

Peptides of the N-terminal Tail of human Galectin-3 interact with its Carbohydrate Recognition Domain in a Phosphorylation-dependent Manner

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Abbreviations used: Gal-3, galectin-3; CRD, carbohydrate recognition domain; NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence.

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Abstract

Galectin-3 (Gal-3) is a multifunctional effector acting extracellularly after non-classical secretion, in the cytoplasm and the nucleus. Its modular display of a carbohydrate recognition domain (CRD) attached to a tail of collagen-like repeats (nine in the human protein) and an N-terminal peptide is unique in this lectin family and not yet fully explored, as is the physiological significance of serine and tyrosine phosphorylation. Using a series of nine synthetic (phospho)peptides and the ^{15}N -labeled CRD of human Gal-3 as well as measuring chemical shift perturbations in mixtures, potential for peptide reactivity was revealed in Gal-3's backface. Tyrosine phosphorylation reduced the affinity, while serine phosphorylation of the N-terminal peptide was essential. Controls with sequence scrambling underscored inherent specificity. These results detect capacity for distinct sites of intramolecular recognition in Gal-3, adjustable by phosphorylation, and thus prompt analysis using the full-length protein.

1. Introduction

The members of the galectin family are potent effectors in diverse cellular mechanisms such as growth regulation or motility, by virtue of glycan or protein recognition acting in the cell, at the cell surface and extracellularly [1-3]. In structural aspects, the modular design of galectin-3 (Gal-3) is unique. It is composed of the common β -sandwich-type carbohydrate recognition domain (CRD) linked to collagen-like repeats (nine in human Gal-3) and an N-terminal 12-mer peptide with two sites for serine phosphorylation, the explanation why Gal-3 is called a chimera-type galectin [4, 5]. Since every animal species studied so far harbors Gal-3 despite marked inter-species differences in the total number of galectin genes and the sequences in the two non-CRD regions appear conserved [4-6], the trimodular structure likely bears special physiological significance.

At present, the non-CRD portion is implicated in non-classical secretion [1, 7, 8], serine phosphorylation of the starting peptide (at S6 and S12 [9-11]) in nuclear export [1, 12, 13]. Although an impact on binding lactose, ganglioside GM1 and N-glycans was excluded [11, 14, 15], this phosphorylation reduced Gal-3 reactivity to laminin and asialomucin but enhanced both complex formation with the cell adhesion molecule L1 and its association with Thy-1-rich microdomains in neurons [16, 17]. Equally intriguing, tyrosine phosphorylation by non-receptor kinases cAbl/Arg (at Y79, Y107 and Y118 [17-19]) appears to establish a routing signal for Gal-3 to reach the cell periphery with ensuing secretion in mouse embryonic fibroblasts [20]. It is an open question whether intramolecular recognition, influenced by the status of phosphorylation, may contribute to the structural basis of these processes.

Of note, the N-terminal portion appears rather flexible, although the transition temperature at 39°C during thermal denaturation and the bimodal charge distribution in nano-ESI mass spectrometry provide evidence for an interconverting mixture of conformers, tentatively between extended and compact forms [21, 22]. Importantly, chemical shift analysis of hamster Gal-3 inferred data interpretable as transient contacts with the CRD, for residues in the first part of the N-terminal tail (F5, W22, W26) and for L109 [23]. The delineation of a nuclear export signal, probably interacting with a transporter such as nucleoporin Nup98 *A, in the distal section of the CRD, i.e. around 240-255 in murine Gal-3 [24], in conjunction with these NMR-based observations and the switch-like impact of serine phosphorylation on

nuclear export noted above, raise the intriguing possibility that serine phosphorylation can trigger so far unknown structural consequences, directly and/or indirectly. On this basis, we have tested the hypothesis of the existence of intramolecular binding with synthetic peptides and ^{15}N -labeled CRD. Looking at secretion, tyrosine phosphorylation may play into this process by favoring a conformer, most suitably in extended form to make the tail fully accessible. Testing intramolecular reactivity followed the described strategy. To complete this study line, we have also examined the spectral properties of the CRD in the presence of peptides comprising one and two collagen-like repeats.

2. Material and methods

2.1. Gal-3

Full-length Gal-3 and its CRD were recombinantly produced with $[^{15}\text{N}]\text{NH}_4\text{Cl}$ as medium additive, purified by affinity chromatography on lactosylated Sepharose 4B as crucial step, and routinely checked for purity and activity as described [11, 25, 26].

2.2. Peptides

The peptides (shown in fig. 1) were synthesized in an automated multiple synthesizer Syro II (MultiSyn Tech, Germany) with Fmoc chemistry under atmospheric conditions at room temperature using HMPB-ChemMatrix® resins. For amino acid coupling, a fivefold excess of the Fmoc-protected amino acid was activated *in situ* with five equivalents 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and DIPEA (0.5 M) in DMF. The coupling time was 40 min. The Fmoc-group was cleaved with piperidine (20%) in DMF for periods of 3 min and 10 min. After each step, the resin was washed five times with DMF. After completion of the synthesis, the resin was washed three times with DMF, dichloromethane and finally isopropanol, then dried. The following release of the peptide from the resin and of the side-chain protecting groups was performed with trifluoroacetic acid (95%), as scavenger triethylsilan/water (2.5%/2.5%) for 2.5 h at room temperature was used.

The resulting material was purified by preparative HPLC on a Kromasil 100–10C 18 μm reverse phase column (30' x 250mm) using an eluent of 0.1% trifluoroacetic acid in water (A)

and 80% acetonitrile in water (B). Peptide was eluted with a successive linear gradient of 10% B to 80% B in 30 min at a flow rate of 23 ml/min. The fractions containing the purified peptide were lyophilized. The purified material was characterized with analytical HPLC (Shimadzu) and MS (Thermo Finnigan LCQ).

2.3. NMR spectroscopy

All experiments were performed at 298 K in a Bruker AVANCE 600 MHz spectrometer equipped with a cryogenically-cooled z-gradient triple resonance probe. Samples for ^1H - ^{15}N HSQC experiments contained ^{15}N -labeled human Gal-3 CRD or ^{15}N -labeled full length human Gal-3 at a concentration of 200 μM , with or without ten equivalents of the tested peptides, in PBS buffer in 90% H_2O plus 10% D_2O for field-frequency lock. Chemical shift perturbations were monitored, using the sequence-specific assignments for the human Gal-3 CRD ^1H and ^{15}N resonances [27].

3. Results and Discussion

3.1. Evidence for intramolecular interaction

NECESITAMOS UN ESQUEMA CON LOS PEPTIDOS

The ^1H - ^{15}N HSQC spectrum of full-length Gal-3 is highly superimposable with that of the truncated version (fig. S1; see also fig. 3 <<<PLEASE START WITH FIG 1>>>). Of note, close inspection reveals differences in chemical shifts for a number of specific signals indicative for intramolecular interaction. The largest differences were observed for residues located on the convex side of the β -sandwich, at the opposite face of the sugar-binding site. This observation suggests that the tail is oriented towards this region. In structural terms, the NMR signals of the aminoacids at the tail appeared in the region characteristic for random-coil conformation, excluding stable formation of a secondary structure.

To delineate potential for binding to the CRD, three sets of structurally relevant tail-derived peptides were tested by NMR spectroscopy in mixtures with ^{15}N -labeled CRD (fig. 1). The first (P1-P3) represents the N-terminal peptide, without and with Ser phosphorylation. The

second (P4-P7) is constituted by the stretch encompassing the sites Y107/Y118 for tyrosine phosphorylation. In the third set, we tested two peptides (P8, P9) from the collagen-like part.

3.2. *N-terminal peptides*

When testing P1-P3, a strong dependence of their interaction abilities was noted when phosphorylation was present (fig. 2, fig. S2), since chemical shift perturbations were evident. Many of the most markedly shifted signals belong to residues located on the backside of the lectin, at the opposite face of the sugar-binding site (fig. 3). Thus, the contact area to lactose remained unaffected. The observed chemical shift perturbations in the Gal-3 CRD resonances in the presence of lactose had been previously deduced in a blank experiment [11]. The strong chemical shift perturbation of residues 207-211 suggests K210 as a likely contact site for phosphates in the tail.

To exclude non-specific effects, two scrambled control peptides were also tested. Despite the presence of two phosphate groups in SCR2 <<<<PLEASE USE SCR INSTEAD AF SP>>>>>, the NMR spectra of Gal-3 CRD showed only very small chemical shift variations (fig. S3 – PLEASE SHIFT S3 BEFORE S4). When the distance between the two phosphates was maintained, as in SCR1, some minor chemical shift perturbations were observed (fig. S4). Altogether, these results strongly suggest that the N-terminal peptide of human Gal-3 can interact with the CRD in a phosphorylation-dependent and sequence-specific manner.

3.3. *Tyr-P peptides*

In contrast to the observations for non-phosphorylated N-terminal peptides, addition of peptides P4-7 to ¹⁵N-labeled Gal-3 CRD yielded selective chemical shift perturbations for a good number of cross-peaks (figs. S5 and S6). Again, many of the most highly shifted NMR signals belonged to residues located on the backside of the β -sandwich structure (fig. 3). The magnitudes of the resonance shiftings were found to be larger for the longer peptides (P6 and P7). In this case, the presence of phosphorylation decreased the magnitude of the observed chemical shift perturbations in the Gal-3 CRD resonance signals. Thus, the affinity of these phosphorylated peptides towards the CRD seems to be significantly reduced. Thus, serine and tyrosine phosphorylations modulate the peptide-protein interaction process in different manners. The detailed comparison of the perturbation profile permitted to assess that the

formed complex somehow resemble the existing interactions the full-length protein (fig. 4). Indeed, for the perturbed resonances, the observed chemical shifts for the peptide/Gal-3 CRD complex were in between those existing in the Gal-3 CRD and in the full length protein. OK?

3.4. Pro/Gly-rich tandem-repeat peptides

Finally, we tested peptides containing one and two repeats from the tail, specifically with residues 42-50 (P8) and 42-59 (P9). The observed chemical shift perturbations permitted to assess that the affected Gal-3 CRD region was rather similar, reflecting the sequence similarity on the peptide level (fig. 3, Fig. S7). Competition experiments with mixtures of peptides P3 (N-terminal) and P9 (tandem-repeat) indicated that both peptides share interactions within an analogous Gal-3 CRD region. This fact was evidenced by the observed shifts for the L203, A212 and L219, HSQC signals (fig. 2B). However, the profile of apparent contacts indicate that the interaction region is larger than for peptide P4. OK?

4. Conclusions

The interaction of the Gal 3 CRD with synthetic (phospho)peptides of its N-terminal tail has been monitored by using NMR experiments. The peptide probes have been useful to detect the existence of a molecular recognition process. In fact, distinct peptide motifs have capacity for providing contacts to specific regions of the Gal-3 CRD. The comparison of the available NMR data for full length Gal-3 (for which a complete assignment is not yet available) with those obtained for the different peptide/protein complexes suggest that the N-terminal region is back-folded towards the CRD domain in the full-length protein. The obtained NMR data for the different peptides has permitted to show that serine and tyrosine phosphorylation act as on/off switches. Serine phosphorylation promotes peptide/protein association, while tyrosine substitution decreases affinity. These results give respective experiments on the level of the full-length protein a clear direction, herein then analyzing unsubstituted and phosphorylated proteins as well as variants with shortened tails.

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Figure 1. Top panel: sequence of the synthetic peptides used in this study, with phosphoserine (pS) and phosphotyrosine (pY) residues highlighted in red. Bottom panel: location of the peptides in the whole protein sequence. Red: peptides P1-P4; orange: peptides P5 and P6; blue: peptides P7-P9. The sequence of the CRD is indicated on a green background. On the right, a schematic representation of the crystal structure of the Gal-3 CRD (green), with the ND (at scale) is shown.

Figure 2. A: Top panel: superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of P2 (green) or P3 (red), with labels indicating some of the most perturbed signals. Bottom panel: ^1H - ^{15}N -weighted chemical shift differences ($\Delta\delta$) for Gal-3 CRD backbone NH signals upon addition of 10 equivalents P1 (yellow), P2 (red) or P3 (blue). B: Superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD with different P9/P3 ratios. Blue: Gal-3 alone; black: in the presence of ten equivalents of P9; orange: after the addition of 3.3 equivalents of P3; green: after the addition of 5 equivalents of P3; red: in the presence of ten equivalents of P3.

Figure 3. Mapping of the Gal-3 CRD residues with the most shifted signals for a number of peptides. In all cases, the structure of the LacNAc-loaded human Gal-3 CRD (PDB: 1A3K) is shown, with residues highlighted in red for the most shifted resonances, followed by orange and yellow.

Figure 4. Superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD (blue), full length Gal-3 (red), and Gal-3 CRD in the presence of ten equivalents of P4, with labels indicating some of the most perturbed signals, and arrows indicating the linear progression of a number of chemical shift variations.

Supplementary figure captions

Figure S1. Bottom panel: superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD (blue) and full length Gal-3 (red), with labels indicating some of the most perturbed signals. Top panel: structure of the LacNAc-loaded Gal-3 CRD (PDB: 1A3K), with residues highlighted in red for the most shifted resonances, followed by orange and yellow.

Figure S2. Superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of P1 (red).

Figure S3. Superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of SP1 (red).

Figure S4. Superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of SP2 (red).

Figure S5. Top panel: superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of P4 (red) or P5 (green). Bottom panel: ^1H - ^{15}N -weighted chemical shift differences ($\Delta\delta$) for Gal-3 CRD backbone NH signals upon addition of 10 equivalents P4 (blue) or P5 (red).

Figure S6. Top panel: superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of P6 (red) or P7 (green). Bottom panel: ^1H - ^{15}N -weighted chemical shift differences ($\Delta\delta$) for Gal-3 CRD backbone NH signals upon addition of 10 equivalents P6 (blue) or P7 (red).

Figure S7. Top panel: superimposition of ^1H - ^{15}N HSQC spectra of the Gal-3 CRD, in the absence (blue) and in the presence of ten equivalents of P8 (green) or P9 (red). Bottom panel: ^1H - ^{15}N -weighted chemical shift differences ($\Delta\delta$) for Gal-3 CRD backbone NH signals upon addition of 10 equivalents P8 (blue) or P9 (red).

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