

## Galectin-1: biphasic growth regulation of Leydig tumor cells

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**Galectin-1 (Gal-1) is a widely expressed  $\beta$ -galactoside-binding protein that exerts pleiotropic biological functions. To gain insight into the potential role of Gal-1 as a novel modulator of Leydig cells, we investigated its effect on the growth and death of MA-10 tumor Leydig cells. In this study, we identified cytoplasmic Gal-1 expression in these tumor cells by cytofluorometry. DNA fragmentation, caspase-3, -8, and -9 activation, loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ), cytochrome c (Cyt c) release, and FasL expression suggested that relatively high concentrations of exogenously added recombinant Gal-1 (rGal-1) induced apoptosis by the mitochondrial and death receptor pathways. These pathways were independently activated, as the presence of the inhibitor of caspase-8 or -9 only partially prevented Gal-1-effect. On the contrary, low concentrations of Gal-1 significantly promoted cell proliferation, without inducing cell death. Importantly, the presence of the disaccharide lactose prevented Gal-1 effects, suggesting the involvement of the carbohydrate recognition domain (CRD). This study provides strong evidence that Gal-1 is a novel biphasic regulator of Leydig tumor cell number, suggesting a novel role for Gal-1 in the reproductive physiopathology.**

**Key words:** apoptosis/galectin-1/Leydig cells/proliferation

### Introduction

Galectins are a family of proteins defined by at least one characteristic carbohydrate recognition domain (CRD) with affinity for  $\beta$ -galactosides and by certain conserved sequence elements (Barondes *et al.*, 1994). The best characterized member of this family is galectin-1 (Gal-1), a homodimer of 14 kDa subunits. Although biochemical features of Gal-1 are typical of nonsecreted proteins, such as

the lack of secretion signal sequence, acetylated N-terminus, and the requirement of reducing conditions, Gal-1 may be targeted for secretion by a nonclassical secretory pathway (Cooper and Barondes, 1990; Seelenmeyer *et al.*, 2005). Once exported from cells, Gal-1 is free to bind to appropriately glycosylated proteins or lipids on cell surfaces or to the extracellular matrix (ECM).

Gal-1 has been shown to be expressed in different tissues/organs, including thymus (Baum *et al.*, 1995), colon (Hittel *et al.*, 2003), spleen (Ahmed *et al.*, 1996), smooth muscle (Moiseeva *et al.*, 2000), ovary (Van den Brule *et al.*, 2003), and the nervous system (Akazawa *et al.*, 2004). According to this wide distribution, Gal-1 has pleiotropic biological functions, such as promoting proliferation (Adams *et al.*, 1996; Vas *et al.*, 2005), adhesion (Moiseeva *et al.*, 2003), migration (Moiseeva *et al.*, 1999), differentiation (Sasaki *et al.*, 2004), processing of pre-mRNA (Vyakarnam *et al.*, 1997), neoplastic transformation (Paz *et al.*, 2001), and induction of apoptosis in many cells, such as immature thymocytes (Perillo *et al.*, 1997), activated T-cells (Nguyen *et al.*, 2001), human mammary cancer cells (Wells *et al.*, 1999), and prostate cancer cell line LNCaP (Ellerhorst *et al.*, 1999).

Gal-1 expression has been well reported in immune privileged sites: eye (Fautsch *et al.*, 2003), placenta (Hirabayashi and Kasai, 1984; Iglesias *et al.*, 1998a), and testis (Dettin *et al.*, 2003). Within testis, immunohistochemical studies have revealed Gal-1 presence in Sertoli, peritubular, and Leydig cells (Wollina *et al.*, 1999). Leydig cells are the main source of testosterone, essential to maintain germ cell maturation through its paracrine effect on Sertoli cells. The level of testosterone is due to both the steroidogenic capacity and total Leydig cell number in the testis, being the luteinizing hormone (LH) the main regulator of androgen biosynthesis. Alterations of either cell number or steroidogenic activity lead to different pathologies, such as infertility and tumor progression.

Recently, we demonstrated the effect of exogenous Gal-1 on the viability of rat Leydig cells *in vitro* (Martínez *et al.*, 2004). Relatively high concentrations of the lectin were required to induce apoptosis, as demonstrated by caspase-3-like activation and fragmentation of DNA. Besides, the presence of lactose or the glycoprotein laminin prevented cells from undergoing apoptosis, indicating the participation of the CRD and an alternative cellular mechanism, based on Gal-1 and laminin-1 interaction, to regulate Leydig cell function and survival. On the contrary, a strong cytoplasmic and a patched membrane expression were demonstrated, suggesting the possibility of either autocrine or paracrine regulation of the lectin. No modulation of Gal-1 on the steroidogenic capacity of Leydig cells was observed.

Different lectin expression profiles have been demonstrated in cancer cells compared with normal cells (Van den

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Brule *et al.*, 2004; Liu and Rabinovich, 2005). Several studies have shown increased Gal-1 expression in different tumors, including astrocytomas (Camby *et al.*, 2001), melanomas (Rubinstein *et al.*, 2004), and thyroid carcinomas (Xu *et al.*, 1995). Moreover, a strong correlation between Gal-1 expression and malignancy of gliomas has been documented (Yamaoka *et al.*, 2000). On the contrary, a decreased expression of the lectin was documented in head and neck cancers (Choufani *et al.*, 1999). MA-10 is a clonal strain of cultured mouse Leydig tumor cells, which secrete progesterone rather than testosterone as a major steroid. Because the expression of Gal-1 is differently regulated in many tumor cells, in this study we describe the expression of Gal-1 on MA-10 cells and evaluate the effect of the lectin on growth and programmed cell death or apoptosis. We report for the first time a biphasic effect of the lectin on the growth of MA-10 cells. Furthermore, we provide insights into the molecular mechanism leading to cell death.

## Results

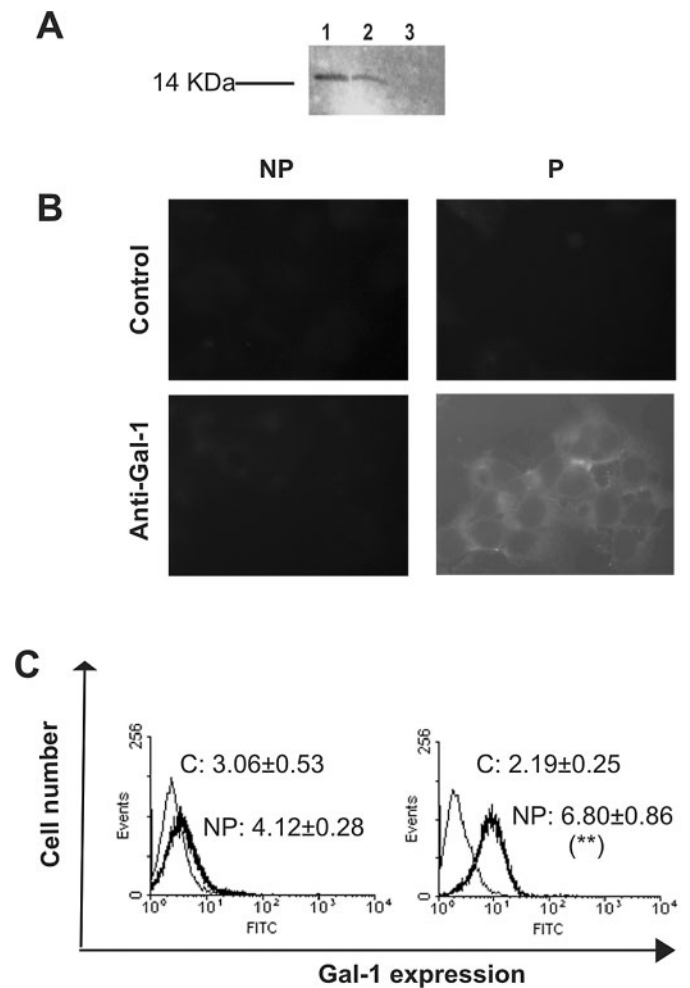
### Cytosolic expression of Gal-1 in MA-10 Leydig tumor cells

To analyze Gal-1 expression and secretion, immunoreactive Gal-1 was detected by western blot with specific antibody. As shown in Figure 1A, Gal-1 was present in MA-10 cell lysates, whereas no immunoreactive band was found in concentrated serum-free medium from MA-10 cultures.

To determine Gal-1 subcellular localization, MA-10 cells were subjected to indirect immunofluorescence staining using a purified goat anti-human Gal-1 polyclonal antibody. As shown in Figure 1B, only a diffuse cytoplasmic localization was revealed in permeabilized cells while no membrane-associated Gal-1 was detected in non-permeabilized cells. To quantify the expression of the lectin, flow cytometric analysis was performed after immunofluorescence staining (Figure 1C). According to these data, no significant cell surface staining was obtained in non-permeabilized cells compared with control fluorescence. On the contrary, Gal-1 was present in very significant concentration in permeabilized cells (fluorescence intensity means:  $6.80 \pm 0.86$  versus  $2.19 \pm 0.25$ ), suggesting a predominant cytoplasmic expression of the lectin.

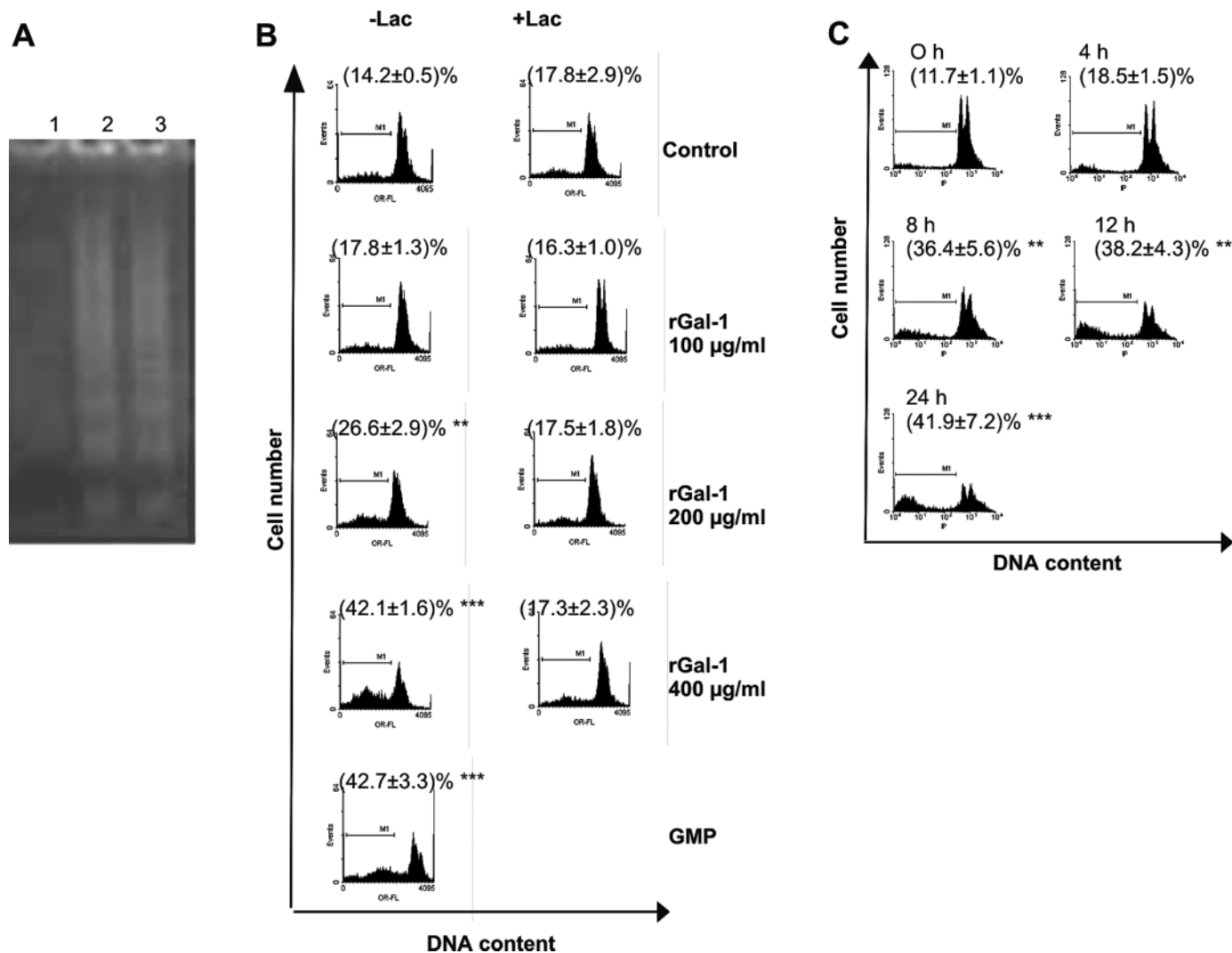
### Induction of apoptosis mediated by Gal-1

Apoptosis, or programmed cell death, is a cell suicide mechanism that enables organisms to control cell number in tissues and to eliminate harmful individual cells. It is formally defined by morphological criteria, but biochemical events provide additional information to characterize the cell death (Hengartner, 2000). To evaluate whether cell death is induced by Gal-1, we explored the effect of recombinant Gal-1 (rGal-1) on DNA fragmentation by agarose gel electrophoresis (Figure 2A). As a positive control of apoptosis, a mixture of 50  $\mu\text{g}/\text{mL}$  genistein and 100  $\mu\text{M}$  methylprednisolone (GMP) was simultaneously used. The genomic DNA extracted from cells treated with GMP (lane 2) or 400  $\mu\text{g}/\text{mL}$  rGal-1 (lane 3) was subjected to electrophoresis in 1.8% agarose gel, and DNA was visualized by ethidium bromide staining. As expected, the typical DNA



**Fig. 1.** Cytosolic expression of Gal-1 in MA-10 cells. (A) Immunoreactive Gal-1 was detected in 50  $\mu\text{g}$  proteins from cellular lysate (lane 2) or serum-free medium (lane 3) by western blot. Thirty nanograms of human rGal-1 was used as a control for Gal-1 immunoreactivity (lane 1). (B) The cellular localization of Gal-1 was assessed by indirect immunofluorescence. Both permeabilized (P) and nonpermeabilized (NP) cells were probed for Gal-1 expression using an affinity-purified polyclonal antibody followed by a secondary antibody conjugated with FITC. Images were visualized at 1000 $\times$  by fluorescent microscopy. (C) Quantification of Gal-1 expression was performed by flow cytometry after immunofluorescence staining. Histograms of control (C), permeabilized (P), and nonpermeabilized (NP) cells are plotted and the fluorescence intensity means  $\pm$  SD are shown.  $**p < 0.01$ .

cleavage into internucleosomal fragments was induced after either GMP or rGal-1 treatment, whereas no DNA fragmentation was visible in control cells (lane 1). Once DNA fragmentation was demonstrated, dose response and time course were evaluated. In an attempt to quantify rGal-1-induced apoptosis, nuclei were subjected to flow cytometry after propidium iodide (PI) staining. As Figure 2B shows, MA-10 cells treated with different concentrations of rGal-1 (100–400  $\mu\text{g}/\text{mL}$ ) for 24 h resulted in a dose-dependent increase of apoptotic nuclei present in the subdiploid region (sub *G0/G1* peak), achieving its maximal inhibitory effect ( $42.1 \pm 1.6\%$ ) at the maximal concentration probed. Notably, the presence of lactose completely prevented rGal-1-mediated apoptosis, leading to the assumption that the CRD of the lectin was involved in the apoptotic effect. On



**Fig. 2.** Induction of apoptosis mediated by Gal-1. (A) Internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. MA-10 cells were cultured for 24 h in the presence of different stimuli and DNA was extracted as described under *Materials and methods*. DNA laddering was examined from cells left untreated (lane 1), cultured with GMP (lane 2) or 400 µg/mL rGal-1 (lane 3). The result is representative of three independent experiments. (B) MA-10 cells were studied by flow cytometry after PI staining as described in *Materials and methods*. Cells were incubated with increasing concentrations of rGal-1 for 24 h, in the presence or absence of 20 mM lactose, or (C) with 400 µg/mL rGal-1 for different periods of time. A positive control of apoptosis was performed with GMP. The percentage of apoptotic MA-10 cells (hypodiploid DNA peak) ± SD was calculated by WinMDI 2.8 software program. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

the contrary, the time course of Gal-1-mediated DNA fragmentation (Figure 2C) revealed that addition of 400 µg/mL rGal-1 caused a significant increase of apoptotic nuclei only after 8 h incubation. The subsequent analyses carried out to elucidate the pathways involved in this apoptotic effect were performed for 8 h with 400 µg/mL rGal-1, concentration comparable with that used in other cells, which induces significant apoptosis at the earliest time possible.

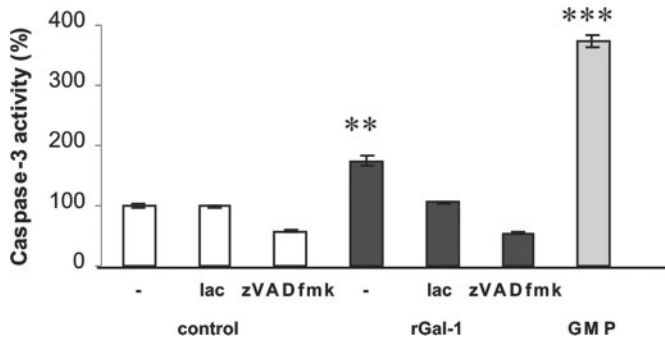
#### *Involvement of caspase-3 in Gal-1-induced apoptosis*

We investigated the involvement of the main effector caspase in Gal-1-mediated apoptosis. Apoptosis-associated caspase-3-like activity was measured by a fluorometric assay based on Asp-Glu-Val-Asp (DEVD)-7-amino-4-trifluoromethyl coumarin (AFC) cleavage activity (Figure 3). After 8 h exposure

to 400 µg/mL rGal-1 or GMP, a 1.7- or 3.7-fold increase, respectively, in caspase-3-like activity was detected compared with control cells. Moreover, caspase-3-like activation was prevented by addition of lactose. As a control, pretreatment of cells with 20 µM pan caspase inhibitory peptide (z-VAD-fmk) completely inhibited caspase-3 activation.

#### *Death receptor pathway dependence of Gal-1-mediated apoptosis*

The death receptor or extrinsic pathway is triggered by the activation of the members of tumor necrosis factor (TNF) receptor superfamily by their ligands, such as Fas/FasL and TNF $\alpha$ R/TNF $\alpha$ , leading to the recruitment of adaptor molecules, such as Fas-associated death domain (FADD) which then recruit other cellular proteins, including the initiator

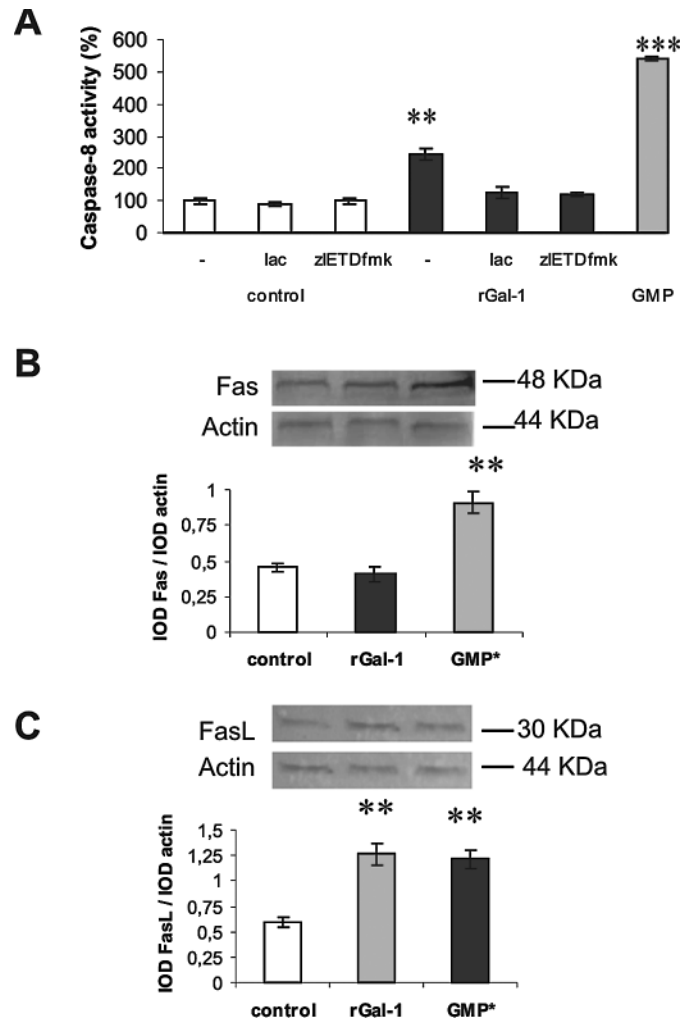


**Fig. 3.** Involvement of caspase-3 in Gal-1-induced apoptosis. Caspase-3 activity was measured by a fluorometric assay after treatment with 400  $\mu$ g/mL rGal-1, with or without 20 mM lactose or 20  $\mu$ M pan caspase inhibitory peptide (z-VAD-fmk), or GMP, for 8 h. Cell lysates were subjected to incubation with DEVD-AFC substrate. Free AFC was recorded fluorometrically. Results are expressed as a percentage of caspase activity relative to control  $\pm$  SD of three independent experiments. \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001.

procaspases-8 and -10 with their subsequent activation and induction of the signaling leading to cell death (Mor *et al.*, 2002). To evaluate the involvement of the death receptor pathway in Gal-1-mediated apoptosis, our attention was focused on the activation of caspase-8. To aim this, caspase-8-like activation was measured by Ile-Glu-Thr-Asp (IETD)-AFC cleavage activity (Figure 4A). Exposure to 400  $\mu$ g/mL rGal-1 for 8 h resulted in a significant 2.4-fold increase in caspase-8-like activity. Furthermore, this effect was completely prevented by addition of lactose or by incubation with 20  $\mu$ M caspase-8 inhibitor (z-IETD-fmk) before rGal-1 treatment. Again, the mixture of GMP was used as a positive control (5.1-fold increase). To assess whether the Fas/FasL system was involved in Gal-1-mediated apoptosis, immunoreactive Fas, FasL, and actin as loading control were analyzed by western blot. In these experiments, a mixture of 50  $\mu$ g/mL genistein and 200  $\mu$ M methylprednisolone (GMP\*) was used as a positive control. Although no alteration in Fas expression was observed (Figure 4B), a 2.1-fold increase of FasL expression resulted from rGal-1 treatment (Figure 4C).

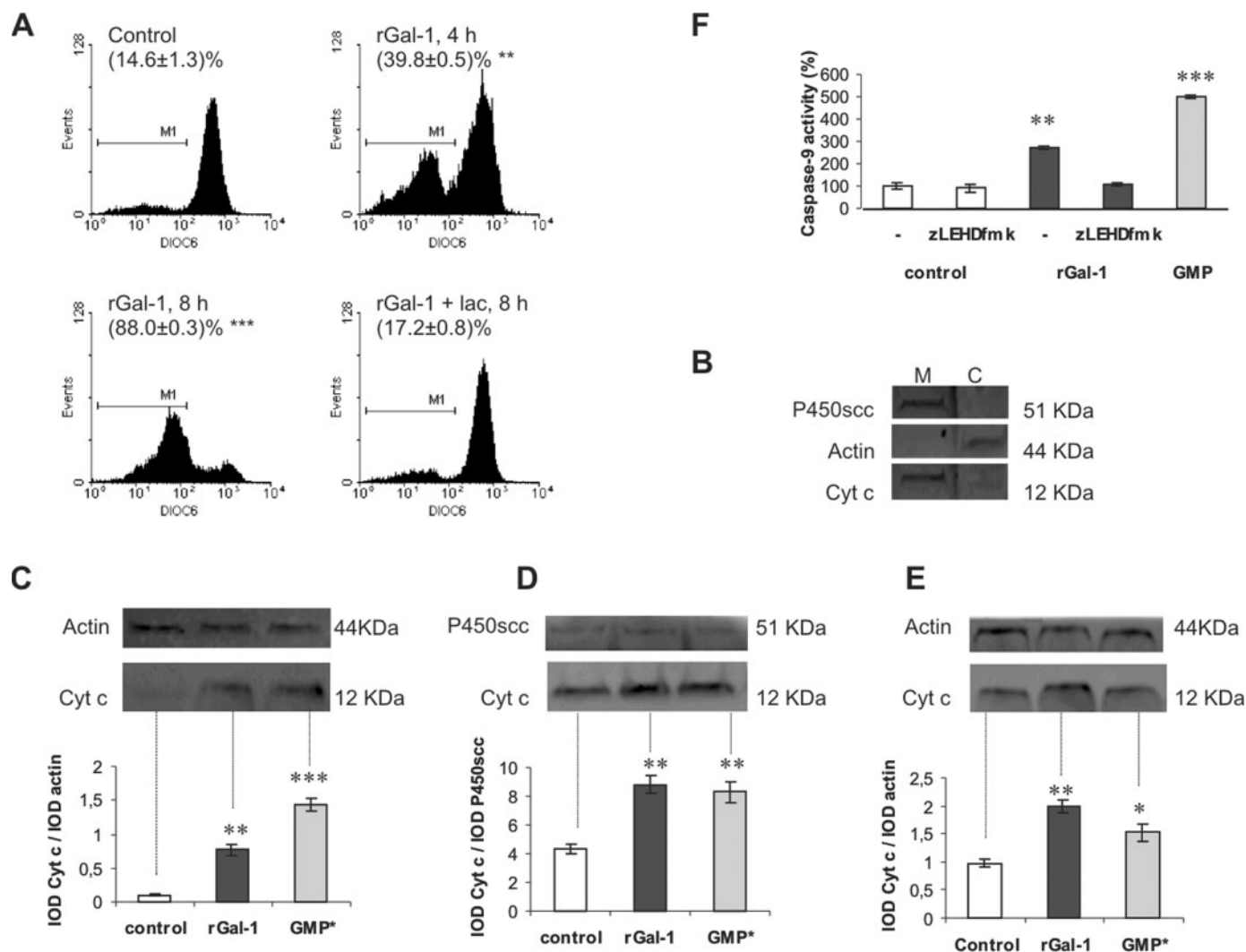
#### Involvement of the mitochondrial pathway in Gal-1-mediated apoptosis

In the mitochondrial or intrinsic pathway of apoptosis, the key event consists on the mitochondrial outer membrane permeabilization, accompanied or not by a loss of inner transmembrane potential ( $\Delta\Psi_m$ ), leading to the release of proteins, such as cytochrome c (Cyt c), which activate downstream caspase-9 (Green and Reed, 1998; Green and Kroemer, 2004). To evaluate whether Gal-1-mediated apoptosis was associated with this pathway, changes in the mitochondrial membrane potential ( $\Delta\Psi_m$ ) were assessed by flow cytometry of cells stained with the potential-sensitive cationic lipophilic dye 3, 3'-dihexyloxadecarbocyanine iodide (DiOC<sub>6</sub>(3)) (Figure 5A). A significant loss of  $\Delta\Psi_m$  was observed following 4 h treatment with 400  $\mu$ g/mL rGal-1 ( $39.8 \pm 0.5$  versus  $14.6 \pm 1.3\%$ ). Longer incubation (8 h) increased the percentage of cells with loss of  $\Delta\Psi_m$  to  $88.0 \pm 0.3\%$ , whereas addition of lactose completely inhibited this effect ( $17.2 \pm 0.8\%$ ).



**Fig. 4.** Death receptor pathway dependence of Gal-1-mediated apoptosis. (A) Caspase-8 activity was measured as described for caspase-3 activity. The cell lysates after treatment were incubated with IETD-AFC substrate, and free AFC was measured by fluorometry. To inhibit caspase-8 activity, cells were pretreated with 20  $\mu$ M specific inhibitory peptide (z-IETD-fmk) for 1 h. Results are expressed as percentage of activity relative to control  $\pm$  SD of three independent experiments. (B) Immunoreactive Fas and (C) FasL were detected by western blot in cell lysates (50  $\mu$ g) after 8 h treatment with 400  $\mu$ g/mL rGal-1 or GMP\*. Immunoreactive actin was used as a loading control. A representative of three experiments is shown. Densitometric analysis was carried out and results are plotted as a ratio of Fas or FasL to actin  $\pm$  SD of three independent experiments. \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001.

Release of Cyt c following loss of  $\Delta\Psi_m$  was also investigated. As Figure 5B shows, a control of cellular fractionation was performed by analyzing immunoreactive Cyt c, actin, and P450<sub>scc</sub> in the mitochondrial and cytosolic fractions of untreated cells. As expected, both Cyt c and P450<sub>scc</sub> were found mainly in the mitochondrial fraction, although a slight contamination of <5% was detectable. As shown in Figure 5C, a significant release of Cyt c into the cytosol was observed after 8 h incubation with 400  $\mu$ g/mL rGal-1 or GMP\* (7.5- or 14-fold increase, respectively). Surprisingly, the release of Cyt c was not accompanied by a concomitant decrease of mitochondrial Cyt c. Moreover, as



**Fig. 5.** Involvement of the mitochondrial pathway in Gal-1-mediated apoptosis. (A) Loss of  $\Delta\Psi_m$  was assessed by DiOC<sub>6</sub>(3) staining as described in *Materials and methods*, after treatment with 400  $\mu\text{g}/\text{mL}$  rGal-1 for 4 or 8 h, in the presence or absence of 20 mM lactose. The percentage of cells undergoing loss of  $\Delta\Psi_m \pm \text{SD}$  of three independent experiments is plotted in each histogram. (B) Control of cellular fractionation was carried out with untreated cells by analysis of immunoreactive Cyt c, actin, and P450scc in the mitochondrial (M) and cytosolic (C) fractions. (C) Immunoblot analysis of cytosolic, (D) mitochondrial, and (E) total Cyt c. Cellular fractions were obtained at 8 h after treatment with 400  $\mu\text{g}/\text{mL}$  rGal-1 or GMP\*. Cytosolic (20  $\mu\text{g}$ ), mitochondrial (20  $\mu\text{g}$ ), and total (50  $\mu\text{g}$ ) proteins were analyzed for immunoreactive Cyt c and protein loading (*Materials and methods*). Densitometric scanning of bands was carried out and results were plotted as a ratio of Cyt c to actin or P450scc  $\pm \text{SD}$  of three independent experiments. (F) Caspase-9 activation in MA-10 cells subject to 400  $\mu\text{g}/\text{mL}$  rGal-1 or GMP treatment for 8 h was analyzed by a fluorometric assay with LEHD-AFC substrate (*Materials and methods*). To inhibit caspase-9, cells were pretreated with 20  $\mu\text{M}$  caspase-9 inhibitor (z-LEHD-fmk) for 1 h. Data are expressed relative to untreated cells and represent the mean  $\pm \text{SD}$  of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

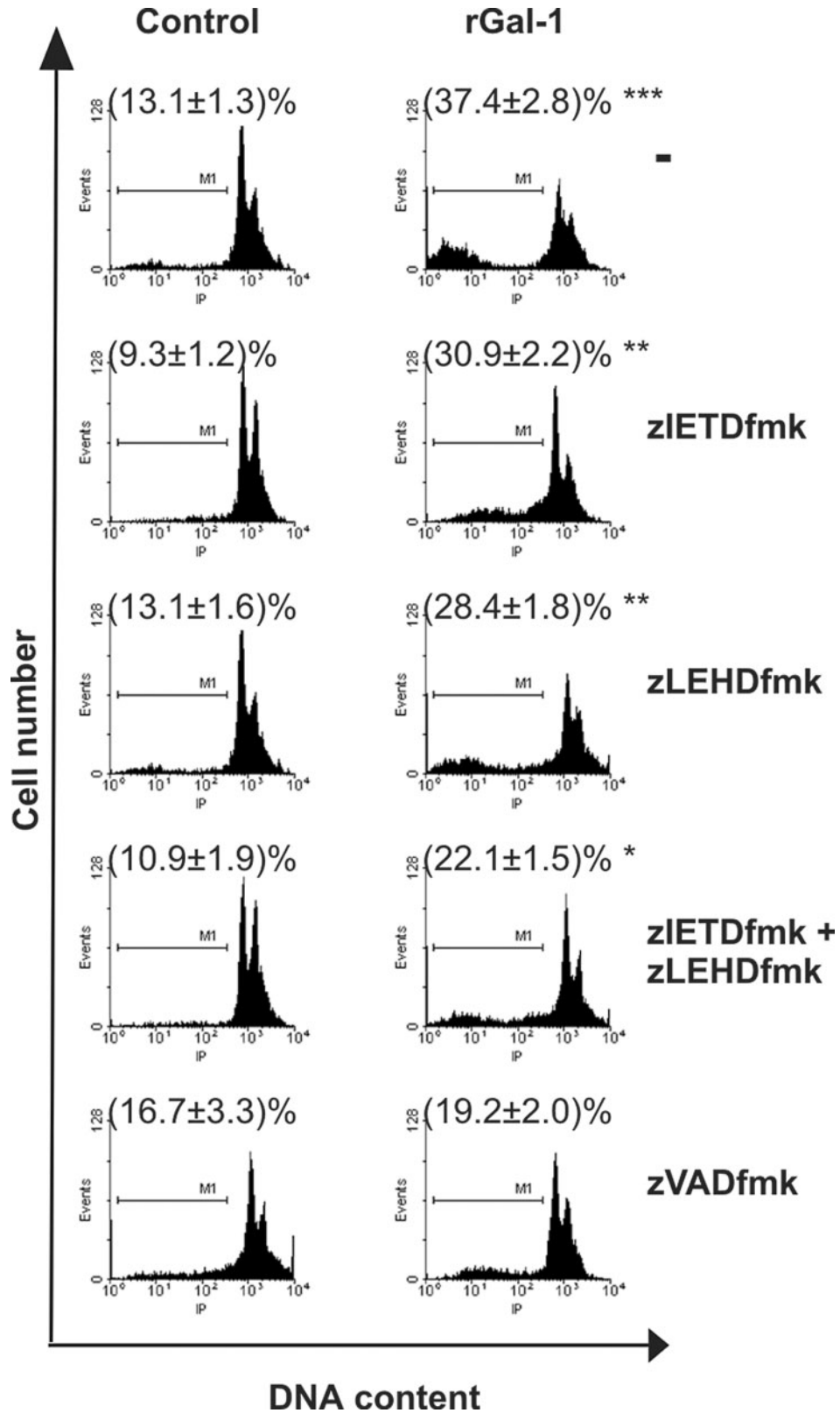
Figure 5D shows, an increase of mitochondrial Cyt c was observed following rGal-1 or GMP\* treatment (2.0- or 1.9-fold increase, respectively). To confirm this data, we measured total Cyt c present in MA-10 cells (Figure 5E). Consistent with the increase in cytosolic and mitochondrial Cyt c, a significant increase of total Cyt c was observed after rGal-1 or GMP\* incubation (2- or 1.6-fold increase, respectively).

The induction of caspase-9-like activation was next evaluated by a fluorometric assay based on Leu-Glu-His-Asp (LEHD)-AFC cleavage activity (Figure 5F). As shown, 400  $\mu\text{g}/\text{mL}$  rGal-1 or GMP treatment for 8 h caused a considerable caspase-9-like activation (2.7- and 5-fold increase, respectively) compared with untreated cells. As a control of specificity, cells were pretreated with 20  $\mu\text{M}$  caspase-9 inhibitor

(z-LEHD-fmk) before rGal-1 exposure, and caspase-9 activation was completely inhibited.

#### *Partial prevention of Gal-1-mediated apoptosis by caspase-8 or caspase-9 inhibitor*

To estimate the dependence of caspase-8 and/or caspase-9 activation on Gal-1-mediated apoptosis, we performed a set of experiments in the presence of their specific inhibitors (z-IETD-fmk or z-LEHD-fmk, respectively). As previously shown, the presence of the inhibitors prevented the activation of caspase-8 or caspase-9 by rGal-1. Subsequent analysis of apoptotic nuclei after 8 h treatment with rGal-1 was carried out in the presence or absence of these inhibitors (Figure 6).



**Fig. 6.** Partial prevention of Gal-1-mediated apoptosis by caspase-8 or caspase-9 inhibitor. MA-10 cells treated with 20  $\mu$ M caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), or pan caspase inhibitor (z-VAD-fmk) before incubation with 400  $\mu$ g/mL rGal-1 for 8 h were subjected to PI staining and flow cytometry. The percentage of apoptotic cells  $\pm$  SD of three independent experiments is shown in each histogram. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Incubation of MA-10 cells with 400  $\mu\text{g}/\text{mL}$  rGal-1 resulted in  $37.4 \pm 2.8\%$  nuclei in the subdiploid region, while this effect was prevented only partially in the presence of z-IETD-fmk ( $30.9 \pm 2.2\%$ ) or z-LEHD-fmk ( $28.4 \pm 1.8\%$ ). Interestingly, although the exposure to both inhibitors together increased their protective effect, it did not totally prevent rGal-1-induced apoptosis ( $22.1 \pm 1.5\%$ ), whereas the presence of the pan caspase inhibitory peptide did.

#### Protein expression dependence of Gal-1-induced apoptosis

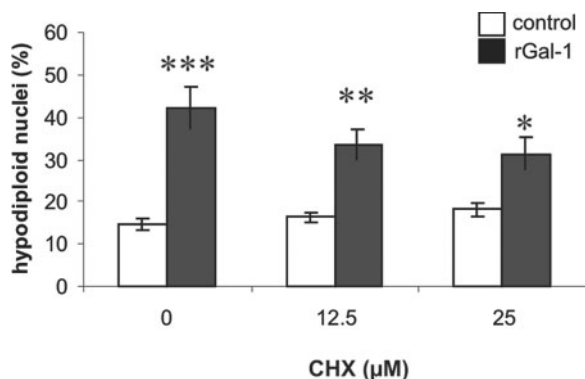
To evaluate whether Gal-1-mediated apoptosis is dependent of *de novo* protein synthesis, MA-10 cells were incubated with different concentrations of cycloheximide (CHX), a potent protein synthesis inhibitor, before 8 h exposure to 400  $\mu\text{g}/\text{mL}$  rGal-1. As Figure 7 shows, the presence of CHX partially prevented the effect of rGal-1 on DNA fragmentation at the maximal concentration probed, as higher concentrations of CHX were toxic for the cells.

#### Growth stimulation of MA-10 cells by Gal-1

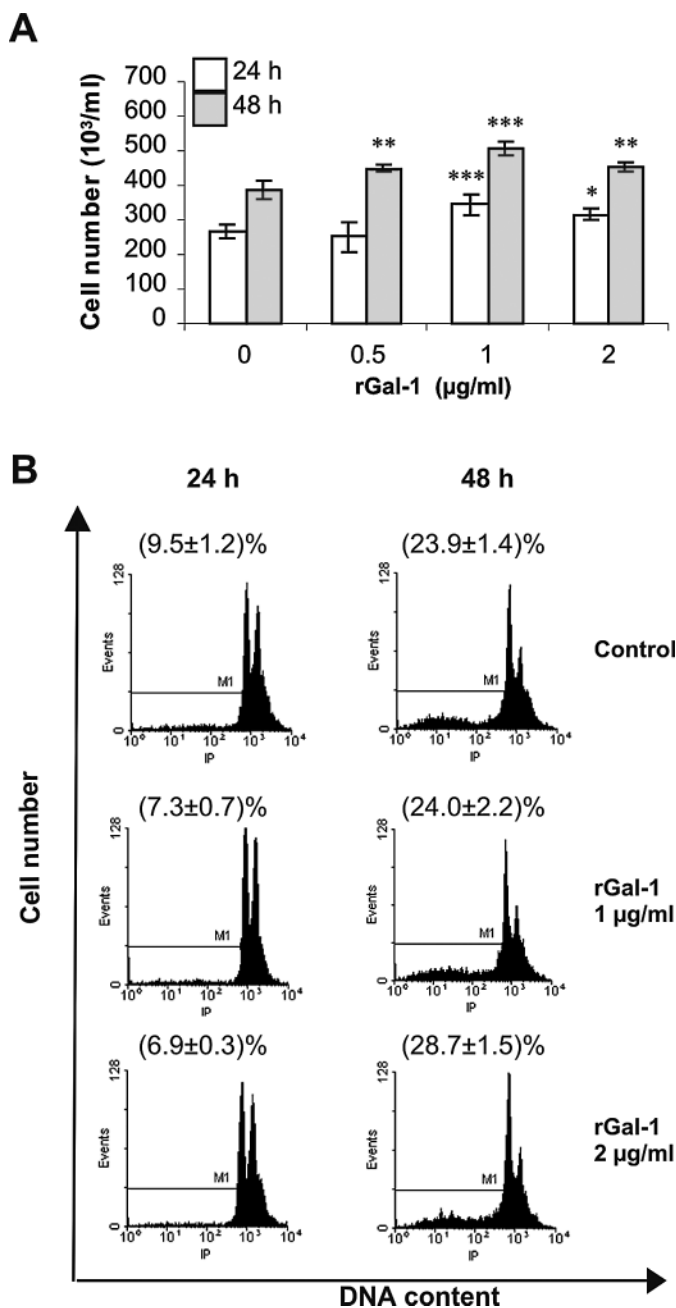
To examine the possibility of a biphasic effect of Gal-1 on cell growth, cells were incubated in the presence of lower concentrations of the lectin. To evaluate a possible stimulatory effect, cells were counted by trypan blue exclusion method after addition of 0.5, 1, or 2  $\mu\text{g}/\text{mL}$  rGal-1 for 24 or 48 h incubation (Figure 8A). While a slight but significant 1.3-fold increase in cell number was observed after 24 h of treatment with 1  $\mu\text{g}/\text{mL}$  rGal-1, the stimulatory effect became significant after 48 h incubation with all the concentrations probed. Analysis of apoptotic nuclei was performed to evaluate the effect of the lectin at lower concentrations on cell death (Figure 8B). As expected, no significant induction of apoptosis was detected after 24 or 48 h incubation with the lectin as compared with untreated cells.

#### Discussion

In a previous publication, we demonstrated that while Gal-1 is diffusely expressed in the cytosol of cultured rat



**Fig. 7.** Protein expression dependence of Gal-1-induced apoptosis. MA-10 cells were pretreated with increasing concentrations of CHX before incubation with 400  $\mu\text{g}/\text{mL}$  rGal-1 for 8 h. Hypodiploid nuclei were analyzed by flow cytometry after PI staining. Results are expressed as a percentage relative to controls  $\pm$  SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Fig. 8.** Growth stimulation of MA-10 cells by Gal-1. (A) Cells were plated and cultured in growth medium for 6 h before incubation with increasing concentrations of rGal-1 in assay medium for additional 24 or 48 h. Cell number was assessed by trypan blue exclusion method and data are shown as mean  $\pm$  SD of three independent experiments. (B) Untreated and treated MA-10 cells incubated for 24 or 48 h were studied by flow cytometry after PI staining as described in *Materials and methods*. The percentage of apoptotic MA-10 cells (hypodiploid DNA peak)  $\pm$  SD of three independent experiments is plotted in each histogram. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Leydig cells, it is also present in discrete sites of the cell membrane (Martinez *et al.*, 2004). In this study, we show that tumor MA-10 cells exhibit a weak, diffuse cytosolic expression of Gal-1 but, unlike normal Leydig cells, these cells do not express membrane-associated Gal-1. Consistent with this data, Gal-1 is not detected in serum-free medium, suggesting

that these cells do not secrete Gal-1 to the medium, at least in the conditions probed. This finding might be relevant for the tumor progression or survival since these cells die in response to Gal-1. However, even if they do not secrete it themselves, these cells can be exposed to the lectin as other cell types might be the source of Gal-1. The regulation of Gal-1 expression and secretion remains to be elucidated.

Leydig cells rarely proliferate or undergo apoptosis in normal adult testis. Induction of apoptosis has been well documented after treatment with different toxics, such as ethylene dimethane-sulfonate (EDS) (Taylor *et al.*, 1999) or ethanol (Jang *et al.*, 2002), glucocorticoids, such as corticosterone (Gao *et al.*, 2003), or the phytochemical isoflavonoid genistein (Kumi-Diaka *et al.*, 1999). As we previously demonstrated, relatively high concentrations of Gal-1 (50–400  $\mu\text{g}/\text{mL}$ ) exert an inhibitory effect on cultured Leydig cells viability by induction of apoptosis (Martinez *et al.*, 2004). Although MA-10 tumor cells are shown to be sensitive to Gal-1 treatment, higher concentrations of the lectin (200–400  $\mu\text{g}/\text{mL}$ ) and longer periods of incubation (8 h) are required to obtain the same inhibitory effect compared to normal cells. The relatively high amounts of Gal-1 required for apoptosis induction (400  $\mu\text{g}/\text{mL}$ ; 14  $\mu\text{M}$ ) are comparable with the concentrations of the lectin (10–20  $\mu\text{M}$ ) used for inducing cell death in immature thymocytes, human T leukemia cell lines, and activated T-cells (Perillo *et al.*, 1995, 1997). In contrast, apoptotic activity is also observed at lower concentrations in early hematopoietic cells (Vas *et al.*, 2005) and mammary cancer cells (Wells *et al.*, 1999). Like in normal Leydig cells, the inhibitory effect on MA-10 cells can be attributed to induction of apoptosis, as demonstrated by DNA fragmentation.

The extrinsic pathway of apoptosis is triggered by activation of the members of TNF receptor superfamily by their ligands (Mor *et al.*, 2002). The Fas/FasL system is constitutively expressed in Leydig cells under physiological conditions and mediates induction of apoptosis after EDS or glucocorticoid treatment (Taylor *et al.*, 1999; Gao *et al.*, 2003), as both toxics up-regulate Fas and FasL expression levels. The present results indicate that Gal-1 elicits up-regulation of FasL expression. Gal-1 treatment results in the activation of caspase-8, the major pro-caspase activated by death receptors, suggesting the implication of this pathway in Gal-1-mediated apoptosis. However, there are, at least, two other possible mechanisms for caspase-8 activation. First, TNF-alpha-related apoptosis-inducing ligand (TRAIL) is a member of TNF-alpha family that is known to induce apoptosis upon binding to its death domain-containing receptors, DR4/TRAIL-R1, and DR5/TRAIL-R2. As described by Grataroli *et al.* (2002), TRAIL ligand is permanently expressed in Leydig cells from fetal stages to adulthood, whereas the receptor DR5 is detected only after the pubertal period. Second, it has been recently demonstrated that unligated integrins promote apoptosis by recruiting caspase-8 to the membrane, where it becomes activated in a death-receptor-independent manner (Stupack *et al.*, 2001). Both TRAIL and integrins are suitable for Gal-1 interaction and their involvement in Gal-1-induced apoptosis needs further investigation.

On the contrary, the key event in the intrinsic pathway of apoptosis is the release of mitochondrial molecules, from

coupled or uncoupled mitochondria, which activate initiator caspases, such as caspase-9, leading to cell death (Green and Reed, 1998; Green and Kroemer, 2004). In this study, evidence is provided that Cyt c is released from uncoupled mitochondria, because loss of  $\Delta\Psi_m$  preceded this release. Surprisingly, there is not a subsequent decrease in mitochondrial Cyt c. Moreover, the results indicate an increase in its expression level following Gal-1 treatment. This fact was also described in other systems, such as the early stages of camptothecin-induced apoptosis in Jurkat cells, where Cyt c and other respiratory complex proteins are up-regulated (Sánchez-Alcázar *et al.*, 2000). Consistent with these results, caspase-9 activity is increased after Gal-1 treatment.

In an attempt to elucidate whether these pathways may be activated independently, Gal-1-mediated apoptosis was analyzed in the presence of caspase-8- or caspase-9-specific inhibitors. This analysis clearly indicates that independent pathways may be activated, as the presence of one of the inhibitors only partially prevents Gal-1-induced apoptosis. It is noteworthy that treatment of these cells with pan caspase inhibitor completely prevents Gal-1-induced cell death, providing evidence that this effect is caspase dependent. It has been well established that Gal-1-mediated T-cell death occurs independently of Fas- or glucocorticoids-triggered pathways (Perillo *et al.*, 1995, 1997). Hahn *et al.* (2004) demonstrated that T-cell death proceeds through a caspase- and Cyt c-independent pathway but is mediated by translocation of endonuclease G from mitochondria to nuclei. On the contrary, activation of AP-1 transcription factor and down-regulation of proapoptotic Bcl-2 were described in freshly isolated rat T-cell death (Rabinovich *et al.*, 2000). Interestingly, consistent with the long time required for Gal-1-mediated Leydig cell death and increased expression of apoptotic proteins such as FasL, the apoptotic effect also requires *de novo* protein synthesis, as demonstrated by treatment with the protein inhibitor CHX. However, the partial prevention observed with this treatment suggests that independent pathways are activated.

Interestingly, Gal-1 also exerts a growth-promoting effect on MA-10 cells. Although MA-10 cells undergo apoptosis in response to relatively high concentrations of Gal-1, a slight but significant increase in proliferation is observed after treatment with lower concentration of the lectin (0.5–2  $\mu\text{g}/\text{mL}$ ). This is a very important finding, since it has been described that Gal-1 acts as mitogen for only a few cells, including fibroblasts (Adams *et al.*, 1996), vascular endothelial cells (Moiseeva *et al.*, 2000), and early hematopoietic cells (Vas *et al.*, 2005). The mechanism appears to be cell-type specific, as it can promote cell proliferation in carbohydrate-dependent or independent fashion, and it elicits its effect at a wide range of concentrations.

The biphasic effect of Gal-1 on cell viability may be due to the different impact of Gal-1 concentration on protein-protein interactions. The monomeric and dimeric forms of Gal-1 are in equilibrium with a  $K_d$  of 7  $\mu\text{M}$  (Cho and Cummings, 1995). While Gal-1 is primarily present as a dimer at the concentrations required to trigger cell death, the monomeric form is predominant at the growth promoting concentrations. Thus, depending on the concentration, Gal-1 may induce cross-linking of proteins or conformational changes leading either to inhibition or to



promotion of a cellular response. Alternatively, Gal-1 may enhance or sterically inhibit the binding of cell surface receptors to their ligands. Therefore, the presence of lactose, a Gal-1 specific ligand, is shown to prevent Gal-1 activities, suggesting that Gal-1 exerts its modulatory effect by binding to glycoproteins present in either the cell surface or the ECM.

In conclusion, this work provides evidence that Gal-1, an endogenous protein, might play a key role in Leydig cell biology. Normal Leydig cells might be exposed to Gal-1 in an autocrine or paracrine manner. Alternatively, other cell types might be responsible for Gal-1 regulation of Leydig cell functions, such as Sertoli or peritubular cells. Importantly, Leydig cells are in intimate contact with interstitial macrophages which, as previously described, express and secrete Gal-1 to the medium once they become activated (Rabinovich *et al.*, 1998). Further investigation should be addressed to elucidate the physiological and pathological implication of Gal-1 in the testis.

## Materials and methods

### Materials

Waymouth MB 752/1 medium, HEPES buffer, RNase A, RNase T, PI, ethidium bromide, DiOC<sub>6</sub>(3), bovine serum albumin (BSA), lactosyl-agarose column, lactose, genistein, methylprednisolone, horse heart Cyt c, CHX, and rabbit anti-actin polyclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Horse serum, gentamycin, and trypsin/ethylenediaminetetraacetic acid (EDTA) were from Gibco BRL, Life Technologies (Grand Island Biological, NY). Phase Lock Gel was obtained from Eppendorf AG (Hamburg, Germany). Mouse anti-human Cyt c (clone 7H8.2C12) monoclonal antibody was purchased from BD Pharmingen (San Diego, CA). Purified goat anti-human Gal-1, rabbit anti-human Fas, and rabbit anti-human FasL polyclonal antibodies; mouse anti-goat IgG coupled to fluorescein isothiocyanate (FITC); and goat anti-mouse IgG and mouse anti-goat IgG coupled to horseradish peroxidase (HRP), pan caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were obtained from SantaCruz Biotechnology, Inc. (Santa Cruz, CA).

### Preparation of recombinant human Gal-1

rGal-1 was expressed according to Couraud *et al.* (1989) in *Escherichia coli* strain BL21DE3 transformed with the plasmid that encodes Gal-1 and pT7IML-1 (a generous gift from Dr. Linda G. Baum, University of California, Los Angeles, School of Medicine) and purified as previously described (Perillo *et al.*, 1997; Iglesias *et al.*, 1998b). To maintain maximal lectin activity, a reducing agent (0.2 mM dithiothreitol) was added.

### MA-10 Leydig cells

The origin of the MA-10 cells (kindly provided by Mario Ascoli, University of Iowa) has been previously described (Ascoli, 1981; Pignataro and Ascoli, 1990). MA-10 cells were cultured in 75 cm<sup>2</sup> tissue culture flasks in growth

medium (Waymouth MB752/1 modified to contain 1.1 g/L NaHCO<sub>3</sub>, 20 mM HEPES, 50 µg/mL gentamycin, and 15% horse serum, pH 7.4) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. For most experiments, cells were cultured in 24-well plates at a density of 150,000 cells per well in growth medium to allow adhesion. After 48 h, the medium was changed to serum-free culture medium supplemented with 1 mg/mL BSA (assay medium). Cells were harvested by trypsin/EDTA treatment.

### Assessment of DNA hypodiploidy by flow cytometry

Cells were seeded in 24-well plates and treated with rGal-1, in the presence or absence of 20 mM lactose. Positive control of apoptosis with a mixture of 50 µg/mL genistein and 100 µM methylprednisolone (GMP) was simultaneously performed (Kumi-Diaka *et al.*, 1999; Gao *et al.*, 2003). In some experiments, cells were pre-incubated for 1 h with 20 µM caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), pan caspase inhibitor (z-VAD-fmk), or different concentrations of CHX (Ramnath *et al.*, 1997). After treatment, cells were harvested, washed, and fixed with 70% ethanol for 24 h. The fixed cells were centrifuged, washed, and incubated with RNase A 1 µg/mL for an additional 1 h at 37°C. Cells were washed and resuspended in 1 mL hypodiploidy solution (2.5 µg/mL PI in 0.1% sodium citrate and 0.1% Triton X-100) as described by Nicoletti *et al.* (1991). After overnight incubation, the stained cells (10,000 events) were analyzed in a fluorescence activated cell sorting (FACS) Calibur cytometer (Becton Dickinson, CA).

### DNA fragmentation analysis by agarose gel electrophoresis

Agarose gel electrophoresis was performed to detect damage to nuclear chromatin. DNA was extracted by the Phase Lock Gel procedure according to manufacturer's instructions. Briefly, cells were collected after 24 h treatment with rGal-1 or GMP. The samples were centrifuged at 200 × g for 5 min and the pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.8; 10 mM EDTA; and 0.5% sodium dodecyl sulfate [SDS]). Proteins were degraded using 0.25 mg/mL proteinase K at 56°C for 1 h. RNase A was added at a final concentration of 0.1 mg/mL and incubated for 1 h at 37°C. The solutions were transferred to a Phase Lock Gel tube and DNA was purified by successive phenol-chloroform extractions. The resultant aqueous phase was mixed with 3 M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at -20°C overnight and then washed with 70% (v/v) ethanol. The samples were resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, and mixed with loading buffer. DNA was resolved on a 1.8% agarose gel, stained with ethidium bromide, and bands were visualized by UV fluorescence.

### Determination of caspase-3-, caspase-8-, and caspase-9-like activities

To determine caspase activity associated with apoptosis, fluorometric protease assays (caspase-3 and caspase-8 apoptosis detection kits, SantaCruz Biotechnology, Inc, and caspase-9 fluorometric substrate, Calbiochem, La Jolla, CA) were used. After treatment with rGal-1, with or

without lactose, or GMP, cells were harvested, washed, and homogenized in lysis buffer. The appropriate substrate (DEVD-AFC, IETD-AFC, or LEHD-AFC) was added in reaction buffer and incubated for 1 h at 37°C. Free AFC, generated as a result of cleavage of the aspartate–AFC bond, was measured using an Aminco Bowman Series-2 spectrofluorometer (Sim-Aminco Spectronic Instrument) with 360 nm excitation and 450 nm emission filters. To inhibit caspase activity, cells were pre-incubated for 1 h with 20  $\mu$ M caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), or pan caspase (z-VAD-fmk) inhibitory peptides.

#### Immunofluorescence labeling

The indirect immunofluorescence assay was carried out as previously described (Martinez *et al.*, 2004). Cells were cultured on glass coverslips for 24 h and fixed in cold acetone for 7 min at  $-20^{\circ}\text{C}$  and then washed with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min (permeabilized cells) or in 2% *p*-formaldehyde in PBS for 10 min at room temperature followed by a wash with PBS for 5 min (nonpermeabilized cells). Both were incubated first with 1% BSA in PBS for 30 min and then with anti-Gal-1 polyclonal antibody (1:20) for 60 min, washed, and incubated for an additional 1 h with FITC-conjugated anti-goat IgG (1:80). After washing, cells were mounted in buffered glycerin and analyzed by fluorescent microscopy.

For FACS analysis, cells were harvested, washed, and resuspended in PBS. For cell surface expression of Gal-1, cells were incubated with anti-Gal-1 polyclonal antibody in 0.1% BSA in PBS (1:20) for 60 min at  $4^{\circ}\text{C}$ , washed, and incubated for 30 min with FITC-conjugated anti-goat IgG (1:80). The stained cells (10,000) were analyzed on FACS Calibur cytometer.

#### Protein extraction and western blot analysis

Gal-1 expression and secretion were analyzed as described by Rabinovich *et al.* (1999). Cells were grown in 25  $\text{cm}^2$  tissue culture flasks for 48 h before 24 h incubation in serum-free medium. The supernatant was collected and centrifuged at  $1000 \times g$  for 5 min. Then, 0.5% SDS was added and the sample was boiled for 5 min. Proteins were precipitated with 9 vol of methanol, incubated overnight at  $-20^{\circ}\text{C}$  and centrifuged at  $21,000 \times g$  for 30 min. On the contrary, cells were harvested and resuspended in PBS containing 1% NP-40, 10 mM EDTA, and a protease inhibitor cocktail for 30 min on ice, followed by centrifugation at  $10,000 \times g$  for 10 min. Proteins (50  $\mu$ g) were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 16% polyacrylamide gels.

For Cyt c release, cells were cultured for 8 h in the presence of rGal-1 or 50  $\mu$ g/mL genistein and 200  $\mu$ M methylprednisolone (GMP\*), harvested, and resuspended in sucrose buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM [ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid] [EGTA], and 1 mM dithiothreitol). After 15 min incubation on ice, cells were homogenized by 15 strokes using a Douncer homogenizer. Homogenates were centrifuged twice at  $1000 \times g$  for 10 min to

remove debris and nuclei and then at  $10,000 \times g$  for 10 min to obtain the mitochondrial fraction (pellet). The supernatant was centrifuged at  $100,000 \times g$  for 60 min to obtain the cytosolic fraction (supernatant). Proteins (20  $\mu$ g) were submitted to SDS–PAGE on 16% polyacrylamide gels.

For Fas, FasL, or Cyt c expression, cells were treated with rGal-1 or GMP\* for 8 h and then incubated in PBS containing 1% NP-40, 10 mM EDTA, and a protease inhibitor cocktail for 30 min on ice. Proteins (50  $\mu$ g) were resolved by SDS–PAGE on 12% polyacrylamide gels.

In all cases, polyacrylamide gels were electrotransferred onto nitrocellulose membranes and probed with the appropriate antibody: goat anti-Gal-1 (1:250), mouse anti-Cyt c (1:1000), or rabbit anti-Fas (1:750), anti-FasL (1:400), anti-actin (1:1000), or anti-P450sc (1:500) antibodies overnight at  $4^{\circ}\text{C}$ , followed by a 60 min incubation with HRP-conjugated secondary antibody—anti-goat IgG (1:3000), anti-mouse IgG (1:20,000), or anti-rabbit IgG (1:15,000). Immunoreactive bands were detected by a chemiluminescence procedure (ECL plus Western Blotting Detection System, Amersham Bioscience). Quantification of bands was achieved by densitometry using Storm 840 (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

#### Assessment of $\Delta\Psi_m$

Loss of  $\Delta\Psi_m$  was assessed by cytofluorometric analysis using the dye DiOC<sub>6</sub>(3) (Gao *et al.*, 2003). Cells treated with rGal-1, in the presence or absence of 20 mM lactose, for 4 or 8 h, were incubated with 80 nM DiOC<sub>6</sub>(3) for 15 min at  $37^{\circ}\text{C}$  followed by analysis in FACS Calibur cytometer (10,000 events).

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#### Conflict of interest statement

None declared.

#### Abbreviations

AFC, 7-amino-4-trifluoromethyl coumarin; BSA, bovine serum albumin; CHX, cycloheximide; CRD, carbohydrate recognition domain; Cyt c, cytochrome c; DEVD, Asp-Glu-Val-Asp; DiOC<sub>6</sub>(3), 3, 3'-dihexiloxadicarbocyanine iodide; EGTA, Ethylene glycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence activated cell sorting; FADD, Fas-associated death domain; FITC, fluorescein isothiocyanate; Gal-1, galectin-1; GMP, genistein and methylprednisolone; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; rGal-1,

recombinant Gal-1; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRAIL, TNF-alpha-related apoptosis-inducing ligand;  $\Delta\Psi_m$ , mitochondrial membrane potential.

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