to favor the growth of astrocytes with only a very little oligodendroglial and microglial contamination. Cells were maintained at 37°C in a 5% CO_2/95% air humidified atmosphere for 2 weeks, and the medium was removed every 3 days. Astroglial cells were characterized at 15 DIV, that is, when confluent, by immunofluorescent staining with the glial marker GFAP, as previously reported (Avola et al., 1988a). All efforts were made to minimize both the suffering and number of animals used. All experiments conformed to the guidelines of the Ethical Committee of the University of Catania, Italy.

Serum Deprivation and Growth Factor and/or DEX or E_2 Treatments

Astroglial cell cultures at 13 DIV were maintained in serum-supplemented medium and then switched to serum-free medium containing bovine serum albumin (1 mg/mL) for a 24-hr starvation period before the addition of growth factors (EGF, 10 ng/mL; IGF-I, 10 ng/mL; or INS, 10 μg/mL). When GFs were used in combination with steroids, they were added in the last 24 hr of the 48-hr pretreatment with 1 nM DEX or 5 nM E_2.

Subcellular Fractionation of Treated Astrocyte Cultures

Cell fractionation into different subcellular fractions (cytosol, mitochondria, and nuclei) was carried out as previously reported by Avola et al. (1986). To better demonstrate the chance of subcellular localization of TG-2 under our experimental conditions, Western blot analysis of the subcellular fraction proteins was performed.

Western Blot Analysis

After treatment, cells were harvested in cold PBS, collected by centrifugation, resuspended in a homogenizing buffer [50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin, leupeptin, and pepstatin] and sonicated on ice. The protein concentration of the homogenates was diluted to 1 mg/mL with 2× reducing stop buffer [0.25M

Tris-HCl (pH 6.8), 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, and 10% glycerol with bromophenol blue as the tracking dye]. Proteins (30 μg) were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked overnight at 4°C with 5% nonfat dry milk dissolved in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.5% Tween 20. TG-2 was detected by incubation for 1 hr with monoclonal antimouse against the protein Ab CUB 7402 (1:1,000 in PBS), followed by incubation for 1 hr with horseradish peroxidase–conjugated antimouse IgG (1:1,500 in PBS). TG-2 expression was visualized by chemiluminescence with an ECL kit after autoradiography film exposure. Blots were scanned and quantified with an AlphaImager 1200 System (Alpha Innotech, San Leandro, CA). The results were expressed as the percentage of TG-2 increase over that in the control.

Confocal Laser Scanning Microscopy

The expression and distribution of TG-2 protein in cell compartments were investigated by confocal microscopic analysis after a 1-hr incubation of the control and treated astroglial cultures with 1% normal blocking serum in PBS, followed by incubation with an anti-TG-2 antibody (1:100 in PBS) for 1 hr and then with Texas Red–conjugated antimouse IgG (1:100 in PBS) for 1 hr. Then the cultures were briefly washed three times with PBS, mounted with 90% glycerol, and observed by confocal laser scanning microscopy.

Monodansyl-Cadaverine Labeling of Living Cells

Total transglutaminase (TGase) activity was evaluated by DC uptake into living cells after steroid/GF treatment was assayed according Ientile et al. (2002) and observed by confocal laser scanning microscopy (CLSM).

Analysis by Confocal Laser Scanning Microscopy

The expression and distribution of TG-2 protein in cell compartments were investigated by confocal microscopic analysis after 1 hr of incubation of the control and treated astroglial cultures with 1% normal blocking serum in PBS, followed by incubation with an anti-TG-2 antibody (1:100 in PBS) for 1 hr and then with Texas Red–conjugated antimouse IgG (1:100 in PBS) for 1 hr.

Then, the control and treated cultures were briefly washed three times with PBS, mounted with 90% glycerol, and observed by CLSM.

First, the optical fields were visualized using a 40 × 1.0 NA oil immersion objective in a Leica DM IRB inverted fluorescence microscope (Leica Microsystems, Heidelberg, Mannheim, Germany). Then they were examined with a Leica TCS SP2 CLSM by green or red fluorophore excitation at 488 and 543 nm, using He/Neon and Ar/Kr laser sources. The fluorescence emitted from specimens was sent through a Leica acoustic optical turnable filter mechanism and pinhole to two different photomultiplier tubes. Image outputs electronically generated by the same parameter settings (frame scanning, pinhole aperture, gain voltage, pixels, and spatial resolution) were measured and compared for intensity (treated vs. control) after subtraction of nonspecific fluorescence.

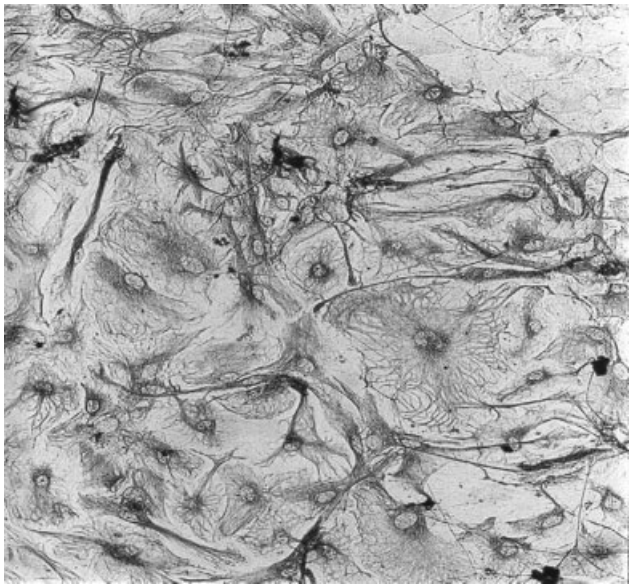

Fig. 1. GFAP-positive astrocyte cultures at 15 DIV; GFAP immunostaining analysis with immunoperoxidase using a biotinylated secondary antibody.

Finally, sequential optical sections, obtained using the CLSM *z*-axis stepping capability, were combined to form an extended depth-of-focus image, and standard image processing using Leica Confocal Software (version 2.0, build 0585) was performed to enhance brightness and contrast.

Statistical Analysis

All values are presented as means ± SEMs of data obtained from five dishes. The Student *t* test was used to compare values from densitometric analysis of the immunoblots. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Astroglial cultures at 15 DIV, that is, when confluent, were characterized by immunofluorescent staining with the glial marker GFAP, as previously reported (Avola et al., 1988a). About 90% of cells were GFAP positive, indicating that cultures were highly enriched in astrocytes (Fig. 1). Nonetheless, the other 10% were oligodendroglial, microglial, and ependymal cells showing very weak and not significant expression of TG-2.

Growth Factors and Steroids Increase TG-2 Levels in Cultured Astrocytes

We assessed TG-2 expression in 15 DIV astroglial cell cultures by Western blot analysis. Control cultures showed low levels of TG-2. Cultures treated for 24 hr with EGF, IGF-I, and INS after 24 hr of starvation showed a marked increase in TG-2 protein levels (Fig. 2A). Priming of the cultures with DEX per se for 48 hr induced increased TG-2 expression, albeit to a lesser extent than in the GF-treated

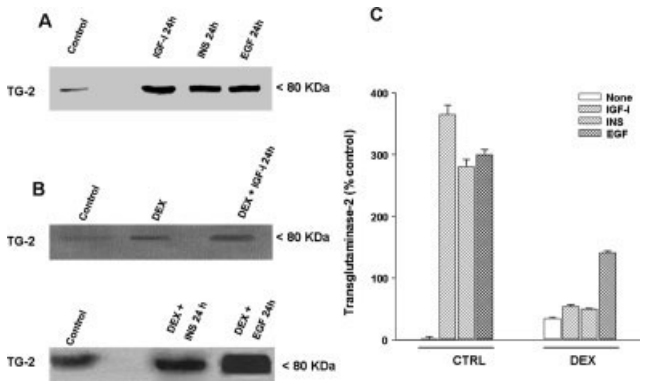

Fig. 2. Representative immunoblots showing increased expression of TG-2 in astrocytes exposed to IGF-I (10 ng/mL), INS (10 μg/mL), and EGF (10 ng/mL) for 24 hr (**A**) alone or during the last 24 hr of the 48-hr priming with 1 nM dexamethasone (DEX; **B**), as assessed by Western blot analysis. Thirty milligrams of proteins was loaded in each lane. **C:** Densitometric analysis of TG-2 expression was calculated as the percentage of increase over the control (untreated cultures); bars show mean ± SEM of 4–6 determinations ($n = 3$); $\star P < 0.05$ versus the respective control (Ctrl) by one-way ANOVA + Fisher's PLSD.

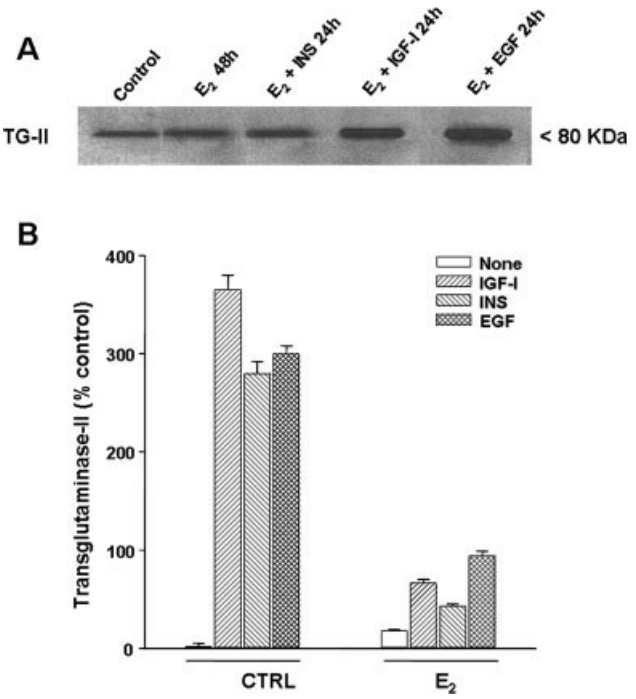

Fig. 3. Representative immunoblot showing increased expression of TG-2 in astrocytes exposed to IGF-I (10 ng/mL), insulin (INS, 10 μg/mL), and EGF (10 ng/mL) during the last 24 hr of the 48-hr priming with 5 nM 17β-estradiol (E_2; **A**), as assessed by Western blot analysis. Thirty milligrams of proteins were loaded in each lane. **C:** Densitometric analysis of TG-2 expression has been calculated as % increase over control (untreated cultures); bars represent mean ± SEM of 4–6 determinations ($n = 3$); $\star P < 0.05$ versus the respective control (Ctrl) by one-way ANOVA + Fisher's PLSD.

cultures. When cultures were primed with DEX followed by the addition of IGF-I or INS in the last 24 hr of steroid treatment, there was a dramatic reduction in the GF-induced increase in TG-2 levels (Fig. 2B). DEX pretreatment only resulted in blocking 50% of the effect of EGF on TG-2 expression (Fig. 2B). The results of densitometric analysis are expressed as the percentage of increase in TG-2 over that in the control (Fig. 2C).

We then assessed the effect of E_2 pretreatment on GF-induced TG-2 expression. We did not find any significant effect on TG-2 expression in 48-hr E_2-treated cultures; however, when E_2-treated cultures were exposed to IGF-I, INS, or EGF, the GF-induced increase in TG-2 expression was almost completely abolished. EGF treatment was least affected by E_2 treatment (Fig. 3A,B).

To further investigate the influence of GFs and steroids on TG-2 expression, we performed a CLSM analysis in order to evaluate both morphological changes in astrocytes in response to the treatments and the subcellular distribution of TG-2. Control cultures showed faint staining for TG-2, which was localized in the cytosol and mitochondria [Fig. 4B(a)], as confirmed by Western blot analysis of TG-2 in subcellular fractions obtained from treated astrocyte cultures (Fig. 8). A marked increase in TG-2 fluorescence levels was observed after a 24-hr exposure to IGF-I, INS, or EGF, as calculated from the relative fluorescence intensity values (Fig. 4A). In particular, in INS-treated cultures TG-2 was localized in the cytosol, mitochondria, and nuclei; in IGF-I-treated astrocytes, TG-2 distribution was prevalent in the cytosolic and mitochondrial compartments; whereas mitochondria, cytosol and cytosolic projections were stained when cultures were treated with EGF (not shown). Glial cultures pretreated for 48 hr with DEX showed a slight increase in the expression of TG-2 [Fig. 4B(b)]; in this condition, TG-2 staining was found in the cytosol and mitochondria and also in nuclei and nucleoli, as confirmed by Western blot analysis of TG-2 in subcellular fractions obtained from treated astrocyte cultures (see below). Moreover, the morphology of the astrocytes had changed, as they appeared smaller and round shaped. When cultures were challenged with DEX and IGF-I or EGF [Fig. 4B(c,d)], the GF-induced increase in TG-2 expression was reduced but remained higher than that in DEX-treated cultures; the protein was distributed only in the cytosol and mitochondria, as confirmed by Western blot data (see below); and cells appeared similar in shape to those in the control cultures (especially in the presence of IGF-I). When INS was added to DEX-treated cultures, the INS-induced increase in TG-2 levels was completely abolished, no change in cellular morphology compared with that of cells in DEX-treated cultures could be observed, and as with IGF-I and EGF, fluorescence was localized only in the cytosol and mitochondria [Fig. 4B(e)]. This last result was in agreement with the results of Western blot analysis of TG-2 in subcellular fractions (see below). In Figure 4C, the relative fluorescence intensity values are shown.

When glial cultures were pretreated for 48 hr with E_2, no significant modifications in TG-2 staining or cellular

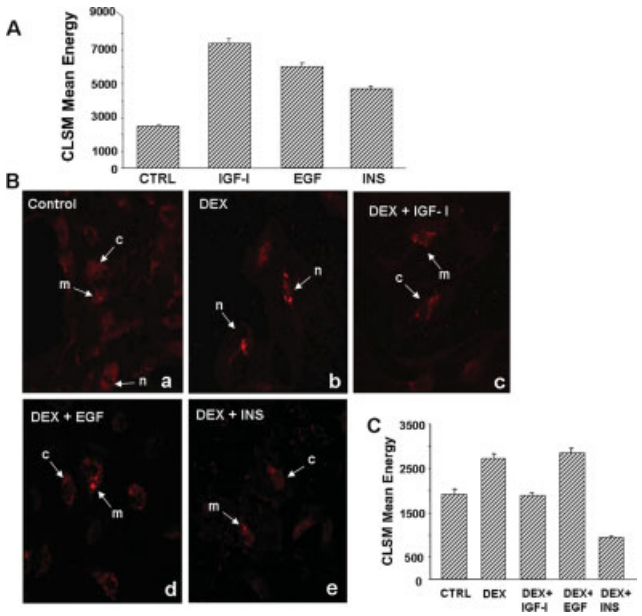

Fig. 4. Confocal laser scanning microscopic (CLSM) analysis of TG-2 expression in 15 DIV astrocyte cultures using anti-TG-2 antibodies and a Texas Red–conjugated antimouse secondary antibody. **A:** Relative florescence intensity (expressed as arbitrary units) in 15 DIV cultures exposed for 24 hr to IGF-I (10 ng/mL), INS (10 μg/mL), and EGF (10 ng/mL). Bars represent mean ± SEM of 9–12 determinations; $\star P < 0.05$ versus control (Ctrl) by one-way ANOVA + Fisher's PLSD). **B:** Representative CLSM images of TG-2 expression in untreated cultures (Ctrl, a), in cultures exposed to 1 nM DEX for 48 hr in the absence (b) or in the presence (in the last 24 hr) of IGF-I (c), EGF (d), or INS (e). (f) Relative fluorescence intensity values. Bars represent mean ± SEM of 9–12 determinations; $\star P < 0.05$ versus control (Ctrl) by one-way ANOVA + Fisher's PLSD. The white arrows show the exact fluorescence distribution of the protein in different subcellular compartments (c, cytoplasm; m, mitochondria; n, nucleus).

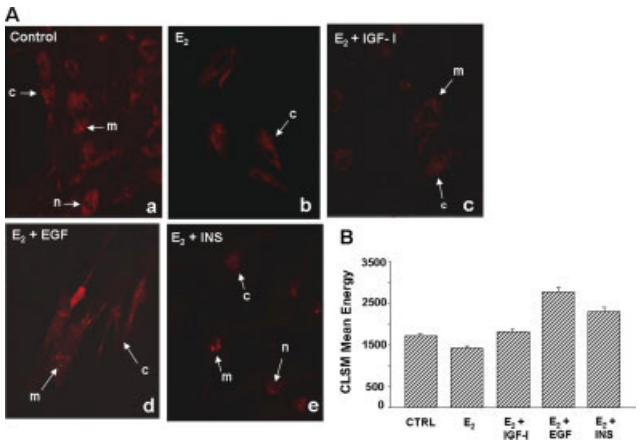

Fig. 5. Confocal laser scanning microscopic (CLSM) analysis of TG-2 expression in 15 DIV astrocyte cultures, using anti-TG-2 antibodies and a Texas Red–conjugated antimouse secondary antibody. **A:** Representative CLSM images of TG-2 expression in untreated (Ctrl) cultures (a), in cultures exposed to 5 nM E_2 for 48 hr, in the absence (b) or in the presence (in the last 24 hr) of IGF-I (c), INS (d), or EGF (e). (f) Relative fluorescence intensity values. Bars represent mean ± SEM of 8–12 determinations; $\star P < 0.05$ versus control (Ctrl) by one-way ANOVA + Fisher's PLSD. The white arrows show the exact fluorescence distribution of the protein in different subcellular compartments (c, cytoplasm; m, mitochondria; n, nucleus).

morphology was found compared with that in the controls (Fig. 5a,b). The E_2 treatment almost completely abolished the slight IGF-I-induced (Fig. 5c) and INS-induced (Fig. 5e) increases in TG-2 fluorescence levels, whereas TG-2 levels remained higher than in E_2-treated cultures when EGF was used (Fig. 5d). However, differences in TG-2 subcellular distribution were found among the different GFs, resembling what was observed in cultures treated only with GFs. In the presence of IGF-I, E_2-treated cultures showed TG-2 fluorescence with a punctuated pattern only in the cytosol and mitochondria, whereas in the INS condition, the same pattern was also found in the nuclei and nucleoli (Fig. 5c,d). This finding was confirmed by Western blot analysis of TG-2 in subcellular fractions (see below). In the presence of EGF, the protein was found to be distributed in the cytosol, mitochondria, and nuclei and also along cellular ramifications. This subcellular localization of TG-2 was confirmed by Western blotting (see below).

Furthermore, the cells had lengthened, probably because of an increased gap junctions (Fig. 5d). In Figure 5B, the relative fluorescence intensity values are shown.

Effect of Growth Factors and Steroids on TG-2 Activity

To assess whether TG-2 is functionally active in cultured astrocytes, we performed confocal microscopic analysis of total TGase activity, which was evaluated by how much DC was incorporated into individual astrocytes. Untreated astroglial cultures at 15 DIV showed low fluorescence, and TGase activity was primarily found in the cytosol and mitochondria [Fig. 6B(a)]. An increase in TG-2 activity was observed after a 24-hr exposure to IGF-I, INS, or EGF, as calculated from the relative fluorescence intensity (Fig. 6A). Forty-eight-hour pretreatment with DEX increased TGase activity [Fig. 6B(b)], which was cytosolic and mitochondrial, suggesting that DEX induced translocation of TG-2 in the nuclei and nucleoli [Fig. 5B(b)] or did not lead to an active form of the enzyme or may have led to a change in the function of the enzyme, which may have lost its transamidating action. Cells appeared round in shape, and no ramifications were visible. When cultures were cotreated with IGF-I [Fig. 6B(c)], TGase activity remained elevated compared with that in DEX-treated cultures, although it was lower than that in IGF-I-treated cultures; fluorescence was evident in the cytosol and mitochondria and also appeared in cellular projections. Moreover, cells regained their normal morphology. Also, in DEX+EGF-cotreated cells, the EGF-induced increase in TGase activity was reduced, although TGase activity remained higher than that in DEX-treated cultures [Fig. 6B(d)], and astrocytes showed distribution of fluorescence in the cytosol and mitochondria and along cellular ramifications and had a normal morphology. In addition, this treatment stimulated connections between astrocytes [Fig. 6B(d)]. In contrast, in

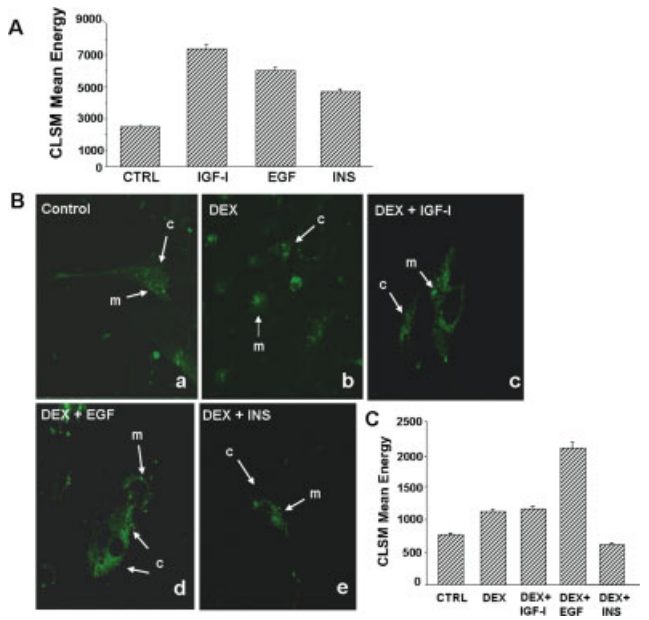

Fig. 6. Confocal laser scanning microscopic (CLSM) analysis of total TGase activity in 15 DIV astrocyte cultures, evaluated by the incorporation of monodansyl-cadaverine (DC) in a single cell. **A:** Relative fluorescence intensity (expressed as arbitrary units) in 15 DIV cultures exposed for 24 hr to IGF-I (10 ng/mL), INS (10 μg/mL), and EGF (10 ng/mL). Bars represent mean ± SEM of 7–9 determinations; $\star P < 0.05$ versus control (Ctrl) by one-way ANOVA + Fisher's PLSD. **B:** Representative CLSM images of TGase activity in untreated (Ctrl) cultures (a), in cultures exposed to 1 nM DEX for 48 hr, in the absence (b) or in the presence (in the last 24 hr) of IGF-I (c), EGF (d), or INS (e). (f) Relative fluorescence intensity values. Bars represent mean ± SEM of 8–10 determinations; $\star P < 0.05$ versus the control (Ctrl) by one-way ANOVA + Fisher's PLSD. The white arrows show the exact fluorescence distribution of the protein in different subcellular compartments (c, cytoplasm; m, mitochondria; n, nucleus).

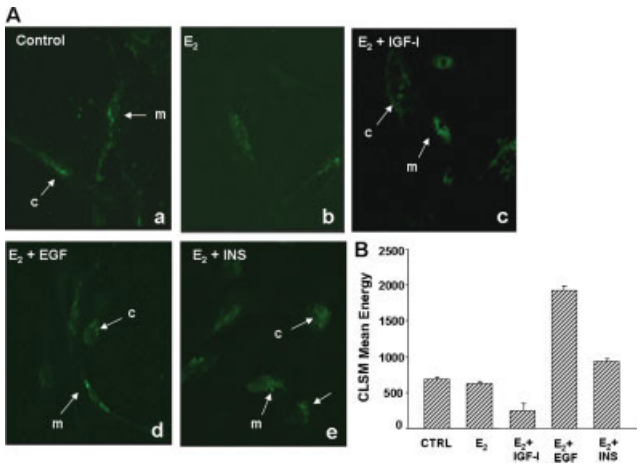

Fig. 7. Confocal laser scanning microscopic (CLSM) analysis of TGase activity in 15 DIV astrocyte cultures evaluated by incorporation of monodansyl-cadaverine (DC) in a single cell. **A:** Representative CLSM images of TGase activity in untreated (Ctrl) cultures (a), in cultures exposed to 5 nM E_2 for 48 hr in the absence (b) or presence (in the last 24 hr) of IGF-I (c), INS (d), or EGF (e). (f) Relative fluorescence intensity values. Bars represent mean ± SEM of 8–10 determinations; $\star P < 0.05$ versus control (Ctrl) by one-way ANOVA + Fisher's PLSD. The white arrows show the exact fluorescence distribution of the protein in different subcellular compartments (c, cytoplasm; m, mitochondria; n, nucleus).

DEX+INS-treated cells, the INS-induced increase in TGase activity was completely abolished, and the distribution of fluorescence appeared to be localized in the cytosol and mitochondria, where it was most prevalent [Fig. 6B(e)]. In Figure 6C, the relative fluorescence intensity values are shown.

When astroglial cultures were pretreated for 48 hr with E_2, a reduction in TGase activity was found [Fig. 7A(b)]. In E_2+EGF-treated astrocytes, the EGF-induced increase in TGase activity was reduced [Fig. 7A(d)], although levels remained higher than in the E_2 condition. Fluorescence localized in the cytosol and mitochondria and also in cellular projections. The same results were obtained with INS and IGF-I, although in the E_2+INS condition [Fig. 7A(e)], fluorescence appeared localized in the cytosol, mitochondria, and nuclei, whereas in E_2+IGF-I-cotreated cells, fluorescence was only localized in the cytosol and mitochondria [Fig. 7A(c)]. In Figure 7B, relative fluorescence intensity values are shown.

DISCUSSION

In the present study we assessed the possible interaction between steroids (E_2 and DEX) and GFs and the effects of this interaction on TG activity and expression in astroglial cells during differentiation in culture. It has recently been shown that the effects of E_2 and GFs on neuronal survival and regeneration are similar, suggesting that estrogens may exert their effects via signaling pathways common to GFs (Singer et al., 1999; Singh et al., 1999). In cerebral cortex explants, estrogen induces activation of mitogen-activated protein kinase (MAPK; Singh et al., 1999), and this pathway mediates estrogen neuroprotection from glutamate toxicity in primary cortical neurons (Singer et al., 1999). Both estrogen and IGF-I promote neuronal survival and differentiation in primary hypothalamic cultures (Azcoitia et al., 1999).

Some reports, demonstrating that physiologically relevant concentrations of E_2 can mimic the activity of EGF (Improta-Brears et al., 1999), support the hypothesis that estrogen-mediated activation of MAPK is likely to be biologically important.

Some intracellular signaling pathways triggered by E_2 may depend on insulin (Patrone et al., 1996) or bFGF and also may interact with neurotrophins and their receptors (Smith, 1998).

In addition, as estrogens have been reported to elicit tyrosine phosphorylation of the EGF receptor, some authors (Singh et al., 1999) addressed the possibility that exposure of cortical explants to E_2 may have first elicited tyrosine phosphorylation of Trk either directly or as a result of estrogen-induced endogenous neurotrophin release. Moreover, Singh et al. (1999) also observed the possibility that the ER may be part of a multimeric

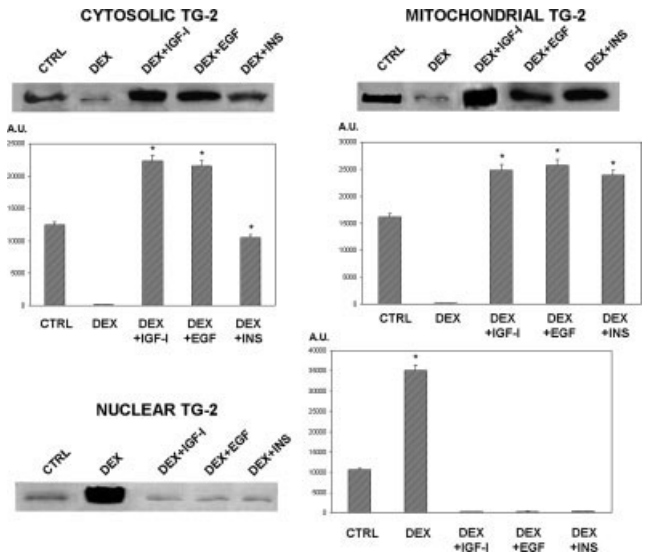

Fig. 8. Western blot analysis of TG-2 in subcellular fractions obtained from astroglial cell cultures treated with 1 nM DEX for 48 hr in the absence or in the presence (in the last 24 hr) of IGF-I, EGF, or INS. Densitometric analysis of TG-2 expression was calculated as the percentage of increase over the control (untreated cultures); bars show mean ± SEM of 4–6 determinations ($n = 3$); $^{\star}P < 0.05$ versus the respective control (Ctrl) by one-way ANOVA + Fisher's PLSD.

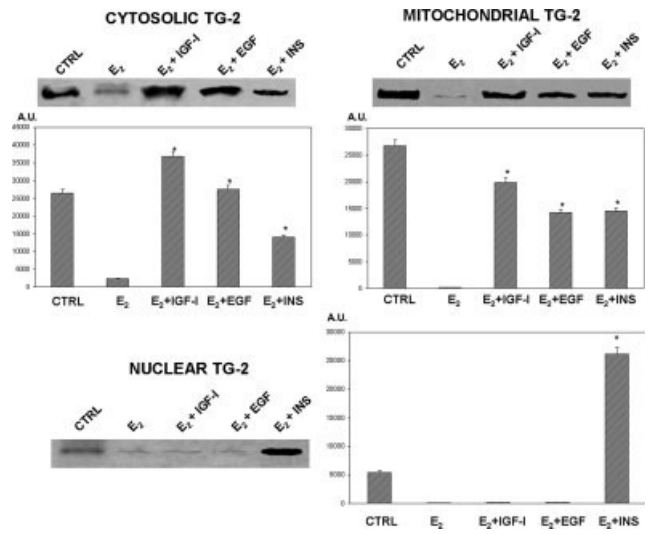

Fig. 9. Western blot analysis of TG-2 in subcellular fractions obtained from astroglial cell cultures exposed to 5 nM E_2 for 48 hr in the absence or in the presence (in the last 24 hr) of IGF-I, EGF, or INS. Densitometric analysis of TG-2 expression was calculated as the percentage of increase over the control (untreated cultures); bars show mean ± SEM of 4–6 determinations ($n = 3$); $^{\star}P < 0.05$ versus the respective control (Ctrl) by one-way ANOVA + Fisher's PLSD.

complex consisting of at least B-Raf, MEK1, and HSP90. Such a putative multimeric complex could serve as an intracellular junction linking the estrogen and trophic factor signaling pathways.

On the other hand, previous studies have shown that various factors are able to increase TG-2 expression depending on the species and cell type (Katoh et al., 1996). It has been reported that TG-2 expression can be up-regulated by interleukin-6 in human hepatoblastoma HepG2 cells (Suto et al., 1993), by interleukin-1B in rat astrocytes (Monsonego et al., 1997), and by the glucocorticoid dexamethasone in hamster fibrosarcoma cell lines (Lesort et al., 2000).

Our results concerning the priming of the cultures with DEX for 48 hr demonstrated a significant increase in TG-2 expression, albeit to a lesser extent than in GF-treated cultures; in particular, TG-2 staining was found in the cytosol and mitochondria and also in nuclei and nucleoli, demonstrating TG-2 translocation to different subcellular compartments, where it exerted a differentiating effect. These findings were confirmed by Western blot analysis of TG-2 in subcellular fractions, as shown in Figures 8 and 9 for subcellular fractionation data.

Furthermore, DEX-primed astrocyte cultures treated with IGF-I or INS in the last 24 hr of steroid pretreatment showed a significant reduction in the GF-induced increase in TG-2 level.

CLSM analysis showed protein localization only in the cytosol and mitochondria, suggesting important involvement of TG-2 also in the energy transduction pathway and in mitochondrial calcium pumping.

In addition, no significant effect on TG-2 expression in 48-hr E_2-treated cultures was found; however, the E_2 treatment of cultures exposed to IGF-I, INS, or EGF completely abolished the GF-induced increase in TG-2 expression, demonstrating that EGF treatment was least affected by E_2 treatment.

CLSM analysis showed localization of TG-2 in the cytosol and mitochondria of control cultures. In particular, a slight increase in TG-2 fluorescence levels was observed after a 24-hr exposure to IGF-I, INS, or EGF in E_2-treated cultures.

In E_2+EGF-cotreated cultures, TG-2 was found to be distributed in the cytosol, mitochondria, and nuclei and also along the elongated areas of the cells, probably because of the increase in gap junctions.

In the presence of IGF-I, E_2-treated cultures showed TG-2 fluorescence with a punctuated pattern only in the cytosol and mitochondria, as previously explained, whereas in the INS condition, the same pattern was also present in nuclei and nucleoli.

On the basis of subcellular localization of TG-2, the protein nuclear translocation caused by GF treatment may be associated with a protective role following increasd TG2 expression. Indeed, there may be different substrates for TG-2 in the nucleus, and the activity of TG-2 is multifunctional (Campisi et al., 2003). However, it is possible to hypothesize that GF stimuli promoted nuclear translocation in the functions of cell survival and differentiation.

In E_2-pretreated astroglial cell cultures, TG-2 activity and expression appeared to be reduced in comparison with that in the controls, and the protein was primarily

localized in the cytosol and mitochondria. The addition of both EGF and IGF-I increased TG-2, but the protein remained in the cytosol and mitochondria, indicating this protein has the same implications in energetic and ion-pumping processes.

In contrast, the addition of INS in E_2-treatred astroglial cell cultures caused nuclear translocation of TG-2 and might lead to alternative mechanisms of action of TG-2.

The addition of GFs (Katoh et al., 1996) increases Ca^{2+} and in turn TG-2, and both p53 and TG-2 can be colocalized in nuclear compartments (Mishra and Murphy, 2006).

Other reports (Milakovic et al., 2004) clearly demonstrate that the transamidating activity of TG-2 is essential for its proapoptotic effects, and nuclear localization of TG-2 is protective against apoptosis when it is not active as a transamidating enzyme. The antiapoptotic effects of TG-2 in the nucleus may be in the function of its increased interaction with nuclear retinoblastoma.

In conclusion, our results demonstrate that the balance between corticosteroids/estrogens and GFs may affect astrocyte function and morphology through a modulatory role of TG-2 on various transcription signaling factors and other activities of this multifunctional protein.

Recent findings have provided evidence that dysregulation of TG-2 may contribute to the pathology of several neurodegenerative conditions including Alzheimer's and Huntington's diseases. Although intriguing, many issues remain to be addressed to definitively establish a role for TG-2 in these neurodegenerative diseases.

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