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The α1 Subunit of $GABA_A$ Receptor Is Repressed by c-Myc and is Pro-Apoptotic

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Abstract The c-myc oncoprotein plays a critical role in the regulation of cellular proliferation and apoptosis. To mediate these biological functions, a variety of target genes are activated or repressed by c-myc, but few genes have yet been identified that directly mediate c-myc's role in proliferation or apoptosis. During a screen for genes that are repressed by c-myc, we identified the α1 subunit of γ aminobutyric acid receptor ($GABA_AR$-α1) as a novel target of c-myc. $GABA_AR$ is the major inhibitory neurotransmitter receptor in the mammalian central nervous system and is involved in developmental events in the brain, such as neurite outgrowth, neuronal survival, neuronal migration, and proliferation. We show here that $GABA_AR$-α1 expression is rapidly and directly repressed by c-myc. $GABA_AR$-α1 expression is elevated in *c-myc* null cells and upregulation of $GABA_AR$-α1 correlates with downregulation of c-myc protein expression during neuronal cell differentiation. We also show that overexpression of $GABA_AR$-α1 causes apoptosis, which is blocked by the coexpression of Bcl-2 or Bcl-X_L. Induction of apoptosis is specific for the α1 subunit, since neither the β1 or β2 subunits of $GABA_AR$ induced apoptosis. Derepression of $GABA_AR$-α1 expression upon downregulation of c-myc represents a unique apoptotic mechanism and a distinct function for the α1 subunit, independent of its role as a component of the $GABA_AR$ in the plasma membrane. In addition, the regulation of $GABA_AR$-α1 expression by c-myc provides a potential direct role for the Myc proteins in neurological processes and neurodegenerative disorders. J. Cell. Biochem. 97: 1094–1103, 2006.

Key words: c-Myc; $GABA_A$ receptor; apoptosis; gene repression

The *c-myc* proto-oncogene is a critical regulator of cellular proliferation and apoptosis. Deregulated c-myc expression is found in a variety of cancers in many species, including humans [Nesbit et al., 1999]. Its role in cellular proliferation is demonstrated by the slow proliferation rate of *c-myc* null fibroblasts, and the increased proliferation and anchorage-independent growth caused by c-myc overexpression [Oster et al., 2002]. In the absence of survival factors, c-myc causes apoptosis [Oster et al., 2002]; however, in some cell types the downregulation of c-myc induces apoptosis [Thompson, 1998]. While c-myc functions as a transcriptional regulator, it is not known whether the activation and/or repression of c-myc target genes mediate these diverse functions.

Using differential display, we identified the α1 subunit of γ aminobutyric acid receptor ($GABA_AR$-α1) as a gene that is repressed by c-myc. $GABA_AR$ is the major inhibitory neurotransmitter receptor in the mammalian central nervous system [Lambert and Grover, 1995; Whiting et al., 1995]. The receptor is a pentameric complex built from a pool of at least 19 different subunits, which can be grouped into six subfamilies (α, β, γ, δ, ε, ρ). These subunits form a chloride channel, which is allosterically regulated by GABA and other natural and synthetic agents [Macdonald and Olsen, 1994]. $GABA_AR$ is involved in a variety of developmental events in the brain, such as neurite outgrowth, neuronal survival, and migration

Abbreviations used: $GABA_AR$-α1, alpha 1 subunit of γ aminobutyric acid receptor; OHT, 4-hydroxytamoxifen, DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; FCS, fetal calf serum.

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[Luk and Sadikot, 2001; Maric et al., 2001]. During development, changes in subunit composition of GABA$_A$R determine the pharmacological properties and the function of the receptor [McKernan and Whiting, 1996; Carlson et al., 1998]. The mechanism(s) that regulate the expression of GABA$_A$R subunit expression are unknown. We show here that the α1 subunit of GABA$_A$R is directly repressed by c-myc. We also show that overexpression of GABA$_A$R-α1, but not the β1 and β2 subunits, leads to induction of apoptosis. This novel pro-apoptotic role of GABA$_A$R-α1 is independent of its role as a subunit of GABA$_A$R in the plasma membrane.

MATERIALS AND METHODS

Cell Lines and Plasmids

The construction of pBabe-MycER retroviral vectors and CMV-c-myc has been described previously [Xiao et al., 1998; Gregory et al., 2003]. HO16 (c-*myc* null) cells were grown in DMEM supplemented with 1 mM sodium pyruvate and 10% fetal calf serum (FCS) and 250 μg/ml neomycin. Rat1a, TGR (HO16 parental Rat1 cells), NIH-3T3 cells, and NIH-3T3 cells overexpressing Bcl-2 or Bcl-X$_L$ were grown in DMEM supplemented with 10% calf serum (CS). To obtain stable cell lines, HO16 cells were infected with viral supernatant from ψ2 packaging cells containing pBabe-Myc2ER or pBabe-MycSER and selected with 2.5 μg/ml puromycin as described previously [Xiao et al., 1998]. NIH-3T3 cells were transiently transfected with 2 μg CMV-GABA$_A$R-α1 expression vector using TransFectin (Bio-Rad) in DMEM with 10% CS. Cells were analyzed 48 h after transfection. P19 embryonal carcinoma cells (ATCC) were grown in alpha MEM (Invitrogen) supplemented with 10% FCS and induced to differentiate with 0.5 μM retinoic acid (Sigma) for the indicated times as described elsewhere [McBurney et al., 1998]. GABA$_A$R-α1 promoter (−747 to −142 relative to the ATG start site) was PCR amplified from mouse genomic DNA (Sanger Institute) using 5′-GAGGACAGAGCGCTCTCC-3′ as the sense primer and 5′-CACTCACACG-GTCTGAGG-3′ as the antisense primer. The ΔE-Box promoter (−721 to −142 relative to the ATG) was amplified using 5′-GGTACCAAGCC-TACATCTCCCTGC-3′ as the sense primer. The amplified promoter regions were then subcloned into pGL3-basic Firefly Luciferase reporter vectors (Promega).

RNA Isolation and Northern Blot Analysis

Total RNA was isolated using Trizol (Invitrogen). Ten micrograms of RNA was separated on 1% agarose-2% formaldehyde denaturing gels and transferred to a nitrocellulose membrane (Nytran, Schleicher & Schuell), which was then UV cross-linked and pre-hybridized with ExpressHyb (Clontech), and hybridized with probe. Probes were labeled with [^{32}P]-dCTP (ICN) with Prime-It II random primer labeling kit (Stratagene). Blots were probed with GABA$_A$-α1 cDNA from the pcDNA-GABA$_A$R-α1 vector.

Western Blot Analysis

Cells lysates were prepared and Western blot analysis was performed as described previously [Gregory et al., 2003]. Briefly, cells were lysed in Ab lysis buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% Triton X-100, 0.5% DOC, and 0.5% SDS, 1 mM EDTA) and proteins were resolved by 15% SDS–PAGE and subjected to immunoblot analysis using anti-c-mycfl (06-340; Upstate) or anti-actin (I19: Santa Cruz) and enhanced chemiluminescence for detection (Western Lightning, NEN) according to the manufacturer's instructions.

Differential Display Analysis

HO16 cells expressing c-mycSER or empty pBabe vector alone were treated with 2 μM hydroxytamoxifen (OHT) for 1 or 4 h. Two microgram total RNA harvested from these cells was subjected to first strand cDNA synthesis using RNAimage kit (GenHunter) according to manufacturer's protocol. The cDNA was used as a template for PCR amplification using random primers and oligo dT with one base anchor provided in the kit. PCR amplification was carried out in the presence of 0.25 μl [^{33}P]dATP (2,000 Ci/mmole; ICN), then PCR products were separated on a 6% denaturing polyacrylamide gel.

Immunofluorescence

The indicated cells were grown on glass coverslips, fixed and permeablized as described previously [Gregory et al., 2003]. Cells were incubated with anti-GABA$_A$R-α1 (Santa Cruz or Upstate) or anti active-caspase-3 (BD Pharmingen), and then incubated with the appropriate fluorescent-labeled secondary antibodies, Alexa Fluor 594 donkey anti-rabbit, or AlexaFluor

488 donkey anti-goat; (Molecular Probes). Fluorescence microscopy was performed as described previously using a 40X objective [Gregory et al., 2003].

Reporter Assay

Four micrograms of either $GABA_AR$-α1-luc reporter or $GABA_AR$-α1-ΔE-Box-luc reporter were cotransfected with 4 μg of CMV-c-myc or empty vector into NIH-3T3 cells. pRL-TK (500 ng) was included as an internal control. Luciferase assays were carried out according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System; Promega). Results were normalized for expression of pRL-TK as measured by Renilla luciferase activity. Readings were equalized for thymidine kinase promoter activity used as an internal control. Luciferase activity from cells transfected with reporter gene alone was standardized to 100%. Normalized values from triplicate samples were reported as the mean ± SD Each assay is representative of at least three independent experiments.

Quantitative Real-Time PCR (qPCR)

qPCR was performed using an iCycler (BioRad) and an Quantitect SYBR green PCR kit (Qiagen). Primers were designed by Primer Express software (ABI prism) directed to generate a 100 bp amplicon. First strand cDNA was generated using Omniscript RT kit (Qiagen). The amplification of a single PCR product was verified by 1.5% agarose gel electrophoresis and by melting curve analysis. Fold induction was calculated as described elsewhere [Pfaffl, 2001]. Briefly, amplification efficiency of both $GABA_A$ R-α1 and β actin primer pairs were determined by generating a linear standard curve, using the primers with increasing dilutions of the respective linear cDNA in qPCR. Crossing points of each dilution was plotted against the log of template concentration. Analysis of the $GABA_A$ R-α1 and β actin expression ratio was performed as described elsewhere [Pfaffl, 2001]. Each assay is representative of at least three independent experiments.

RESULTS

Identification of the α1 Subunit of $GABA_A$ as a c-Myc-Repressed Gene

To identify target genes that are regulated by c-myc, we used differential display analysis. To increase the probability of identifying genes that are repressed by c-myc we used c-mycS, an N-terminally truncated alternative translational form that lacks two-thirds of the transcriptional regulatory domain (TRD). We had previously shown that c-mycS cannot transactivate, yet is able to transrepress transcription and retains most of the biological functions of the full-length c-myc [Xiao et al., 1998]. To eliminate the influence of endogenous c-myc activity, we used Rat1 fibroblasts with homozygous deletion of *c-myc* (HO16) [Hanson et al., 1994]. The activity of c-mycS was controlled in these cells using the inducible chimera c-mycSER (c-myc fused to a modified estrogen receptor). The c-mycER protein is constitutively expressed in these cells, but is inactive until hydroxytamoxifen (OHT) is added. c-mycSER expression was confirmed by Western blot analysis and by immunofluorescence (data not shown). Translocation of c-mycSER to the nucleus, enhanced proliferation, and apoptosis in low serum was observed following treatment with OHT, confirming that c-myc was activated (data not shown).

We examined changes in gene expression at early times (1 and 4 h) after OHT-induction of c-mycSER activity to increase the probability of identifying direct target genes. Differential display PCR products amplified from cells after c-mycSER activation were compared to those of control cells with empty vector. The differentially expressed genes were further analyzed by Northern blot analysis. There was a dramatic difference in expression of one of those genes in cells with activated c-mycSER compared to cells with empty vector (Fig. 1a). A BLAST database search revealed that this gene is the α1 subunit of γ aminobutyric acid receptor ($GABA_AR$-α1). To characterize the kinetics of the repression of $GABA_AR$-α1 by c-mycS, its expression was monitored over time following c-mycSER activation in c-myc null cells. We found that $GABA_AR$-α1 mRNA is expressed at high levels in untreated HO16.c-mycSER cells and drops 2.5-fold within 1 h and fivefold after 26 h of activation of c-mycSER (Fig. 1b). $GABA_AR$-α1 mRNA levels also dropped proportionally with increasing concentrations of OHT after 4 h of treatment (Fig. 1c). Full-length c-myc (c-myc2) also repressed $GABA_AR$-α1 expression with or without the ER fusion (data not shown). Taken together these data suggest that $GABA_AR$-α1 mRNA expression is rapidly repressed by c-myc in a dose-dependent manner.

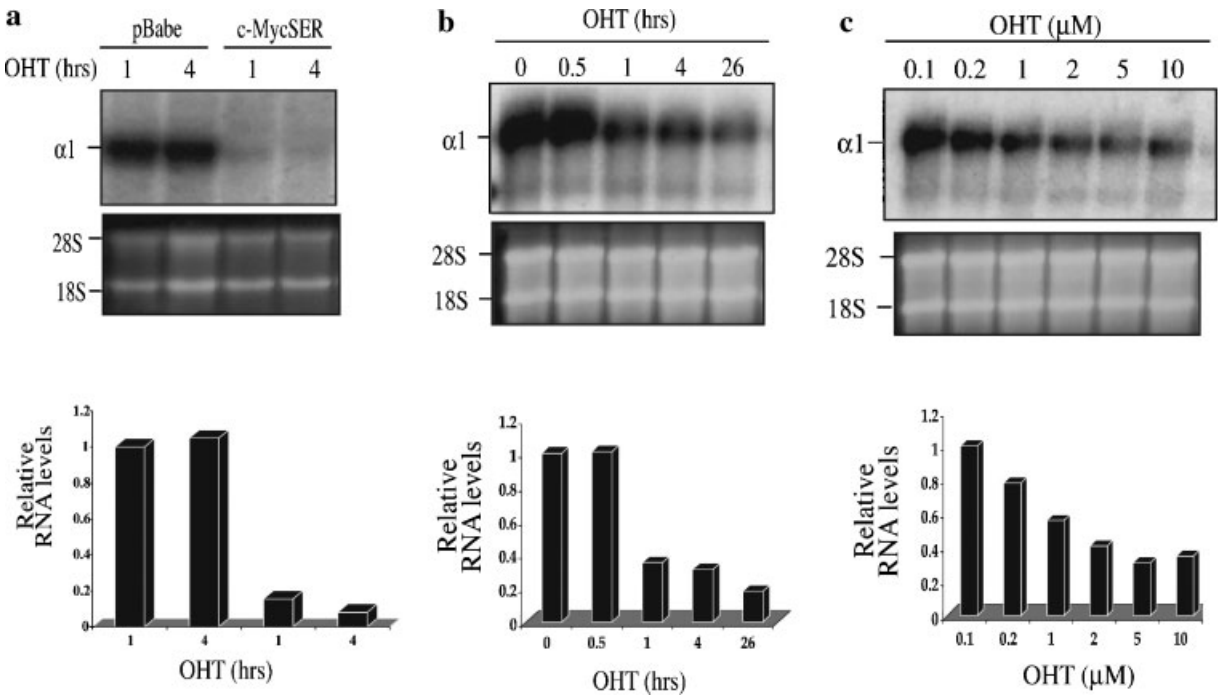

Fig. 1. GABA$_A$R-α1 mRNA expression is repressed by c-Myc. **a**: HO16 cells (*c-myc* null fibroblasts) stably expressing either c-MycSER or empty vector alone were analyzed for GABA$_A$R-α1 mRNA expression following treatment with 2 μM hydroxytamoxifen (OHT) for 1 or 4 h by Northern blot as described in the Experimental Procedures. Equal loading was verified by ethidium bromide staining of the gels. **b**: HO16 cells expressing c-MycSER were analyzed for GABA$_A$R-α1 mRNA expression following treatment with 2 μM OHT for the indicated times by Northern blot. Equal loading was verified by ethidium bromide staining of the gels. **c**: HO16 cells expressing c-MycSER were analyzed for GABA$_A$R-α1 mRNA expression following treatment with increasing concentrations of OHT for 4 h by Northern blot. Equal loading was verified by ethidium bromide staining of the gels. The bar graphs represent densitometric scanning analysis of the data.

Expression of GABA$_A$R-α1 mRNA Inversely Correlates With Endogenous c-Myc Expression

To determine whether repression of GABA$_A$ R-α1 by c-myc is an effect of c-myc overexpression, we examined GABA$_A$R-α1 levels in cells expressing endogenous c-myc compared to c-myc null cells. Northern blot analysis revealed that GABA$_A$R-α1 expression is high in cells without c-myc (HO16), while the parental Rat1 cells (TGR), Rat1a, and mouse NIH-3T3 cells showed low expression (Fig. 2a). Since GABA$_A$R-α1 is normally restricted to neuronal cells, we also wanted to examine its expression in a neuronal-like cell line. Previous reports demonstrated that c-myc expression is downregulated during differentiation of P19 embryonal carcinoma cells into neuronal-like cells by retinoic acid [St-Arnaud et al., 1988]. We examined whether this loss of c-myc expression during differentiation of P19 cells correlates with upregulation of GABA$_A$R-α1 expression. Western blot analysis of differentiating P19 cells confirmed that c-myc is dramatically downregulated to undetectable levels within 2 days after retinoic acid treatment (Fig. 2b). In contrast, c-myc mRNA shows a biphasic response (data not shown), suggesting post-translational regulation. To monitor GABA$_A$R-α1 mRNA levels during P19 differentiation, we used quantitative real-time PCR analysis (qPCR). As shown in Figure 2b, GABA$_A$R-α1 mRNA expression increased 10.5-fold after 4 days of retinoic acid treatment, and 12.5-fold after 6 days, compared to untreated cells. This increase in GABA$_A$R-α1 mRNA levels correlated well with the decrease in c-myc protein expression during differentiation of P19 cells. Taken together, these results suggest that GABA$_A$R-α1 expression is regulated by endogenous c-myc.

GABA$_A$-α1 Is a Direct Target of Repression by c-Myc

To test whether GABA$_A$R-α1 mRNA is directly repressed by c-myc, we used two different approaches. First, we determined whether

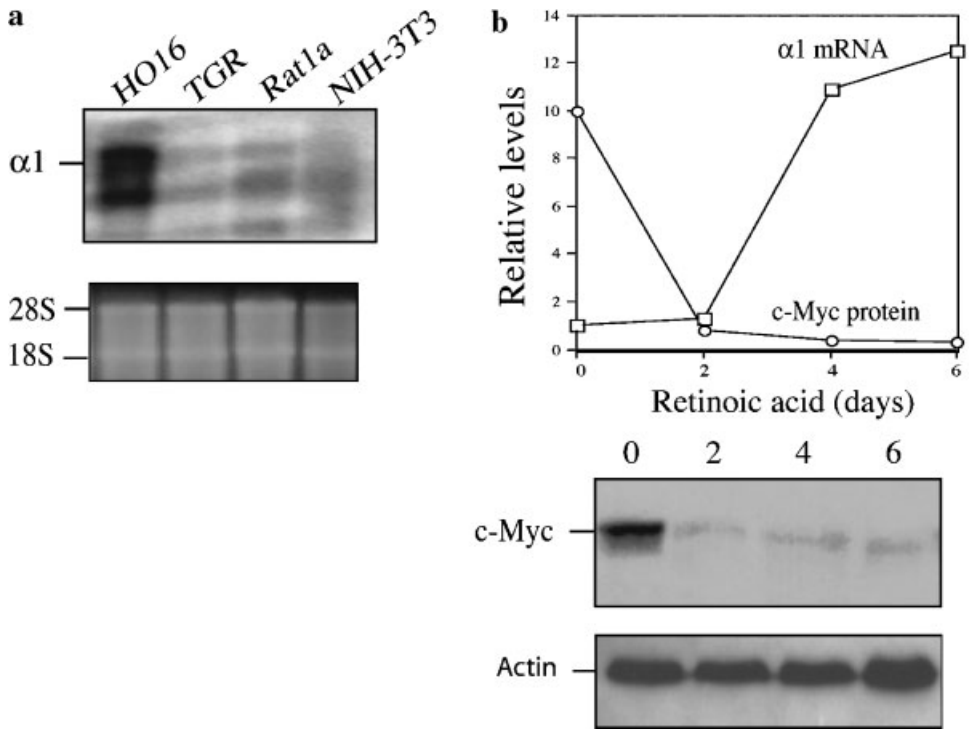

Fig. 2. Endogenous $GABA_AR$-α1 mRNA expression is inversely correlated with endogenous c-Myc protein levels. **a**: $GABA_AR$-α1 expression was determined by Northern blot analysis in HO16 *c-myc* null cells, TGR, NIH-3T3, and Rat1a. Equal loading was verified by ethidium bromide staining of the gels (**bottom panel**). **b**: P19 embryonal carcinoma cells were induced to differentiate to neuronal like cells with 0.5 μM retinoic acid for the indicated days. The cells were then subjected to Western blot analysis of c-Myc protein or actin as a loading control (**lower panels**) and quantitative real-time PCR (qPCR) analysis of $GABA_AR$-α1 mRNA as described in the Experimental Procedures. A schematic representation of the relative expression levels of $GABA_AR$-α1 mRNA and c-Myc protein in differentiating P19 cells is shown.

$GABA_AR$-α1 repression by c-myc requires new protein synthesis. Northern blot analysis of $GABA_AR$-α1 in c-myc null cells expressing c-mycER, revealed that $GABA_AR$-α1 mRNA levels were repressed upon c-mycER activation in the absence or presence of cycloheximide (Fig. 3a). The inhibition of protein synthesis by cycloheximide was confirmed by metabolic labeling of the cells (data not shown). In addition, we examined whether c-myc is capable of repressing the $GABA_AR$-α1 promoter in a reporter assay. Overexpression of c-myc with $GABA_AR$-α1-luc reporter in NIH-3T3 cells resulted in a threefold repression of promoter activity compared to cells with empty vector (Fig. 3b). A smaller promoter fragment without the canonical E-Box c-myc binding site was still repressed threefold by c-myc, although the basal activity of this smaller promoter fragment was significantly lower. This suggests that the canonical E-Box c-myc binding site is not involved in the repression of $GABA_AR$-α1, but may be involved in basal transcription. Taken together, these results indicate that the $GABA_AR$-α1 promoter is directly repressed by c-myc at a site within −441 to +159 relative to the start site of transcription.

$GABA_A$-α1 Induces Apoptosis

To determine whether there is an effect of constitutive expression of the α1 subunit of $GABA_AR$ on proliferation or apoptosis, we attempted to generate NIH-3T3 cell lines stably expressing $GABA_AR$-α1. However, we were unable to generate stable cell lines expressing $GABA_AR$-α1, suggesting that $GABA_AR$-α1 caused growth inhibition or apoptosis. To determine whether the cells expressing $GABA_AR$-α1 were undergoing apoptosis, we performed immunofluoresence analysis to detect apoptosis in transient transfections using a caspase-3 antibody that is specific for the active form. Following transfection of NIH-3T3 cells, we found high levels of active caspase-3 in cells expressing $GABA_AR$-α1 (Fig. 4a). More than 90% of the cells overexpressing $GABA_AR$-α1 expressed caspase 3. Cells transfected with the empty vector showed no detectable active

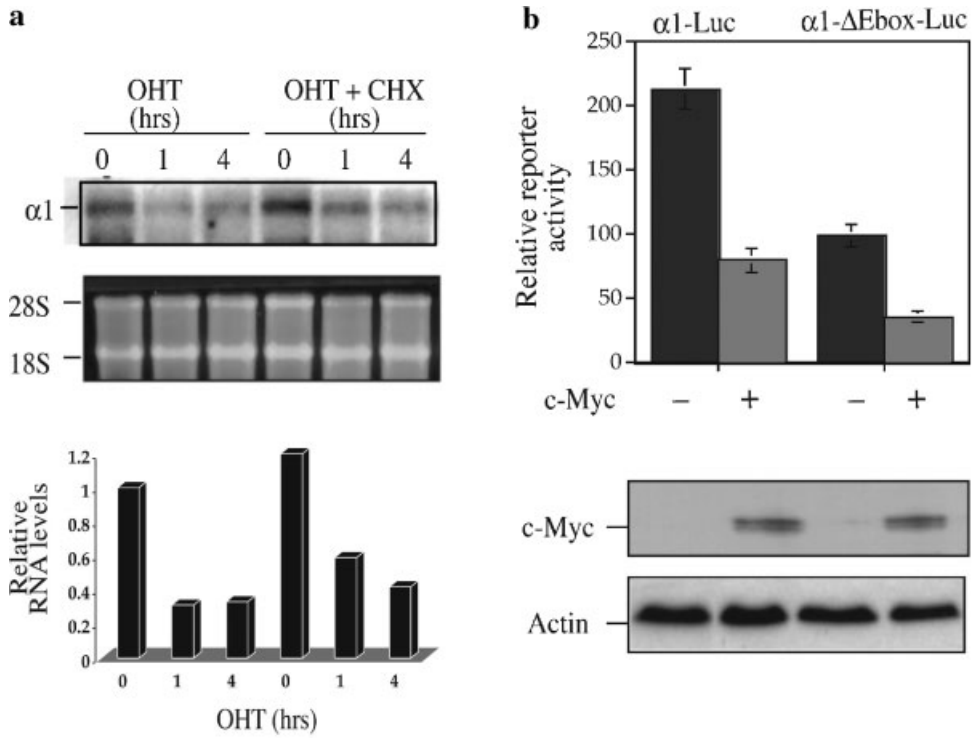

Fig. 3. GABA$_A$R-α1 expression is directly repressed by c-Myc. **a**: HO16 cells expressing c-MycER were pretreated with 50 μg/ml cycloheximide for 5 min to inhibit protein synthesis. c-MycER was then activated with 2 μM hydroxytamoxifen for 1 or 4 h and GABA$_A$R-α1 mRNA expression was analyzed by Northern blot analysis. The bar graph represent densitometric scanning analysis of the data. **b**: GABA$_A$R-α1-luc or GABA$_A$R-α1-ΔE-Box-luc reporter vectors were coexpressed with CMV-c-Myc or empty vector and a thymidine kinase control promoter. Luciferase activity was equalized to thymidine kinase promoter activity and relative reporter activity was measured in triplicates. The cells were also subjected to Western blot analysis of c-Myc protein or actin as a loading control (**lower panels**).

caspase-3 (data not shown). The induction of apoptosis by GABA$_A$R-α1 appears to be specific, since transfection of either the β1 or the β2 subunit of GABA$_A$R did not result in activation of caspase-3 (Fig. 4a). Identical results were found with the neuronal-like P19 cell line (Fig. 4b), suggesting that the apoptosis induced by the α1 subunit is not cell type specific.

To determine whether the anti-apoptotic factors, Bcl-2 or Bcl-X$_L$, can protect cells from apoptosis caused by GABA$_A$R-α1, we transfected GABA$_A$R-α1 into NIH-3T3 cells constitutively overexpressing either Bcl-2 or Bcl-X$_L$. Expression of GABA$_A$R-α1 in either of these cell lines did not result in apoptosis as assessed by the absence of caspase-3 activation (Fig. 5a,b), indicating that both Bcl-2 and Bcl-X$_L$ are able to block GABA$_A$R-α1-induced apoptosis. The ability of Bcl-2 and Bcl-X$_L$ to block GABA$_A$R-α1-induced apoptosis allowed the generation of stable cell lines constitutively expressing GABA$_A$R-α1. The GABA$_A$R-α1/Bcl-2 expressing cells did not show any discernable morphological or proliferation changes. The α1 subunit was not localized to the plasma membrane (Fig. 5), as previously shown [Connolly et al., 1996]. Taken together, these data demonstrate that GABA$_A$R-α1 has a role in mediating apoptosis that is independent of its role as a subunit in the GABA$_A$R.

DISCUSSION

Transcriptional Repression of GABA$_A$R-α1 by c-Myc

Several lines of evidence suggest that the α1 subunit of GABA$_A$R is repressed by c-myc. In addition to the inverse correlation of their expression during the differentiation of neuronal-like P19 cells, expression of *c-myc* and GABA$_A$R-α1 have opposing patterns of expression in adult tissues and during development. The expression of GABA$_A$R-α1 is highest in the brain, whereas c-myc expression is absent in the adult brain [Vaknin and Hann, unpublished observations; Zimmerman et al., 1986; Brooks-Kayal

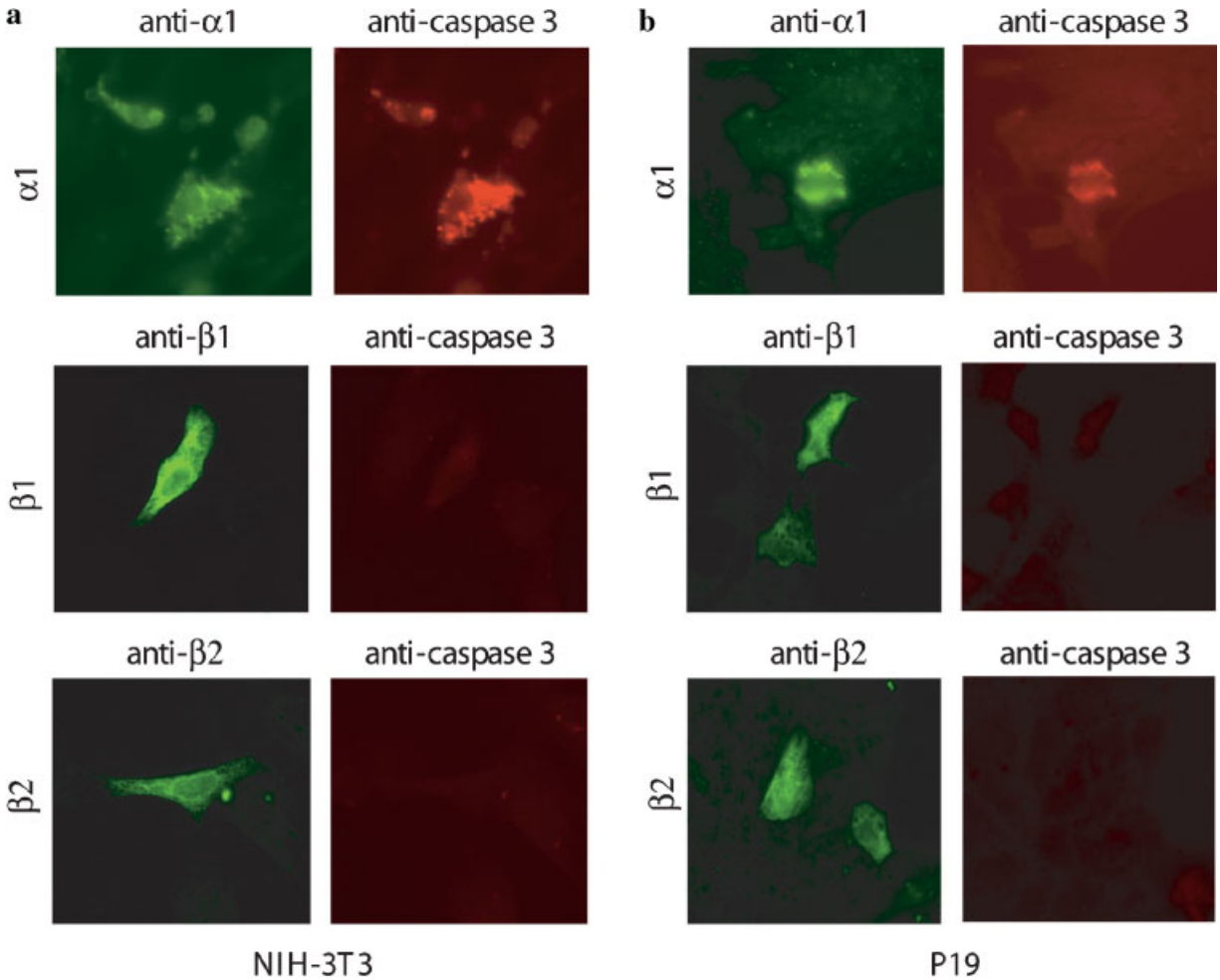

Fig. 4. $GABA_AR$-α1 induces apoptosis. NIH-3T3 (**a**) and P19 (**b**) cells were transiently transfected with CMV-$GABA_AR$-α1. The cells were fixed and costained with antibodies against $GABA_AR$-α1 (green) and caspase-3 (red). As a control, two other $GABA_AR$ subunits, β1 and β2, were transfected and probed for caspase-3 activity.

and Prichett, 1993]. During embryogenesis c-myc mRNA expression in the cerebellum is very high, while shortly after birth it drops to very low levels [Ruppert et al., 1986]. In contrast, $GABA_AR$-α1 is expressed at low levels before birth in the cerebellum and significantly increases after birth [Brooks-Kayal and Prichett, 1993].

We have shown that the repression of $GABA_AR$-α1 by c-myc is rapid, occurs directly without the need for protein synthesis and occurs at the promoter level. The relatively high expression of $GABA_AR$-α1 in fibroblasts upon ablation of c-myc was unexpected, since these cells have little or no detectable $GABA_AR$-α1 expression. The absence of $GABA_AR$-α1 in non-neuronal cells appears to be due to active transcriptional repression, rather than a silencing genetic event. This dynamic regulation of $GABA_AR$-α1 also appears to be controlled by factors other than c-myc, since we have found that its expression varies during proliferation of the c-myc null fibroblasts (Vaknin and Hann, unpublished observations). While there have been several mechanisms proposed for both the activation and repression of target genes by c-myc, the mechanisms are not well understood. Activation of target gene expression is known to occur through direct binding of c-myc/Max to a canonical E-box DNA binding site present in promoters [Oster et al., 2002]. Although $GABA_AR$-α1 does have a canonical E-box c-myc DNA binding site, deletion of this sequence did not have any effect of the ability of c-myc to repress $GABA_AR$-α1, although it did lower basal promoter activity. Repression of some target genes

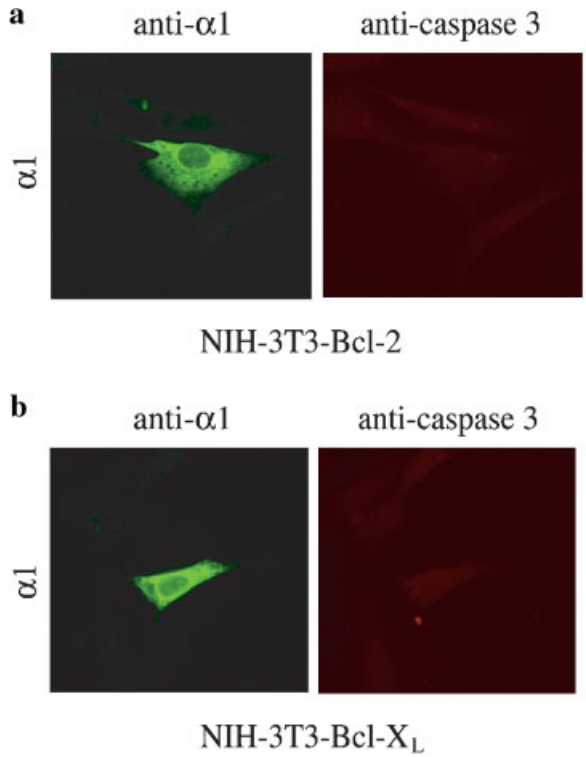

Fig. 5. GABA$_A$R-α1-induced apoptosis is blocked by Bcl-2 and Bcl-X$_L$. NIH-3T3 cells overexpressing pBabe-Bcl-2 (**a**) or pBabe-Bcl-X$_L$ (**b**) were transiently transfected with CMV- GABA$_A$R-α1. The cells were then fixed and costained for GABA$_A$R-α1 and active caspase-3 as described above.

by c-myc appears to be through an Initiator (Inr) element [Oster et al., 2002], but since the GABA$_A$R-α1 promoter sequence that is repressed by c-myc does not contain Inr elements, it appears that it is repressed by an Inr-independent mechanism. Repression of several c-myc target promoters appears to be through interaction and inhibition of transcriptional activators, including Miz-1, Sp1/Sp3 and NF-Y[Oster et al., 2002]. GABA$_A$R-α1 does have a consensus Sp1 site, but further studies will be required to determine the mechanism of repression.

Functional Implications of GABA$_A$R-α1 Repression by c-Myc

One implication of our findings that c-myc controls the levels of GABA$_A$R-α1 is that c-myc may influence the activity of the GABA$_A$R in neurological processes and play a novel role in brain development. The dramatic increase in the α1 subunit after postnatal day 6 in cerebellar granule cells, correlates with a change in GABA$_A$R affinity to its ligands and a change of GABA$_A$R function [Carlson et al., 1998; Ganguly et al., 2001]. These developmental changes may be directly attributed to downregulation of GABA$_A$R-α1 by c-myc in undifferentiated cerebellar cells and to its upregulation following c-myc downregulation after birth. In addition, alterations in the GABA$_A$R-α1 gene have been observed in epilepsy and bipolar disorders in humans [Fisher, 2004]. Our results suggest a possible direct link between Myc and neurological processes and neurodegenerative disorders.

Another implication of our results is that GABA$_A$R-α1 may have an additional role independent of its role as a subunit of the GABA$_A$R in the plasma membrane, as a mediator of apoptosis. We have shown that the α1 subunit causes apoptosis in fibroblasts and neuronal-like cells, which can be blocked by Bcl-2 or Bcl-X$_L$. This appears to be a specific event, since the overexpression of either the β1 or β2 subunits of GABA$_A$ did not cause apoptosis. Since it has been previously shown that the GABA$_A$R subunits are localized to the endoplasmic reticulum [Connolly et al., 1996], GABA$_A$R-α1 may induce apoptosis through the ER stress pathway. Disruption of calcium homeostasis due to prolonged ER stress is sufficient to induce apoptosis, and has been shown to contribute to neuronal apoptosis in pathogenesis of numerous neurodegenerative disorders, including Alzheimer's disease and stroke [Mattson et al., 2000; Paschen, 2001]. Since there is a change in GABA-mediated biochemical signaling leading to an activation of calcium channels during the postnatal period [Ganguly et al., 2001], perhaps elevated GABA$_A$R-α1 in the ER may lead to induction of apoptosis by influencing the calcium channels. In an opposing manner, Bcl-2 has been shown to block ER-mediated apoptosis by modulating calcium uptake into the ER [Kuo et al., 1998].

The induction of apoptosis by GABA$_A$R-α1, which is repressed by c-myc, suggests that the downregulation of c-myc is necessary for GABA$_A$R-α1-mediated apoptosis. While the overexpression of c-myc has been shown to induce apoptosis in low serum [Oster et al., 2002], apoptosis can also occur as c-myc expression is reduced. For example, in B-cells, downregulation of c-myc has been closely correlated with apoptosis induced by a variety of agents [Thompson, 1998]. This has also been shown in non-hematopoietic cells, including the epithelial cancer cell line NA and the MCF-7 breast

adenocarcinoma cell line [Thompson, 1998]. Reducing c-myc expression with antisense oligonucleotides led to apoptosis of an esophageal cancer cell line, melanoma cell lines, HL60 monomyeloid cells, ovarian cancer cell lines, lymphoma cell lines, CEM-C7 acute lymphoblastic leukemia cell line, MCF-7 cells, and CHP-100 Ewing's sarcoma cell line [Thompson, 1998]. This suggests that a pro-apoptotic target gene that is typically repressed by c-myc, such as $GABA_AR$-α1, is derepressed by the loss of c-myc expression. Whereas $GABA_AR$-α1 caused apoptosis in NIH-3T3 and P19 cells, the derepression of $GABA_AR$-α1 in c-myc null fibroblasts did not cause apoptosis. One possible explanation for this observation is that levels of Bcl-2 are higher in c-myc null cells, since it has been shown that c-myc represses the expression of Bcl-2 [Eischen et al., 2001]. It is likely that the repression of pro-apoptotic target genes may be necessary in some cells for c-myc to cause tumorigenesis. In support of this idea, expression of $GABA_AR$ is strongly correlated to the grade of tumor malignancy. $GABA_AR$ expression is restricted to low-grade, non-invasive glioma, whereas cells from glioblastoma do not have functional $GABA_AR$ [Synowitz et al., 2001]. Established glioma cells, most likely selected for their high proliferative rate and high expression of myc, are devoid of functional $GABA_AR$ [Synowitz et al., 2001]. Therefore, the ability of c-myc to induce apoptosis or repress apoptosis depending on the cell type may be dependent on the dynamic balance of specific pro-apoptotic and anti-apoptotic genes in different cellular contexts.

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