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Structural basis of redox-dependent modulation of galectin-1 dynamics and function

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Abstract

Galectin-1 (Gal-1), a member of a family of multifunctional lectins, plays key roles in diverse biological processes including cell signalling, immunomodulation, neuroprotection and angiogenesis. The presence of an unusual number of six cysteine residues within Gal-1 sequence prompted a detailed analysis of the impact of the redox environment on the functional activity of this lectin. We examined the role of each cysteine residue in the structure and function of Gal-1 using both experimental and computational approaches. Our results show that: (i) only three cysteine residues present in each carbohydrate recognition domain (CRD) (Cys2, Cys16 and Cys88) were important in protein oxidation, (ii) oxidation promoted the formation of the Cys16-Cys88 disulfide bond, as well as multimers through Cys2, (iii) the oxidized protein did not bind to lactose, probably due to poor interactions with Arg48 and Glu71, (iv) *in vitro* oxidation by air was completely reversible and (v) oxidation by hydrogen peroxide was relatively slow (1.7 ± 0.2 M^{-1} s^{-1} at pH 7.4 and 25 ºC). Finally, an analysis of key cysteines in other human galectins is also provided in order to predict their behaviour in response to redox variations. Collectively, our data provide new insights into the structural basis of Gal-1 redox regulation with critical implications in physiology and pathology.

Keywords: circular dichroism / cysteine / galectin-1 / molecular dynamics / oxidation

Introduction

Galectins are members of a family of multifunctional lectins widely distributed in the animal kingdom (Cooper. 2002). They are defined by their specificity for β-galactoside-containing glycans and carbohydrate recognition domain (CRD) (Cooper. 2002). Galectins participate in diverse functions, including cell signaling and death, immunomodulation, host-pathogen interactions, neuroprotection and angiogenesis (Ilarregui et al. 2009; Dam and Brewer 2010; St-Pierre et al. 2011; Rabinovich and Croci 2012; Starossom et al., 2012; Thijssen et al. 2013). In humans, about 16 different galectins' CRDs have been discovered and identified (Guardia et al. 2011), being galectin-1 (Gal-1) the first and most studied so far. However, there are still members of the family that are not completely characterized, such as Gal-12 and galectin-related protein folds such as hGRPC (C-terminal of human galectin-related protein, previously known as HSPC159 for hematopoietic stem cell precursor), PP13 (placental protein 13, also known as Gal-13) and PPL13 (placental protein 13-like, or Gal-14). These proteins display a high degree of sequence identity with members of the galectin family, although their lectin activity is uncertain.

Since their discovery, it was established that most galectins require a reducing microenvironment in order to fulfill their function (Vasta and Ahmed 2009). The relevance of protein oxidation in galectin structure and function has been demonstrated by biochemical characterization (Pande et al. 2003; Shahwan et al. 2004; Ashraf et al. 2011) and the contribution of cysteine residues to lectin inactivation has been demonstrated by site-directed mutagenesis (Abbott and Feizi 1991; Hirabayashi and Kasai 1991) and chemical modification (Oda and Kasai 1983; Whitney et al. 1986; Hirabayashi et al. 1987). However, in spite of considerable evidence showing the importance of oxidation in Gal-1 function, a clear consensus on the importance of each cysteine residue and the molecular basis of this oxidative mechanism has not been reached.

Human Gal-1 is a small lectin composed of 135 amino acids which folds into a three-dimensional structure in the form of a β-sandwich, consisting of two slightly bent sheets with variable long connecting loops. Gal-1 has been widely used as a model of ligand binding and multimerization, but it has also emerged as an interesting model to explore other molecular hallmarks of the galectin family such as the

presence of a high number of cysteine residues in its sequence (six cysteines per monomer). This biochemical property makes this glycan-binding protein highly sensitive to oxidation leading to loss of lectin activity (Tracey et al. 1992). Interestingly, the first reported X-ray structure of human Gal-1 (Lopez-Lucendo et al. 2004) revealed not only the spatial distribution of the cysteines but also modifications such as sulfenic acid formation and mixed disulfide formation with 2-mercaptoethanol (ME). In addition, the intramolecular disulfide bonds present in oxidized Gal-1 have been characterized (Tracey et al. 1992; Inagaki et al. 2000). Whereas the reduced form of this lectin appears to be critical for its immunoregulatory and pro-apoptotic activity, oxidized Gal-1 has been postulated to function as a growth factor during axonal regeneration in peripheral nerves (Inagaki et al. 2000; Kadoya and Horie 2005). Thus, fluctuations in the redox status of Gal-1 may control the range and diversity of biological functions displayed by this lectin in physiologic and pathologic settings.

It is known that inactivation of Gal-1 by air is a very slow process that can be catalyzed by traces of heavy metals such as Cu. However, the kinetics of the oxidation process has not been characterized. This is in part because the air-exposed protein is subjected to various oxidants, which operate through still poorly understood mechanisms. In this regard, hydrogen peroxide (H_2O_2) is one of the most important reactive oxygen species (ROS) (Stone and Yang 2006), not only because of its involvement in microbicidal activities, aging process and oxidative stress, but also because of its critical function as a signaling molecule (Veal and Day 2011; Brigelius-Flohé and Flohé 2011). To verify whether hydrogen peroxide is a suitable candidate for the oxidation of Gal-1 *in vivo*, we measured the kinetics of oxidation of this lectin.

Here, using an interdisciplinary approach involving experimental and computational strategies, we investigated the structural and molecular determinants of redox-dependent Gal-1 inactivation.

Results

Selected cysteines are involved in the Gal-1 oxidation process

One of the most striking features of Gal-1 is the high proportion of cysteine residues, being cysteine one of the rarest amino acids employed for biosynthesis (Pe'er et al. 2004). In fact, there are six cysteines in each Gal-1 monomer, namely Cys2, Cys16, Cys42, Cys60, Cys88 and Cys130. Their spatial distribution in the 3D structure of the protein is shown in Figure 1. These cysteine residues exhibit a wide range of solvent accessibility; hence their reactivity towards oxidizing agents is expected to vary according to the solvent environment. To verify this hypothesis, free thiols were measured using Ellman's reaction (DTNB assay). The experimental analysis of exposed sulfhydryl groups showed a molar ratio of 3.9 ± 0.6, suggesting the presence of about four moles of free thiol per mole of protein. In order to identify the cysteine residues that are exposed, we employed molecular dynamics simulations to compute the radial distribution functions *g(r)* of water molecules around the sulfur of the six cysteines and the solvent accessible surface area (SASA) per residue and sulphur atom, showing a differential profile of solvent exposure (Figure 2). The results are in accordance with previous works employing a different type of calculation (Lopez-Lucendo et al. 2004). Cys2 and Cys130 are the most highly exposed (higher SASA) and solvated cysteines in Gal-1 monomer, since both residues are located near the terminal ends of the protein. Cys16 and Cys88 show a similar profile with lower exposure than Cys2 and Cys130, while the absence of a typical profile of solvation (negligible SASA) for Cys42 and Cys60 indicates no solvent accessibility for these internal cysteines. These results suggest that reactivity towards oxidation and/or any kind of functionalization of these cysteines is markedly different, being the Cys42 and Cys60 hardly accessible by water-soluble reagents, as previously suggested (Whitney et al. 1986). Therefore, the Gal-1 redox chemistry associated with oxidation of cysteine residues is associated with the presence of the four solvent-exposed cysteines: Cys2, Cys16, Cys88 and Cys130.

Using PROPKA and the results of the MD of wild-type Gal-1, we calculated the pK_a values for the six cysteines present in the monomeric structure of the protein (and also for the C2S mutant as a control for single cysteine-to-serine mutants) (Table II). The cysteines that undergo the greatest change in

pK_a with respect to the free amino acid are Cys16, Cys42 and Cys60. Since Cys42 and Cys60 are buried in the core of the protein, we may expect a significant disturbance in their side chain pK_a value with respect to free cysteine in water. The positive value of this difference confirmed that these cysteines are excluded from solvent and surrounded by hydrophobic residues. On the other hand, Cys16 displayed the greatest change (with negative sign), being Cys16 more acidic than free cysteine in water. Since cysteine oxidation often involves the nucleophilic attack of the thiolate to the oxidant, the lower pK_a of Cys16 suggests that among all the solvent-exposed cysteines, this residue might control the onset of the oxidation process as it is the most reactive cysteine due to increased thiolate availability at physiologic pH. However, it is well known that other factors might also affect cysteine reactivity (Ferrer-Sueta et al. 2011). In addition, despite being slightly acidic, other solvent exposed cysteines display pK_a values similar to a free cysteine.

Oxidation and reduction of Gal-1 is a reversible process

Although the redox status appears to be critical for the functional activity of Gal-1 and oxidation has been proposed as a regulatory mechanism to limit the immunoregulatory activity of this lectin (Rabinovich and Ilarregui, 2009), it has not been established whether this process is reversible. When we exposed a 5 μM Gal-1 solution in PBS to oxidation, either through exposure to air (5 days) or by treatment with 30 mM H_2O_2 for 30 min, similar far-UV circular dichroism spectra were obtained in both cases, which were clearly different from that generated by the reduced protein (Figure 3A). This change occurred in the intensity and position of the minimum of ellipticity, indicating a conformational change towards an alternative folded state. Spectra deconvolution using DichroWeb rendered for reduced Gal-1 a β-strand content of 43 %, which diminished to 39 % upon air oxidation. In addition, these changes were accompanied by a slight increment of turn components (from 11.5 to 13 %). Similar results were obtained for this galectin in particular (Kadoya and Horie 2005) and for other galectins from other species (Pande et al. 2003; Shahwan et al. 2004; Ashraf et al. 2011). Interestingly, when the air-oxidized protein was treated for 15 min with 2 mM DTT, we recovered a spectrum that was similar to that obtained with the reduced protein (Figure 3A), suggesting that oxidation was a reversible process. Furthermore, when an

excess of hydrogen peroxide was added again to the samples, it was possible to recover the spectrum of the oxidized protein (data not shown). In addition, CD spectra on the near-UV showed a flattening of the positive band centered at 280 nm upon oxidation, suggesting the loss of the native packing around the aromatic residues (Figure 3B). Since these experiments required higher concentrations of the protein (70 μM), oxidation also induced substantial aggregation of Gal-1. For this reason, upon adding DTT to the oxidized sample only a partial recovery of the initial Gal-1 spectrum was achieved. Finally, when we performed a similar analysis using fluorescence spectroscopy (Figure 3C), we found identical emission spectra when the protein was reduced after being oxidized by air, even in the presence of lactose. Taken together, these results indicate that both the secondary and tertiary structures of the reduced form as well as the lactose-binding capacity were recovered after reducing the oxidized protein.

Cysteines 2, 16 and 88 play key roles in the conformational change experienced by Gal-1 during oxidation

To fully dissect the contribution of each cysteine to the oxidation process, the six single cysteine mutants CXS as well as two selected double mutants were expressed and purified. These mutated proteins were exposed to the same reduction and oxidation procedures previously used for wild-type Gal-1. Conformational changes were followed by CD (Figure 4). Similarly to the wild-type protein, mutation of the internal cysteines (Cys42 and Cys60) did not prevent the protein to reach the oxidized conformation. A similar behavior was observed with the C130S mutant, suggesting that this exposed cysteine is not essential for promoting oxidation-driven conformational changes. By contrast, proteins mutated in Cys16, Cys88 and surprisingly Cys2 showed little changes on their circular dichroism spectra after the air oxidation protocol. However, C2S mutant underwent a conformational change when exposed to hydrogen peroxide. In addition, no significant changes were observed in the double mutants C2SC16S and C2SC88S, as they lack two of the critical cysteines involved in the conformational change (Figure 4).

Based on these findings it was difficult to understand why the absence of some particular cysteines prevents the conformational transition, as it has been proposed that the final state of oxidation requires the presence of six cysteine residues to form three disulfide bridges (Tracey et al. 1992; Inagaki et

al. 2000). Although changes that do not directly influence the CD spectra might also occur, in our experimental conditions only Cys16 and Cys88 (and Cys2 if the oxidation is performed in air) appeared to be essential for inducing conformational changes, as their mutation prevented structural alterations evidenced by CD.

Oxidation of Gal-1 by H_2O_2

When reduced Gal-1 (4 thiols/protein as measured by the DTNB assay) was exposed to increasing concentrations of hydrogen peroxide, biphasic time courses of protein thiol oxidation were observed (Figure 5A). Bi-exponential curves were fitted to the experimental data at each hydrogen peroxide concentration, where the amplitude of each exponential was consistent with the oxidation of ~ two thiol groups. From the slope of the plot of the observed rate constants of the first exponential versus hydrogen peroxide concentration, the rate constant for the most reactive thiol in Gal-1 was 1.7 ± 0.2 M^{-1} s^{-1} at pH 7.4 and 25 ºC in PBS (Table III). In the presence of lactose this value was slightly higher but was still within the error bars of the method. At pH 6.8, the rate constant was ~ 6 folds lower, as expected for a reaction where thiolate is the reactive species. Since the second exponential corresponds to a process that is not fully resolved in time, the value of the constant obtained from these experimental data has larger errors, and can only be estimated as ~ 10^{-3} M^{-1} s^{-1} at pH 7.4 and 25 ºC. When the three most relevant CXS mutants were subjected to similar experiments, all rate constants were lower than that obtained for Gal-1 (Table III). The value for the rate constant of the C2S mutant provided the real consumption rate for the Cys16-Cys88 pair formation, resulting in a value of 0.4 ± 0.1 M^{-1} s^{-1}.

Regarding the nature of the first disulfide bond formed, the most reasonable possibility was the formation of a Cys16-Cys88 intramolecular disulfide bond, due to the reasons mentioned above and because any reaction of Cys2 with some cysteine residue from another Gal-1 monomer would generate dimers. Although dimers are indeed formed during the reaction of Gal-1 and hydrogen peroxide, the kinetics of dimer formation did not coincide with the first phase of thiol consumption, which revealed a half life of 40 s at 10 mM hydrogen peroxide. On the contrary, dimer formation was not found to be

complete after two hours (Figure 5B). Moreover, the monomer band from the samples subjected to oxidation migrated faster than the same band under reducing conditions, most likely showing a disulfide bond formation that occurs within the monomer (Figure 5B; lanes 9 and 11). This observation was confirmed by testing the mobility of C16S and C88S mutants (Figure 1S, Supplementary data). Under non-reducing conditions, both mutants showed the same mobility (slightly lower than that observed for oxidized Gal-1), supporting the hypothesis that Cys16 and Cys88 are responsible for the formation of an intramolecular disulfide bond. When similar oxidation assays were performed using C2S or C130S mutants (Figure 5C), we observed formation of dimers not only in the wild-type (Figure 5B), but also in both mutants. Interestingly, dimers were reduced by ME and they were formed even when iodoacetamide (IAM) was added after hydrogen peroxide treatment (to avoid undesired thiol oxidation during sample manipulation). However, the band corresponding to the dimer was more evident in the C130S mutant**,** suggesting that the Cys2 is primarily responsible for the formation of these dimers and also for the formation of higher molecular weight aggregates.

To obtain an estimation of the rate of the conformational change experienced by Gal-1 during oxidation by H_2O_2, kinetics experiments were performed following the changes in intrinsic fluorescence signal. Fluorescence intensity at 345 nm of a 7 μM Gal-1 solution was registered as function of time at various concentrations of H_2O_2 (Figure 6). As noted above, the fluorescence intensity of samples increased upon oxidation (Figure 3C). However, the most striking trend was the decline in final fluorescence intensity using increasing concentrations of the oxidant. Once reactive cysteines reached the intermediate sulfenic acid, the conformational change has been shown to be independent of the concentration of H_2O_2 (Ferrer-Sueta et al. 2011), so we did not expect this trend in fluorescence spectra. As hydrogen peroxide can induce not only the two-electron oxidation of thiol groups to sulfenic acids, but also the two-electron oxidation of sulfenic acids to sulfinic acids, in a process termed overoxidation (Baker and Poole 2003; Georgiou and Masip 2003; Pascual et al. 2010), we propose a kinetics model that supports an overoxidation process of cysteine, thus providing an explanation for the above-mentioned behavior and allowing the calculation of the rate constant for the conformational change. This reaction competes with

the formation of disulfide bonds, thus inhibiting the associated conformational change (Supplementary data). After fitting the model, we obtained a constant for conformational change independent of H_2O_2 of 0.003 s^{-1} and an overoxidation constant of 0.3 M^{-1} s^{-1}, comparable with the rates obtained for the kinetics of thiol consumption. At low H_2O_2 concentration, the initial speed of conformational change was greater than that of overoxidation (in 5 mM H_2O_2, the conformational change was about twice faster). On the other hand, at high H_2O_2 concentration, the sulfenic intermediate overoxidation can compete with the reaction that drives the conformational change. Importantly, these oxidant concentrations were beyond any physiologically relevant range.

Oxidized Gal-1 does not bind lactose and formation of Cys16-Cys88 disulfide bond is sufficient to disrupt Gal-1-lactose binding

We monitored the intrinsic fluorescence intensity of Gal-1 at 363 nm (a wavelength at which the difference in the fluorescence of the free and the ligand-bound protein is maximal) as a function of lactose concentration and found a binding constant (K_b) of 4.7 x 10^3 M^{-1} (Figure 7A). When the same test was performed with Gal-1 previously incubated in the presence of air or 10 mM H_2O_2, the fluorescence spectra remained constant upon lactose addition (Figure 7A). Therefore, the oxidized protein did not bind lactose at any of the concentrations tested (from 0 to 1.3 mM). In order to clarify the molecular basis of this effect, we performed a computational thermodynamic analysis of the lactose-binding process by comparing the free energy of lactose binding of the protein with the Cys16-Cys88 disulfide bond (Gal-1 oxidized) versus the wild-type protein (Gal-1 reduced), *i.e.* with all the cysteines reduced. Although during the simulation time (80 ns), the lactose molecule remained in the binding site of oxidized Gal-1, the $\Delta G_{binding}$ calculated with the MM/QM-COSMO method ($\Delta G_{binding}$ (Gal-1 reduced) = -34.21 kJ/mol) increased as the simulation time increased, indicating the onset of dissociation, consistent with the fact that the binding process is unfavorable when Gal-1 bears the Cys16-Cys88 disulfide bond ($\Delta G_{binding}$ (Gal-1 oxidized) = -17.49 kJ/mol). This tendency indicates that lactose binds with higher affinity to the reduced form of Gal-1 as expected from the experimental data. The computational method used, based on a continuum model

approach, affords binding ΔG values that are useful only on a qualitative basis, since absolute values are typically overestimated (Kongsted et al. 2009; Hou et al. 2011). On the other hand, the simulation of the oxidized protein would probably require longer times to statistically converge than those accessible within the time scale of our simulations. In this context, our results suggest that the ΔG for the oxidized protein is less negative than the results computed for the reduced protein, consistent with the experimental data. The amino acids located at the binding site that were more affected by the formation of this disulfide bond were Arg48, Glu71 and Arg73 (Figure 7B) as calculated with MM/GBSA amino acid decomposition approach. Arg48 and Arg73 (and Asn61 and Arg111 to a lesser extent) increased the electrostatic component that weakened lactose binding, while Asn46, Ala51, Lys63, Glu71 and Asp123 promoted the opposite effect. Moreover, the His44, Ala51 and Trp68 residues increased the vdW energy component by breaking the dispersive interactions with the ligand. Finally, Arg48 and Glu71 contributed to the binding in the oxidized form through a reduction in vdW energy contribution, where the total energy (sum of all energy inputs) indicated a positive overall contribution to the binding in the oxidized protein. This trend was also reflected at the level of distances of these amino acids to the ligand. When Gal-1 was oxidized, the distances to O3 of the glucose moiety to Glu71(CD) and Arg48(NH2) increased 0.06 Å and 0.3 Å in 80 ns of simulation, respectively. In addition, Arg48(NH1) approached to O3 lactosamine moeity by approximately 0.10 Å, reflecting a significant displacement of the ligand within the binding site. This approach allowed the discrimination of the contribution of each amino acid to ligand binding of Gal-1 upon formation of the disulfide bond between Cys16 and Cys88.

Oxidized Gal-1 does not impair T cell viability

Gal-1 has been reported to selectively alter the viability of activated T cells under reducing conditions (Perillo et al. 1995; Toscano et al., 2007). This effect involves the binding to specific glycosylated ligands on the surface of T cells and a predominant presentation of this lectin in a dimeric form. We studied the impact of oxidation on the structure and function of Gal-1 using cell death assays. We exposed activated Jurkat T cells to different Gal-1 concentrations under reducing conditions, i.e. adding 0.55 mM ME to the

medium (Figure 2S, Supplementary data). We used an optimal concentration of wild-type Gal-1 and mutants corresponding to 3 μM to promote T cell apoptosis in culture (Toscano et al. 2007). In contrast to the effects observed under reducing conditions, we found that oxidation of wild-type Gal-1 and mutants C42S, C60S and C130S resulted in gradual loss of the pro-apoptotic activity of this lectin (Figure 8). On the other hand, C2S, C16S, C88S single mutants and C2SC16S and C2SC88S double mutants did not change the viability of T cells irrespective of the prevalent redox condition. These results confirmed the relevance of Cys2, Cys16 and Cys88 in Gal-1 inactivation and reinforced the hypothesis that only selected cysteine residues are essential for regulating Gal-1 function. Interestingly, double mutants successfully circumvented oxidative inactivation and were capable of triggering the same degree of apoptosis as wild-type Gal-1 exposed to reducing conditions. Thus, double mutants of Gal-1 are substantially more resistant to oxidative inactivation than single mutants of this protein.

Discussion

Although protein oxidation was originally believed to be part of an inactivating process responsible of attenuating or eliminating galectin activity, recent studies suggested that oxidized Gal-1 may display alternative functions independently of glycan ligand recognition, including enhancement of peripheral nerve regeneration (Kadoya and Horie 2005). During oxidation, it has been proposed that each subunit of Gal-1 forms three intramolecular disulfide bridges that result in profound conformational changes, thereby preventing Gal-1 dimerization and ligand recognition (Kadoya and Horie 2005; Stowell et al. 2009). Stability under non-reducing conditions is considerably improved in cystein-to-serine mutants, while glycan-binding specificity and affinity are barely affected (Hirabayashi and Kasai 1991; Nishi et al. 2008). In this regard, it has been demonstrated that ligand engagement partially protects Gal-1 from oxidation (Stowell et al. 2009). Stowell and colleagues found that binding to specific ligands may control Gal-1 sensitivity to oxidation by shifting the monomer-dimer equilibrium toward dimerization, suggesting that glycan binding protects Gal-1 from oxidative inactivation. Dimerization may therefore limit the conformational freedom required to successfully form intramolecular disulfide bonds, thereby protecting

Gal-1 from oxidation. Supporting these findings, a mutant form of Gal-1 that exhibits impaired dimerization, showed enhanced sensitivity to oxidation and failed to induce cell surface phosphatidylserine exposure, a biological activity commonly exerted by reduced Gal-1 (Stowell et al. 2009). These results suggested that binding of Gal-1 to specific glycans enhanced dimerization and reduced sensitivity to oxidative inactivation. Accordingly, mutations that impair dimerization and therefore increase monomer formation favour oxidation of the protein.

Here, using a combination of *in vitro* and *in silico* experiments we studied the molecular mechanisms underlying Gal-1 oxidation. We established a hierarchy of reactivity and importance of each cysteine residue and characterized the kinetics of oxidation with hydrogen peroxide, yielding an atomistic vision of this process with the aim of integrating the structural information available. The first surprising result was the high degree of reversibility of the oxidation-reduction process. The secondary structure followed by circular dichroism and the intrinsic fluorescence spectra reflected the potential of Gal-1 to reversibly move between two structures, a balance dictated by the redox potential of the environment (Figure 2). Since only 4 of the 6 thiols present in Gal-1 are exposed to solvent, we postulated that the cysteine residues responsible for triggering the oxidation-driven conformational change of the protein are among these four residues. This hypothesis was verified by analyzing the CXS single mutants. We found that only Cys2, Cys16 and Cys88 are important for this process, since the C130S mutant displayed a similar behavior to that observed with the wild-type protein (Figure 4). Furthermore, given their proximity and the particular acidity of one of these residues (Table II), Cys16 and Cys88 are good candidates to form a disulfide bridge. This finding is also supported by experimental evidence (Tracey et al. 1992). In this regard, the formation of three disulfide bonds, involving the six cysteine residues of Gal-1, has been reported. However, the conformational change induced by oxidation when Cys42, Cys60 or Cys130 were mutated indicates almost no relevance of these residues in the overall oxidation process (Figure 4).

The critical role of Cys2 in Gal-1 oxidation and stability was first established by the pioneering work of Hirabayashi and Kasai (Hirabayashi and Kasai 1991). The results presented here support the idea that the presence of this particular amino acid is essential for the conformational stability against oxidation

in vitro. When replacing the Cys2 by serine, a disturbance in the interactions with the surrounding residues was expected. However, molecular dynamics simulations and pK_a calculations (Table II) showed that this mutation did not significantly affect the structure and energetics of the reactive cysteines. Since the C2S mutant was oxidized in the presence of H_2O_2, we argue that the difference with air oxidation could be caused by the different concentrations of oxidants in solution. Hence, other possibilities to assign differences in reactivity between C2S and wild-type Gal-1 must be explored. Moreover, our results on the the stability of Gal-1 mutants in oxidized microenvironments correlated well with their ability to promote T cell apoptosis, thus substantiating the relevance of oxidation in the biological activity of this lectin.

Quantification of thiols consumption versus time was used to follow the kinetics of oxidation of Gal-1 by H_2O_2 (Figure 5A). Gal-1 reacted with H_2O_2 with a biphasic kinetics and this phenomenon displayed a specific rate constant of 1.7 ± 0.2 M^{-1} s^{-1}, a value very similar to the oxidation rate constant of free cysteine amino acid (2.9 ± 0.1 M^{-1} s^{-1} at pH 7.4) (Winterbourn and Metodiewa 1999) or cysteine residues in other proteins such as human serum albumin (2.26 M^{-1} s^{-1} at pH 7.4) (Carballal et al. 2003) or thioredoxin (1.05 M^{-1} s^{-1} at pH 7.4) (Goldman et al. 1995). This value is low in comparison with the typical values recorded for thiol-containing proteins specialized on hydroperoxide reduction, *i.e.* thiol-dependent peroxidases, an effect which may be associated with the particular folding of these proteins (10^5 -10^8 $M^{-1}s^{-1}$) (Flohé et al. 2011; Trujillo et al. 2007).

In the presence of lactose, the value of the rate constant of hydrogen peroxide-mediated Gal-1 oxidation was not significantly affected. In this regard, a previous study (Stowell et al. 2009) suggests that sensitivity to oxidation involves changes in dimerization and ligand-binding equilibria. We could not observe these differences probably because of the concentration of Gal-1 used in our experiments, which was higher than the dimerization equilibrium constant (K_d of 7 μM) (Cho and Cummings 1996). Moreover, no changes could be detected in the presence of lactose. In addition, the reversibility of the oxidation process suggests that oxidation may represent a transient factor that limits the biological activity of the protein in inflammatory microenvironments where the risk of oxidation is high but its function could be restored when oxidative stimuli are eliminated or attenuated. Hence, the biological effects

observed with the oxidized form of the protein could also be due, at least in part, to a balance between the reduced and the oxidized forms of Gal-1.

The low oxidation rate constant measured with H_2O_2 suggests that Gal-1 oxidation *in vivo* may be associated with a specific enzymatic catalysis or strictly controlled by the cell and its surroundings. To gain biological relevance in a more complex regulatory circuit, these structural and functional changes should occur faster than those observed here using air or H_2O_2. Indeed, Gal-1 oxidation may be provoked by these or other oxidants indirectly, through thiol-disulfide exchange reactions with more reactive, peroxide-sensing proteins. Such interactions were firstly demonstrated for the yeast transcriptional factor Yap, whose response to hydrogen peroxide was mediated by glutathione peroxidase 3, and then confirmed for other factors (Delaunay et al. 2002; Flohé and Ursini 2008).

The oxidation kinetics and electrophoretic analysis (Figure 5) showed the time-dependent formation of intra and intermolecular disulfide bridges. The lower molecular weight protein band migrated from the initial position at early stages of the kinetics (Figure 5C), indicating the formation of an intramolecular disulfide bond. Simultaneously, species displaying the molecular weight of a dimer were also apparent but were almost absent under reducing conditions; these species correspond to an intermolecular disulfide bridge between Gal-1 monomers. Formation of this dimer was slower than the first phase of oxidation, which according with the rate constant reported here and a H_2O_2 concentration of 10 mM, must be complete in less than 10 min (half life of 40 s).

The kinetics of the conformational change observed upon Gal-1 oxidation with H_2O_2 followed by intrinsic fluorescence revealed that the final signal intensity decreased as the concentration of oxidant increased (Figure 6). A plausible model to explain this phenomenon (Supplementary data) required the consideration of a peroxide concentration-dependent reaction which competed with the conformational change: overoxidation of cysteine. Using this model we were able to obtain a rate constant for the conformational change of 0.003 s^{-1}, which at high H_2O_2 concentration competes with the overoxidation (formation of sulfinic and sulfonic acids) of the particular cysteines that trigger the conformational change.

These data are relevant when considering a strict regulation of disulfide bonds formation in Gal-1 in order to generate timely structural modifications according to the requirements of the tissue microenvironment

Finally, to gain a more integrated picture, we evaluated the relevance of cysteine residues present in other members of the human galectin family. We hypothesized that galectins displaying the key cysteines in the proper environment might exhibit the same profile of oxidation demonstrated for Gal-1 because of the high conserved folding (Guardia et al. 2011). To address this question, we conducted a multiple sequence alignment using Clustal-X program, and incorporated structural information for the gaps in the alignment (Figure 9). The alignment revealed that: 1) the most conserved cysteine is Cys60; 2) the most commonly substituted cysteine is Cys42 (mostly by alanine); 3) the only family members that have the pair of relevant cysteines Cys16 and Cys88 (contained in the Gal-1 sequence) are PP13 and PPL13. Those with Cys88 but without Cys16 are Gal-3 and the *N*-terminus of Gal-9 (Gal-9N). Thus, the occurrence of Cys60 in this lectin family could play a role in their structural/folding stability, as it is present and conserved in most members of the family. Furthermore, our results suggest that this effect could not be related to a redox function. The same could be concluded for Cys42 but in the opposite way: its role in the oxidation of the protein may not be crucial as it is the most commonly replaced cysteine within the galectin family. It is also possible that both PP13 and PPL13 could exhibit a similar redox behavior to that observed for Gal-1, as they have the key cysteine residues in the same topological location. Interestingly, Gal-1, as well as PP13 and PPL13 have been proposed to play roles in pregnancy maintenance, suggesting a cooperative or redundant role for these lectins in mammalian placentation (Than et al. 2008, Blidner and Rabinovich, 2013). On the other hand, Gal-3 and Gal-9N, might reflect the the opposite situation: these galectins could have become insensitive to oxidation, probably as a consequence of an evolutionary pressure to eliminate Cys16. Alternatively, redox regulation governed by the Cys16-Cys88 disulfide bond would not be required for these particular galectins.

In conclusion, our data provide new insights into the understanding of redox regulation in the structure-function relationship of Gal-1, a key regulatory lectin that plays central roles in the control of immune, neural and vascular signaling circuits

Materials and methods

All experiments were performed at 25 °C in 100 mM phosphate-buffered saline (PBS) containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.4, unless otherwise stated.

Expression and purification of recombinant Gal-1 and CXS mutants

Recombinant human Gal-1 was produced as outlined previously (Pace et al., 2003). A similar protocol was adopted for the production of the six single cysteine mutants. Briefly, *Escherichia coli* BL21 (DE3) cells were transformed with each plasmid containing different genes inserted into the expression vector pET22b (Novagen), and production of the recombinant galectin was induced at the log phase by addition of 1 mM isopropyl β-D-thiogalactoside. Cells were separated by centrifugation, washed, and disrupted by sonication. Debris was eliminated after centrifugation at 15,000 x g, and soluble fractions were obtained for subsequent purification by affinity chromatography on a lactosyl-Sepharose column (Sigma-Aldrich), using 0.1 M lactose in PBS supplemented with 4 mM ME. Eluted Gal-1 was further purified using a HiPrep Sephacryl S-100 HR gel filtration column (GE Healthcare). After gel filtration, lectin-containing fractions were subjected to extensive dialysis against PBS containing 4 mM ME at 4 ºC to remove lactose bound to the protein. In order to prevent mixed disulfide bridge formation between cysteine residues and ME, prior to any analysis, ME was removed from protein structure by incubating the lyophilized sample in PBS with 10 mM dithiothreitol (DTT) on ice during 30 min and desalted with a NAP-5 column (GE Healthcare). This procedure removes the excess of DDT and ME. The reduced protein samples were immediately purged with argon in a closed vessel and the solution was kept on ice until use.

Generation of mutants

Cysteine residues on Gal-1 were mutated to Ser (CXS) using the inverse PCR method (Clackson et al. 1991). The forward sense primer contained a mismatch that changes the appropriate Cys to Ser. These

primers were used in combination with antisense primers that start at the beginning of the sense primers. The insert and the vector were amplified on the same step with KOD Hot Start polymerase (Novagen) and the resulting product was ligated with T4 DNA Ligase (Promega). Double cysteine mutants C2SC16S and C2SC88S were generated using the single mutant C2S as starting material and the mutations were introduced using the primers previously employed to generate the single mutants C16S and C88S. Mutations were checked by DNA sequencing of the entire insert. Primers used are listed on Table I.

Oxidants, protein and thiol quantification

The concentration of H_2O_2 (Mallinckrodt Chemicals) stock solutions was measured at 240 nm (ε_{240} = 43.6 M^{-1} cm^{-1}). Protein concentration after reduction treatment was measured spectrophotometrically using an absorption coefficient at 280 nm of 8,480 M^{-1} cm^{-1} for Gal-1 and the single cysteine mutants, as assessed from their primary sequences (http://www.expasy.ch/tools/protparam.html). Thiols were determined with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) after incubating Gal-1 samples with an excess of DTNB in PBS for 30 min in the dark at room temperature. An absorption coefficient at 412 nm of 14,150 M^{-1} cm^{-1} (Riddles et al. 1979) was used to quantify the 5-thio-3-nitrobenzoate anion with the absorbance of the DTNB solution and the intrinsic low absorbance of Gal-1 at this wavelength accounted for.

Spectroscopic measurements

Far- and near-UV circular dichroism (CD) spectra were recorded using a Jacso J-815 spectropolarimeter equipped with a Peltier temperature control. Spectra shown are averages of at least 8 scans, with background corrected by the subtraction of respective buffer blanks. They were acquired over the wavelength range of 190–360 nm, using a 1 mm path length polarimetrically certified cell (Hellma). Spectra deconvolution was performed using DichroWeb (http://dichroweb.cryst.bbk.ac.uk) with the CONTIN analysis program and the reference set SP175**.** Intrinsic fluorescence emission spectra were measured at 25 °C in a Jasco FP-6500 spectrofluorometer. Excitation wavelength was set to 280 nm (295 nm for the experiments related to the kinetics of conformational change), and spectra were recorded

between 300 and 420 nm. Excitation and emission bandpasses were set to 1 and 3 nm, respectively. An average of at least six scans was used for final calculations. Spectra were corrected for dilution effects, and the final dilution of the sample was always <10%.

Kinetics of the reaction between hydrogen peroxide and Gal-1

The rate constants of oxidation of Gal-1 or CXS mutants by hydrogen peroxide were determined using pseudo-first-order conditions as described (Carballal et al. 2003). Reactions were conducted at increasing concentrations of H_2O_2 and the same protein concentration (67 μM). At different time intervals, aliquots of the reactions were mixed with catalase (100 U mL^{-1}) and the samples thus obtained were measured for thiol content. Observed rate constants of thiol consumption (*k*') were determined by fitting time courses of thiol oxidation data at each oxidant concentration to a double exponential function.

SDS-PAGE

SDS-PAGE was performed using 15:1 polyacrylamide gels containing sodium dodecyl sulfate (SDS) further stained with silver or Coomassie blue.

Binding of Gal-1 to lactose

The Gal-1:lactose binding constant under reducing or oxidizing conditions was determined by fitting the fluorescence emission spectrum change in the presence of DTT or H_2O_2, respectively. Gal-1 (8 μM) was titrated by adding aliquots of a 100 mM lactose stock solution. The intensity of the emission spectrum at 363 nm was recorded and fitted as function of lactose concentration. Binding constant (K_b) at 25 ºC was calculated by fitting a single binding site model to the fluorescence data.

Computational experiments

Initial structures of Gal-1 CRDs were retrieved from the Protein Data Bank when available: 1GZW for the wild-type protein (X-ray, 1.65 Å resolution) and 1W6N for the C2S Gal-1 mutant. For those CRDs whose

structures were not available or required editing of the structure (oxidized form of Gal-1 -Cys16-Cys88 disulfide bridged-, bound or unbound to lactose) (all initial structures available in the Supplementary Data), we generated them using Amber9 package of computational simulation programmes (Case et al. 2006). In all cases, all crystallographic water molecules were deleted, and a single subunit was then solvated with explicit three-site point charge modelled (TIP3P) water molecules in an octahedral box, localizing the box limits as far as 10 Å from the protein surface. Molecular dynamics (MD) simulations were performed at 1 atm and 300 K, maintained with the Berendsen barostat and thermostat (Berendsen et al. 1984; van Gunsteren and Berendsen 1990), using periodic boundary conditions and Ewald sums (grid spacing of 1 Å) for treating long-range electrostatic interactions with a 10 Å cut-off for computing direct interactions. The SHAKE algorithm was applied to all hydrogen-containing bonds, allowing employment of a 2 fs time step for the integration of Newton's equations. The Amber f99SB force field parameters (Hornak et al. 2006) were used for all residues. GLYCAM parameters (Kirschner et al. 2008) were employed for lactose. The equilibration protocol involved an optimization of the initial structure, followed by 500 ps constant volume MD run heating the system slowly to 300 K. Finally, 1 ns MD run at constant pressure was performed to achieve proper density. Different production MD runs (60 ns for the structures experimentally determined and 80 ns for the models) were performed. Frames were collected at 1-ps intervals for the last 20 ns of each simulation, and were subsequently saved on disk for further analyses.

Using the MD simulation of the wild-type Gal-1 (one monomer on its reduced form) and C2S Gal-1 mutant, 50 frames were taken at random and for each one, we calculated the value of all cysteines lateral chain pK_a using PROPKA (Bas et al. 2008), an empirical method for structure-based protein pK_a prediction which takes into account desolvation effects and intra-protein interactions. The results were expressed as ΔpK_a, the difference between the average value obtained from all frames and the reference value used by the program (9.00 for cysteine). Data are expressed with the standard deviation. For the same frames, a solvent accessible surface area (SASA) calculation of the lateral chain and the sulphur SG atom of each cysteine was performed using the vmdICE plug-in implemented in VMD (Humphrey et al. 1996; Knapp et al. 2010).

Thermodynamic parameters for MD simulations of CRD:lactose complexes were calculated using two different strategies: the single trajectory molecular mechanics/generalized Born surface area (MM/GBSA) approach (Jorgensen et al. 1983; Bashford and Case 2000; Zou et al. 1999) implemented in the Amber 9 package (Case et al. 2006) and the linear-scaling quantum mechanical-based end-point method developed by Anisimov and Cavasotto and termed MM/QM-COSMO (Anisimov and Cavasotto 2011). The former method combines molecular mechanical energies, continuum solvent approaches, and solvent accessibility in order to elicit free energies from structural information avoiding the computational intricacy of free energy simulations. The molecular mechanical energies were determined with the *sander* program from Amber and represented the internal energy (bond, angle, and dihedral) and van der Waals and electrostatic interactions. An infinite cut-off for all the interactions was used. The electrostatic contribution to the solvation free energy was calculated with a numerical solver for the generalized Born method (Still et al. 1990). Energetic contributions were computed corresponding to the electrostatic energy (ELE) and van der Waals contribution (vdW). Solvation-free energy was estimated using the generalized Born approximation (GB_{Solv}), which is based on the use of a cavitation and electrostatic energy components. The total free energy contribution computed by the generalized model is also presented (GB_{Tot}). With this method we obtained the binding energy characterization per residue. In the MM/QM-COSMO approach, MD trajectories are re-evaluated using a semi-empirical Hamiltonian and a continuum solvent model to calculate an enhanced binding-free energy description where translational and rotational entropies are calculated using configurational integrals, and internal entropy is calculated using the harmonic oscillator approximation.

Multiple sequence alignment of 15 publicly available human sequences was performed, using the CLUSTAL-W multiple alignment method and software (Thompson et al. 1994): *LGALS-1* to *-12*, *LGALS-13* (or *PP13* for placental protein 13), *LGALS-14* (or *PPL13* for placental protein 13-like) and *HSPC159* (hGRPC), all the three last genes correspond to galectin-related proteins. Structural information from the Gal-1 CRD structure deposited in the 1GZW PDB entry was used to set local gap penalties.

T cell death assay

Jurkat T cells (5 x 10^5) were cultured and activated as described (Toscano et al., 2007; Lange et al., 2009) and incubated with or without 3 μM Gal-1 or its variants in RPMI medium supplemented with 5% fetal bovine serum (FBS), penicillin (100 mU/mL) and streptomycin (50 μg/mL) in 24-well culture plates at 37 °C in 5% CO_2. To generate reducing conditions, 0.55 mM ME (final concentration) was added to complete the medium before adding the cell suspension. To test the functional activity of oxidized galectins, galectins were cultured in RPMI and treated with 10 mM H_2O_2 for 20 min before assays. The excess of ROS was quenched by using catalase (100 U / $ml.^{-1}$) and the oxidation reaction was stopped. Then, medium was completed with FBS and antibiotics and cells were added to each well. After 14 h of exposure to Gal-1 or its variants, cells were washed with PBS. Cell death was determined by annexin V-FITC/propidium iodide (PI) in staining buffer (100 mM HEPES, 1.4 M NaCl, 25 mM $CaCl_2$) as previously described (Toscano et al., 2007). Fluorescence (FITC and PI) were analyzed on a FACSCanto (BD Biosciences). Cell death was calculated as the % of annexin V-positive cells in galectin-treated cells minus the % of annexin V-positive control-treated cells.

Statistical analysis

Data are expressed as mean ± S.D. Prism software (GraphPad Software) was used for statistical analysis. Two groups were compared with the Student's *t* test for unpaired data. *P*-values of 0.05 or less were considered significant.

Abbreviations

CD, circular dichroism; CRD, carbohydrate recognition domain; CXS, serine-to-cysteine galectin-1 mutants; DTNB, 5,5'-dithiobis-(2-nitrobenzoic) acid; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; Gal-1, galectin-1; IAM, iodoacetamide; MD, molecular dynamics; ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate.

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Figure Legends

Figure 1: Spatial distribution of the six cysteine residues in the monomer and dimer structure of Gal-1. Six cysteines residues are distributed in each Gal-1 monomer, namely Cys2, Cys16, Cys42, Cys60, Cys88 and Cys130. While Cys2 and Cys130 are located at the *N*- and *C*-terminal ends of the protein respectively, the Cys42 and Cys60 are localized in the core of Gal-1 β-sandwich folding. The Cys16 and Cys88, one close to another, are found in the *F* sheet, diametrically opposite to the ligand binding groove where lactose binds to Gal-1.

Figure 2: Radial distribution functions of water molecules O(H_2O) around the sulfur atom of each cysteine residue S(Cys) in Gal-1. The radial distribution function, *g(r)*, indicates the probability of finding a water molecule particle in the distance *r* from sulfur atom of lateral chain of each highlighted cysteine. It is a measure of the probability that a water soluble oxidant could approach a reactive sulfur atom of cysteine. Results corresponding to solvent accessible surface area (SASA) calculation per residue and per sulphur are included (inset). The decreasing order of solvent exposure evidenced is Cys2 □ Cys130 □ Cys16 ≈ Cys88 □□ Cys60 ≈ Cys42.

Figure 3: **Reversibility of Gal-1 structure and function upon redox-dependent environmental changes**. A) Far-UV and B) near-UV circular dichroism (CD) spectra of Gal-1: The freshly prepared reduced form of Gal-1 spectrum is shown in black solid line. When Gal-1 was oxidized by air (red solid line) or by 10 mM H_2O_2 during 15 min (orange dot-dash line), similar final spectra were obtained, that differed from the reduced Gal-1 spectrum. After treatment of air-oxidized Gal-1 with 2 mM DTT, the reduced Gal-1 CD spectrum form was almost fully recovered (blue dashed line). C) Reduced apo Gal-1 (black solid line) and lactose bound (fuchsia solid line) showed different emission fluorescence spectra.

When oxidized by air (red dot-dash line), an increase of intensity of intrinsic emission fluorescence was observed. By addition of DTT to the oxidized protein, identical emission spectra to that of the reduced protein was recovered (apo-Gal-1 in blue dashed line), even in the presence of lactose (green dashed line). All the samples contained DTPA as a metal chelating agent.

Figure 4: **Circular dichroism spectra of different redox states of Gal-1 and CXS mutants**. All the reduced forms of Gal-1 and its CXS mutants (black solid line) showed similar circular dichroism (CD) spectra. When oxidized in air (red solid line) or with hydrogen peroxide (orange dot-dash line), different spectra were obtained as a function of the absence of particular cysteine residues. In air, only Cys2, Cys16 and Cys88 seemed to be key cysteines critical to generate the conformational state of the oxidized form of wild-type Gal-1. When hydrogen peroxide was used to induce protein oxidation, only C16S and C88S mutants kept the reduced protein conformation.

Figure 5: Kinetics of oxidation of Gal-1 with H_2O_2. A) Gal-1 (67 μM) was incubated with hydrogen peroxide at concentrations of 2.81 mM (squares), 4.65 mM (circles), 5.56 mM (triangles) and 8.28 mM (diamonds) in PBS buffer (100 mM, 0.1 mM DTPA, pH 7.4) at 25 ºC. Aliquots were removed at increasing time points and the reactions were stopped with catalase (100 U mL^{-1}). A bi-exponential function was fitted to the results of Gal-1 free thiols concentration as a function of time to determine the pseudo-first-order rate constants (*k*'). Then, the pseudo-first-order rate constants for the first exponential decay were plotted as a function of hydrogen peroxide concentration (inset) to obtain the rate constant for the most reactive thiol in Gal-1. B) SDS−PAGE of aliquots obtained from the reaction of Gal-1 with 10 mM H_2O_2. The first nine lanes contain non-reducing SDS buffer, while lane 11, corresponding to the last aliquot (time = 120 min), includes 2-mercaptoethanol (ME) in the SDS buffer (reducing conditions). C) Wild-type Gal-1 as well as C2S and C130S mutants were subjected to oxidation by 10 mM H_2O_2 for 2 h. Non-reducing and reducing SDS-PAGE were performed. Iodoacetamide (IAM) was added after hydrogen

peroxide treatment to analyze the effect of sample manipulation in the oxidation of the remaining free thiols after ceasing the reaction.

Figure 6: **Kinetics analysis of conformational changes of Gal-1 upon oxidation with H_2O_2.** Gal-1 (7 μM) was incubated with hydrogen peroxide at concentrations of 5 mM (squares), 10 mM (circles), 15 mM (triangles) and 20 mM (diamonds) in PBS buffer (100 mM, 0.1 mM DTPA, pH 7.4) at 25 ºC. The intensity of the emission spectrum at 345 nm was recorded as function of time. A kinetics model taking into account the consumption of reduced Gal-1, the formation of the different oxidized Gal-1 species and the concentration of hydrogen peroxide (Supplementary Data) was fitted (line) in order to obtain the rate of conformational changes and the reactions corresponding to overoxidation of cysteines.

Figure 7: **Thermodynamic analysis of Gal-1 binding to lactose**. A) Gal-1 (8 μM) (squares) was titrated by adding aliquots of a 100 mM lactose stock solution. The intensity of the emission spectrum at 363 nm was recorded and fitted as function of lactose concentration. Binding constant (K_b) 25 ºC was calculated by fitting a single binding site model to the fluorescence data. No significant change of emission fluorescence was observed for Gal-1 oxidized by H_2O_2 (circles) at any of the lactose concentrations tested. B) Differences of $\Delta E_{binding}$ ($\Delta(\Delta E)$) between Gal-$1_{oxidized}$:lactose and Gal-$1_{reduced}$:lactose systems per residue, using the classical mechanics MM/GBSA approach. Energetic contributions were computed to the van der Waals contribution (vdW) (black solid line) and electrostatic energy (ELE) (red solid line) as well as the total free energy contribution computed by the generalized model (GB_{Tot}) (green solid line). Using this method, we obtained the detailed differences in binding energy per residue between reduced and oxidized Gal-1. Although the structural modification was located in the *F*-sheet of Gal-1 folding (*i.e.*, the formation of the Cys16-Cys88 disulfide bond), the principal amino acids that suffered from that structural modification were mostly located in the ligand-binding groove in contact with lactose. These amino acids are: Arg48, Glu71 and Arg73 as they presented the greatest changes in $\Delta E_{binding}$.

Figure 8: **Effect of Gal-1 and its CXS mutants in T cell death.** A) Activated Jurkat T cells (5 x 10^5) were incubated with or without 3 μM of Gal-1 or its mutants under different redox conditions. For reducing conditions, 0.55 mM ME (final concentration) was added to complete the medium before adding the cell suspension. For non-reducing conditions, galectins were previously treated with 10 mM H_2O_2 for 20 min. The oxidation reaction was stopped by using catalase (100 U mL^{-1}). After 14 h of exposure to Gal-1 or its variants, cells were washed with PBS. Cell death was determined by annexin V-FITC/propidium iodide (PI) in staining buffer by flow cytometry using a FACSCAnto. A) Representative data for wild-type Gal-1 presented as the peak fluorescence intensity profile (log scale) of cells stained with annexin V-FITC (control, gray line; reduced Gal-1, black line; oxidized Gal-1, red line). B) Δ Cell death (%) observed for each recombinant Gal-1 tested (wild-type, CXS and two double CXS mutants), reduced (black bars) or oxidized (grey bars). Notably, Cys2, Cys16 and Cys88 appear to be key cysteines that are involved in Gal-1 pro-apoptotic function under different redox conditions. The results shown are representative of three independent experiments (mean ± S.D.; *, $P < 0.05$).

Figure 9: **Multiple sequence alignment of all human galectins domains.** Structural information for the gaps penalties in the alignment was incorporated (template: Gal-1). Secondary structure annotation and numbering on top correspond to the folding and cysteine residues of Gal-1. Beta strands are represented by arrows and the loops connecting them by lines. Cysteine residues shared with Gal-1 are indicated in bold, while all remaining cysteines are highlighted by white letter in a black box. For the tandem-repeat galectins Gal-4, -8, -9 and -12, the *N*- or *C*-terminus sequence is denoted by N or C, respectively. A continuous numbering is placed at the bottom of the alignment for clarity. Conserved residues (~80%) are highlighted in grey.

Table I. Primers used to generate the Cys to Ser (CXS) mutants. Mutated codons are in bold.

Mutation	Direction	Primer
C2S	Forward	5'-ATATGGCT**TCT**GGTCTGG-3'
C2S	Reverse	5'- GTATATCTCCTTCTTAAAGTTAAAC -3'
C16S	Forward	5'- CTGGAGAG**TCC**CTTCGAGTG -3'
C16S	Reverse	5'- GTTTGAGATTCAGGTTGCTGG -3'
C42S	Forward	5'- CAACCTG**TCC**CTGCACTTC -3'
C42S	Reverse	5'- TTGCTGTCTTTGCCCAGGTTC -3'
C60S	Forward	5'- CCATCGTG**TCC**AACAGCAAG -3'
C60S	Reverse	5'- TGTTGGCGTCGCCGTG -3'
C88S	Forward	5'- CAGAGGTG**TCC**ATCACCTTC -3'
C88S	Reverse	5'- CAACACTTCCAGGCTGGAAG -3'
C130S	Forward	5'- CAAGATCAAA**TCT**GTGGCCTTTG -3'
C130S	Reverse	5'- AAGTCACCGTCAGCTGC -3'

Table II. Estimation of the shift in cysteines pK_a values of wild-type Gal-1 and C2S mutant

	ΔpK_a	
Residue	Wild-type Gal-1	Gal-1 C2S
Cys2	-0.34 ± 0.82	-
Cys16	-1.02 ± 0.79	-0.80 ± 0.63
Cys42	0.66 ± 0.43	0.83 ± 0.40
Cys60	0.84 ± 0.68	0.67 ± 0.49
Cys88	-0.26 ± 0.27	-0.20 ± 0.56
Cys130	-0.43 ± 0.48	-0.52 ± 0.26

Table III. Kinetics of thiol consumption upon oxidation of Gal-1 and selected CXS mutants by H_2O_2.

Protein	Condition (T = 25 °C in PBS)	k [M^{-1} s^{-1}]
Gal-1	pH 7.4	1.7 ± 0.2
	pH 7.4 + 100 mM lactose	2.1 ± 0.2
	pH 6.8	0.3 ± 0.1
C2S	pH 7.4	0.4 ± 0.1
C16S		0.8 ± 0.1
C88S		1.0 ± 0.1