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The novel lectin KM+ detects a specific subset of mannosyl-glycoconjugates in the rat cerebellum

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KM+ is a D(+)mannose-specific lectin with a carbohydrate structure-affinity relationship different from those of most mannose-binding lectins. KM+ elicits carbohydrate-dependent biological effects in several mammalian cell types, but it has not yet been employed as a probe for the detection of its specific ligands. We show here for the first time the screening and partial identification of cerebellar mannosyl-glycoconjugates recognized by KM+, by means of lectin-histochemistry and lectin-blotting. Biotinylated KM+ stained most cellular structures in the adult rat cerebellum, particularly Purkinje cells bodies and the surface of granule cells, but not cellular processes. Capillaries in the choroid plexus were also strongly decorated, while blood vessels in the cerebellar parenchyma remained unstained. D(+)mannose, but not D(+)galactose, abolished the staining of all cerebellar structures. Higher inhibitory potencies were found for mannosyl-glycans such as mannotriose (man-α1,3-[man-α1,6]-man) and the biantennary heptasaccharide carried by the enzyme horseradish peroxidase. After separation of cerebellar proteins by SDS-PAGE, KM+ recognized three major unidentified mannosyl-glycoproteins of 132, 83 and 49 kDa. KM+ also detected high-Mw bands corresponding to the light and heavy chains of Type-I laminin, but not a 160-kDa cleavage product of laminin. We conclude that KM+ binds preferentially to a specific subset of mannose-containing glycoproteins in cerebellar tissue, thus being much more restricted than other mannose-specific lectins. KM+ can be used as a novel probe to screen the central nervous system for this specific subset of complex mannosyl-glycoconjugates.
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Introduction

Lectins are proteins recognized by the presence of at least one non-catalytic domain that reversibly binds to specific mono- or oligosaccharides [1]. The use of plant lectins as probes to analyse glycoconjugates from animal cells has a long-standing and productive history [1]. Lectins have proved particularly useful in the study of recognition molecules that are present on the surface of neural cells. Most of these cell-surface molecules are carbohydrate-bearing glycoproteins, glycolipids or proteoglycans. They are involved in cell migration and neurite outgrowth during central nervous system (CNS) development (reviewed in [2,3]) as well as in the maintenance of synaptic activity in the adult CNS (see for instance [4,5]). The mammalian cerebellum provides a well-characterized model to study the expression of cell-cell recognition molecules in the CNS. It has a relatively simple cortical laminar organization and clear cell-cell recognition events during the postnatal formation of the cortical layers [6–8]. In addition, glycoconjugate recognition in cerebellar tissue has attracted particular attention since the discovery of endogenously secreted cerebellar lectins involved in development and pathology [9–11]. The presence and identity of glycoconjugates in the rat cerebellum has been previously studied with plant lectins [12–15]. However, most studies have been restricted to particular cellular types or have not provided histological evidence of glycoconjugate distribution.

KM+ is a lectin isolated from the seeds of the jackfruit (*Artocarpus integrifolia*, Moraceae) that specifically binds α-D(+)-mannose and α-D(+)-glucose, but has no detectable affinity for α-D(+)-galactose [16]. KM+ and the lectin artocarpin purified from the same plant share the same carbohydrate-recognition properties and ~94% identity in primary structure, thus likely being isoforms of the same protein [17–19]. The name KM+,

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however, has been maintained over time to avoid confusion with a functionally different lectin also named artocarpin [20,21] and with the flavonoid artocarpin isolated from several *Artocarpus* species [22–25]. The carbohydrate-mediated biological effects of KM+ on animal cells include stimulation of mitosis in mouse spleen cells [16], macrophage activation [26] and haptotactic induction of neutrophil migration [27]. KM+ belongs to a small lectin group with a carbohydrate recognition pattern different from those of most mannose/glucose binding lectins, including Concanavalin A (ConA) and *Dioclea glandiflora* lectin [28,29]. KM+ has a carbohydrate specificity that allows the recognition of complex biantennary N-linked glycans, even when they contain a β2-substituted mannose motif that hinders recognition by other mannose/glucose lectins [17,18].

We show here the first screening and partial identification of adult rat cerebellar mannosyl-glycoconjugates recognized by KM+, using quantitative lectin-histochemistry and lectin-affinity blotting. Our results suggest that KM+ can be employed to detect a specific subset of mannose-containing glycoproteins, including Type-I laminin, in the CNS.

Materials and methods

Purification and biotinylation of KM+

A crude saline extract of *Artocarpus integrifolia* seeds was prepared as previously described [16]. The lectin KM+ was purified from this extract to apparent electrophoretic homogeneity by affinity chromatography using agarose-D-galactose and agarose-D-mannose columns (Pierce, USA). Purified KM+ was biotinylated using 6-((6-((biotinoyl)amino)hexanoyl)amino) hexanoic acid, sulfosuccinimidyl ester (biotin-XX SSE, Molecular Probes, USA). Briefly, 20 μl of biotin-XX (10 mg/ml) in dimethylsulfoxide were added to 0.5 mg KM+ in 200 μl 0.2 M sodium bicarbonate buffer, pH 8.3. The reaction was stopped after 1 h by adding 200 μl 2 M ethanolamine-HCl, pH 7.4, and incubating for 1 h at room temperature. The reaction mixture was dialyzed at 4°C overnight against 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4 (PBS) and stored at −70°C. Purified KM+ and biotinyl-KM+ were compared electrophoretically, showing homogeneity after purification and no evident changes in molecular mass after biotinylation.

Lectin-histochemistry

Adult male Wistar rats were anesthetized with pentobarbital (40 mg/kg ip), and perfusion-fixed through the left heart with 300 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 4% w/v paraformaldehyde and 0.1% w/v glutaraldehyde, at room temperature. The cerebellar *vermis* was dissected and immersion-fixed in the same fixative for 4 h. Fixed tissues were embedded in paraffin and cut in 5 μm sections. Sagital sections were mounted on gelatin-coated microscope slides and processed for histochemistry.

The detection of KM+ ligands was enhanced by treating dewaxed sections in 10 mM sodium citrate buffer, pH 6.0, with a microwave oven at 900 watt for 20 min [30]. Sections were rinsed and incubated for 30 min in 0.1 M Tris-Glycine, pH 7.4, followed by a 4-h incubation in blocking buffer (20 mM sodium phosphate buffer, pH 7.4, containing 0.45 M NaCl, 0.3% w/v Triton X-100 and 5% w/v bovine serum albumin). Endogenous biotin was blocked with the Biotin Blocking System (Dako) following the specifications of the manufacturer. Sections were subsequently incubated overnight with 0.8 μg/ml biotinyl-KM+ diluted in blocking buffer. After each incubation step, sections were washed with 20 mM sodium phosphate buffer, pH 7.4, containing 0.45 M NaCl and 0.3% w/v Triton X-100. Biotinyl-KM+ was detected using an alkaline phosphatase kit (Vectastain ABC kit, Vector) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate in the presence of 2 mM levamisole. All incubations were performed at room temperature. Stained sections were dehydrated, cleared and coverslipped with Permount (Fischer), and subsequently analysed using an Olympus BX60F3 microscope.

Quantification and inhibition of KM+ staining

KM+ staining intensity was estimated using the NIH Image (v.1.6) analysis system. Tissue images were collected by a high resolution CCD monochrome camera and digitized using a frame grabber. Tissue structures between the *intrapyramidal sulcus* 1 and the depth of the *fissura secunda* were outlined for quantification. The system automatically integrated the total optical density for areas of equal size between treatments. Illuminance was uniformly maintained and checked after every ten measurements using an optical density calibration tablet (Kodak). In all cases there was a linear relationship between gray value and alkaline phosphatase reaction time, ranging from 1 to 20 min, for all cerebellar layers.

Inhibition of KM+ binding was studied in the presence of several concentrations (from 1 μM to 0.8 M) of the following carbohydrates, dissolved in MilliQ water: man-α1,3-[man-α1,6]-man (mannotriose), methyl-α-D-mannopyranoside, D(+)mannose, D(+)glucose and D(+)galactose (all from Sigma). Horseradish peroxidase isozyme C (HRPc type VIa, Sigma) was also dissolved in water and added to KM+ at final concentrations ranging from 50 ng/ml (=1 nM) to 0.5 mg/ml (10 μM). HRPc contains eight N-linked sugar chains per molecule, of which six to seven (i.e., ~80%) correspond to a heptasaccharide with the following structure: α-Man-(1→6)[α-Man-(1→3)][β-Xyl-(1→2)]-β-Man-(1→4)-β-GlcNAc-(1→4) [α-Fuc(1→3)]-GlcNAc [31]. HRPc was used as a carrier of the inhibitory heptasaccharide since the isolated carbohydrate was not available. Concentration-dependent inhibitory curves were adjusted with a sigmoidal model and inhibition constants (IC_{50})

were calculated using the Prism program (v3.02, Graphpad Inc.).

Lectin-affinity bloting

Adult male Wistar rats selected for biochemical procedures were killed by decapitation in compliance with institutional guidelines. Cerebella were quickly dissected and homogenized on ice in 25 mM Tris-HCl buffer, pH 7.4, containing 0.32 M sucrose, 10 mM EDTA, 0.3 mM phenylmethane sulfonyl fluoride, 1 mM benzamidine and 0.3 μM aprotinin, using a Potter-Elvehjem homogeneizer at 1,000 rpm. Soluble and total particulate fractions were separated by centrifugation of the homogenate at 48,000 *g* for 2 h at 4°C. Samples were boiled with 1× gel loading buffer for 5 min and separated by SDS-PAGE in 6–15% gradient gels. Proteins were transferred to nitrocellulose membranes and non-specific binding was blocked using 50 mM TrisHCl buffer, 150 mM NaCl, pH 7.4, containing 0.2% w/v Tween 20 and 5% w/v bovine serum albumin (2 h at room temperature). KM+ ligands were detected using biotinyl-KM+ (0.08 μg/ml) followed by streptavidine-alkaline phosphatase. Biotinyl-ConA (0.04 μg/ml, Vector) was used in the same conditions as a "typical" manose-binding lectin for comparative detection of mannosyl glycoproteins.

To improve the detection of high Mw proteins, samples were fractionated in 3–10% gels and detection of biotinyl-KM+ was performed by chemiluminescence. Laminin bands were identified using a rabbit antibody produced against murine Engelbreth-Holmes-Swarm (EHS) sarcoma laminin (1 μg/ml, Sigma) and purified EHS-laminin (Type-I laminin [32], Sigma) as a positive control. Protein concentrations were determined according to Lowry's method using bovine serum albumin as standard.

Results

KM+ stained neurons from all layers of the cerebellar cortex, as shown in Figure 1A. The staining in the molecular and granular cell layers corresponded mostly to detection of glycoconjugates in the extracellular space and cell sufaces, while the cytoplasm of the cells was only moderately stained (Figure 1D and F). In contrast, Purkinje cells exhibited a strong cytoplasmatic decoration by KM+ (Figure 1A, D and E). This staining was predominantly punctate, suggesting that KM+ bound mostly to glycoprotein precursors in the endoplasmic reticulum of the Purkinje cells. Interestingly, we never observed KM+ staining the neuronal processes of any cell type (Figure 1A and 1C–F). Regarding non-neuronal components, cells from the ependymal lining and the choroid plexus were also very strongly stained (Figure 1G), as well as the blood vessels of the choroid plexus (Figure 1G). In stark contrast, the blood brain barrier capillaries within the cerebellar parenchyma were never decorated by KM+ (Figure 1A).

In agreement with the carbohydrate specificity reported for KM+, D(+) mannose (0.4 M) abolished or strongly reduced KM+ staining of all cerebellar cortical layers (Figure 1C). Mannosyl-glycans and the mannose-containing glycoprotein HRPc also inhibited the binding of KM+ to cerebellar sections, as indicated in the Table 1. The rank of inhibitory potency was the same for all cerebellar layers: HRPc $\gg$ man-α1,3-[man-α1,6]-man $>$ methyl-α-D-mannopyranoside $>$D(+) mannose ($\geq$D(+)glucose, not shown). As expected, D(+) galactose (0.4 M) did not affect KM+ staining (not shown). It is important to note that the extremely high inhibitory potency of HRPc on KM+ staining cannot be directly compared to the inhibition by the other glycans since it is likely due to additional effects of the core protein. HRPc carries eight oligosaccharide chains, of which at least six correspond to a specific heptasaccharide

Table 1. Mannosyl-glycoconjugates inhibit KM+ staining of cerebellar cortical layers

Inhibitor	*ML*	*PL*	*GL*	*WM*
IC_{50} (M):				
HRPc ($Man_3(Xyl)GlNac_2F$)	9.9×10^{-8}	6.8×10^{-8}	9.5×10^{-8}	6.0×10^{-8}
Man-α1,3-[man-α1,6]-man	9.3×10^{-5}	9.4×10^{-5}	3.2×10^{-4}	2.2×10^{-4}
Methyl-α-D-mannopyranoside	1.2×10^{-2}	1.2×10^{-2}	2.6×10^{-2}	2.5×10^{-2}
D(+)mannose	2.4×10^{-2}	3.0×10^{-2}	7.0×10^{-2}	3.3×10^{-2}
Inhibitory potency (D(+)mannose = 1):				
HRPc ($Man_3(Xyl)GlNac_2F$)	~37200	~67800	~113000	~84600
Man-α1,3-[man-α1,6]-man	258.1	319.1	218.8	150.0
Methyl-α-D-mannopyranoside	2.0	2.5	2.7	1.3
D(+)mannose	1	1	1	1

Biotinyl-KM+ was preincubated with the carbohydrates or HRPc for 1 h before adding to the tissue sections. Quantification of tissue staining inhibition was performed as indicated in the methods section. Results in the table are average values from three independent experiments performed by quadruplicate, with less than 10% SD. Inhibitory potencies are indicated relative to the IC_{50} for D(+)mannose. Abbreviations: ML, molecular layer; PL, Purkinje cell layer; GL, granule cell layer; WM, white matter. Molar IC_{50} values for HRPc were calculated based in a Mw = 44,000 Da for carbohydrate-containing HRPc. The inhibitory potency for this glycoconjugate is an estimate value based on a stoichiometry of ~6.5 mols heptasaccharide/mol HRPc.

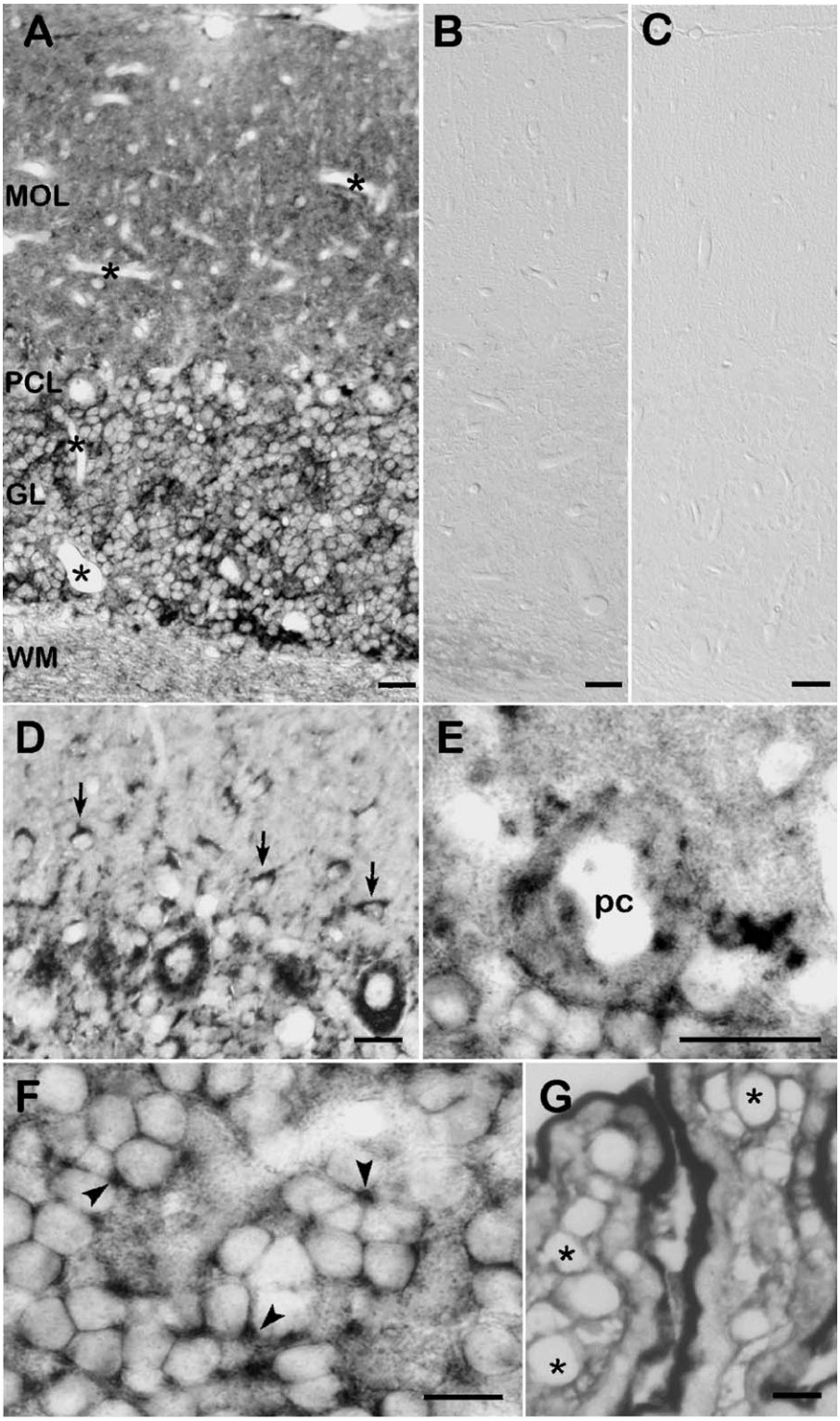

Figure 1. KM+stains most cellular types of the rat cerebellar cortex and choroid plexus. Cortical cerebellar layers were stained in the presence (A) or absence (B) of biotinyl-KM+ as described in the methods section. Staining was abolished by 0.4 M D(+)mannose (C). Higher magnification of the molecular layer (D) shows that interneurons (arrows) close to the Purkinje cells were the most strongly stained cells in this layer. The bodies of Purkinje cells exhibited a strong and largely punctate staining (E), whereas granule cells presented a well-stained rim (F). Arrowheads in (F) point to the accumulation of extracellular staining in the granular layer. KM+ also stained the blood vessels (asterisks) and epithelial cells in the choroid plexus (G), but not the blood vessels in the cerebellar cortex (asterisks in A). Panels B and C, Nomarski optics. Bar size is 10 μm for E and 20 μm for all other panels.

recognized by KM+. This adds cooperative effects and steric hindrance by the HRPc core protein to the direct sugar-mediated inhibition of KM+ staining. However, the observed result is not unexpected since the isolated HRPc heptasaccharide has an inhibitory potency on KM+ binding that is several orders of magnitude higher than that of D(+)manose [17], thus indicating the complexity of the carbohydrate motif preferentially recognized by KM+.

Cerebellar glycoproteins were screened with KM+ using a lectin-affinity blotting technique (Figure 2A). KM+ detected three major bands with apparent Mw of 132, 83 and 49 kDa, a weaker band at ~220 kDa and a few less conspicuous bands. All the major bands failed to be detected after preincubation of KM+ with 0.4 M mannose, but not with 0.4 M galactose (data not shown), indicating that the detection of these proteins was mediated by the carbohydrate recognition domain of KM+. The 132-kDa band predominated as a soluble protein and the 83 kDa band as a membrane-associated protein, although they were observed in both subcellular fractions. In contrast, the 49-kDa band was only detected in the particulate fraction. Proteins from total cerebellar homogenates were probed for comparison with the typical mannose-binding lectin ConA, resulting in the detection of several more mannosyl-glycoproteins than with KM+ (Figure 2B). However, three of the detected bands migrated at positions indistinguishable from the major bands recognized by KM+, suggesting that they may be the same mannosyl-glycoconjugates recognized by both lectins.

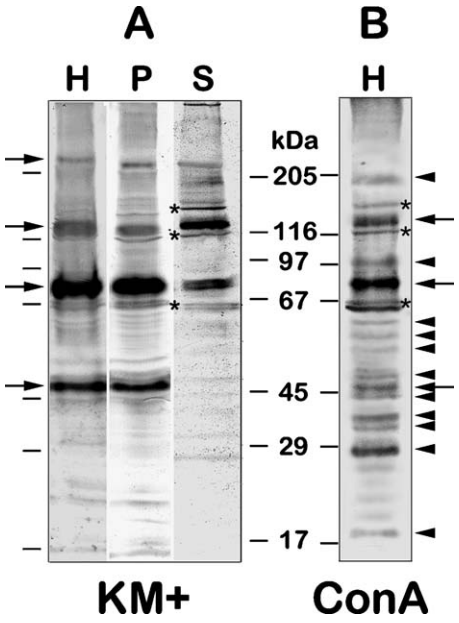

Figure 2. KM+ recognizes a specific subset of cerebellar glycoproteins. (A) Samples of total homogenate (*H*), particulate (*P*) and soluble (*S*) fractions of adult rat cerebellum (20 μg total protein) were fractionated in 6–15% acrylamide gels and probed with biotinyl-KM+. Arrows indicate specific 220-, 132-, 83- and 49-kDa bands detected by KM+. (B) Total homogenate was fractionated as in (A) and probed with biotinyl-ConA. ConA recognized a large number of glycoproteins (arrowheads). Three of the observed bands (132-, 83- and 49-kDa) migrated at positions indistinguishable from the bands previously detected by KM+ (arrows). Asterisks indicate non-specific bands observed in the absence of the lectins.

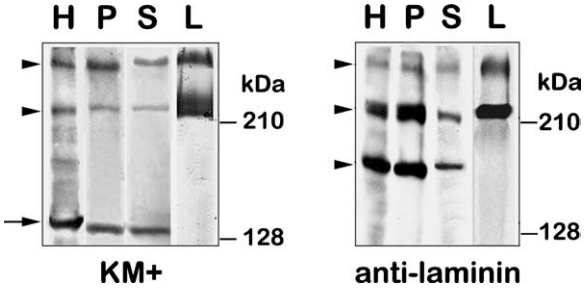

Figure 3. KM+ binds Type-I laminin-like proteins in the adult rat cerebellum. Total homogenate (H), particulate (*P*) and soluble (*S*) fractions of adult rat cerebellum (20 μg total protein), as well as purified Type-I laminin (*L*, 1 μg) were fractionated in 3–10% acrylamide gels and probed with biotinyl-KM+ (*KM+*) or with a rabbit antibody against Type-I laminin (*anti-laminin*). KM+ recognized the heavy and light chains of purified laminin as well as two high Mw cerebellar proteins of ~440 and ~220 kDa that co-migrated at positions indistinguishable from the laminin chains. These same bands were also detected by the anti-laminin antibody. Arrowheads indicate specifically detected bands at ~440, ~220 and ~160 kDa, the latter not detected by KM+. The arrow indicates the 132-kDa band previously observed in Figure 2A.

Previous results suggest that KM+ may bind to Type-I laminin in capillaries from non-neural tissues (LG and ARM, unpublished data). Here, we observed that KM+ recognized a ~220-kDa protein, with Mw similar to that of Type-I laminin light chains. Using an acrylamide gradient improved for electrophoretic resolution of high Mw proteins, we were able to show that KM+ recognized both the heavy (alpha, ~440 kDa) and light (beta/gamma, ~220 kDa) chains of purified Type-I laminin (Figure 3, KM+). In addition, lectin-blotting of cerebellar fractions showed that KM+ recognized two bands migrating at positions indistinguishable from the heavy and light chains of Type-I laminin. Western blotting of the same fractions (Figure 3, *anti-laminin*) showed that those bands corresponded to Type-I laminin chains, thus confirming that KM+ detects Type-I-like laminin in the cerebellar tissue. However, a cerebellar 160-kDa band, likely corresponding to a laminin cleavage product recognized by the anti-laminin antibody, failed to be recognized by KM+.

Discussion

KM+ is a novel D(+)mannose-specific plant lectin that binds cell-surface glycoconjugates in several mammalian cell types, thus being useful to promote biological effects such as lymphocyte mitosis, neutrophil migration and macrophage activation [16,26,27]. Here, we sought to extend the use of this lectin as

a probe to detect and characterize glycoconjugates from the central nervous system.

KM+ exhibits a widespread staining of all layers of the cerebellar *vermis*, with a particularly strong decoration of Purkinje cell and granule cell bodies (Figure 1A–F). Remarkably, however, the cellular processes were never found to be stained, thus suggesting that the glycoconjugates detected by KM+ may be spatially restricted to the cell soma or, alternatively, blocked from recognition in the cell processes.

The staining of all the cerebellar layers and structures by KM+ was consistenly inhibited by D(+)mannose, but not by D(+)galactose, in agreement with the properties of the carbohydrate recognition domain of this lectin [19]. More complex glycans, including the oligosaccharide mannotriose and the mannosyl-heptasaccharide carried by the glycoprotein HRPc, inhibited KM+ tissue staining with much higher affinity than D(+)mannose (Table 1). This inhibition ranking was similar to that previously estimated for this lectin by solid-phase binding assays [17]. KM+ has been demonstrated to recognize biantennary N-linked glycans by binding to their mannotriose core motif [18,19]. Interestingly, KM+ exhibits increased binding affinity to these glycans when the central mannose of the mannotriose core has an additional glycosydic linkage [17,19,28,29]. In particular, KM+ binds with high affinity to biantennary β2-xylosyl-substituted mannosyl-glycoconjugates, thus behaving very differently from "typical" mannose/glucose-specific lectins that are hindered by this substitution. Although the glycoconjugates detected in the cerebellum by KM+ cannot be core β2-xylosylated due to the lack of the corresponding glycosyltransferase in mammals [3], the unique binding ability of this lectin suggests that it may be a useful probe to detect similar unusually substituted mannosyl-glycoconjugates. The results from our inhibition studies as well as the restricted subset of glycoproteins detected by KM+ (see below) strongly suggest that the carbohydrates bound by this lectin may be a subset of N-linked glycans of the complex type.

In addition to the staining of neuronal structures, KM+ strongly decorated ependymal cells as well as blood/cerebrospinal fluid brain barrier capillaries in the choroid plexus. However, surprisinghly, blood brain barrier capillaries in the cerebellar cortex remained unstained (Figure 1A and G). It is known that blood brain barrier capillaries in the parenchyma of the CNS differ from blood vessels of other tissues in that they contain several laminin types that undergo functional cleavage [33,34]. In particular, endothelial cells have been demonstrated to stimulate astrocytary secretion of Type-V laminin followed by cleavage to lower Mw laminin chains that are deposited in the extracellular matrix [34]. In addition, Type-I laminin-like immunoreactive proteins with Mw 120–150 kDa have been previously detected in the rat cerebellum [35] and likely correspond to cleavage products of the beta or gamma ($\sim$220 kDa) laminin chains. Here we have shown that KM+ can bind Type-I laminin like-proteins in the cerebellum, but fails to recognize a $\sim$160 kDa band that may be one of the cleavage products of Type-I laminin chains (Figure 3). Since laminins are deposited around all blood vessels in the CNS, KM+ should be expected to decorate the capillaries within the cerebellar parenchyma by binding to Type-I laminin. Therefore, the absence of KM+ staining of cerebellar blood vessels suggests that Type-I laminin deposited around these capillaries could be differentially glycosylated in a manner that prevents it from being recognized by the lectin. Alternatively, the deposited Type-I laminin surrounding these capillaries could be mostly in the cleaved form undetectable by KM+.

In addition to the laminin-like protein bands, our results disclosed three other major mannose-containing glycoproteins detected by KM+ in the cerebellum, with Mw 132, 83 and 49 kDa. We also observed three bands of the same Mw by lectin-blotting using the typical mannose-specific lectin ConA (Figure 2B). However, ConA detects not only these three proteins but many other protein bands separated by SDS-PAGE, in agreement with previous literature showing ConA-binding glycoproteins in the forebrain and cerebellum [36,37]. This difference between the extensive glycoprotein detection by ConA and the restricted subset detected by KM+ suggests that KM+ binds glycoconjugates containing a complex mannose-bearing core motif that is preferentially recognized by this lectin. KM+ may thus be a specific screening probe for this subset of glycoproteins once their identity can be determined. Although we have no suitable identity yet for these three main KM+ cerebellar glycoligands, the use of purified KM+ may provide in a near future a useful tool for the specific purification of these glycoproteins by lectin-affinity chromatography.

In sum, we show here for the first time the efficient use of the lectin KM+ in combination with histochemistry and protein blotting to study the expression and distribution of its glycoligands in the CNS. The unique carbohydrate specificity of KM+, its differential recognition of cerebellar structures and its clear detection of major glycoprotein ligands encourage us to use this lectin as a novel probe to screen the CNS for subsets of complex mannosyl-glycoconjugates.

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