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# Molecular mechanisms implicated in galectin-1-induced apoptosis: activation of the AP-1 transcription factor and downregulation of Bcl-2

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## Abstract

**Galectins are emerging as a new class of bioactive molecules with specific immunomodulatory properties. Galectin-1 (Gal-1), a member of this family, has been shown to induce apoptosis of mature T cells and immature thymocytes. To gain insight into the intracellular signals transduced by Gal-1 upon binding to mature T cells, we investigated whether this protein triggered activation of the dimeric AP-1 transcription factor. A marked increase in the binding of nuclear extracts to synthetic oligonucleotides containing the AP-1 consensus sequence, could be detected by an electrophoretic mobility shift assay, when T cells were cultured for 30 min in the presence of Gal-1. This DNA-binding activity was preceded by a rapid increase in the levels of *c-Jun* mRNA, as determined by Northern blot analysis. Requirement of AP-1 for Gal-1-induced apoptosis was confirmed by the dose-dependent reduction on the level of DNA fragmentation observed when cells were pre-treated with curcumin (an inhibitor of AP-1 activation) before exposure to Gal-1. Finally, evidence is also provided by Western blot analysis, showing that Gal-1 inhibits Concanavalin A (Con A) induction of Bcl-2 protein. Results presented in this study provide the first experimental evidence regarding AP-1 and Bcl-2 as targets of the signal transduction pathway triggered by Gal-1 and set the basis for a more in depth understanding of the molecular mechanisms of T-cell death regulation.** *Cell Death and Differentiation* (2000) **7,** 747 – 753.

**Keywords:** galectin-1; apoptosis; AP-1; *c-Jun*; Bcl-2

**Abbreviations:** AP-1, activating protein-1; Con A, concanavalin A; CRD, carbohydrate recognition domain; EMSA, electrophoretic mobility shift assay; Gal-1, Galectin-1; PCD, programmed cell death; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labeling

## Introduction

Galectin-1 (Gal-1) is a member of a growing family of animal $\beta$-galactoside-binding proteins, which are highly conserved throughout animal evolution[1–3] and share sequence similarities in the carbohydrate recognition domain (CRD).[4] Its presence has been localized within the central and peripheral immune compartment in thymic epithelial cells,[5] activated T cells[6,7] and activated macrophages.[8,9] It has also been found in immune privileged sites of the body such as placenta,[10,11] cornea[12] and testis.[13]

Recent observations suggest that Gal-1 could play an important role in generating and maintaining central and peripheral immune tolerance.[14–16] This homodimeric protein, composed by subunits of $\sim$134 amino acids,[2] has been shown to induce apoptosis of activated mature T cells[9,17,18] and particular subsets of non-selected and negatively selected CD4$^{lo}$ CD8$^{lo}$ immature thymocytes.[19] The apoptotic effect of Gal-1 depended upon the activation state of T cells and was mediated by engagement of CD43 or CD45, particularly the polylactosamine-enriched CD45RO splicing product.[17,19]

We have recently validated this hypothesis *in vivo*, using gene and protein therapy strategies, showing that Gal-1 was able to suppress the inflammatory and autoimmune response via T-cell apoptosis in collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis.[16]

However, despite considerable progress, there is still scarce information concerning the intracellular downstream signals transduced by Gal-1 upon binding to mature T cells and the molecular events underlying Gal-1-induced apoptosis.

Activating protein-1 (AP-1) is a collective term referring to dimeric transcription factors composed by Jun, Fos or activating transcription factor (ATF) subunits.[20] Homodimers of c-Jun or heterodimers of c-Jun and c-Fos modulate several biological responses by binding to the DNA consensus sequence TGA(C/G)TCA (12-*o*-tetradecanoyl-phorbol 13-acetate-responsive element of TRE) found in the promoter region of several genes.[21] There is ample evidence that the activity of AP-1, especially that of c-Jun, is essential for cell proliferation and differentiation. Recently, it has been suggested that certain components of AP-1 may also be involved in programmed cell death (PCD).[20,21] However, the available information is contro-

versial, showing a scenario where AP-1 activity is essential for cells to undergo apoptosis under certain conditions such as cytokine withdrawal,[22] but may not be linked to cell death in the case of TNF-$\alpha$ or Fas-induced apoptosis.[23,24] In the present study we investigated whether binding of Gal-1 to mature T cells triggers activation of AP-1 and modulates expression of the *c-Jun* proto-oncogene. Furthermore, we explored whether AP-1 activation is indeed an essential step in Gal-1-induced T cell apoptosis and not a side effect in the signaling process leading to PCD.

Since the response of a cell to a death signal is determined by a preset rheostat, represented in many cell types by the relative protein levels of members of the Bcl-2 family,[25] we also investigated whether Gal-1 was able to modulate Bcl-2 protein synthesis in the context of stimulated T cells.

## Results

### Gal-1-induced apoptosis is preceded by an early activation of AP-1

We have recently reported that activated macrophages produce a Gal-1-like protein, which induces apoptosis of T cells.[9] Cells exposed to Gal-1 (2, 4 and 8 $\mu$g/ml) underwent apoptosis after 6 h of incubation, as evidenced by DNA cleavage into oligonucleosomal-sized fragments of $\sim$180–200 bp and the typical morphological changes of apoptosis, including reduction of the cytoplasmic volume, loss of surface microvilli and chromatin condensation. As summarized in Table 1, incorporation of biotinylated dUTP by exogenous terminal deoxynucleotidyl transferase (TdT) into DNA strand breaks increased in $\sim$28%, when cells were exposed for 6 h to this protein. This effect was specific since addition of lactose, a $\beta$-galactoside-specific sugar, to the culture medium was able to induce a marked decrease in the percentage of TUNEL positive cells.[9]

Transcription factors play a central role in converting extracellular signals into changes in the expression of specific genes and thereby regulate complex biological processes.[20] Since the downstream intracellular signals transduced by Gal-1 upon binding to mature T cells still remain to be elucidated, we first investigated whether Gal-1-induced apoptosis is preceded by an early activation of AP-1, a transcription factor which was found to be essential for many biological processes. By an electrophoretic mobility shift assay (EMSA) (Figure 1), we found that Gal-1 increased the binding of nuclear extracts to synthetic oligonucleotides containing the AP-1 consensus sequence (lane 3), in comparison with nuclear extracts prepared from T cells cultured in the absence of any stimuli (lane 4). The DNA-protein complex induced by Gal-1 had the same electrophoretic mobility, as well as a comparable intensity, as the AP-1 complex induced by 5 $\mu$g/ml Con A (lane 1), as previously shown.[26] No additive effects were observed in the abundance of the AP-1 complex using protein extracts from cells stimulated with both Gal-1 and Con A (lane 2). The prominent increase of AP-1 DNA-binding activity was detected as early as 30 min after treatment with 4 $\mu$g/ml Gal-1 (lane 3), being undetectable 120 min after treatment (data not shown). In order to determine the specificity of this DNA-binding activity, we carried out competition experiments with homologous and heterologous unlabeled oligonucleotides. These experiments demonstrated that the observed complex was specific, since the binding was displaced by the addition of 100-fold molar excess of unlabeled AP-1 consensus oligonucleotides (lane 5), while the addition of 100-fold molar excess of the unlabeled mutated AP-1 oligonucleotide did not affect the formation of the Gal-1-induced complex (lane 6). Constitutive SP-1

**Table 1** Incorporation of biotinylated dUTP by exogenous TdT into DNA strand breaks generated after Gal-1-treatment

| Cell treatment | Gal-1 (4 $\mu$g/ml) | Percentage of apoptotic cells (TUNEL reaction) |
|---|---|---|
| Medium | – | 5.0 |
| Medium | + | 33.4 |
| Con A (2.5 $\mu$g/ml) | – | 9.5 |
| Con A (2.5 $\mu$g/ml) | + | 38.0 |
| Lactose (100 mM) | + | 11.8 |

T cells ($2\times10^7$/well) were exposed to different stimuli for 6 h at 37°C. Samples were harvested, fixed, permeabilized and the percentage of apoptotic cells in each sample was determined by flow cytometric analysis after TUNEL labeling

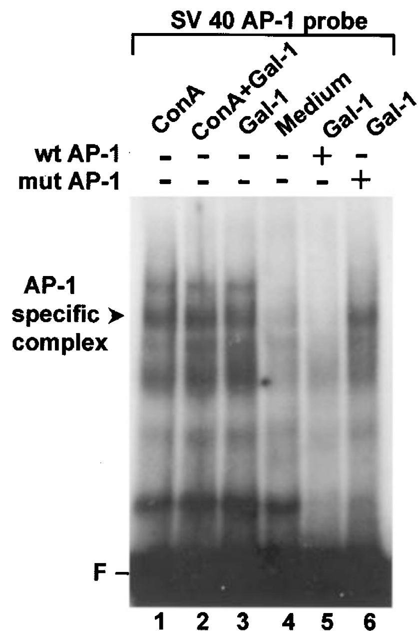


**Figure 1** Effect of Gal-1 on AP-1 DNA binding activity. Electrophoretic mobility shift assay. T cells were cultured for 30 min in the presence of 5 $\mu$g/ml Con A (lane 1), Con A plus 4 $\mu$g/ml Gal-1 (lane 2), 4 $\mu$g/ml Gal-1 (lane 3) or medium alone (lane 4). Cells were collected and nuclear extracts were prepared as described in Materials and Methods. Protein DNA-binding reactions were carried out in the absence of competitors or in the presence of 100-fold molar excess of an unlabeled AP-1 consensus oligonucleotide (lane 5) or an AP-1 mutant oligonucleotide (lane 6). The arrow indicates the specific AP-1 nucleoprotein complexes, which were efficiently competed with a 100-fold molar excess of unlabeled specific oligonucleotides. A representative of three independent experiments is shown

DNA-binding activity was present in all of the extracts suggesting that no protein degradation occurred in our experimental conditions (data not shown). These results demonstrate that Gal-1 binding to mature T cells triggers an early AP-1 DNA-binding activity.

Since AP-1 transcription factor is canonically a heterodimer of Fos-Jun or a homodimer of Jun-Jun subunits,[21] we next examined whether Gal-1 binding to mature T cells was able to modulate expression of the proto-oncogene *c-Jun*. The mRNA levels of this immediate early gene were examined by Northern blot analysis. As shown in Figure 2, Gal-1 treatment (4 $\mu$g/ml) resulted in a rapid increase of *c-Jun* mRNA at the expected 3.2 kb molecular size (lane 3), in comparison with cells cultured in the absence of any stimuli (lane 1) or cultured in the presence of Con A (lane 2). Maximal induction of this immediate early gene was achieved at 30 min (Figure 2), being undetectable 120 min after Gal-1 addition (data not shown). Equal loading and RNA integrity were checked by the amount of 18S and 28S ribosomal RNA stained with methylene blue dye. The disappearance of *c-Jun* transcript was coincident with the initiation of the apoptotic program of T cells triggered by Gal-1.[9,17,18] Hence, *c-Jun* transcription and AP-1 DNA-binding activity were both induced in the early phases after the message of death has been delivered to cells, as has been previously suggested for other stimuli.[22,27]

## Rescue of Gal-1-induced apoptosis by inhibition of the c-Jun/AP-1 pathway

To challenge the question whether AP-1 DNA-binding activity plays a crucial role and constitutes a pre-requisite for the initiation of apoptosis induced by Gal-1, we tried to block AP-1 activation by treatment with curcumin (1,7-bis [4-hydroxy-3-methoxy-phenyl]-1,6-heptadiene-3,5-dione). Curcumin, a dietary pigment responsible for the yellow color of curry, was reported to inhibit *c-Jun* expression and to block AP-1 activation induced by other agents such as phorbol esters and ceramide.[27,28] When cells were treated with curcumin (0.5, 1 and 2 $\mu$M) for 12 h before adding Gal-1, a dose-dependent decrease on the level of DNA fragmentation was observed (Figure 3, lanes 3, 4 and 5 respectively) in comparison with T cell cultures exposed to Gal-1 in the absence of curcumin (lane 2). Cells cultured in the presence of medium alone were used as negative controls (Figure 3, lane 1). Moreover, exposure of T cells to curcumin in the absence of Gal-1 did not induce any change on the DNA fragmentation pattern (data not shown). The dose-dependent inhibitory effect induced by the AP-1 inhibitor was reproduced when cells were analyzed by the TUNEL assay adapted to the flow cytometric protocol, showing a decrease from 33.4 to 12.5% of TUNEL positive cells when curcumin was added to cell cultures at its optimal concentration of 1 $\mu$M before addition of Gal-1 (data not shown). These results clearly indicate that AP-1 DNA-binding activity is indeed an essential step in Gal-1-induced apoptosis and not a side effect in the signaling process leading to PCD.

## Gal-1 binding to mature T cells downregulates Bcl-2 protein expression

It has been suggested that potent T-cell mitogenic stimuli, such as Con A, markedly increase transcription of the *bcl-2* proto-oncogene.[29] Hence, we investigated whether Gal-1 was able to modulate Con A-stimulated induction of Bcl-2. For this purpose, T cells were stimulated for 3 h in the presence of the indicated stimuli and the whole cell extracts were then processed for Western blot analysis

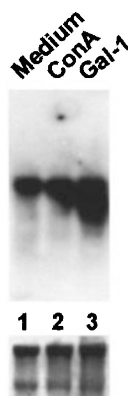


**Figure 2** Induction of c-Jun expression by Gal-1. Northern blot analysis. T cells were cultured for 30 min in the presence of medium alone (lane 1), 5 $\mu$g/ml Con A (lane 2) or 4 $\mu$g/ml Gal-1 (lane 3). Cells were then collected and total RNA was extracted, subjected to formaldehyde-agarose gel electrophoresis and examined for *c-Jun* gene expression by Northern blot analysis. Membranes were hybridized with a *c-Jun* probe and exposed for 24 h at $-70^{\circ}$C with intensifying screens. Equal loading and RNA integrity were checked by the amount of 18 S and 28 S ribosomal RNA stained with methylene blue dye. A representative of two independent experiments is shown

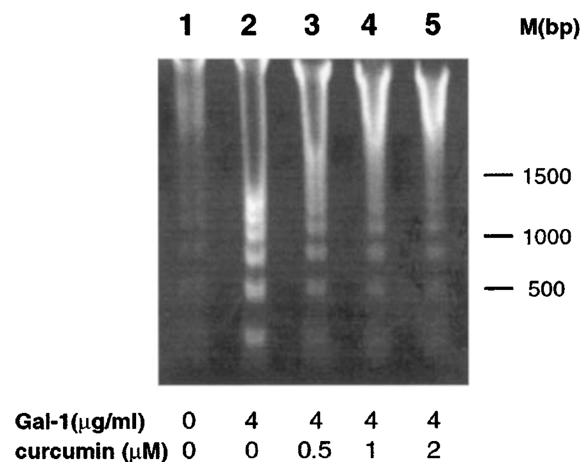


**Figure 3** Rescue of Gal-1-induced apoptosis by the AP-1 inhibitor curcumin. T cells were cultured in 24-well plates at a density of $2 \times 10^7$ cells/well for 6 h in medium alone (lane 1) and in medium containing 4 $\mu$g/ml Gal-1 (lane 2). To investigate the requirement of AP-1 for Gal-1-induced apoptosis, cells were pre-treated with curcumin (0.5, 1 and 2 $\mu$M) for 12 h, before exposure to Gal-1 (lanes 3, 4 and 5 respectively). Samples were then harvested, washed and genomic DNA was extracted as described above. Samples were diluted in loading buffer and resolved on a 1.5% agarose gel. The relative mobility of oligonucleosome-length DNA fragments reflects integer multiples of $\sim$180–200 bp. Molecular weight standards (100-bp DNA ladder) are indicated on the right. A representative of three independent experiments is shown

using the anti-Bcl-2 antibody (Figure 4A) and further analyzed by densitometry (Figure 4B). As expected, Bcl-2 expression was increased in Con A-stimulated T cells (5 $\mu$g/ml) (lane 2), compared to cells cultured in the presence of medium alone (lane 1). Treatment of T cells with Gal-1 (4 or 8 $\mu$g/ml) was able to downregulate Bcl-2 expression induced by the miotogenic stimulus (lanes 4 and 5 *vs* lane 2). We could not detect any significant change in Bcl-2 expression, when cells were exposed to Gal-1 in the absence of Con A (lane 3 *vs* lane 1). Moreover, no changes in Bcl-2 protein levels could be detected when cell cultures were performed for periods shorter than 3 h (data not shown), suggesting that downregulation of Bcl-2 was tightly correlated with the kinetics of initiation of apoptosis.

## Discussion

Galectins have emerged as a new class of bioactive molecules with specific immunomodulatory properties.[1–4] Gal-1, a proto-type member of this family, has shown immunosuppressive,[14–16] pro-apoptotic,[9,17–19] anti-inflammatory[16] and anti-adhesive[30] properties.

Gal-1 has been proposed to exert its functions by cross-linking specific oligosaccharide ligands on cell surface glycoconjugates.[4,17] It has recently been suggested that Gal-1 triggers early upstream events in T cell physiology, such as generation of inositol-1,4,5-triphosphate and tyrosine phosphorylation of phospholipase C$\gamma$1.[31] However, the intracellular 'downstream' signals triggered by this protein upon binding to cell surface receptors still remain to be elucidated. In the present study, we have demonstrated that apoptosis induced by Gal-1 is preceded by an increase in *c-Jun* mRNA levels, which leads to an early AP-1 DNA-binding activity. Immediate early genes such as *c-Fos* and *c-Jun* are considered as a 'master switch' for turning on other genes in response to a wide range of stimuli.[21] These genes may act as a step in the chain of events that convert signals at the cell membrane to long-lasting responses that require gene activity.

Three possible scenarios could be envisaged concerning the role of AP-1 in Gal-1-induced apoptosis: (a) AP-1 expression may play a crucial role and constitute a pre-requisite for the initiation of apoptosis; (b) apoptosis is triggered by activation of certain cell death specific signal transduction mechanisms and some of these signaling modifications may induce AP-1 expression as a side effect in the signaling process leading to PCD; and (c) AP-1 expression may constitute a side-effect for the initiation of apoptosis due to the fact that the intracellular signal transduction mechanism involved in apoptosis may result, through cross-talk of signaling processes, in an accidental induction of AP-1, without a direct connection between both events.[20,32] To discriminate between these possibilities and define whether AP-1 is an essential step in Gal-1 induced apoptosis, we used curcumin which has shown an inhibitory effect on 12-*o*-tetradecanoylphorbol 13-acetate-induced AP-1 activity.[27,28] This compound has been shown to inhibit the transactivating activity of c-Jun/AP-1, presumably by blocking the binding of this transcription factor to a single TRE site.[28] A dose-dependent inhibition of apoptosis was observed when T cells were pre-incubated for 12 h with curcumin, before exposure to Gal-1, which is consistent with a requirement for this transcription factor in the execution of PCD induced by this carbohydrate-binding protein.

Apoptosis occurs during lymphopoiesis to remove potentially autoreactive cells and those lymphocytes that have failed to produce a functional antigen receptor. Activated mature lymphocytes also undergo apoptosis by a propriocidal mechanism to prevent immunopathology associated with an overactive immune response.[33] As to the controversial information concerning the role of AP-1 in promoting or blocking the apoptotic program, it has been suggested that AP-1 may be activated in some cell types, when protein synthesis is required to initiate PCD. Withdrawal of cytokines in lymphoid cells has been associated with a rapid and transient induction of *c-Fos* and *c-Jun* transcripts.[22] Moreover, AP-1 activation has been reported to be an essential step in dexamethasone- and ceramide-induced PCD.[26,27] Indirectly, the balance

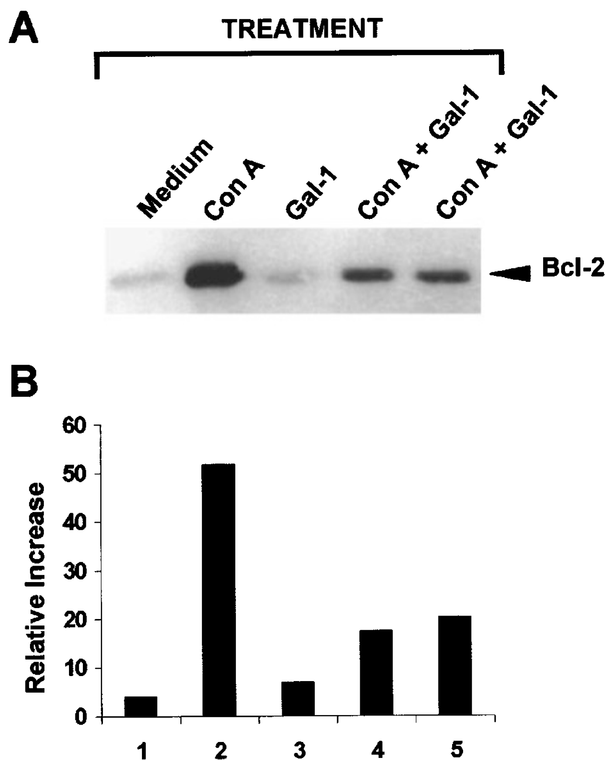


**Figure 4** Inhibition of Con-A-stimulated induction of Bcl-2 by Gal-1. Western blot analysis. (**A**) T cells were cultured for 3 h in medium alone (lane 1) or in the presence of 5 $\mu$g/ml Con A (lane 2), 4 $\mu$g/ml Gal-1 (lane 3), Con A plus 4 $\mu$g/ml Gal-1 (lane 4) or Con A plus 8 $\mu$g/ml Gal-1 (lane 5). Samples were lysed and equal amounts of protein were subjected to SDS–PAGE on a 15% polyacrylamide slab gel and immunoblotted with a rabbit anti-Bcl-2 polyclonal Ab (1 $\mu$g/ml). Equal loading and absence of extract degradation were checked using an anti-$\alpha$-tubulin (DM1A) mAb. (**B**) The immunoreactive protein bands indicated by the arrow were quantified by densitometry using a Shimadzu Dual Wavelength Cromato Scanner.® A representative of three independent experiments is shown

between ERK and JNK-p38 MAPK pathways has been shown to determine cell survival or death.[34] In contrast, TNF-$\alpha$ and Fas-mediated apoptosis did not require AP-1 activation.[23,24] Finally, AP-1/*c-Fos* has been shown to play a vital role in protecting immature double positive thymocytes from signal-induced apoptosis and therefore promoting cell survival.[35]

In connection with the therapeutic potential of Gal-1 in CIA and the results presented herein, Wakisaka *et al*[36] have recently reported that transfection of *Jun D*, a member of the AP-1 family of transcription factors, corrected the abnormal synovial cell function and inhibited the production of pro-inflammatory cytokines in patients with rheumatoid arthritis.

Finally, evidence is also provided in our study, showing that Gal-1 inhibited Con A-stimulated induction of Bcl-2 protein in mature T cells. Bcl-2 protein can be upregulated by cytokines, mitogenic stimuli (Con A, PHA) and other death-preventing signals or downregulated by death-promoting signals at different levels.[25,37] It has been shown to protect against diverse cytotoxic insults, such as dexamethasone, ultraviolet-irradiation and cytokine withdrawal.[25] However, deletion of autoreactive T cells in the thymus has not been blocked by a *bcl-2* transgene.[25] Furthermore, Fas-induced apoptosis of mature T cells bypasses the Bcl-2-inhibitable step common to most stress stimuli.[38] Here again, many forms of PCD are mediated by Bcl-2, while many others are not associated with the modulation of this anti-apoptotic protein. To connect both intracellular modulators, a recent study reported a link between c-Jun-induced apoptosis and the levels of Bcl-2, but not Bcl-xL gene product.[39] In our study, no difference could be found in the expression of the Bcl-xL protein after Gal-1 treatment (data not shown). Suggestively, Gal-3, an anti-apoptotic member of the galectin family, showed a significant sequence similarity with the BH1 domain of the Bcl-2 family of proteins containing the NWGR motif.[40]

Taken together, our results and previous observations, suggest that Gal-1 and Fas (CD95) antigen use different signal transduction pathways leading to apoptosis. In this sense, Gal-1 contributes to activation-induced cell death (AICD) through a Fas-independent mechanism.[17,19,41] It has been reported that this $\beta$-galactoside-binding protein triggered apoptosis in the T lymphoblastoid cell line MOLT-4, which is insensitive to Fas-induced apoptosis. In contrast, Gal-1 did not trigger PCD in the T lymphoblastoid cell line CEM, which is highly susceptible to the lethal effects of Fas-Fas L interaction.[17,19] Concerning the molecular mechanisms involved in Gal-1-induced apoptosis, it has been determined that this carbohydrate-binding protein is able to modulate T-cell receptor (TCR) signals to enhance antigen-mediated apoptosis.[42] Moreover, Pace *et al*[43] have recently reported that Gal-1-induced apoptosis in mature T cells involves a spatial redistribution of glycoprotein receptors (CD45, CD43) into specific microdomains.

The results presented in this paper provide the first experimental evidence regarding AP-1 and Bcl-2 as targets of the signal transduction pathway triggered in Gal-1-mediated apoptosis. In addition, they set the basis for a more in depth understanding of the molecular mechanisms of Gal-1-induced cell death regulation and its implications in T-cell physiopathology.

# Materials and Methods

## Reagents

RPMI 1640 medium, Con A, curcumin (1,7-bis [4-hydroxy-3-methoxy-phenyl]-1,6-heptadiene-3,5-dione), protease inhibitors cocktail, iodoacetamide, lactose, Sepharose 6B, horseradish peroxidase-conjugated anti-rabbit IgG, NP-40 and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). FCS and L-glutamine were from Gibco Lab (Scotland, UK). [$\gamma$-$^{32}$P]-dATP and [$\alpha$-$^{32}$P]-dATP (3000 Ci/mmol) were from DuPont (Boston, MA, USA). All other chemical reagents were commercially available analytical grade.

## Animals

Female 8- to 12-week-old Wistar rats (average weight 250 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba, Argentina, in accordance with institutional guidelines.

## Gal-1 purification

Gal-1 was purified from rat peritoneal macrophages previously activated with PMA (1 $\mu$g/ml), by affinity chromatography on a lactosyl-Sepharose matrix, as previously described.[9] The purified protein was identified by SDS–PAGE on a 15% polyacrylamide slab gel, by Western blot analysis using a specific anti-Gal-1 Ab and by microsequencing tryptic peptides.[8,9]

## Cell culture

T cells were obtained from normal rats as previously described.[18] Cell viability assessed by means of the trypan blue exclusion test was consistently greater than 95%. Cultures were performed in 24-well plates at a density of $2 \times 10^7$ cells per well in 1 ml of complete medium (RPMI 1640 plus 10 mM HEPES, 2 mM L-glutamine and 100 $\mu$g/ml gentamicin, supplemented with 10% heat-inactivated FCS) in the absence or in the presence of the following stimuli: (a) Con A (5 $\mu$g/ml), (b) Gal-1 (4 or 8 $\mu$g/ml) or (c) Con A plus Gal-1, for 30, 60, 120 and 360 min at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After the indicated periods, cells were processed for apoptotic cell detection, RNA purification and protein extracts preparation.

## Nuclear extract preparation

Nuclear extracts were prepared from $4 \times 10^7$ cells according to Schreiber *et al.*[44] Briefly, cells were collected, washed once in PBS, resuspended in 400 $\mu$l cold buffer ‘A’ (10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.7 $\mu$g/ml pepstatin, 1 $\mu$g/ml leupeptin and 10 $\mu$g/ml aprotinin), allowed to swell for 15 min on ice, and then lysed by adding 25 $\mu$l NP-40 10% and gently passing through a 27-gauge needle. The nuclei were collected by centrifugation at $400 \times g$ for 5 min and resuspended in 50 $\mu$l of buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.7 $\mu$g/ml pepstatin, 1 $\mu$g/ml

leupeptin and 10 μg/ml aprotinin). The tube was vigorously rocked on a shaking platform at 4°C for 30 min and centrifuged at 12 000 × *g* for 5 min at 4°C. The supernatant was used as the nuclear extract and frozen at −70°C until used. Protein concentration of the extracts was determined by using the Bio-Rad protein assay.

## Electrophoretic mobility shift assay (EMSA)

AP-1 consensus oligonucleotide (5′-TCGACATCTCAATTAGTCAG-CAAG-3′) was 5′-end labeled using T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]-dATP. Nuclear extracts (6 μg) were mixed with 2 or 4 μg of poly (dI-dC) (Pharmacia Biotech Inc.) in EMSA buffer (20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 250 mM EDTA, 0.125 mM EGTA, 0.25 mg/ml BSA, 20% glycerol, 0.5 mM PMSF, final volume, 20 μl) for 15 min on ice. Then, the labeled probe was added ($2.5 \times 10^4$ c.p.m.) and the reaction mixture was incubated for 20 min on ice. Finally, the samples were separated by 6% native polyacrylamide gel electrophoresis in 0.25 × TBE buffer. The gel was dried under vacuum and exposed overnight to X-ray film (Kodak XAR, Eastman Kodak, Rochester, NY, USA) at −70°C with intensifying screens. Competition studies were performed by adding 100-fold molar excess of unlabeled AP-1 consensus oligonucleotide or AP-1 mutant oligonucleotide (5′-TCGACATCTCAATTAGGAAGCAAG-3′).

## Northern blot assay

Total RNA was isolated from $4 \times 10^7$ cells by the guanidine isothiocyanate method as described by Chomczynski and Sacchi.[45] Ten μg of total RNA were analyzed by electrophoresis through 1.2% (w/v) agarose-formaldehyde gels in 20 mM MOPS buffer (pH 6.8), followed by Northern blot transfer to Hybond-N-nylon membranes (Amersham). Cross-linking by UV radiation and pre-hybridization were performed as described.[46] Membranes were subsequently hybridized overnight at 42°C with 5 ng/ml [$\alpha$-$^{32}$P]-dATP random primer-labeled[47] *c-Jun* cDNA probe prepared with a Prime-a-Gene® Labeling System kit (Promega), according to the protocol recommended by the manufacturer. After hybridization, membranes were washed three times in a buffer containing 0.2 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate) and 0.2% SDS at 65°C for 20 min and exposed for autoradiography at −70°C with intensifying screens. RNA integrity and equal loading were confirmed by methylene blue staining.

## Apoptosis assays

T-cell apoptosis was determined by DNA fragmentation and TUNEL assay essentially as described.[9] Briefly, T cells ($2 \times 10^7$/well) cultured for 6 h in the presence of 4 μg/ml Gal-1 (25 μM) or pre-treated with increasing concentrations of curcumin (0.5, 1 and 2 μM) for 12 h before exposure to Gal-1, were harvested and washed with TNE buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, pH 8). T cells were then lysed by addition of 0.5% SDS and cell lysates were incubated at 56°C for 3 h in the presence of 100 μg/ml proteinase K. After digestion, DNA was purified by successive phenol-chloroform extractions and 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 the ethanol-precipitated DNA was washed with 70% (v/v) ethanol. The purified DNA was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5), and treated with 5 μl of 1 mg/ml DNase-free RNase A for 1 h. Samples were finally prepared in loading buffer and resolved on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was conducted in TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.4) and DNA visualization was accomplished under UV light. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end-labeling (TUNEL) assay was performed essentially as described,[9] by using the MEBSTAIN Apoptosis Kit (Immunotech, Marseille, France, Cat No. 1946), according to the manufacturer's recommended protocol for flow cytometry.

## Western blot analysis

To analyze Bcl-2 expression, cells were collected at the indicated periods by centrifugation at 1500 r.p.m. for 5 min, the medium was carefully removed and T-cells were washed twice with PBS at room temperature. Then, cell pellets were mixed gently with 1 ml ice-cold lysis buffer (PBS containing 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2) with freshly added protease inhibitor cocktail (0.2 mM PMSF, 0.1% aprotinin, 0.7 μg/ml pepstatin and 1 μg/ml leupeptin) and incubated for 30 min on ice. Cells were further disrupted by five cycles of sonication at 100 W for 30 s at 4°C. Samples were finally centrifuged at 15 000 × *g* for 20 min at 4°C and the supernatant fluid, representing the whole cell lysate, was stored at −70°C until use. Protein concentration was estimated as described above.

SDS–PAGE was performed in a Miniprotean II electrophoresis apparatus (Bio-Rad) as described.[48] Briefly, total cell lysates (~40 μg) were diluted in electrophoresis sample buffer and boiled for 90 s. Equal amounts of protein for each lysate were then resolved on a 15% separating polyacrylamide slab gel. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and probed with anti-Bcl-2 (Bcl-2 ΔC21; Santa Cruz Biotechnol., UK) or anti-Bcl-xL (Bcl-xL H62; Santa Cruz Biotechnol., UK) polyclonal antibodies, diluted 1 μg/ml in blocking buffer (1 × TBS, 5% (w/v) non-fat dry milk and 0.05% Tween-20). Blots were then incubated with a 1:2000 dilution of a horseradish peroxidase-conjugated anti rabbit IgG and developed with 4-chloro-1-naphthol. Equal loading and extracts degradation were checked using an anti-α-tubulin (DM1A) mAb. The immunoreactive protein bands were quantified by densitometry scanning comparing the total integrated areas under the respective peaks using a Shimadzu Dual Wavelength Cromato Scanner.®

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