0026-895X/07/7204-1015–1023$20.00
MOLECULAR PHARMACOLOGY
Vol. 72, No. 4
38687/3255881
Mol Pharmacol 72:1015–1023, 2007
Printed in U.S.A.

γ-Glutamylcysteine Synthetase Mediates the c-Myc-Dependent Response to Antineoplastic Agents in Melanoma Cells[S]

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Received June 1, 2007; accepted July 12, 2007

ABSTRACT

This study aims to investigate the role of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme for glutathione (GSH) synthesis, in the c-Myc-dependent response to antineoplastic agents. We found that specific c-Myc inhibition depleted cells of GSH by directly reducing the gene expression of both heavy and light subunits of the γ-GCS enzyme and increased their susceptibility to antineoplastic drugs with different mechanisms of action, such as cisplatin (CDDP), staurosporine (STR), and 5-fluorouracil (5-FU). The effect caused by c-Myc inhibition on CDDP and STR response, but not to 5-FU treatment, is directly linked to the impairment of the γ-GCS expression, because up-regulation of γ-GCS reverted drug sensitivity, whereas the interference of GSH synthesis increased drug susceptibility as much as after c-Myc down-regulation. The role of γ-GCS in the c-Myc-directed drug response depends on the capacity of drugs to trigger reactive oxygen species (ROS) production. Indeed, although 5-FU exposure did not induce any ROS, CDDP- and STR-induced oxidative stress enhanced the recruitment of c-Myc on both γ-GCS promoters, thus stimulating GSH neosynthesis and allowing cells to recover from ROS-induced drug damage. In conclusion, our data demonstrate that the γ-*GCS* gene is the downstream target of c-Myc oncoprotein, driving the response to ROS-inducing drugs. Thus, γ-GCS impairment might specifically sensitize high c-Myc tumor cells to chemotherapy.

c-*myc* is a transcriptional factor involved in many cellular processes such as proliferation, differentiation, transformation, apoptosis, and drug sensitivity (Nilsson and Cleveland, 2003). Nevertheless, the role of c-Myc in apoptosis has not yet been convincingly defined, with the outcome depending on the circumstances, cell type, and local somatic environment; the mechanisms that mediate c-Myc-induced cell death in response to various types of stress are still largely unknown (Biroccio et al., 2001, 2004; Pelengaris et al., 2002). The pleiotropic effects of c-Myc can be explained by the large number of genes set under its control (Dang, 1999). A continuously updated c-Myc Target Gene Database, assembling all the documented c-Myc-responsive genes, has been launched (*http://www.myccancergene.org/site/mycTargetDB.asp*).

Our group has recently singled out a further function of c-Myc in determining cellular redox balance and has identified glutathione (GSH), the most important low-molecular thiol involved in cellular detoxification, redox balance, and stress response, as the leading molecule mediating this process (Benassi et al., 2006). The link between c-Myc and GSH is represented by the γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme catalyzing GSH biosynthesis. γ-GCS is a heterodimer composed of a heavy catalytic (γ-GCS_H) and a light regulatory (γ-GCS_L) subunit, encoded by two different genes in both rat and human (Galloway et al., 1997). The γ-GCS enzyme plays a key role in the maintenance of intracellular redox balance and in determining cellular response to several different stimuli, including oxidative stress, xenobiotic and drug exposure, hormones, and growth factors (Wild and Mulcahy, 2000).

The 5′-flanking regions of both γ-GCS subunits have been cloned and sequenced, identifying putative nuclear factor-κB, simian virus 40 promoter factor 1, AP-1, AP-2, Nrf1, and Nfr2 binding sites together with metal response and antioxidant response elements (Mulcahy et al., 1997; Moinova and Mulcahy, 1998; Wild et al., 1998). We have recently identified the presence of functional c-Myc binding consensus sites on both γ-GCS promoters, defining γ-*GCS* as a new c-Myc target gene (Benassi et al., 2006).

The relationship between high γ-GCS expression levels and drug resistance has been extensively documented in

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (A.I.R.C.) and Ministero della Salute.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.038687.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; AP, activator protein; CDDP, cisplatin; STR, staurosporine; ROS, reactive oxygen species; 5-FU, 5-fluorouracil; CPT, camptothecin; ChIP, chromatin immunoprecipitation.

different cancer cell histotypes (Godwin et al., 1992; Mulcahy et al., 1995; Iida et al., 1999; Pompella et al., 2006). Here, we studied the role played by the γ-GCS expression in the c-Myc-driven response of melanoma cells to some antineoplastic agents. In particular, we evaluated whether γ-GCS is the downstream effector through which c-Myc mediates the response to antineoplastic drugs.

We found that c-Myc inhibition increases the sensitivity of melanoma cells to different antineoplastic drugs, but γ-GCS expression is exclusively involved in cisplatin (CDDP) and staurosporine (STR) treatment. The role of γ-GCS expression in the c-Myc-triggered drug susceptibility depends on drug-induced reactive oxygen species (ROS) production, which enhances c-Myc recruitment to the E-boxes located in both γ-*GCS* genes, thus stimulating GSH neosynthesis in response to drug exposure. The higher susceptibility displayed by the cells showing reduced levels of c-Myc depends on their inability to enhance c-Myc binding to both promoters after drug treatment and, therefore, to recover from ROS-induced damage.

Materials and Methods

Cell Culture Conditions and Transfections. All melanoma cell lines (M14, PLF2, JR1, JR8, SAN, CCH, FRM, and SbCl) were obtained from the patients' biopsy. LP and LM cell lines were derived from a nodular primary cutaneous melanoma (LP) and a supraclavicular metastatic lymph node (LM) of the same patient (Leonetti et al., 1999). Cells were maintained as monolayer cultures in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics at 37°C in a 5% CO_2-95% air atmosphere. c-Myc and γ-GCS stable M14 transfectants (MNeo, MAs, γGas, and MAsγG) were previously generated and characterized (Biroccio et al., 2001, 2004; Benassi et al., 2006).

Stable and transient c-Myc transfectants were obtained in M14, PLF2, JR1, JR8, SAN, CCH, FRM, and SbCl cells as previously reported (Biroccio et al., 2001, 2004). In brief, cells were incubated with Lipofectamine-Plus reagent (Gibco-BRL) and either an antisense/sense c-*myc* cDNA and/or the neomycin selection marker gene in serum-free OPTIMEM medium (Invitrogen). Transfected cells were then either harvested from 24 to 96 h after the end of transfection (transient clones) or grown in neomycin-containing medium for 2 weeks to obtain individual stable clones.

Treatments. Clinical-grade cisplatin and 5-fluorouracil were purchased from Pharmacia (Milan, Italy), and staurosporine and camptothecin were obtained from Sigma (Milan, Italy). Drug dilutions were freshly prepared before each experiment.

For clonogenic and apoptosis assay, cells were seeded in 60-mm Petri dishes at the density of 2×10^5 cells/dish. After 24 h, cells were exposed to the following dose range of each antineoplastic agent: 0.5 to 10 μg/ml CDDP for 2 h, 0.1 to 5 μM STR for 4 h, 0.05 to 1 mM 5-FU for 2 h, and 3.0 μg/ml CPT for 2 h. To evaluate cell colony-forming ability, aliquots of cell suspensions from each sample were seeded into 60-mm Petri dishes with complete medium and incubated from 10 to 12 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (one colony ≥50 cells). Surviving fractions were calculated as the ratio of absolute survival of the treated sample/absolute survival of the control sample. The IC_{50} value was calculated for each cell line as the drug dose able to inhibit cell survival by 50%.

Western Blotting. Western blot analysis was performed as previously reported (Biroccio et al., 2002). In brief, 40 μg of total proteins were loaded from each sample on denaturing SDS-polyacrylamide gel electrophoresis. Immunodetection of c-Myc was performed with an anti-c-Myc antibody (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA). To check the amount of proteins transferred to nitrocellulose membrane, β-actin was used as control and detected by an anti-human β-actin monoclonal antibody (Santa Cruz Biotechnology). Enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used for chemoluminescence detection.

Luciferase Assay. The evaluation of basal and drug-induced transcription activity of γ-GCS_H and γ-GCS_L promoters was carried out by transient cotransfection as previously reported (Benassi et al., 2006).

Glutathione Determination. Intracellular GSH content was measured as described previously (Biroccio et al., 2004) by a colorimetric assay (Bioxytech GSH-400; Oxis International, Inc., Portland, OR), according to the manufacturer's instruction.

Cytofluorimetric Detection of Apoptosis and ROS. Apoptosis and ROS production were assessed as described previously (Biroccio et al., 2004). In brief, cell death was evaluated by a fluorescein isothiocyanate Annexin V-propidium iodide double staining of cells (Vibrant apoptosis assay; Invitrogen), whereas ROS generation was assayed by incubating cells with 4 μM dihydro-ethidium (Invitrogen) for 45 min at 37°C in phosphate-buffered saline.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) assay was performed as previously reported (Benassi et al., 2006) using the Chromatin Immunoprecipitation Assay kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions.

Statistical Analysis. All the results reported are presented as mean values (of at least three independent experiments) with S.D. Each blot shown in the figures represents a typical experiment out of three separate ones, giving comparable results. Significant changes were assessed by using Student's *t* test for unpaired data, and *P* values ≤0.05 were considered significant. In the figure legends, *P* values are represented as follows: *, $P < 0.05$; **, $P < 0.01$.

Results

c-Myc Regulates γ-GCS Expression and Affects Drug Response. We first evaluated the relationship among c-Myc expression, GSH metabolism, and response to CDDP, the most widely clinically administered drug for melanoma treatment, in a panel of patient-derived melanoma cell lines (M14, JR8, PLF2, and SbCl) displaying different degrees of endogenous c-Myc protein expression. The basal transcriptional activity of both γ-GCS_H and γ-GCS_L promoters (Fig. 1B) and the relative intracellular GSH concentration (Fig. 1C) reflected the basal levels of c-Myc protein (Fig. 1A) as the rate of GSH metabolism progressively decreased from the highest to the lowest c-Myc-expressing lines. Moreover, c-Myc protein levels correlated with the response to the antitumoral treatment (Fig. 1D). The analysis of the survival curves to CDDP and the concomitant assessment of the drug-triggered apoptosis revealed that M14 cells were the most resistant to drug treatment and that the CDDP-surviving fractions progressively decreased from M14 to SbCl cells, according to the basal endogenous c-Myc expression (Fig. 1D). By further comparing the two melanoma cell lines originating from a primary (LP) and a metastatic (LM) lesion of the same patient, we found that both the basal promoter activity of γ-GCS_H and γ-GCS_L genes (Fig. 1F) and the intracellular GSH content (Fig. 1G) were significantly enhanced in LM compared with LP cells, in accordance with the different endogenous c-Myc expression (Fig. 1E), and that the altered GSH pool reported in the metastatic lesion correlated with the response to CDDP treatment, the percentage of cells activating apoptosis being reduced by almost 50% in

the LM compared with LP (Fig. 1H). The different levels of c-Myc expression and intracellular GSH content did not exclusively affect cell sensitivity to CDDP. In fact, the exposure to STR and 5-FU triggered a c-Myc level-dependent response in melanoma cells (Fig. 1I), the drug-induced apoptotic rate progressively increasing in M14 and JR8 cells to that of SbCl cells, whereas no difference was reported upon CPT treatment, the percentage of drug-activated cell death being independent of the basal c-Myc expression level.

To assess the direct involvement of c-Myc level in the modulation of γ-GCS and drug sensitivity, c-Myc expression was inhibited in an extended set of patient-derived human melanoma cell lines (Fig. 2). When c-Myc protein levels were reduced by approximately 50 to 60% in the c-*myc* antisense-transfected PLF2, SAN, JR1, and JR8 cells compared with their Neo controls (Fig. 2A, top), a proportional decrease in both γ-GCS promoters' activity ($P < 0.05$) was detected in all the analyzed cell lines, with no change in the c-Myc-nonresponsive cyclin E promoter region (Fig. 2A, bottom). Besides, the diminished γ-GCS transcription rate triggered a significant downstream decrement in the intracellular GSH content by approximately 40% (Fig. 2B). The c-Myc-dependent modulation of γ-GCS transcription directly correlated with the altered sensitivity to CDDP, STR, and 5-FU reported in two representative cell lines (JR8 and PLF2) upon c-Myc inhibition, because the percentage of apoptosis induced by drug exposure underwent a significant ($P < 0.01$) increment in both cell lines after c-*myc* antisense transfection compared with their Neo control clones (Fig. 2C).

In a mirror experiment carried out by transfecting CCH, FRM, and SbCl cells with a c-*myc* sense cDNA, the induced 3- to 4-fold overexpression of c-Myc protein levels (Fig. 3A, top) triggered a significant ($P < 0.05$ and $P < 0.01$) increment in the transcriptional activity of both γ-*GCS* gene promoters and in the inner GSH concentration (Fig. 3B). Moreover, a

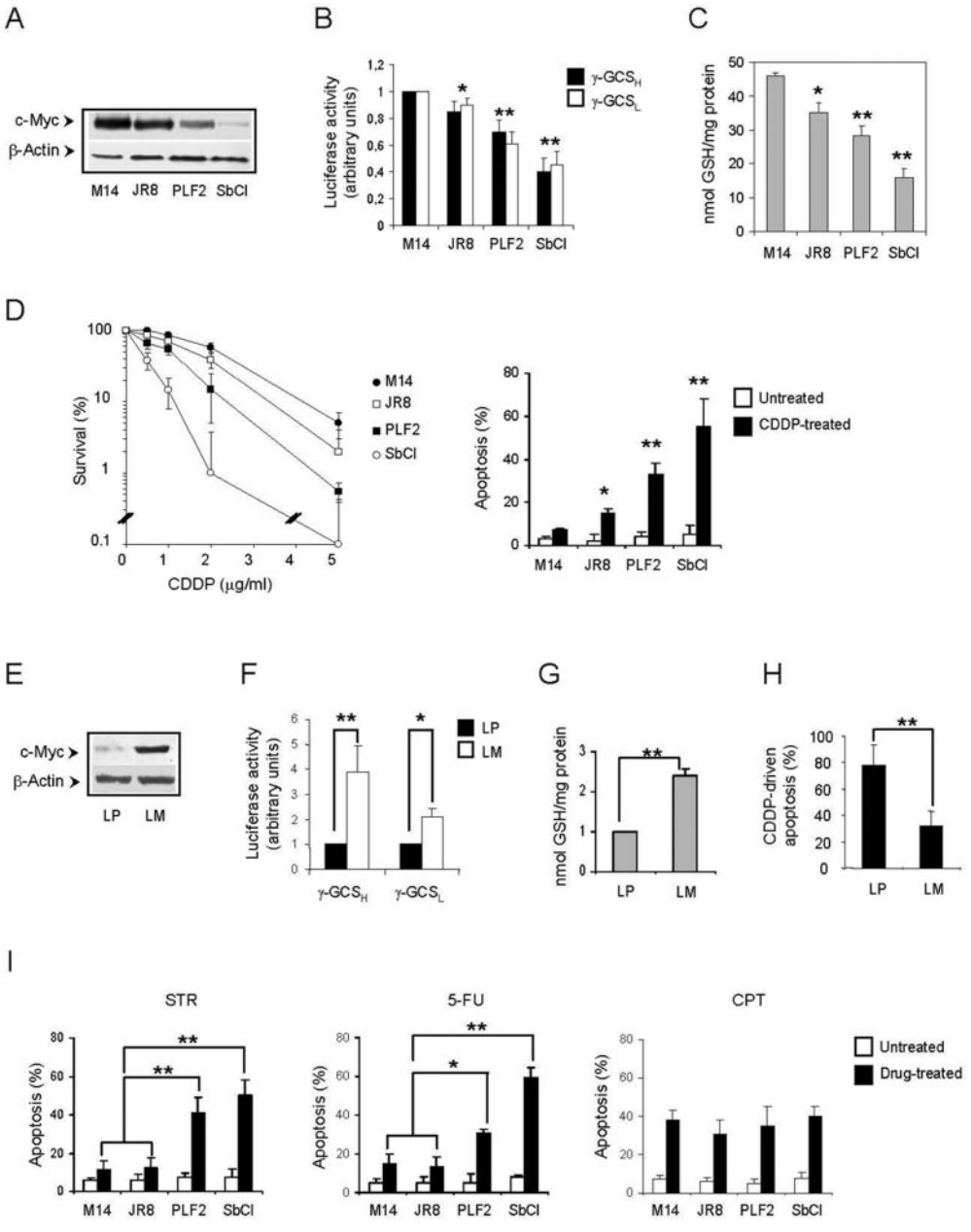


Fig. 1. The experiments have been carried out in the M14, JR8, PLF2, and SbCl (A–D and I) and LP and LM (E–H) patient-derived melanoma cell lines, displaying different endogenous c-Myc expression levels. A, Western blot analysis of c-Myc protein expression; B, $\gamma\text{-GCS}_H$ and $\gamma\text{-GCS}_L$ promoter activity evaluation; C, intracellular GSH content assessment; and D, left, dose-response curve to increasing doses of CDDP (0.5–5 μg/ml for 2 h); and right, analysis of the apoptotic rate performed 48 h after the end of CDDP treatment (2 μg/ml for 2 h). *P* values (asterisks) refer to CDDP-treated JR8, PLF2, and SbCl cells versus CDDP-exposed M14 ones and are calculated as reported under Materials and Methods. E, Western blot analysis of c-Myc protein expression; F, evaluation of $\gamma\text{-GCS}_H$ and $\gamma\text{-GCS}_L$ promoter activity; G, measurement of the intracellular glutathione content; and H, evaluation of the CDDP-induced apoptosis (48 h after the end of the drug treatment, 2 μg/ml for 2 h). I, evaluation of the apoptotic rate carried out in the M14, JR8, PLF2, and SbCl cells 48 h after the end of the treatment with 1 μM STR (4 h), 0.2 mM 5-FU (2 h), or 3.0 μg/ml CPT (2 h).

significant enhancement of cell resistance to drug treatment occurred in all the analyzed c-*myc* sense-transfected cells, the CDDP IC_{50} values undergoing an over 3-fold increase upon c-Myc incubation compared with Neo transfectants (Fig. 3C). Exogenous Myc overexpression achieved in c-*myc*-transfected M14 cells (Biroccio et al., 2004) also led to a marked up-regulation of GSH neosynthesis with a concomitant enhancement of cell resistance to CDDP as well as to STR and 5-FU (data not shown).

c-Myc-Dependent Modulation of the γ-GCS Expression Is Responsible for the Increased Sensitivity to CDDP and STR, but Not to 5-FU. To demonstrate that γ-GCS is the downstream effector of the c-Myc-driving drug response, the sensitivity to CDDP was assessed in a set of previously generated M14 melanoma transfectants (Benassi et al., 2006), including control (MNeo) and a representative c-Myc low-expressing clone (MAs) (Fig. 4A), in which γ-GCS expression was either inhibited (γGAs) or up-regulated (MAsγG), respectively, by stable transfection (Benassi et al., 2006; Fig. 4A). As reported in the survival curves to CDDP (Fig. 4B, left), the down-regulation of γ-GCS expression in the MNeo control cells increased their sensitivity to CDDP. Moreover, the dose-response curve of cells upon interference with the pathway regulating the GSH synthesis resembled the trend reported for the low c-Myc cells, the sensitivity to the drug treatment being enhanced upon γ-GCS inhibition as much as after c-Myc down-regulation. On the other side, restoration of the γ-GCS expression in the low c-Myc cells reverted their sensitivity to CDDP (Fig. 4B, right), the dose-response curve of the γ-GCS-transfected cells completely overlapping the response of the MNeo control cells. It is noteworthy that the modulation of drug sensitivity observed in the γ-GCS clones is not due to changes in the c-Myc levels, because c-Myc protein expression was not affected upon sense/antisense γ-GCS cDNA transfection (Fig. 4A).

The role played by the γ-GCS in the c-Myc-mediated drug response of melanoma cells is not restricted to CDDP. As above reported for other melanoma cell lines (Fig. 2C), specific c-Myc inhibition affected the sensitivity of M14 cells to STR and 5-FU. The surviving fraction of cells treated with either STR (Fig. 4C) or 5-FU (Fig. 4F) dramatically decreased upon c-Myc down-regulation, even at the lowest doses. The inhibition of γ-GCS expression in the control MNeo cells significantly enhanced the survival of STR (Fig. 4D), indicating that the γ-GCS gene is involved in the melanoma susceptibility to this drug. It is surprising that no difference was reported in the dose-response curve of MNeo cells to 5-FU, regardless of the γ-GCS expression (Fig. 4G), the sensitivity of cells being unaffected by the GSH synthesis interference.

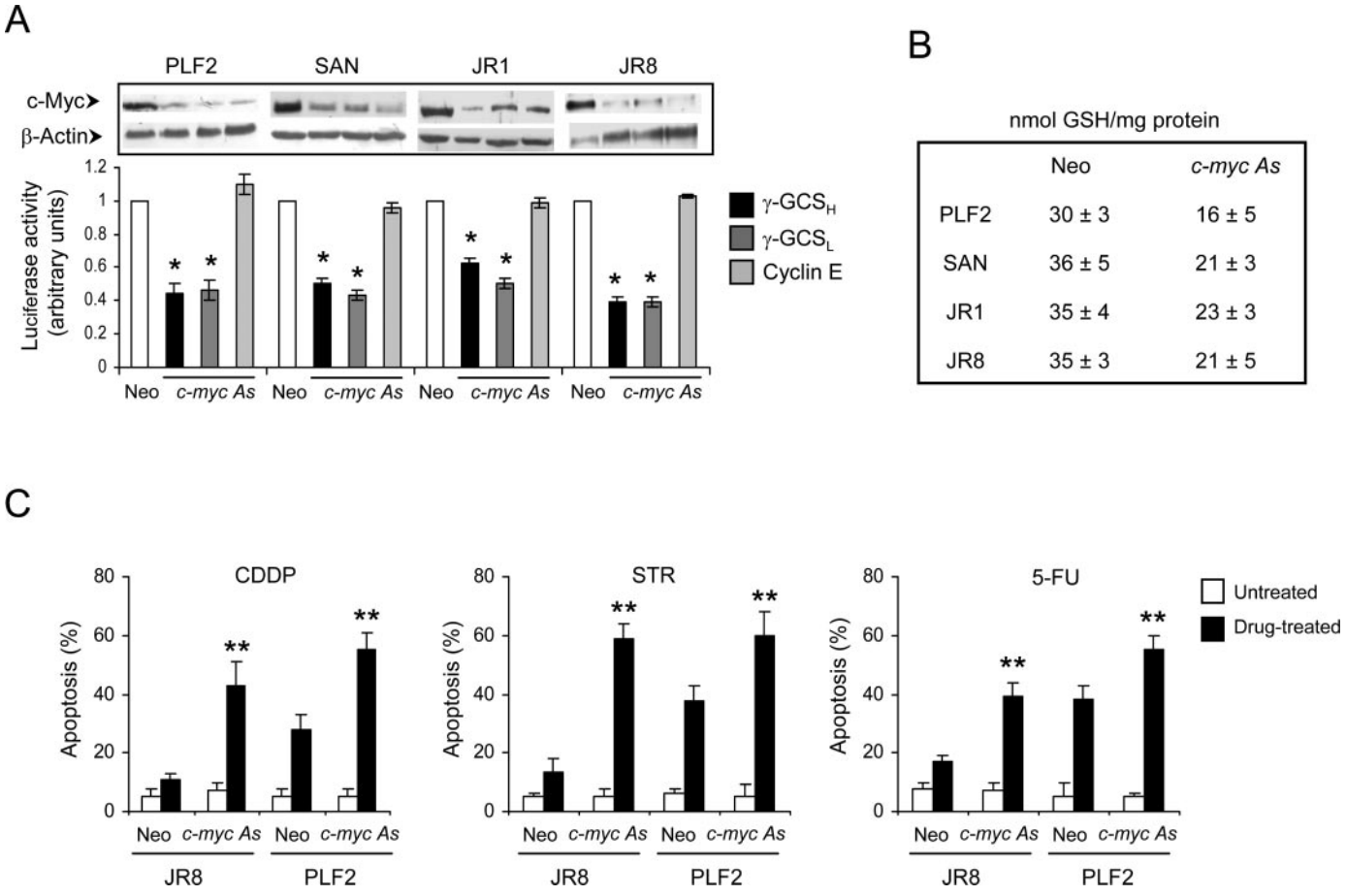


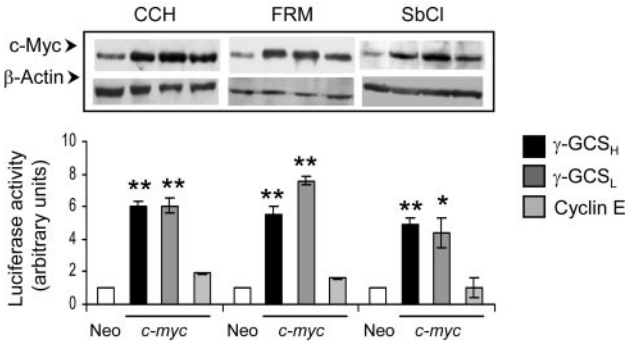
Fig. 2. The reported experiments have been carried out in PLF2, SAN, JR1, JR8 melanoma cells undergoing transfection with c-*myc* antisense cDNA. A, top, Western blot analysis of c-Myc protein expression, and (bottom) evaluation of the γ-GCS_H, γ-GCS_L, cyclin E (c-Myc negative control) promoters transcription rate. *P* values (asterisks) refer to c-*myc* transfectants compared with Neo ones. B, intracellular GSH content assessment. C, evaluation of percentage of apoptosis carried out in the reported transfectants 48 h after the end of treatment with 2 μg/ml CDDP for 2 h, 1 μM STR (4 h), or 0.2 mM 5-FU (2 h). *P* values (asterisks) refer to drug-treated c-*myc* antisense transfectants compared with drug-exposed Neo ones, and are calculated as reported under Materials and Methods.

In accordance with these results, the up-regulation of the γ-GCS expression in the low c-Myc cells enhanced and reverted their sensitivity to STR (Fig. 4E), whereas γ-GCS increment did not have any effect on the enhanced susceptibility to 5-FU displayed by the c-Myc low-expressing cells (Fig. 4H). Results similar to those above reported by modulating the γ-*GCS* expression were also obtained by pretreating the MNeo control cells with the BSO, a specific inhibitor of γ-GCS activity, or by preincubating the low c-Myc cells with an external source of GSH (glutathione ethyl-ester, GSHest) or NAC (*N*-acetyl-L-cysteine), respectively (see Supplemental Data).

ROS-Inducing Drugs Enhance c-Myc Recruitment to γ-GCS Promoters. In the attempt to identify the mechanism(s) responsible for the drug-specific involvement of the γ-GCS in the c-Myc-mediated response to chemotherapy, and on the basis of previously published data (Biroccio et al., 2002, 2004; Benassi et al., 2006), we measured the reactive oxygen species production upon drug administration. CDDP treatment induced oxidative stress, as demonstrated by the marked increase in ROS generation (in over 50% of the population) observed at the end of drug exposure in both MNeo control and MAs low c-Myc cells (Fig. 5A). It is interesting that although control cells displayed the ability to recover from the drug-induced stress and to scavenge ROS accumulation, the c-Myc-depleted cells underwent a progressive increase in reactive species, the percentage of ROS-producing cells reaching almost 90% 24 h after the end of CDDP treatment. As with CDDP, 24 h after the end of STR treatment, a higher drug-triggered oxidative stress was reported in the low c-Myc compared with the control cells, whereas 5-FU exposure did not induce any ROS generation (Fig. 5B).

Since we reported that the oxidative stress can drive the binding of c-Myc protein to specific promoters (Benassi et al., 2006), we had to verify whether ROS-inducing drugs might recruit c-Myc to γ-GCS regulator sequences. ChIP assay revealed that CDDP and STR exposure enhanced c-Myc binding to both γ-GCS_H and γ-GCS_L target genes in the MNeo control cells (Fig. 5C). Basal c-Myc binding levels in both γ-GCS promoters were reduced in c-Myc antisense cells compared with control ones, and no further increment was reported in c-Myc recruitment to both γ-*GCS* genes upon treatment with CDDP and STR. A different behavior was instead reported in cells upon 5-FU treatment. Indeed, exposure to this drug did not trigger any further recruitment of c-Myc protein to γ-GCS promoters in treated cells compared with untreated ones, independently of the c-Myc expression levels. The evaluation of the γ-GCS promoters activity matched with the different c-Myc protein binding to γ-*GCS* genes in response to specific drug treatment. Both light and heavy subunit transcription rates were up-regulated in control cells after treatment with CDDP and STR, whereas only a slightly increased response in their transactivating activity was observed in the CDDP- and STR-treated low c-Myc cells compared with control ones (Fig. 5D). Besides, the inability of 5-FU to involve ROS and to recruit c-Myc protein to the GSH synthesis controlling genes led to a lack of transactivation of both γ-GCS_H and γ-GCS_L promoter activity in treated cells regardless of their c-Myc expression levels (Fig. 5D).

A



B

nmol GSH/mg protein

	Neo	*c-myc*
CCH	22 ± 3	41 ± 3
FRM	23 ± 3	43 ± 2
SbCl	16 ± 5	30 ± 5

C

IC_{50} CDDP (μg/ml)

	Neo	*c-myc*
CCH	0.7 ± 0.2	2.5 ± 0.5
FRM	0.5 ± 0.1	2.5 ± 0.3
SbCl	0.4 ± 0.2	3.0 ± 0.5

Fig. 3. The reported experiments have been carried out in the CCH, FRM, SbCl melanoma cell lines, undergoing transfection with c-*myc* sense cDNA. A, top, Western blot analysis of c-Myc protein expression; bottom, evaluation of the γ-GCS_H, γ-GCS_L, cyclin E (c-Myc-negative control) promoters transcription rate. *P* values (asterisks) refer to c-*myc* transfectants compared with Neo ones, and are calculated as reported under Materials and Methods. B, intracellular GSH content assessment; C, cisplatin IC_{50} values calculated for the reported control Neo and c-*myc* transfectant cells.

Discussion

Role of c-Myc on Apoptosis. The role of c-Myc protein in triggering programmed cell death is controversial because both its repression and overexpression can lead to apoptosis, and the mechanisms that mediate Myc-induced cell death in response to various types of stress are still to be fully determined (Evan et al., 1992; D'Agnano et al., 2001; Nilsson and Cleveland, 2003; Ceballos et al., 2005). Several lines of evidence indicate that the role played by c-Myc on apoptosis may depend on the cellular genetic background, presence/absence of growth factors, or specific mechanism of drug action (Nilsson and Cleveland, 2003; Ceballos et al., 2005). Deregulated c-Myc expression can sensitize cells to some stimuli but not to others (Park et al., 2002; Desbiens et al., 2003; Grassilli et al., 2004), and, in this context, we previously demonstrated that c-Myc inhibition specifically sensitizes cells to CDDP and other alkylating agents without affecting the response to topoisomerase inhibitors (Biroccio et al., 2001, 2004). Here, we report that c-Myc down-regulation can also sensitize cells to other agents with different mechanisms of action, including the protein kinase inhibitor STR and the thymidylate synthase-targeting agent 5-FU.

However, the role of c-Myc on STR and 5-FU-induced apoptosis is not univocally explained because deregulation of c-Myc is reported to either increase the response or limit cancer cell responsiveness to these drugs (Augenlicht et al., 1997; Arango et al., 2001).

The role of c-Myc on drug-triggered cell death may also be affected by the dose or time exposure of the cellular insult. c-Myc seems essential for the induction of apoptosis by sublethal doses of drug but not required in cases when the apoptotic stimuli is sufficient to trigger apoptosis (Grassilli et al., 2004; Albihn et al., 2006). This is consistent with data demonstrating that low drug doses can have a cytostatic rather than a cytotoxic effect, a condition in which endogenous c-Myc expression is usually down-regulated and, if overexpressed, it can trigger apoptosis. In our experiments, the dose and/or time exposure of the oxidative damage were not able to reduce endogenous c-Myc expression or to decrease cell proliferation (data not shown), and in these circumstances, c-Myc plays a protective role on drug-induced damage.

Role of γ-GCS on Drug Resistance. γ-GCS enzyme is a heterodimer composed of a heavy catalytic and a light regulatory subunit, catalyzing the rate-limiting step in the biosynthesis of GSH, which is the most important low-molecular thiol involved in cellular detoxification, redox balance, response to stress, hormones, and growth factor (Deneke and Fanburg, 1989). Moreover, the γ-GCS gene has been associated to chemotherapy response, as cancer cell-acquired resistance to different agents proceeds through multiple pathways including GSH synthesis-mediated drug inactivation, homeostasis, and conjugation to GSH-transferase enzymes (Gately and Howell, 1993). Both transcriptional and post-translational regulation have been reported to be responsible for the γ-GCS-dependent drug resistance. In particular, many nuclear factor-κB, simian virus 40 promoter factor 1, AP-1, AP-2, metal, and antioxidant response elements have been mapped in both γ-*GCS* genes and correlated to antineoplastic treatment response (Godwin et al., 1992; Galloway et al., 1997; Wild et al., 1998; Iida et al., 1999; Pompella et al., 2006). Our group recently reported the presence of functional

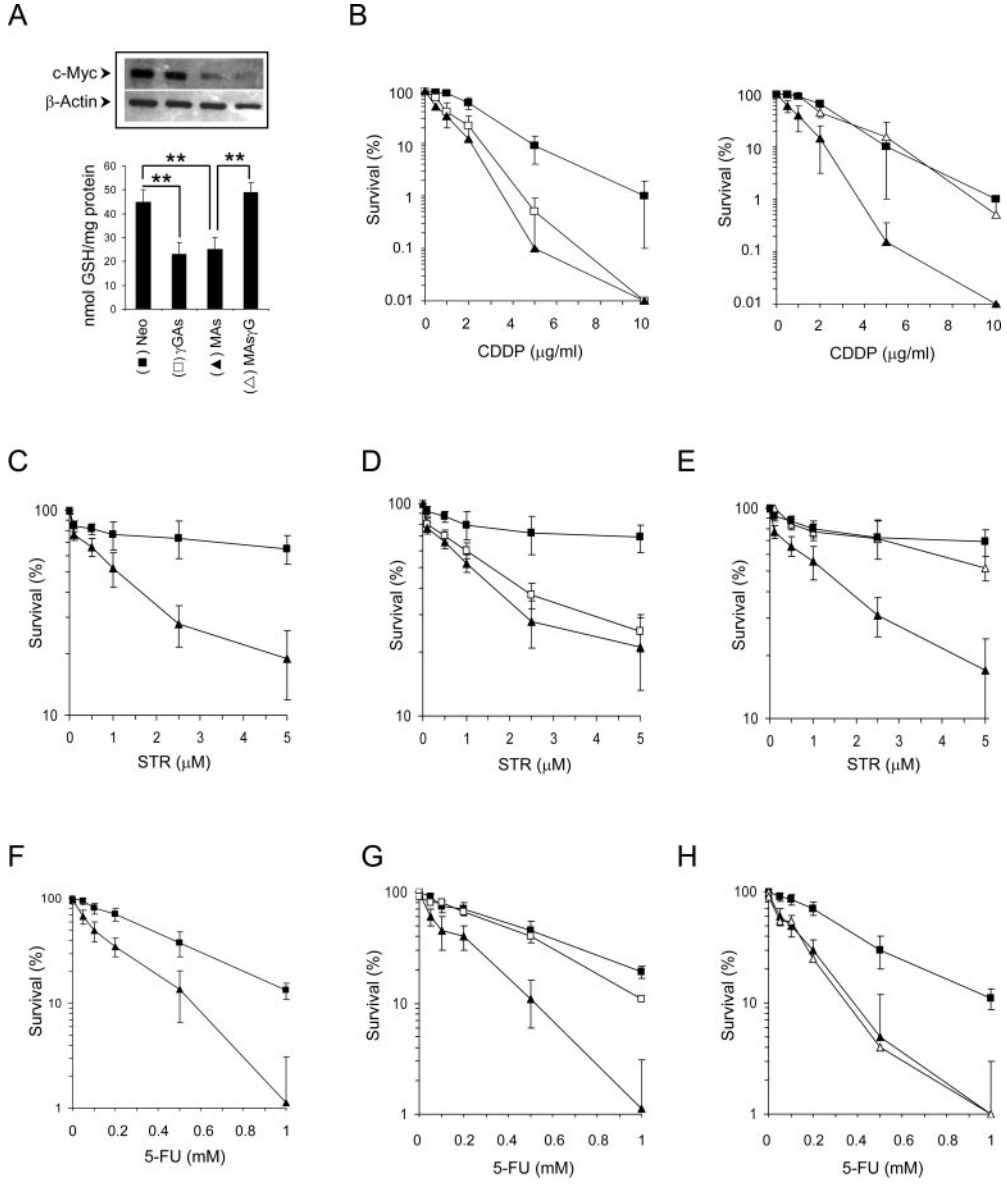


Fig. 4. The analysis of the c-Myc expression levels (A, top), inner GSH content (A, bottom), and drug-survival curves (B–H) have been performed in a set of M14 stable clones previously generated (see Materials and Methods for references), including the control clone (MNeo, ■), the MNeo γ-GCS low-expressing clone (γGAs, □), a representative low c-Myc transfectant (MAs, ▲), and a MAs γ-GCS overexpressing clone (MAsγG, △), exposed to the following treatments: cisplatin (0.5–10 μg/ml for 2 h; B), staurosporine (0.1–5 μM for 4 h; C–E), and 5-fluorouracil (0.05–1 mM for 2 h; F–H).

c-Myc consensus sites on both γ-GCS promoters, identifying *γ-GCS* as a new c-Myc transcriptionally regulated target gene (Benassi et al., 2006), although the role of this effector in the c-Myc-dependent drug response has not been defined yet.

Role of γ-GCS on the c-Myc-Driven Drug-Induced Apoptosis. We here investigated the involvement of γ-GCS expression in the c-Myc-mediated drug response of human melanoma cells, since both c-Myc and GSH homeostasis play a key role in this tumor histotype (Gately and Howell, 1993; Meyskens et al., 2001). In a panel of patient-derived melanoma cell lines, we found a strong correlation among endogenous c-Myc expression, GSH metabolism, and response to CDDP, a highly reactive, nonselective alkylating agent with demonstrated clinical effectiveness against several tumors, including melanoma. In particular, the degree of resistance to CDDP treatment was directly associated to c-Myc expression levels, GSH content, and synthesis rate. The dose-dependent modulation of c-Myc protein expression perfectly correlated with the progressive change of both intracellular GSH content and γ-GCS promoters' activity. Even more interesting is that the percentage of drug-induced cell death reflected the modulation of GSH metabolism by c-Myc when exposed to CDDP treatment. Furthermore, when treated with γ-GCS short interfering RNA, cells survival to CDDP dramatically dropped down to levels comparable with those achieved by c-Myc inhibition. Likewise, the drug effects as a result of the c-Myc inhibition were completely reverted by either transfecting γ-GCS cDNA or preloading cells with an external source of GSH. Taken together, these results clearly demonstrate that γ-GCS is a direct downstream mediator of the c-Myc-triggered apoptosis in response to CDDP.

The onset of CDDP resistance has been already well documented and correlated to GSH intracellular content and γ-GCS expression (Godwin et al., 1992; Polsky and Cordon-Cardo, 2003; Das et al., 2006), but here we are reporting for the first time that c-Myc affects cell response to CDDP through direct regulation of the γ-GCS expression. Our results are in agreement with data demonstrating that most c-Myc phenotypes can be explained by the activation/repression of the target genes set under its control (Iida et al., 2001; Zeller et al., 2003). In the context of drug response, Park et al. (2002) demonstrated that overexpression of c-Myc can promote survival through activation of the *ornithine decarbox-*

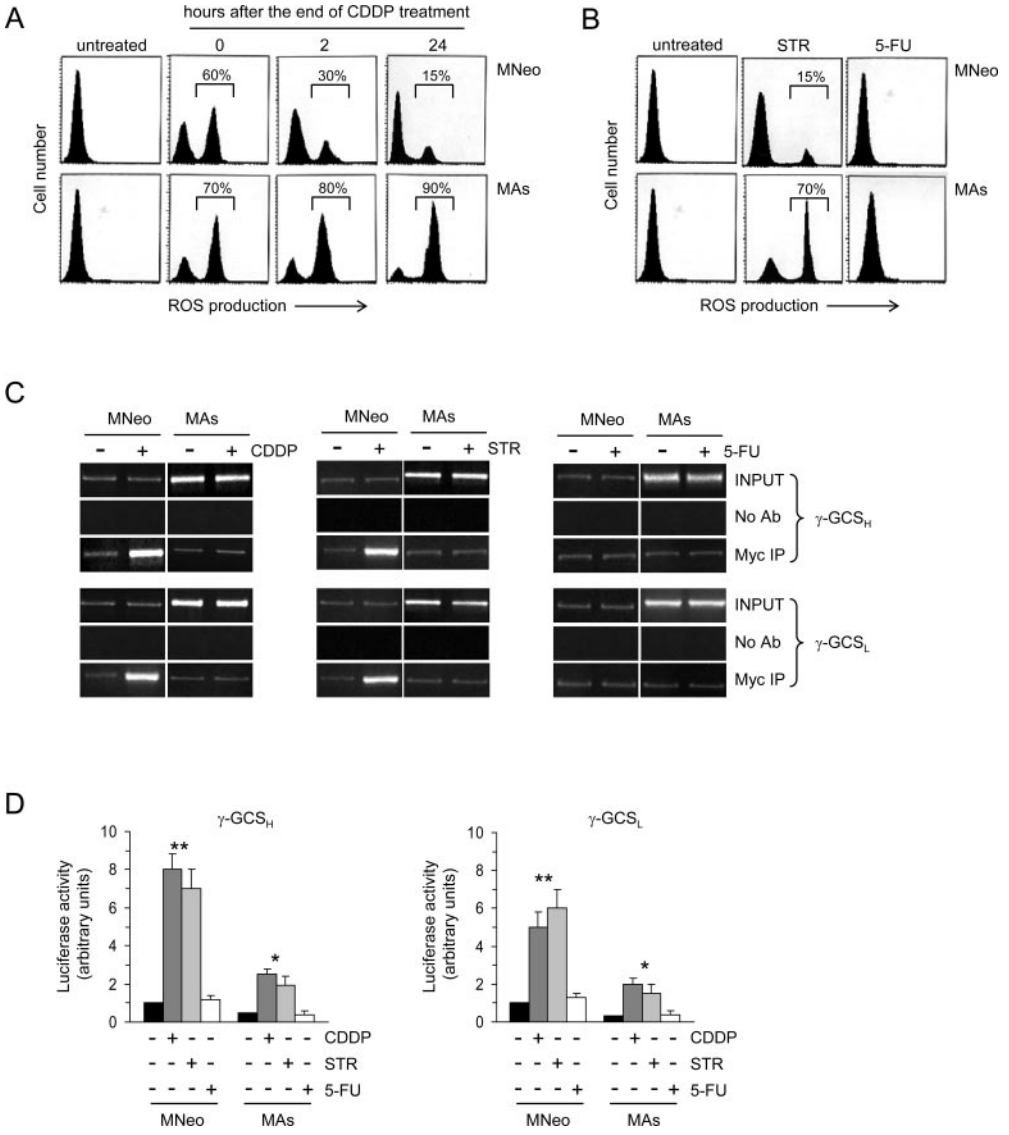


Fig. 5. The reported data have been carried out in the M14 control clone (MNeo) and in a representative low-c-Myc transfectant (MAs) undergoing treatment with 5 μg/ml CDDP (2 h), 2.5 μM STR (4 h) or 0.5 mM 5-FU (2 h). Cytofluorimetric evaluation of ROS production performed at 0, 2, and 24 h after the end of the CDDP treatment (A) or 24 h after the end of the exposure to either STR or 5-FU (B). C, chromatin immunoprecipitation assay carried out in the indicated cells treated with each different agent. Formaldehyde cross-linked chromatin fragments have been immunoprecipitated either with no antibody or c-Myc antibody and amplified by PCR analysis by means of specific forward and reverse primers chosen for each indicated promoter. D, evaluation of the γ-GCS_H and γ-GCS_L gene promoter activity carried out in the indicated cells after the exposure to each single drug. *P* values (asterisks) refer to CDDP- and STR-treated cells compared with untreated ones, and are calculated as reported under Materials and Methods.

ylase gene, a well known c-Myc target gene. Here, we have identified a new c-Myc target gene mediating the c-Myc-dependent response to CDDP.

The role of γ-GCS on the c-Myc-dependent drug response is not limited to CDDP. We found that inhibition of c-Myc also affected the sensitivity of cells to other drugs, such as STR and 5-FU, but γ-GCS expression is exclusively involved in the STR treatment, indicating that pathways other then the regulation of the GSH synthesis and homeostasis are involved in the c-Myc-dependent susceptibility of cells to 5-FU, in accordance to many reported data indicating that 5-FU-triggered thymidylate synthase inhibition and misincorporation of 5-FU metabolites into RNA result in p53 stabilization-mediated cell death (Adhikary and Eilers, 2005). The different effect exerted by the γ-GCS on the c-Myc-dependent drug survival depends on whether the specific agent generates oxidative stress. Indeed, although 5-FU exposure did not induce any ROS production, CDDP and STR triggered oxidative stress independently of c-Myc expression levels, but the c-Myc/GSH-impaired cells were unable to efficiently scavenge oxygen species. Stress recovery depended on the ability to quickly up-regulate GSH neosynthesis in response to the drug treatment. By ChIP analysis, we found that only ROS-generating drugs, such as CDDP and STR, were able to trigger a higher binding of c-Myc protein to γ-GCS promoters and that this further recruitment can contribute to protect cells from drug-induced oxidative damage. These data are consistent with those demonstrating that STR and CDDP are pro-oxidant agents (Longley et al., 2003; Siomek et al., 2006), but we add further insight to the elucidation of their mechanisms of action by demonstrating that drug-induced ROS generation can be responsible for the different c-Myc recruitment to specific gene promoters. Along with other reported transcription factors, c-Myc protein can therefore be included into the panel of DNA-binding protein specifically recruited to γ-*GCS* gene promoters to mediate the apoptotic cascade in response to different antineoplastic agents. On the basis of our previously published data (Benassi et al., 2006), the ability of c-Myc to be recruited to the GSH synthesis genes upon stress can be specifically driven through regulation of its phosphorylation.

As a whole, our findings highlight a new molecular mechanism by which c-Myc mediates the response to ROS-inducing drugs and identify in γ-GCS the direct effect of this process. They also drive the use of innovative γ-GCS-specific inhibitors (Seleznev et al., 2006) in the treatment of tumors displaying deregulated c-Myc expression, specifically with oxidative stress-inducing drugs.

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