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Review Article

Full-length galectin-8 and separate carbohydrate recognition domains: the whole is greater than the sum of its parts?

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Galectin-8 (Gal-8) is a tandem-repeat type galectin with affinity for β-galactosides, bearing two carbohydrate recognition domains (CRD) connected by a linker peptide. The N- and C-terminal domains (Gal-8N and Gal-8C) share 35% homology, and their glycan ligand specificity is notably dissimilar: while Gal-8N shows strong affinity for α(2-3)-sialylated oligosaccharides, Gal-8C has higher affinity for non-sialylated oligosaccharides, including poly-*N*-acetyllactosamine and/ or A and B blood group structures. Particularly relevant for understanding the biological role of this lectin, full-length Gal-8 can bind cell surface glycoconjugates with broader affinity than the isolated Gal-8N and Gal-8C domains, a trait also described for other tandem-repeat galectins. Herein, we aim to discuss the potential use of separate CRDs in modelling tandem-repeat galectin-8 and its biological functions. For this purpose, we will cover several aspects of the structure–function relationship of this protein including crystallographic structures, glycan specificity, cell function and biological roles, with the ultimate goal of understanding the potential role of each CRD in predicting full-length Gal-8 involvement in relevant biological processes.

Introduction

The galectin family is a group of evolutionary-conserved soluble lectins characterized by their ability to recognize β-galactosides through their carbohydrate recognition domains (CRDs) [1–5]. Current classification of these lectins relies on their structure and number of CRDs; thus, prototype galectins (Gal-1, -2, -7) present only one CRD, while chimera type Gal-3 has a single CRD and a non-lectin N-terminal domain. In turn, tandem-repeat type galectins such as Gal-4, -8, -9 and -12 are composed by an N-terminal and a C-terminal CRD, linked by a peptidic sequence of variable length [6].

Synthesized in the cytosol and with a still unknown non-classical secretion pathway, galectins display an army of intracellular and extracellular functions [7,8]. In the extracellular compartment, and due to the bivalent nature of tandem-repeat type and oligomerization of prototype and chimera types, galectins are able to cross-link glycosylated receptors on the cell surface forming supramolecular structures often termed 'lattices' [9,10], that convey glycan-containing information into distinct signaling programs and control cell fate [11]. Interestingly, galectins regulate glycoprotein uptake by inducing their cross-linking into lattices avoiding internalization [12], or by co-clustering them with glycolipids into raft-type membrane nanodomains called clathrin-independent carriers (CLICs) inducing endocytosis in a process termed glycolipid–lectin (GL-Lect) hypothesis [13,14]. It should be

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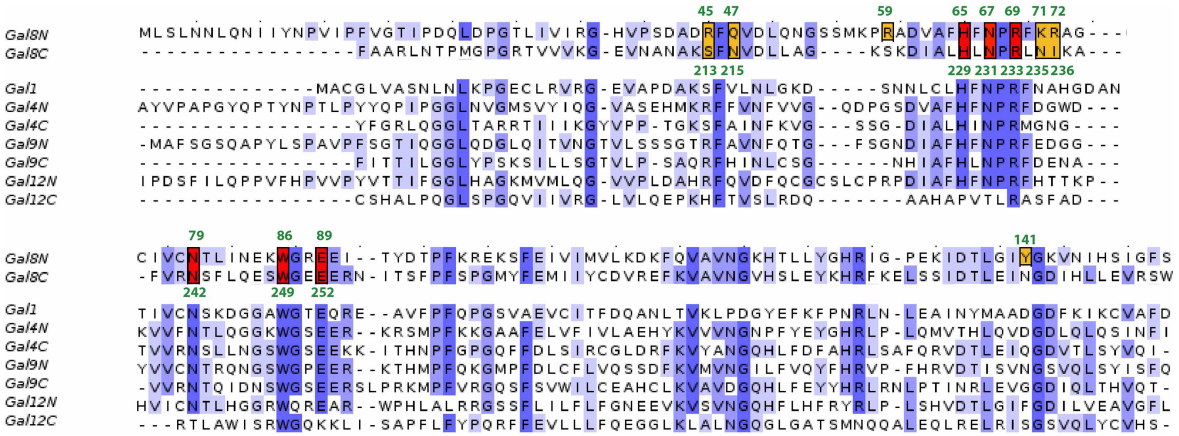

Figure 1. Sequence alignment of selected galectin family members.

Sequences were aligned using Jalview software [22]. Each carbohydrate recognition domain (CRD) consists of 135–165 amino acids, arranged on two antiparallel β-sheets, each composed of six (F′–F5 and S1–S6) β-strands. Identical residues of galectins are highlighted in blue and similar residues in light blue. Tandem-repeat type galectins are composed of two non-identical CRDs, joined by a hinge region. Gal-8 highly conserved residues shared with the galectin family, essential for protein–carbohydrate interactions, are highlighted in red. Key Gal-8 residues for glycan recognition (not usually found in galectins) are depicted in orange. Amino acids pointed out by crystallization studies as relevant for glycan complexes [23,24] are numerated.

noted that carbohydrate-mediated interactions of galectins with glycoproteins and glycolipids seem to induce mild cluster glycoside effects in spite of the presence of multivalent glycan epitopes [2,15]. Moreover, galectin–protein interactions are also acquiring significant relevance [2,16].

The human Gal-8 gene (*LGALS8*) was shown to encode six different isoforms resulting from alternative splicing [17–19]; three of these isoforms are tandem-repeat type, which have been named according to the length of the linker peptide as: Gal-8S (‘*small*’), Gal-8M (‘*medium*’) or Gal-8L (‘*long*’). The other Gal-8 isoforms are isolated CRDs that have been only found at the transcription but not protein level [20,21]. Gal-8 N- and C-terminal domains (Gal-8N and Gal-8C, respectively) share 35% homology, and their sequence motifs are conserved among most galectin members (Figure 1) [17,25]. Recombinant expression of each separate CRD showed that Gal-8N exhibits strong affinity for α(2-3)-sialylated and 3′-sulfated oligosaccharides [26,27]. In contrast, Gal-8C exhibits preferential binding for non-sialylated oligosaccharides, including poly-*N*-acetyllactosamine ([-3)Galβ(1-4)GlcNAcβ(1-]$_n$, poly-LacNAc) and A and B blood group antigens [23,28]. However, the reported glycan preferences of full-length Gal-8 do not necessarily reflect the binding preferences of each of its CRDs, showing a broader glycan recognition pattern and tight binding to ligands that present moderate or weak affinity for each separate CRD [29]. In the next sections, we discuss available data on Gal-8 glycan specificity, comparing the full-length lectin to its separate N-CRD and C-CRD domains, and interpret these results from a crystallographic perspective. Finally, we analyze how this particular glycan specificity impacts on cell binding and key biological functions.

Gal-8 carbohydrate-binding specificity is not solely dictated by its separate CRDs

Isolated Gal-8 CRDs differ noticeably in their carbohydrate specificity [26,27]. As a general trend, Gal-8N preferentially binds α(2-3)-sialylated oligosaccharides, while Gal-8C binds neutral β-galactosidic glycans with higher affinity. Indeed, frontal affinity chromatography (FAC) studies showed preferential binding of Gal-8N to α(2-3)-sialylated oligosaccharides (i.e. 3′-sialyllactose, $K_d = 0.62 \times 10^{-6}$ M for Gal-8N; $K_d = 97 \times 10^{-6}$ M for Gal-8C) [26]. Compared with Gal-8C, the N-terminal domain has lower affinity for different branched complex N-glycans ($K_d = 47–290 \times 10^{-6}$ M for Gal-8N; $K_d = 26–52 \times 10^{-6}$ M for Gal-8C). Surface plasmon resonance (SPR) validated these results for Gal-8N, with high affinity for 3′-sialyllactose (3′-SLac), and also demonstrated preferential recognition of 3′-sulfated and Lewis X-containing glycans than for oligosaccharides bearing terminal type I (Galβ(1-3)GlcNAc) or type II (Galβ(1-4)GlcNAc) LacNAc [27]. Conversely, Gal-8C

preferentially bound non-sialylated oligosaccharides like poly-LacNAc and blood group A (BGA; GalNAcα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNAc) and B (BGB; Galα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNAc) determinants [23].

Notably, glycan array studies narrowed down the preferred ligands for Gal-8N to 3′-sialylated/sulfated Galβ(1-4)Glc (lactose, Lac), type I LacNAc or Galβ(1-3)GalNAc (Thomsen-Friedenreich antigen, TF antigen), whereas type II LacNAc was less preferred [29]. Contrariwise, oligosaccharides containing poly-LacNAc or A and B blood group determinants were preferential ligands for Gal-8C [29,30]. Accordingly, sialylation significantly inhibited Gal-8C binding to poly-LacNAc structures [30]. Consistent with these experimental studies, computational analysis by molecular dynamic simulations showed that Gal-8C presented the strongest binding energy for lacto-*N*-*neo*tetraose (Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc), followed by diLacNAc (Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAc), A and B blood group antigens, and, finally, type I and II LacNAc and Lac [31].

When comparing full-length Gal-8 to each isolated CRD, glycan microarray studies showed that Gal-8 binding pattern was dominated by the N-terminal domain, with 3′-sulfated and 3′-sialylated glycans as preferential ligands [30]. Moreover, glycan arrays demonstrated that isoform Gal-8S recognizes a broader scope of oligosaccharides, further beyond the sum of structures bound by each domain. For example, recognition of glycans presenting -at least- two Lac, or type I or type II LacNAc moieties seemed to be mediated by the combined binding of the two CRDs, even if the affinity for each isolated CRD was too weak to be detected on the array [29]. In other glycan microarray studies, when evaluated at low concentrations, Gal-8N and full-length Gal-8 recognized the same 3′-sulfated and 3′-sialylated glycans with similar relative affinity [30]. In contrast, as previously observed, Gal-8C recognized poly-LacNAc and blood group antigens, while it exhibited no binding towards sulfated or sialylated glycans. Furthermore, sialylation significantly inhibited Gal-8C binding to poly-LacNAc structures [30].

Fluorescence anisotropy results, obtained with full-length Gal-8, isolated CRDs and ligands in solution, were consistent with glycan arrays and SPR data: the best ligand for Gal-8N was 3′-sialyllactose with $K_d = 53 \times 10^{-9}$ M [29]. Inhibition experiments confirmed similar K_d values for 3′-sialylated Galβ(1-3)GlcNAc and Galβ(1-3)GalNAc [29]. In each case, Gal-8N bound the 3′-sialylated compound 100-fold stronger than the non-sialylated disaccharide. On the other hand, and consistent with FAC measurements, Gal-8C affinities were in general weaker than for Gal-8N, showing K_d values of 10–100 μM for most probes. Furthermore, binding to Gal-8C was inhibited by 3′-sialylated structures but enhanced by Galα(1-3) ($K_d = 15.5 \times 10^{-6}$ M) or GalNAcα(1-3)[Fucα(1-2)] ($K_d = 8.8 \times 10^{-6}$ M) structures, validating previous data for BGA and BGB structures [29].

In summary, full-length Gal-8 binding preferences in solution were mainly dictated by the overall stronger affinity of the N-terminal CRD. However, the dominance of the N-terminal domain in dictating affinity is not complete, as full-length Gal-8 was able to recognize structures such as blood group A tetrasaccharide (GalNAcα(1-3)[Fucα(1-2)]Galβ(1-4)Glc) with comparable affinity to the C-terminal CRD [29].

Crystallization studies

Gal-8N crystal structure

To understand the main interactions driving glycan affinity, Gal-8N complexes with Lac, 3′-SLac and 3′-sulfolactose were crystallized [23]. First, in the Gal-8N/Lac complex (Figure 2A), the β-galactoside moiety is deeply buried in the binding site formed by β strands S4–S6, where Arg^{45}, His^{65}, Asn^{67}, Arg^{69}, Asn^{79} and Glu^{89} directly interact with Lac via hydrogen bond interactions (Table 1). In addition, Trp^{86} presents hydrophobic interactions with the α-face of the galactose ring. Moreover, Gln^{47}, Arg^{59} and Trp^{86} form water-mediated hydrogen bond interactions with the Lac moiety (Table 1) [26].

Regarding the Gal-8N/3′-sulfolactose complex, the residues involved in lactose moiety recognition are identical with those found in the Gal-8N/Lac complex. Additionally, four of these residues are also involved in sulfate group recognition, namely Arg^{45}, Gln^{47}, Arg^{59} and Trp^{86} (Table 1). Of the three oxygen atoms present in sulfate group, O2S and O3S present a direct hydrogen bond with the lectin: O3S forms hydrogen bonds with Arg^{45}, Gln^{47} and Arg^{59}, whereas O2S only presents a hydrogen bond with Arg^{59}. In addition, Trp^{86} interacts with O2S through water-mediated hydrogen bonds (Table 1).

When comparing Gal-8N/3′-SLac complex (Figure 2B) and Gal-8N/Lac complex (Figure 2A), the residues involved in lactose recognition are the same. Moreover, the Gal-8N residues responsible for sialic acid recognition are those involved in sulfate group recognition in the Gal-8N/3′-sulfolactose complex previously mentioned [23]. Indeed, the carboxylate group of sialic acid forms direct hydrogen bonds with Gln^{47}, Arg^{59} and

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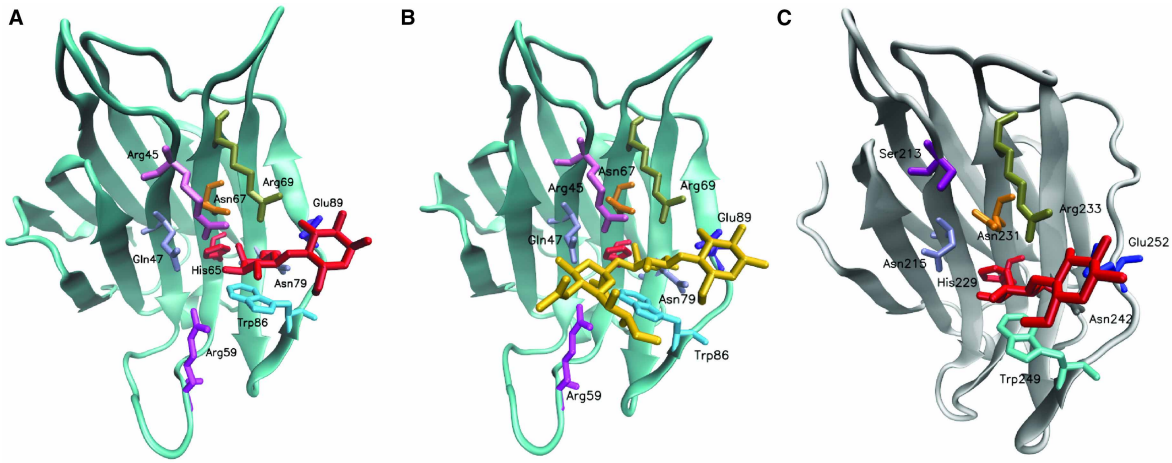

Figure 2. Human Gal-8 CRDs structure in complex with glycans.

Crystal structures of: (**A**) Gal-8N/lactose complex [23]; (**B**) Gal-8N/3′-sialyllactose complex [23]; (**C**) Gal-8C/lactose complex [24]. Homologous amino acid residues in A, B and C are identically coloured according to their sequence alignment.

Trp^{86} (Table 1). Of these, Arg^{59} forms two independent hydrogen bonds including the shortest one, suggesting that Arg^{59} is the key residue for sialic acid recognition. Additionally, Gln^{47} forms a water-mediated hydrogen bond with the O4 oxygen atom of the sialic acid moiety [23]. Furthermore, site-directed mutagenesis followed by SPR studies showed that in Gal-8N, Arg^{45}, Gln^{47} and Arg^{59} are essential, and coordinately contribute to the strong binding to 3′-sialylated and 3′-sulfated oligosaccharides, with Arg^{59} as the most critical residue (Figures 1, 2B) [23,27]. When comparing with other tandem-repeat type galectins, Arg^{59} is only found in Gal-8N and Gal-12N [32].

Gal-8 N-CRD in complex with 3′-sialyllactosamine [24] showed a very similar structure to the Gal-8N/3′-SLac complex previously described [23]: efficient salt bridge interactions are achieved between Arg^{59} side chain, located in the long S3–S4 loop particular to Gal-8N (Figure 1) and the carboxyl group of the sialic acid; Gln^{47} and Trp^{86} also interact with the carboxyl group from both sides via hydrogen bonds [24], and Gal-O3 further contributes to recognition of this moiety via hydrogen bonds with Arg^{45} and Gln^{47}. Notably, Arg^{45} is also present in Gal-4N, Gal-9N/C and Gal-12N (Figure 1), which have also shown significant affinity for α(2-3)-sialylated oligosaccharides (K_d = 3.8–7.4 × 10^{-6} M for Gal-9N, and K_d = 39–79 × 10^{-6} M for Gal-9C) [26,32,33]. In addition, besides tandem-repeat type galectins, Gal-3 also exhibits an arginine residue in the analogous position, namely Arg^{144}, and Salomonsson *et al.* [34] showed that Gal-3 R144S mutant exhibited lower affinity towards 3′-sialylated oligosaccharides, further indicating the key role of this residue for sialic acid recognition.

Furthermore, while the *N*-acetyl group of the GlcNAc residue in 3′-sialyllactosamine forms a water-mediated hydrogen bond with Gal-8N Glu^{89}, its O3 forms two direct hydrogen bonds with Arg^{69} and Glu^{89}. The galactose residue plays a key role: direct hydrogen bonds are found between Gal-O4 and His^{65}, Asn^{67}, and Arg^{69}. Moreover, four additional hydrogen bonds (O3-Arg^{45}, O5-Arg^{69}, O6-Asn^{79} and O6-Glu^{89}) define the ligand position. These amino acids, with the exception of Arg^{45}, are strictly conserved among the galectin family members and are essential for LacNAc recognition.

In conclusion, considering Gal-8N glycan interactions, structural complexes show that Arg^{59} is the critical residue responsible for the strong affinity towards α(2-3)-sialylated oligosaccharides, while Arg^{45} and Gln^{47} are also important in sialic acid moiety recognition [24].

Gal-8C crystal structure

Notably, and in contrast with Gal-8N, there are very few structural studies with the isolated Gal-8 C-terminal domain, and none of them reports Gal-8C in complex with glycans. Structure of Gal-8C CRD was initially reported by Tomizawa and co-workers using nuclear magnetic resonance (NMR) studies (PDB entry: 2YRO), and in 2011, Zhou *et al.* described the crystal structure of apo Gal-8C (PDB entry: 3OJB), but none of these studies were published. Finally, Li *et al.* elucidated the crystal structure of Gal-8 C-CRD in complex with a

Table 1. Amino acid residues involved in Gal-8 glycan specificity, as determined by crystallographic studies [23,24]

Part 1 of 2

Crystal structure	Amino acid	CRD	Interaction	Glycan atom
Gal-8N/lactose	Arg^{45}	N-CRD	H bond	Gal-O3
	Arg^{45}	N-CRD	H bond	Gal-O4
	Asn^{67}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O4
	His^{65}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O6
	Asn^{79}	N-CRD	H bond	Gal-O6
	Trp^{86}	N-CRD	Hydrophobic	Gal α-face
	Arg^{69}	N-CRD	H bond	Glc-O3
	Glu^{89}	N-CRD	H bond	Glc-O3
	Gln^{47}	N-CRD	H_2O-mediated H bond	Gal-O3
	Trp^{86}	N-CRD	H_2O-mediated H bond	Gal-O3
	Arg^{59}	N-CRD	H_2O-mediated H bond	Gal-O3
Gal-8N/3′-sulfolactose	Arg^{45}	N-CRD	H bond	Gal-O3
	Arg^{45}	N-CRD	H bond	Gal-O4
	Asn^{67}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O4
	His^{65}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O6
	Asn^{79}	N-CRD	H bond	Gal-O6
	Trp^{86}	N-CRD	Hydrophobic	Gal α-face
	Arg^{69}	N-CRD	H bond	Glc-O3
	Glu^{89}	N-CRD	H bond	Glc-O3
	Arg^{59}	N-CRD	H bond	O2S
	Arg^{45}	N-CRD	H bond	O3S
	Gln^{47}	N-CRD	H bond	O3S
	Arg^{59}	N-CRD	H bond	O3S
	Trp^{86}	N-CRD	H_2O-mediated H bond	O2S
Gal-8N/3′-sialyllactose	Arg^{45}	N-CRD	H bond	Gal-O3
	Arg^{45}	N-CRD	H bond	Gal-O4
	Asn^{67}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O4
	His^{65}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O6
	Asn^{79}	N-CRD	H bond	Gal-O6
	Trp^{86}	N-CRD	Hydrophobic	Gal α-face
	Arg^{69}	N-CRD	H bond	Glc-O3
	Glu^{89}	N-CRD	H bond	Glc-O3
	Gln^{47}	N-CRD	H bond	Neu5Ac-O1
	Arg^{59}	N-CRD	H bond	Neu5Ac-O1
	Trp^{86}	N-CRD	H bond	Neu5Ac-O1
	Gln^{47}	N-CRD	H_2O-mediated H bond	Neu5Ac-O4
Gal-8N/3′-sialyllactosamine	Arg^{45}	N-CRD	H bond	Gal-O3
	Arg^{45}	N-CRD	H bond	Gal-O4
	Asn^{67}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O4
	His^{65}	N-CRD	H bond	Gal-O4
	Arg^{69}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O5
	Glu^{89}	N-CRD	H bond	Gal-O6
	Asn^{79}	N-CRD	H bond	Gal-O6

Continued

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Table 1. Amino acid residues involved in Gal-8 glycan specificity, as determined by crystallographic studies [23,24] Part 2 of 2

Crystal structure	Amino acid	CRD	Interaction	Glycan atom
	Trp^{86}	N-CRD	Hydrophobic	Gal α-face
	Gln^{47}	N-CRD	H bond	Neu5Ac-O1
	Arg^{59}	N-CRD	H bond	Neu5Ac-O1
	Trp^{86}	N-CRD	H bond	Neu5Ac-O1
	Arg^{69}	N-CRD	H bond	GlcNAc-O3
	Glu^{89}	N-CRD	H bond	GlcNAc-O3
	Glu^{89}	N-CRD	H bond	GlcNAc-O7
	Arg^{59}	N-CRD	Salt bridge	Neu5Ac-O1
Gal-8C/lactose	His^{229}	C-CRD	H bond	Gal-O4
	Asn^{231}	C-CRD	H bond	Gal-O4
	Arg^{233}	C-CRD	H bond	Gal-O4
	Arg^{233}	C-CRD	H bond	Gal-O5
	Asn^{242}	C-CRD	H bond	Gal-O6
	Glu^{252}	C-CRD	H bond	Gal-O6
	Glu^{252}	C-CRD	H bond	Glc-O3
	Arg^{233}	C-CRD	H bond	Glc-O3
	Trp^{249}	N-CRD	Hydrophobic	Gal α-face

H bond: hydrogen bond. O2/3S: oxygen (2/3) in sulfate.

peptide from the autophagy receptor named nuclear domain 10 protein 52 (NDP52), describing the first crystallized protein–protein interaction for Gal-8 [35]. Gal-8 C-terminal domain adopted a bent β-sandwich structural fold formed by two antiparallel β-sheets of five and six strands, comprising the convex and concave surfaces, respectively. In the crystallized structure, this NDP52 peptide bound to Gal-8 convex side, opposite to the concave glycan-binding groove of the lectin [35,36].

Full-length Gal-8 crystal structure

The first X-ray structure of full-length Gal-8 was reported in 2012 by Yoshida *et al.* [24]. This work describes the structure of Gal-8Null, a Gal-8 mutant in which the linker region was replaced by a His-Met dipeptide, complexed with Lac (PDB entry: 3VKL) and 3′-SLac (PDB entry: 3VKM). As expected, each CRD adopted a β-sandwich arrangement formed by two antiparallel β-sheets of six (S1–S6) and five (F1–F5) β-strands, with a short α-helix located between F5 and S2. Oligosaccharides bound to the concave surface created by S3, S4, S5 and S6 strands [24]. In addition, full-length Gal-8 structure complexed with NDP52 peptide was reported by Kim *et al.* [36]. Although the structure of each CRD exhibited high correlation with the corresponding CRD reported by Yoshida *et al.* [24], the superimposition of the full-length structure showed a large rotation of the N-CRD relative to the C-CRD in the Gal-8–NDP52 complex. Different spatial orientation between the two CRDs could be required for a specific biological function and may be a characteristic feature of tandem-repeat type galectins [36].

The structure and glycan–lectin interactions of Gal-8N CRD observed in Gal-8Null complexes with Lac and 3′-SLac [24] were almost identical as those described for the isolated Gal-8N/Lac and Gal-8N/3′-SLac [23] crystal structures (Supplementary Figure S1). In contrast with the N-CRD, in the C-CRD there are no residues capable of sialic acid recognition: Arg^{59} is absent, and Arg^{45} and Gln^{47} are replaced by Ser^{213} and Asn^{215}, which explains the lack of sialic acid recognition by the C-terminal domain (Figure 1). The X-ray crystal structure of Gal-8C bound to Lac in Gal-8Null showed that galactose undergoes stacking interactions with Trp^{249}, and forms five hydrogen bonds with His^{229}, Asn^{231}, Arg^{233}, Asn^{242} and Glu^{252} (Figure 2C) [24]. In addition, glucose residue is recognized by hydrogen bonds from Arg^{233} and Glu^{252}. Apart from the S3–S4 loop and N-terminal region, superimposition of the two Gal-8 CRDs shows another structural difference at the S4–S5 loop, not related to ligand recognition: in the N-CRD, Lys^{71} and Arg^{72} direct their side chains toward the outer side of the protein, possibly because of repulsion by the positively charged $Arg^{45}$and Arg^{69}, whereas Asn^{235} and Ile^{236} in the C-CRD direct their side chains to the bound oligosaccharides [24]. In conclusion, and from a crystallographic perspective, the main difference between the N- and C-terminal Gal-8 CRDs, determining their

glycan-binding preferences, seems to be the presence of Arg^{59} residue in Gal-8N, essential for sialic acid and sulfate groups recognition, which is replaced by Ser^{223} in Gal-8C [24].

From structure to biological activity: cell binding and functional role of Gal-8

Upon secretion, Gal-8 acts as a matricellular protein capable of modulating cell adhesion and migration in various cell types [37,38]. Gal-8 also promotes angiogenesis and lymphangiogenesis on vascular and lymphatic endothelial cells, respectively, through interaction with a selected repertoire of glycosylated receptors [39,40]. In addition, Gal-8 is up-regulated in several carcinomas [41,42], and can promote cell transformation in epithelial cells, inducing enhanced cell migration, invasion, anchorage-independent growth and epithelial–mesenchymal transition [43]. *In vivo*, Gal-8 is pro-tumorigenic in mouse xenografts [44,45], while high levels of circulating Gal-8 have been detected in sera from breast, prostate and colon cancer patients [45–47].

In fact, the impact of specific Gal-8 CRDs–glycan interactions in the biological functions of the full-length lectin is still matter of controversy [2,48]. Protein–protein interactions are crucial for Gal-8, and -although they are beyond the focus of this review- noteworthy associations have been documented for: (i) actin [49]; (ii) a peptide from NDP52, a pathogen-specific autophagy receptor which interacts with Gal-8C, inducing a selective autophagy pathway against bacterial invasion [36,50,51]; and (iii) the farnesylated domain in the C-terminus of the oncogene K-Ras4B, which binds to Gal-8 N-CRD modifying K-Ras-induced cell signalling [52].

In the next sections, we will cover interesting studies pointing out differences between Gal-8 CRDs on cell binding in relation to their glycan specificities, and the promotion of some precise cell functions in which isolated CRDs are compared with full-length Gal-8.

It should be pointed out that carbohydrate-binding studies for Gal-8 such as SPR, FAC, glycan array or fluorescence anisotropy assays are usually performed at room temperature, while Gal-8 cell surface binding (measured by flow cytometry) is assessed on ice (to avoid lectin internalization) and biological functions are evaluated at 37°C.

Case study 1: HL60 promyelocytic leukemia cell desialylation did not alter full-length Gal-8 binding: relevance of phosphatidylserine exposure and lectin dimerization

Both recombinant Gal-8N and Gal-8C domains, as well as full-length Gal-8, bound to HL60 promyelocytic leukemia cells (Table 2), but only full-length Gal-8 was able to elicit phosphatidylserine (PS) exposure, which occurred independently of apoptosis [30]. As expected, treatment of HL60 cells with a pan-neuraminidase increased Gal-8C binding, while reducing Gal-8N affinity. Moreover, enzymatic degradation of poly-LacNAc glycans with endo-β-galactosidase reduced Gal-8C binding to HL60 cells, but did not alter Gal-8N interaction, suggesting that poly-LacNAc recognition by full-length Gal-8 occurs through the C-terminal domain. Importantly, cell desialylation did not alter full-length Gal-8 binding, but enhanced cellular sensitivity to Gal-8-induced PS exposure, indicating that this function is mediated by Gal-8C [30].

Notably, dimerization of full-length Gal-8 at the HL60 cell surface was reported to occur through its N-terminal domain, while isolated Gal-8N, but not Gal-8C, was also shown to form dimers [30]. Accordingly, a recombinant Gal-8 variant (named Gal-8NM) with a mutated N-terminal domain inactive for glycan-binding (R69H) and active Gal-8C also suffered dimerization, induced PS exposure and presented similar binding towards poly-LacNAc glycans as full-length Gal-8. These results suggest that PS exposure is mediated by Gal-8C but requires Gal-8 dimerization [30]. However, the identity of Gal-8N amino acids involved in lectin dimerization is still uncertain.

Case study 2: Gal-8 binding to U937 monocytes and MOLT4 lymphoblastic leukemia cells

In another relevant study, full-length Gal-8L and Gal-8S showed strong carbohydrate-dependent binding to U937 monocytes and MOLT-4 lymphoblastic leukemia cells [29]. Since the preferred ligands for Gal-8N contain α(2-3)-sialylated structures, the role of sialic acid at the cell surface was examined by digestion with a pan-neuraminidase or with a specific α(2-3)-neuraminidase. As expected, both sialidase treatments reduced Gal-8N binding (Table 2). Again, Gal-8C presented stronger binding to both neuraminidase-treated cells, in

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Table 2. Cell surface binding of full-length Gal-8, its separate CRDs or specific mutants

Cell	Treatment	Gal-8N	Gal-8C	Gal-8	Mutant Gal-8	Ref.
HL60 human promyelocytic leukemia cell line	Non-treated (WT)	++++	++	++++		[30]
	Pan-neuraminidase	+	++++	++++		
U937 human monocyte cell line	Non-treated (WT)	++	++	++++	Q47A* mutant: ++++	[29]
	Pan-neuraminidase	+	+++	++++		
	α(2-3)-neuraminidase	+	+++	++++		
CHO hamster ovary cell lines	WT cells	++	+	++++		[28]
	Lec2	–	++	+++		
	Lec1	++	ND	–		
	St3gal4/6 KO cells	–	++	ND		[53]
	Stgal4/6 KO + *St6gal1* KI cells	–	ND	ND		
	Mgat1 KO	–	+	ND		
	Mgat4A/B or *Mgat4A/4B/5* KO cells	+++	++	ND		
WT CHO cell line	Non-treated (WT)	ND	ND	+++	+++ (NN)**	[54]
	Pan-neuraminidase	ND	ND	++++	+ (NN)**	
MDA-MB-231 breast cancer cell line	Non-treated (WT)	ND	ND	+++		[45]
	Pan-neuraminidase	ND	ND	++++		
	α(2-3)-neuraminidase	ND	ND	++++		

WT: wild-type cell. KO: knocked-out cell. KI: knocked-in cell. ND: not determined.
*Full-length Gal-8, bearing a point mutation in glutamine[47] substituted by alanine.
**Mutant chimera Gal-8, composed of two N-domains connected by a linker peptide.

accordance to its glycan specificity, but notably, and similar to what was observed in HL60 cells, average binding of full-length Gal-8 was not significantly altered after desialylation. These results indicate that non-sialylated ligands provide sufficient affinity for cell binding of full-length Gal-8 [29].

Accordingly, Gal-8 Q47A mutant, with decreased affinity for α(2-3)-sialylated galactosides, did not present significantly different binding to the cell surface compared with wild-type (WT) Gal-8. In summary, full-length Gal-8 cell surface binding to U937 monocytes and MOLT4 lymphoblastic leukemia cells is mediated by both CRDs [29].

Case study 3: CHO cells and glycosylation mutants as models for Gal-8 binding

Patnaik and co-workers exhaustively explored the contribution of each CRD using different CHO mutant cells with altered glycosylation. Even though WT CHO cells present strong α(2-3)-sialylation, which is permissive for Gal-8N [28,55], Gal-8 N-terminal domain bound weakly to WT CHO cells and, as expected, Gal-8C exhibited low binding [28]. However, full-length Gal-8 was more efficient than any isolated CRD in CHO cell recognition, even when considering different isoforms with either long or short inter-CRD linkers. Absence of complex or hybrid N-glycans in Lec1 cells - which have a mutation in *Mgat1* gene encoding for β(1-2)-*N*-acetylglucosaminyltransferase I - (Figure 3) decreased full-length Gal-8 binding, while Gal-8N showed similar binding to Lec1 and WT CHO cells. These results suggest that Gal-8N binding to Lec1 cells could be mediated by recognition of gangliosides and sialylated O-glycans, while poor binding of full-length Gal-8 is due to their high mannose N-glycans [28] (Figure 3). Accordingly, mutation in the CMP-sialic acid transporter in Lec2 mutant CHO cells (Figure 3), which eliminates all sialic acid residues, hampered Gal-8N binding, while full-length Gal-8 recognized these cells similar to WT cells [28] (Table 2), probably by interaction with Gal-8C.

Later, Nielsen *et al.* demonstrated the key role of sialic acid linkage in Gal-8 cell binding: as expected, CHO cells lacking α(2-3)-sialylated complex N-glycans (deficient in sialyltransferases *St3gal4/6*) showed reduced

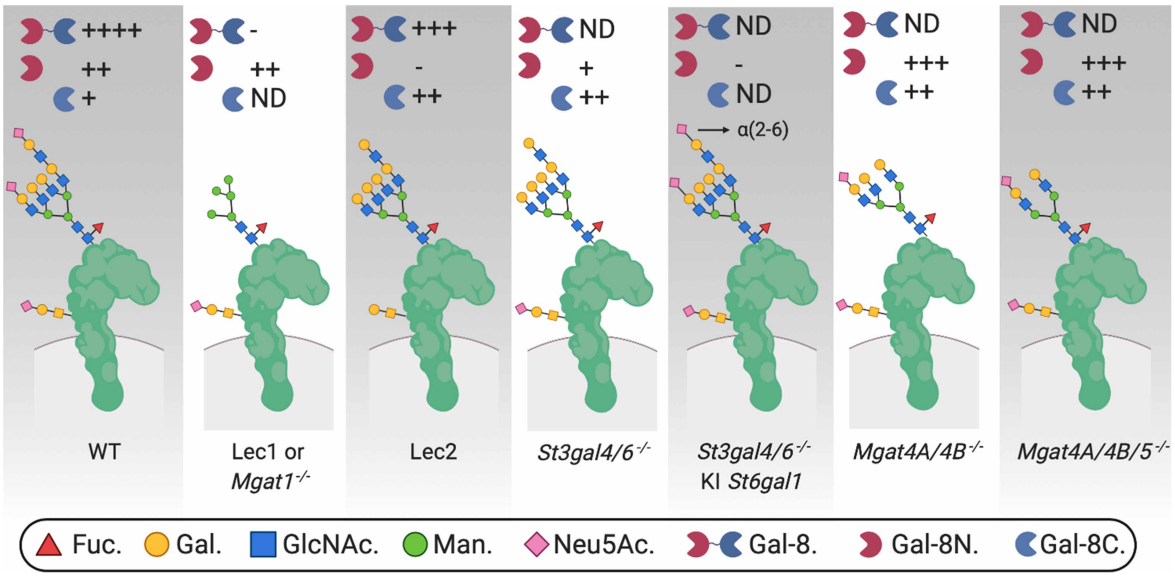

Figure 3. Cell surface Gal-8 binding to CHO cells.

Binding of full-length Gal-8, Gal-8N and Gal-8C to different mutant and wild-type CHO cell lines is represented by: – (no binding), and +(low binding) to ++++ (high binding). A schematic transmembrane glycoprotein is shown in green at CHO cell surfaces, bearing N- and O-glycans. All sialic acids (Neu5Ac) in CHO cells are displaying α(2-3)-linkages. CHO cells transfected with α(2-6)-sialyltransferase 1 (*St6gal1* KI) exhibit α(2-6)-sialic acid in their N-glycans. *St3gal4/6*$^{-/-}$: α (2-3)-sialyltransferase deficient cells; *Mgat*$^{-/-}$: *N*-acetylglucosaminyltransferase deficient cells; Lec1 cell: *Mgat1* gene that encodes β(1-2)-*N*-acetylglucosaminyltransferase I has been mutated; Lec2 cell: CMP-sialic acid transporter has been mutated; WT: wild-type cell; KI: knocked-in cell; ND: not determined. Adapted from [28,53] and made in ©BioRender - biorender.com.

binding of Gal-8N, and recognition was even lower after transfection with *St6gal1* [53], a sialyltransferase responsible for α(2-6)-sialylation, a non-permissive glycan modification for all members of the galectin family. In WT CHO cells, Nielsen *et al.* [53] found higher binding of Gal-8N compared with *Mgat1*$^{-/-}$ cells, measured either by fluorescence anisotropy or cell binding by flow cytometry [53], probably due to binding to oligo- or poly-LacNAc structures present in WT cells, known to be good Gal-8N ligands [29]. On the contrary, Patnaik *et al.* [28] showed similar Gal-8N binding in Lec1 and in WT CHO cells. We attribute these disparities ([53] versus [28]) to different experimental approaches, but we certainly consider that the contribution of oligo- or poly-LacNAc present in WT cells should promote Gal-8N binding as suggested in [53]. Furthermore, CHO cells with only biantennary N-glycans (genetically engineered *Mgat4A/4B/5*$^{-/-}$ cells), possessing less branching in complex N-glycans but normal sialylation in N- and O-glycans, showed higher Gal-8N binding than WT cells, due to less antennarity and typical α(2-3)-sialylation [53]. In fact, Gal-8C also showed higher binding to mutants with less branching in complex N-glycans than to WT CHO cells [53], suggesting that steric hindrance between branches precludes access of C-CRD to internal poly-LacNAc [30].

Gal-8 is rapidly endocytosed at 37°C [56], as also demonstrated for other galectins [13,14,16]. Interestingly, in internalization experiments performed in CHO cells at 37°C, interaction of the N-CRD in full-length Gal-8 with sialylated glycans has been shown to be crucial for lectin intracellular trafficking [56]. In fact, when comparing endocytosis of full-length Gal-8 in CHO cells and in Lec2 mutants, the pathway after endocytosis differed dramatically. While in WT cells, Gal-8 was found along the plasma membrane, near the nucleus and in small vesicles, in Lec2 cells, in contrast, Gal-8 was mainly found in larger vesicles evenly spread in the cell [56]. Therefore, we might speculate that endocytic vesicles carrying full-length Gal-8 could be involved in different intracellular routes and signalling pathways, depending on whether their membranes contain sialylated saccharides or not. Similarly, cytosolic galectins were also shown to modulate cellular responses according to the structure of cell surface glycans located in endocytic vesicles

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[57], which can be exposed to cytosolic galectins in damaged endosomes. Indeed, after experimental induction of vesicle damage, WT CHO cells expressing fluorescently-labelled Gal-8 showed large lectin aggregates, co-localizing with carbohydrates in these endosomes, which finally generated Gal-8-mediated autophagic activation. Conversely, in Lec2 cells, fewer lectin aggregates were observed compared with WT cells, indicating that sialic acid contributes to Gal-8 effects [57]. In conclusion, cytosolic full-length Gal-8 preferentially binds to sialylated glycans on damaged endosomes, highlighting the key role of its N-CRD in intracellular signalling [56].

When measuring adhesive capability of WT CHO cells to Gal-8-coated surfaces, the full-length lectin was able to promote adhesion, with Gal-8N maintaining the properties of full-length Gal-8 when used at high concentrations; however, Gal-8C resulted functionally inactive. Importantly, deletion of the linker peptide partially impaired Gal-8-mediated adhesive properties. Thus, linker flexibility and proper disposition of Gal-8 N- and C-terminal domains are needed to mediate CHO cell adhesion [58].

Finally, Ludwig and colleagues engineered a homodimeric Gal-8 variant with two Gal-8N domains, named Gal-8NN, and compared its cell binding to that of full-length WT Gal-8 (Table 2). In WT CHO cells with abundant α(2-3)-sialylation, binding of Gal-8NN was found slightly reduced by desialylation compared with non-treated cells, while the WT lectin showed enhanced staining of neuraminidase-treated versus control cells [54].

Case study 4: Gal-8 mediates cell adhesion in desialylated breast cancer MDA-MB-231 cells

Full-length Gal-8 induction of cell adhesion in MDA-MB-231 cells has been shown to be mediated by activated leukocyte cell adhesion molecule (ALCAM/CD166), a Gal-8 ligand [39,59]. Notably, both α(2-3,6,8,9) neuraminidase A or α(2-3) neuraminidase treatments significantly increased cell adhesion onto full-length Gal-8-coated surfaces, compared with untreated or to ALCAM-silenced cells. Thus, treatment with neuraminidases unmasks Gal-8 ligands, as reported by others [29], and favors Gal-8-mediated cell adhesion [45]. To understand the relevance of sialylated glycans in Gal-8-induced cell adhesion, we recently analyzed the specific N-glycosylation profile of ALCAM as a Gal-8 ligand in MDA-MB-231 cells, and confirmed a high percentage of permissive structures with neutral terminal *N*-acetyllactosamine residues or α(2-3)-sialylated structures. Notably, when digested with α(2-3,6,8,9) neuraminidase, a considerable increase in permissive structures was obtained, as removal of α(2-6) sialylation unmasked *N*-acetyllactosamine residues. Even though not all permissive N-glycans characterized in ALCAM may be exposed on the surface of these cells, and Gal-8 induction of cell adhesion may also be mediated by other glycosylated ligands such as integrins or CD44, Gal-8 interaction and induction of MDA-MB-231 cell adhesion does not require α(2-3)-sialylation, and can also be mediated by neutral glycans [45]. Interestingly, Renard *et al.* [60] recently showed that Gal-8 acts in concert with endophilin-A3 to control clathrin-independent endocytosis of ALCAM/CD166.

Case study 5: Gal-8 domains and their different roles on immune cells and platelets

When evaluating neutrophil adhesion, Gal-8C seems to have a primordial role, since mutant full-length Gal-8 lacking the carbohydrate-binding activity of N-terminal CRD (Gal-8 R69H) retained neutrophil adhesion activity, while inactivation of the C-terminal domain (Gal-8 R233H) abolished this function [61]. Conversely, platelet adhesion was promoted by isolated Gal-8N and protein chimera Gal-8NN, as well as full-length Gal-8. All of them generated aggregation and secretion of dense and alpha granules, highlighting the central role of the N-terminal domain for this biological function [62].

In T cells, Gal-8NN and Gal-8CC homodimers, but not isolated N- or C-terminal domains, were able to induce proliferation; however, single domains induced T-cell co-stimulation, suggesting that tandem-repeat structure is essential only for the proliferative effect. In both cases, CC chimeras displayed higher activity than WT Gal-8, indicating that the C-CRD was the main domain involved, as was further supported by the strong inhibition of proliferation and co-stimulation in the presence of blood group B antigen [63].

Perspectives

- *Importance of Gal-8 functional and structural studies*: Gal-8 has been intensively studied given its abundance in several carcinomas and its broad pro-tumorigenic effects, including cell adhesion and migration, as well as angiogenesis and lymphangiogenesis. Among members of the galectin family, structure-activity analysis of tandem-repeat type galectins has remained challenging due to difficulties in their crystallization, particularly as full-length galectins. Thus, structural studies on apo galectins and their complexes with specific oligosaccharides is essential for understanding structure–function relationships and delving into their biological roles and evolutionary advantages.

- *Summary of current knowledge on Gal-8 and its CRDs*: Gal-8 CRDs differ noticeably in their carbohydrate specificity. However, studies on the full-length lectin show that Gal-8 recognizes and binds a broader scope of ligands than its separate CRDs. In solution studies, both domains seem to act independently of each other, exhibiting similar specificity and affinity as the isolated domains. Crystallographic studies unveiled critical residues mediating this differential affinity; however, when exploring the biological functions of this lectin at the cell surface, Gal-8 has shown the ability to bind ligands recognized by either CRD but also glycans of low affinity for either of them, indicating that, for this tandem-repeat type galectin (and probably others), prediction of biological function by each separate domain is limited. Although the reasons for this broader specificity still remain unclear, linker flexibility and proper disposition of Gal-8 CRDs should not be overlooked. Certain biological functions are mainly dependent on either CRD, while other specific roles are only mediated by full-length Gal-8, probably as an evolutionary mechanism to preserve function independently of alterations in cellular glycosylation. Assay temperature should be carefully considered in each experimental design: cell surface binding experiments by flow cytometry are performed in the cold to avoid endocytosis, while Gal-8 biological functions are studied at 37°C, a condition in which the lectin is being endocytosed.

- *Future directions*: Gal-8-glycan specificity, as determined by biophysical studies, does not always correlate with cellular binding assays, where results markedly vary within each cell type, its glycome and the specific cell function analyzed. Therefore, studies are needed to elucidate both the contribution of each Gal-8 CRD and the role of the full-length protein. Moreover, other factors that could control cell binding, including dimerization at the cell surface, have not been fully documented and need to be addressed.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

3′-SLac, 3′-sialyllactose; ALCAM, activated leukocyte cell adhesion molecule; BGA, blood group A determinant; BGB, blood group B determinant; CRD, carbohydrate recognition domain; FAC, frontal affinity chromatography; Gal-8, galectin-8; Gal-8M, medium full-length Gal-8; Gal-8C, galectin-8 C-CRD; Gal-8L, long full-length Gal-8; Gal-8N, galectin-8 N-CRD; Gal-8S, small full-length Gal-8; K_d, dissociation constant; KO, knock out; Lac, lactose; LacNAc, *N*-acetillactosamine; Lec1 cell, mutant CHO cell bearing a mutated *Mgat1* gene (encoding β (1-2)-*N*-acetylglucosaminyltransferase I); Lec2 cell, mutant CHO cell bearing a mutated CMP-sialic acid transporter; *Mgat*, *N*-acetylglucosaminyltransferase gene; NDP52, nuclear domain 10 protein 52; Neu5Ac, *N*-acetylneuraminic acid or sialic acid; NMR, nuclear magnetic resonance; PDB, protein data bank; poly-LacNAc, poly-*N*-acetillactosamine; SPR, surface plasmon resonance; *St3gal4*, β-galactoside α(2-3)-sialyltransferase 4 gene; *St3gal6*, β-galactoside α(2-3)-sialyltransferase 6 gene; *St6gal1*, β-galactoside α(2-6)-sialyltransferase 1; TF, Thomsen–Friedenreich antigen; WT, wild-type.

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