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ORIGINAL PAPER

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Application of a lectin from the mushroom *Polysporus squamosus* for the histochemical detection of the NeuAcα2,6Galβ1,4Glc/GlcNAc sequence of *N*-linked oligosaccharides: a comparison with the *Sambucus nigra* lectin

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Abstract The lectin from the mushroom *Polysporus squamosus* (PSL) has an extended carbohydrate combining site, which exhibits a high specificity and affinity toward the NeuAc5α2,6Galβ1,4Glc/GlcNAc trisaccharide sequence of asparagine-linked oligosaccharides. Therefore, PSL should be a superior reagent to the lectin from *Sambucus nigra* (SNA), which does not discriminate between α2,6-linked NeuAc5 present either in asparagine- or serine/threonine-linked oligosaccharides. We have prepared a digoxigenin-conjugated PSL and applied it for histochemistry and blotting. We observed a more restricted staining pattern by PSL as compared to SNA in paraffin sections from different rat organs. Pretreatment of sections with *N*-glycanase F abolished PSL staining indicating that it interacts only with asparagine-linked oligosaccharides. Furthermore, PSL staining was neuraminidase sensitive. In contrast, SNA staining was only partially sensitive to *N*-glycanase F pretreatment demonstrating that it was in part due to α2,6-linked NeuAc5 present in serine/threonine-linked oligosaccharides. The most striking observation in this regard was that PSL, in contrast to SNA, did not stain the mucus of sheep submandibular gland, which is extremely rich in serine/threonine-linked Neu5Acα2,6*N*-acetylgalactosamine. Furthermore, in some tissues neuraminidase pretreatment resulted in increased intensity of SNA staining probably due to binding to exposed terminal *N*-acetylgalactosamine residues. Collectively, these results indicate that PSL is a useful tool for the histochemical detection of α2,6-linked NeuAc5 in asparagine-linked oligosaccharides.

Keywords *Polysporus squamosus* lectin · *Sambucus nigra* agglutinin · *N*-glycans · Histochemistry

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Introduction

Sialic acids occur widely in nature as constituents of oligosaccharide side chains of glycoproteins and glycolipids and many of their functions have emerged during the last decade (Rosenberg 1995; Roth et al. 1993; Varki 1997). Several studies have focused on the cell type-specific expression of sialoglycoconjugates during tissue development and cell differentiation, their distribution pattern in adult tissues, and their expression patterns in malignant tumors (Dennis et al. 1982; Itzkowitz et al. 1989, 1990; Kaneko et al. 1995; Krishna and Varki 1997; Landmesser et al. 1990; Lee et al. 1989; Roth 1993; Roth et al. 1987, 1988, 1994; Yang and Rutishauser 1992). In these histochemical investigations, sialic acid reactive lectins have served as invaluable tools.

Two lectins with broad reactivity towards sialic acids, the *Limulus polyphemus* lectin (Marchalonis and Edelman 1968; Muresan et al. 1982) and the *Limax flavus* lectin (Knibbs et al. 1993; Miller et al. 1982), have been employed in histochemistry (Muresan et al. 1982; Roth et al. 1984, 1985; Schulte et al. 1984; Taatjes and Roth 1991). Since sialic acids occur in specific glycosidic linkages to the penultimate sugar at the non-reducing terminus of oligosaccharide side chains, an important advance for the field was accomplished with the isolation and characterization of lectins with high specificity for sialic acids present in a particular glycosidic linkage. The first reported linkage-specific lectin was the one isolated from Elderberry bark, the *Sambucus nigra* L. lectin (SNA), recognizing *N*-acetylneuraminic

acid (NeuAc5) in α2,6 linkage to galactose or *N*-acetylgalactosamine (Shibuya et al. 1987a, b). Another lectin with specificity toward α2,6-linked NeuAc5 was isolated from the tuberous roots of *Trichosanthes japonica* (Yamashita et al. 1992). To date, two lectins with high specificity to NeuAc5 in α2,3 linkage have been reported, the leukoagglutinin from *Maackia amurensis* (Knibbs et al. 1991; Wang and Cummings 1988) and a lectin from the seeds of *Amaranthus caudatus* (Rinderle et al. 1989, 1990). The suitability of all these lectins recognizing NeuAc5 in a specific glycosidic linkage for the histochemical detection of the respective binding sites in tissues was subsequently demonstrated (Sata et al. 1989, 1990a, b; Taatjes et al. 1988; Yamashita et al. 1995). Noteworthy, all these lectins proved to be of practical importance in cancer research (Benallal et al. 1995; Boland et al. 1991; Lemarer and Stehelin 1995; Murayama et al. 1997; Pousset et al. 1997; Sata et al. 1991, 1992; Toma et al. 1999, 2000; Vierbuchen et al. 1995; Yamashita et al. 1995).

Recently, a lectin has been purified from the mushroom *Polysporus squamosus* (PSL) that has an extended carbohydrate combining site which exhibits a very high specificity and affinity toward the NeuAc5α2,6Galβ1,4Glc/GlcNAc trisaccharide sequence present in asparagine-linked oligosaccharides (Mo et al. 2000). This lectin appears to be a superior probe for the detection of α2,6-linked NeuAc5 at the non-reducing terminus of asparagine-linked oligosaccharides when compared to SNA. In our earlier histochemical studies, we obtained evidence that SNA recognizes not only α2,6-linked NeuAc5 present on asparagine-linked oligosaccharides but also additionally those present on serine/threonine-linked oligosaccharides of sheep and bovine submaxillary glands and colonic goblet cells. Furthermore, after neuraminidase treatment, SNA reacted with exposed *N*-acetylgalactosamine residues *O*-glycosidically linked to serine or threonine in sheep submandibular gland (Sata et al. 1989; Taatjes et al. 1988). In contrast to SNA, the PSL did not react in vitro with purified ovine submaxillary mucin containing high amounts of the *O*-glycosidically linked NeuAc5α2,6*N*-acetylgalactosamine disaccharide (Mo et al. 2000). With these features in mind, we have tested the PSL for its suitability as a histochemical reagent and demonstrate its superiority over SNA for the histochemical detection of α2,6-linked NeuAc5 residues in asparagine-linked oligosaccharides.

Materials and methods

Reagents

PSL was affinity purified as described (Mo et al. 2000). Lectin–digoxigenin conjugates were prepared using a digoxigenin conjugation kit from Roche Molecular Biochemicals (Mannheim, Germany) according to the manufacturer's instructions. Horseradish peroxidase-conjugated sheep anti-digoxigenin antibodies (Fab fragments), SNA–digoxigenin conjugate, neuraminidase from *Vibrio cholerae*, and *N*-glycanase F were also from Roche Molecular Biochemicals, and a 4 nm gold-labeled rabbit anti-horserad-

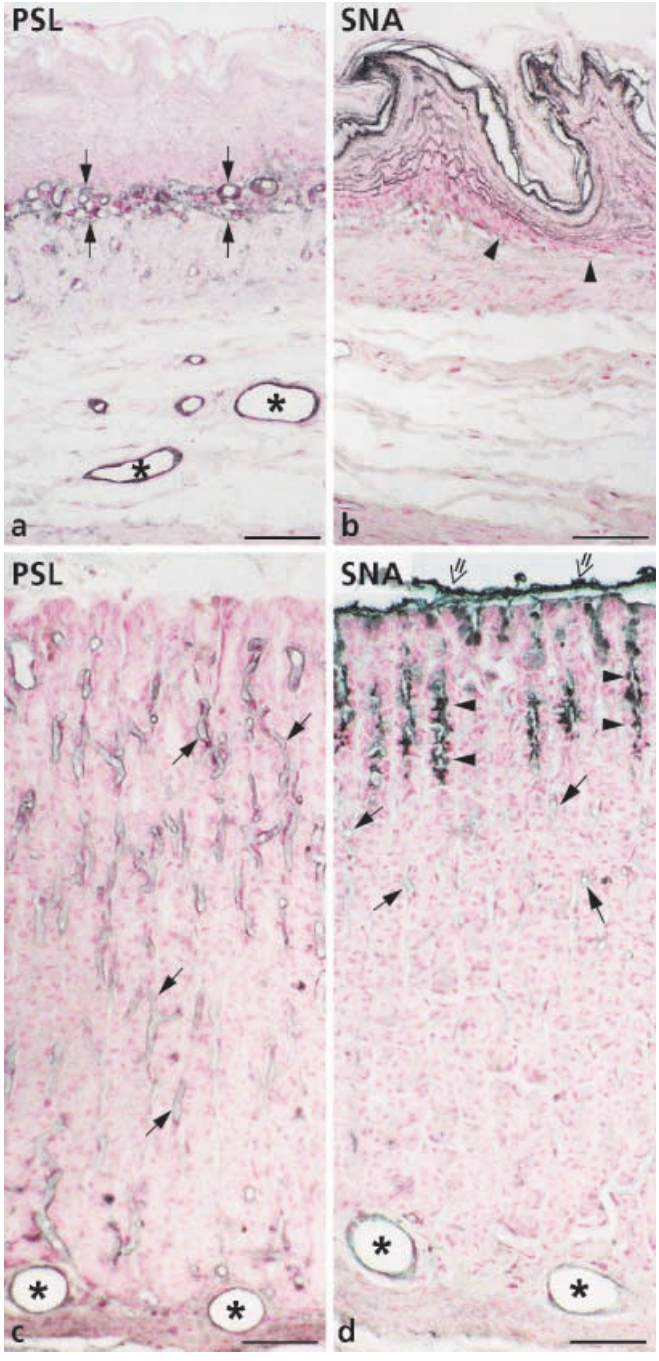

Fig. 1a–d Rat stomach. Silver intensified lectin labeling in this and all subsequent figures appears in *black*; fast nuclear red counterstaining. **a** *Polysporus squamosus* lectin (PSL) staining in the forestomach is restricted to endothelia of capillaries adjacent to the squamous mucosa (*arrows*) and larger blood vessels (*asterisks*). **b** However, *Sambucus nigra* agglutinin (SNA) staining is additionally observed in the stratum spinosum and granulosum with the stratum basale being unstained (*arrowheads*). **c** PSL staining of the glandular stomach is restricted to endothelia (*arrows*). **d** However, SNA stains the mucous layer (*open arrows*) and mucus-producing cells (*arrowheads*) intensely, but capillaries only faintly (*arrows*). *Bars* 36 µm (**a**), 33 µm (**b**), 40 µm (**c**, **d**)

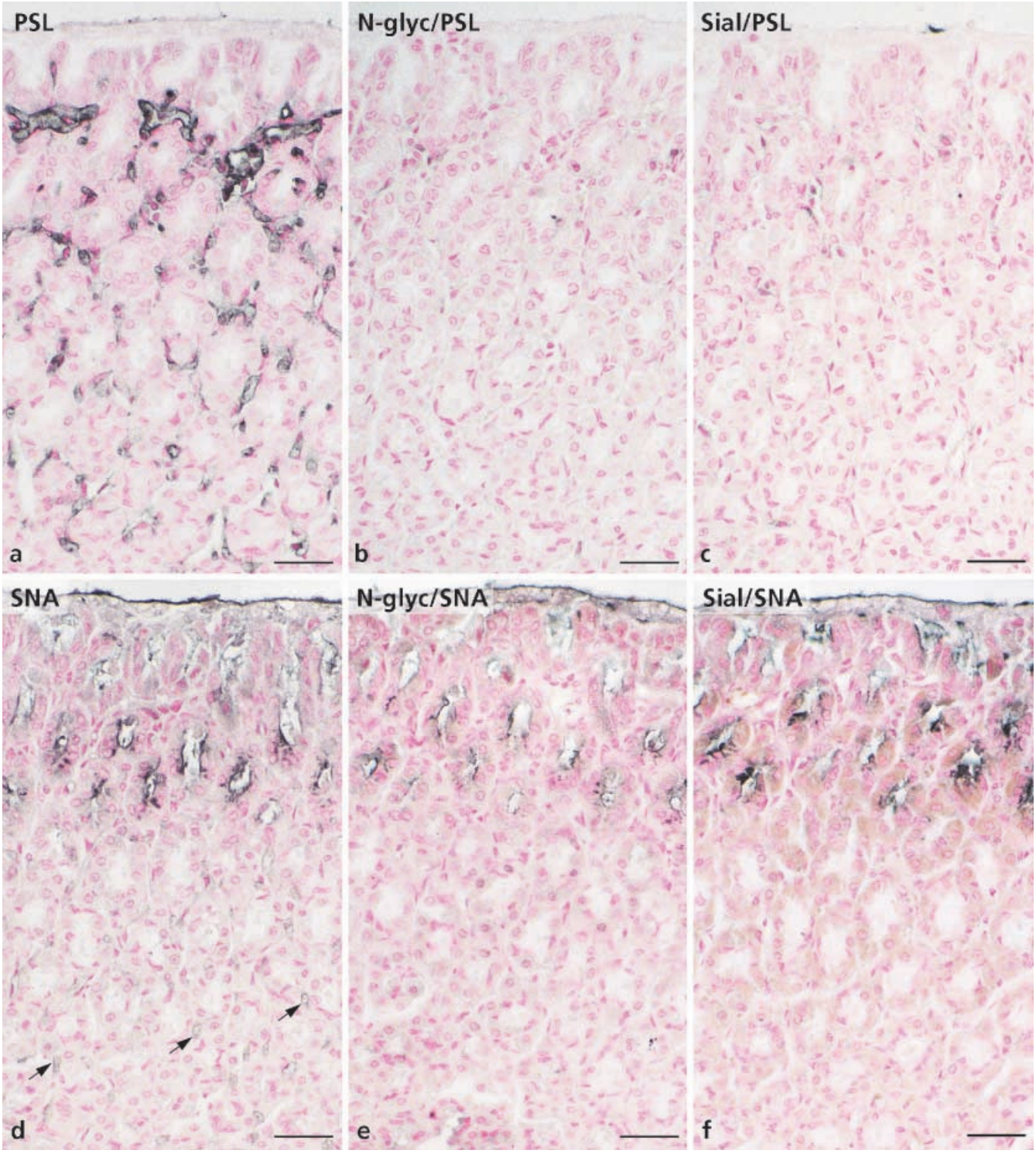

Fig. 2 Detail of PSL (**a**) and SNA (**d**) staining of glandular stomach. PSL reactivity becomes undetectable following section pretreatment with *N*-glycanase F (**b**) and sialidase (**c**). Although SNA staining of endothelia is abrogated following pretreatment with *N*-glycanase F (**e**) and sialidase (**f**), the mucous layer and mucus-producing cells are still positive. *Arrows* in (**d**) point to positive capillary endothelia. *Bars* 26 µm

ish peroxidase antibody from Immunoresearch Laboratories (West Grove, Pa., USA). All other reagents were of analytical grade.

Tissue preparation

Sprague-Dawley rats were obtained from the Central Animal Facility of the University of Zurich. Rat tissues were fixed by vascular fixation or immersion in freshly prepared 3% formaldehyde–0.1% glutaraldehyde or 4% formaldehyde in Hank's balanced salt solu-

tion (pH 7.4) for 2–4 h at ambient temperature. Sheep submandibular gland was immersion-fixed in freshly prepared 4% formaldehyde in Hank's balanced salt solution (pH 7.4) for 2 h. Free aldehyde groups were blocked by immersing the tissues in 50 mM NH_4Cl in PBS (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4) for 1 h. Tissues were embedded in paraffin according to standard protocol and sections of 5 µm thickness were prepared. Low temperature Lowicryl K4M-embedded sheep submandibular gland was available from previous studies (Taatjes et al. 1988).

Lectin histochemistry

A three-step labeling protocol for lectin histochemistry followed by photochemical silver amplification was performed as described in detail previously (Roth et al. 1998). In brief, deparaffinized and rehydrated tissue sections were incubated with digoxigenin-conjugated PSL (1.5 µg/ml) or SNA (1.25 µg/ml) in PBS (pH 7.4) containing 1% BSA, 0.05% Tween 20, and 0.005% Triton X-100 for 2 h at ambient temperature, rinsed in buffer, and incubated with horseradish peroxidase-conjugated sheep anti-digoxigenin Fab (0.3 U/ml) for 1 h. After rinses with buffer, sections were incubated with 4 nm gold-labeled anti-horseradish peroxidase antibody (Roth et al. 1992) adjusted to an absorbance of 0.05 at 525 nm. Finally, silver intensification using silver acetate (Roth et al. 1998) of the lectin–gold labeling was performed followed by counterstaining with nuclear fast red.

In control incubations, PSL was incubated with varying amounts (0.1 mM–0.2 M) of either 6′ or 3′ sialyllactose for 30 min prior to use to stain sections from kidney.

To enzymatically remove oligosaccharide side chains at the asparagine-*N*-acetylglucosamine core linkage, sections were treated with 0.1% SDS in TRIS-buffered saline at 70°C for 30 min and washed in NP40 in TRIS-buffered saline. This was followed by *N*-glycanase F (80 U/ml in 0.1 M phosphate buffer, pH 7.4) in a moist chamber for 18 h at 37°C. Afterwards, tissue sections were conditioned with PBS containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20 for 20 min at ambient temperature and incubated with the lectins as described above. To enzymatically remove sialic acids, sections were treated with *Vibrio cholerae* neuraminidase (0.02 U/ml in 50 mM acetate buffer, pH 5.5) for 18 h at 37°C in a moist chamber, washed, conditioned, and incubated with PSL or SNA as described above. To assess the effect of the incubation buffers on the lectin staining, sections were incubated in the respective enzyme buffers only for 18 h at 37°C before lectin labeling.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and lectin blotting

Rat liver, kidney, and mucosal scrapings from stomach (forestomach and glandular stomach) as well as small and large intestine were homogenized in PBS (pH 7.4) containing protease inhibitor tablets, and proteins were extracted with 1% Triton X-100 for 1 h at 4°C. The homogenates were centrifuged at 100,000 *g* for 1 h at 4°C and aliquots of the supernatants were used for SDS-PAGE. Lectin blotting was performed as described in detail previously (Zuber et al. 1998). In brief, proteins (100 µg/lane) were denatured in Laemmli buffer and then separated by 3–10% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with digoxigenin-conjugated PSL (1.5 µg/ml PBS containing 0.05% Tween 20 and 1% defatted milk) or SNA (2.5 µg/ml 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl containing 0.05% Tween 20 and 1% defatted milk) for 2 h, washed, and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (0.25 U/ml TRIS-buffered saline containing Tween 20 and defatted milk) for 1.5 h. Furthermore, nitrocellulose membranes were incubated with *N*-glycanase F (5 U/ml) or *Vibrio cholerae* neuraminidase (0.5 U/ml) for 18 h at 37°C prior to incubation with the lectins.

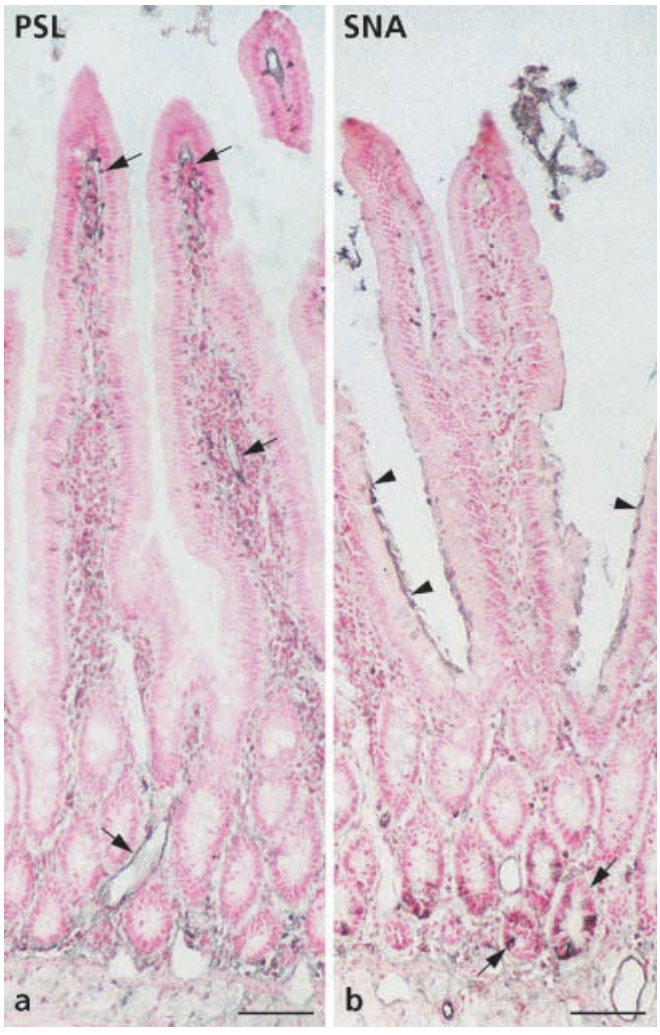

Fig. 3a, b Rat jejunum. **a** PSL stains endothelia of capillaries and various cell types present in the mucosal stroma (*arrows*), but not the villus and crypt epithelium. **b** SNA stains the surface of epithelia present in the base of the crypts (*arrows*) and the mucous layer (*arrowheads*) as well as endothelia, albeit only weakly. *Bars* 50 µm

Results

We have covalently modified the PSL with digoxigenin (Sata et al. 1990b) for application in lectin blotting and histochemistry in conjunction with anti-digoxigenin antibodies. As observed for a spectrum of other lectins (Sata et al. 1990b), the digoxigenin-conjugated PSL retained its activity for lectin blotting and histochemistry performed on formaldehyde-fixed and paraffin-embedded tissues. Since biochemical analyses strongly indicated a higher specificity of the PSL for NeuAc5 in α2,6 linkage in asparagine-linked oligosaccharides as compared to the SNA (Mo et al. 2000), a comparative analysis was performed.

When the PSL staining patterns in tissue sections was compared to that observed with SNA, certain tissues ex-

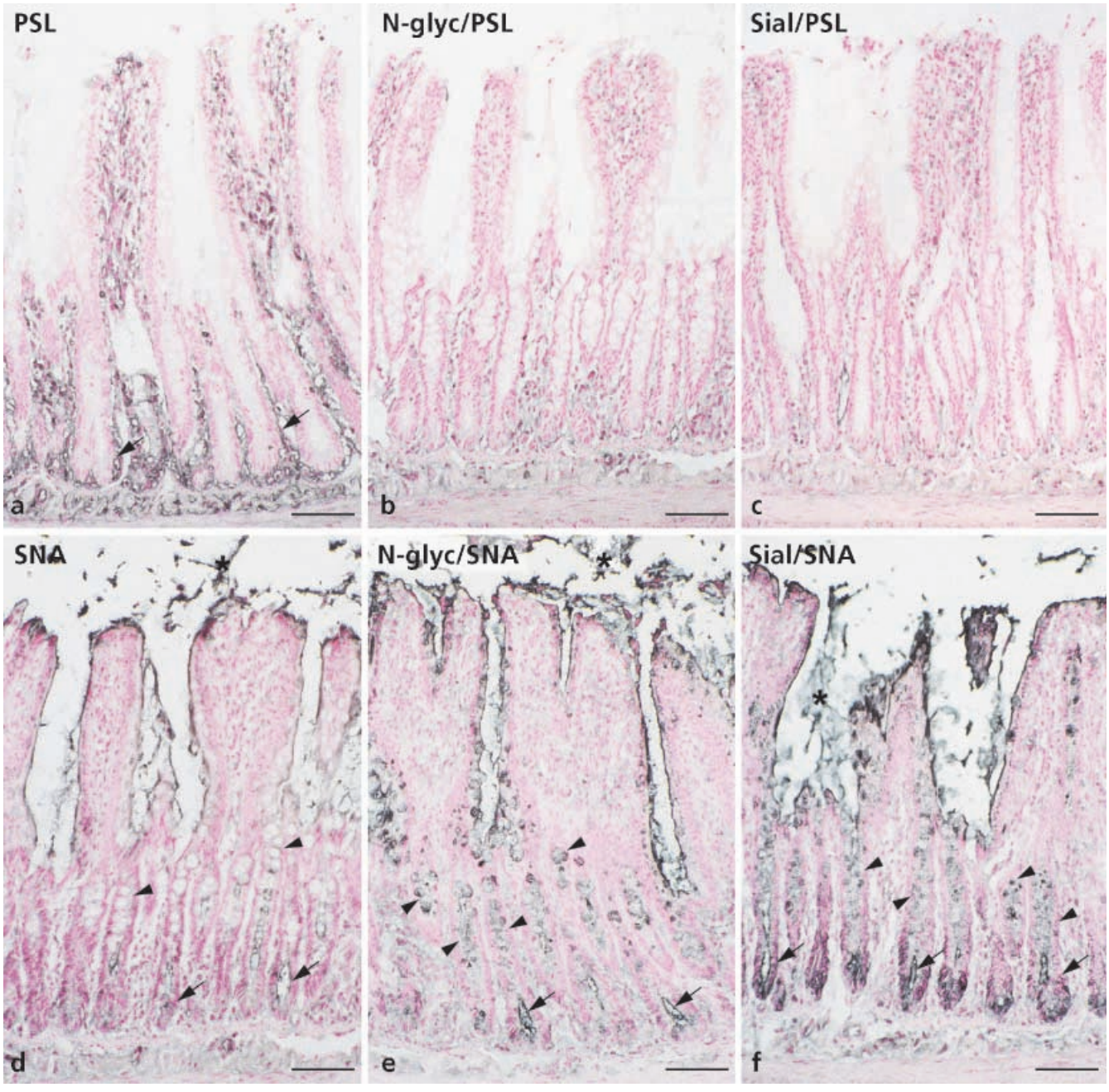

Fig. 4a–f Rat colon. **a** PSL stains the stroma and submucosa, and capillaries (*arrows*) intensely, but not the surface and crypt epithelium. This staining is greatly reduced following pretreatment of sections with *N*-glycanase F (**b**) and sialidase (**c**). **d** SNA predominantly stains crypt (*arrows*) and surface epithelium as well as the mucous layer. Goblet cells are irregularly and only faintly reactive (*arrowheads* in **d**). Although staining of endothelia is abolished following *N*-glycanase F (**e**) and sialidase (**f**) pretreatment, staining of crypt epithelium (*arrows* in **e** and **f**) and goblet cell mucus (*arrowheads* in **e** and **f**) is enhanced and the mucous layer remains positive. *Bars* 45 µm

hibited significantly different labeling patterns at the cellular and subcellular levels. Preincubation of PSL and SNA with 0.1 mM 6′sialyllactose abolished all lectin staining whereas 3′sialyllactose had no recognizable effect up to 0.2 M. In order to clarify whether the staining of the tissue structures was due to NeuAc5α2,6Galβ1,4GlcNAc structures in asparagine-linked oligosaccharides, sections were pretreated with *N*-glycanase F. Lectin staining not affected by this pretreatment should represent bona fide reactivity with α2,6-linked NeuAc5 in serine/threonine-linked oligosaccharides or even terminal *N*-acetylgalactosamine residues in serine/threonine-linked oligosaccharides. Furthermore, neuraminidase pretreatment of the tissue sections was performed to analyze the importance of NeuAc5 on lectin staining. In all studied tissues, pretreatment of the sections with either of the enzyme incubation buffers alone did not affect the lectin staining (not shown). Among the tissues studied, rat kidney, stomach, small

and large intestine, and sheep submandibular gland exhibited different PSL and SNA staining patterns, as described in detail below.

In the cutaneous (non-glandular) area of the rat stomach, no PSL staining of the cornified squamous epithelium was observed (Fig. 1a), whereas SNA stained the stratum spinosum and granulosum (Fig. 1b). The endothelia of all blood vessels of the forestomach wall were reactive with both lectins although the staining was consistently stronger with PSL (compare Fig. 1a and b). Section pretreatment with *N*-glycanase F or neuraminidase abrogated staining with PSL and the staining of the endothelia by SNA (not shown). However, SNA staining of the squamous epithelium was not affected either by *N*-glycanase F or by neuraminidase. Neuraminidase pretreatment also abolished all staining by PSL (not shown) but not that by SNA of the squamous epithelium. Both lectins stained the endothelia of all types of blood vessels of the fundic wall of the stomach (Figs. 1c, d, 2a, d), the staining by PSL being stronger. The entire epithelium of the glandular mucosa in the fundic part of the stomach was unreactive with PSL (Figs. 1c, 2a), whereas mucus-producing cells of the gastric pits and the mucous layer covering the surface of the mucosa were reactive with SNA (Figs. 1d, 2d). Pretreatment with *N*-glycanase F or neuraminidase had no effect on SNA staining of the mucus although it eradicated the staining by both SNA and PSL of the endothelia of all blood vessels (Fig. 2b, c, e, f). Neuraminidase pretreatment abolished all PSL staining (Fig. 2c) Differences in the staining pattern of PSL and SNA and its sensitivity toward *N*-glycanase F section pretreatment were also noticed in the jejunum. Figure 3a illustrates PSL staining of the endothelia of blood vessels of the jejunum wall and of the crypt and villus stroma, and absence of staining in the epithelium covering the crypts and villi. In contrast, the epithelium in the base of the crypts and along the villi was stained with SNA (Fig. 3b), as were the endothelia and the stromal elements, albeit less intense than with PSL. The SNA staining in the crypts and the villi was not affected by *N*-glycanase F pretreatment, although the staining by SNA and PSL of endothelia and the cells present in the stroma was abrogated (not shown). Similar findings were made for the colon since the epithelium of the crypts and the surface was PSL unreactive and the endothelia of blood vessels positive (Fig. 4a). In striking contrast, the epithelium of the crypts and the surface was SNA positive which included the mucus of the goblet cells to a varying degree (Fig. 4d). PSL staining was sensitive to *N*-glycanase F and neuraminidase pretreatment (Fig. 4b, c). This was not the case for SNA staining of the colonic epithelium and the goblet cell mucus. Actually, the intensity of mucus of goblet cell staining increased following *N*-glycanase F and neuraminidase pretreatment (Fig. 4e, f). The most obvious differences were observed in sections from sheep submandibular gland. PSL did not react with the mucus of the acinar cells (Fig. 5a, c), whereas SNA did (Fig. 5b, d), confirming previously reported SNA staining of semithin sections of Lowicryl K4M-embedded sheep submandibular gland (Taatjes et al. 1988).

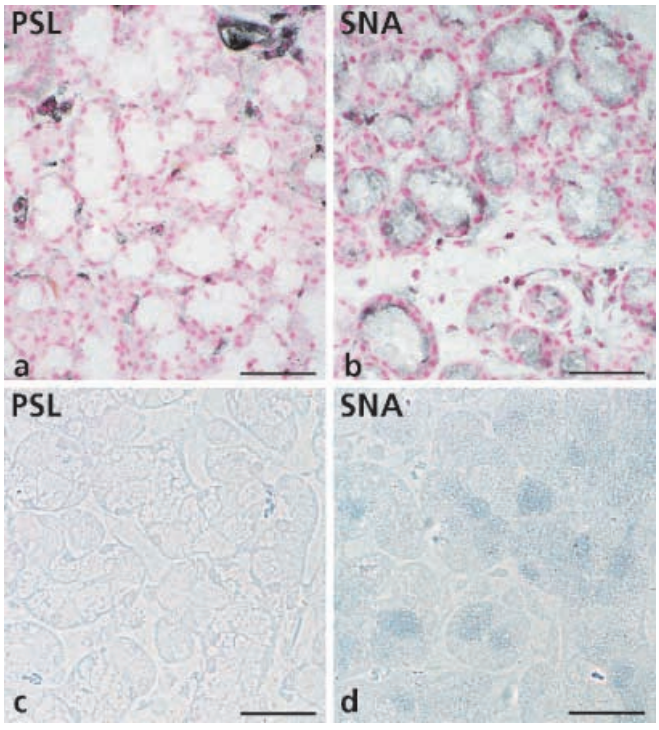

Fig. 5 Sheep submandibular gland, paraffin sections (**a**, **b**) and semithin Lowicryl K4M (**c**, **d**) sections. PSL staining (**a**, **c**) is undetectable in the mucus of the acini although capillaries are positive. In contrast, SNA stains the mucus of the acini as well as capillaries (**b**, **d**). *Bars* 45 µm (**a**, **b**), 40 µm (**c**, **d**)

In kidney, similar to the above-described organs, both lectins reacted with the endothelial cells of the glomeruli, intertubular capillaries, and arteries and veins of various sizes notwithstanding a stronger staining with SNA (Fig. 6). Although pretreatment with neuraminidase or *N*-glycanase F affected the staining of endothelia by both lectins, only the staining with the PSL was eradicated whereas some SNA staining was still observed. The S1 segment of proximal tubules was strongly positive with both PSL and SNA (Fig. 6c, d). Likewise, the S3 segment was strongly PSL positive, but the S2 segment was of intermediate staining intensity (Fig. 6c, e). However, both the S2 and S3 segments of proximal tubules were only faintly stained with SNA (Fig. 6d, f). In the papilla, PSL and SNA staining were indistinguishable although the SNA staining was usually stronger (Fig. 6g, h). Pretreatment with *N*-glycanase F abrogated the PSL staining of all segments of proximal tubules (Fig. 7a, b, e) and of SNA staining of S1 (Fig. 7). However, apical cell surface SNA staining of the S3 segment of proximal tubules was still present and additional staining of the basolateral cell surface was observed (Fig. 7f). SNA staining of thick ascending loops was resistant to *N*-glycanase F (Fig. 7f). Neuraminidase pretreatment abrogated staining by PSL and SNA, with the exception of partially remaining SNA staining of thick ascending loops (Fig. 7g, h) and cortical collecting ducts.

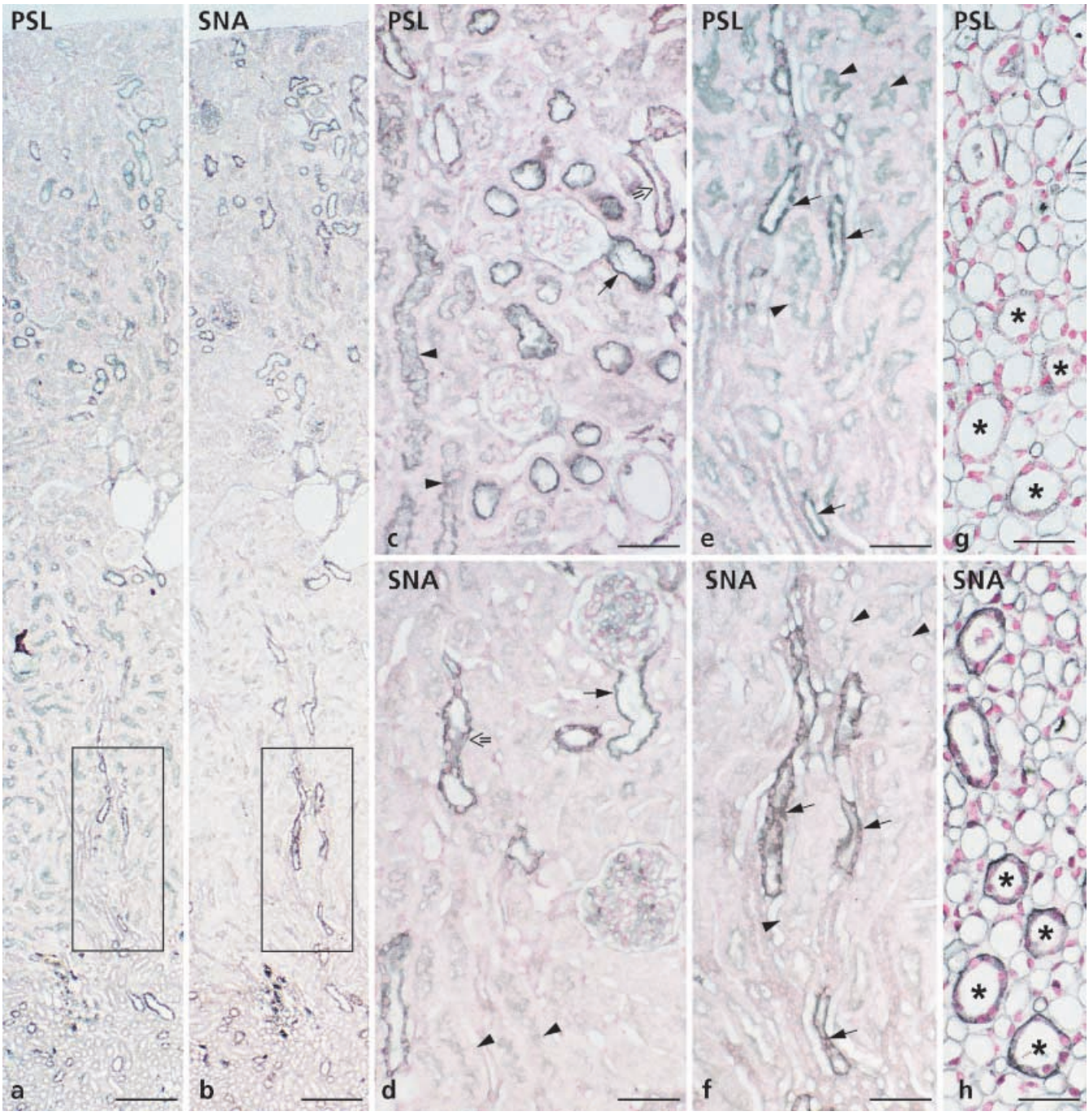

Fig. 6a–h Rat kidney. At low magnification, the differences in staining pattern by PSL (**a**) and SNA (**b**) in cortex and part of outer medulla are visible. Details of the PSL staining in cortex (**c**) and outer medulla (**e**): origin of a proximal tubulus (*arrow* in **c**), S3 segment of proximal tubule (*arrowheads* in **c** and **e**), and thick ascending limb (*open arrow* in **c**, *arrows* in **e**) as well as glomeruli are positive. Details of the SNA staining in cortex (**d**) and outer medulla (**f**): origin of a proximal tubulus (*arrow* in **d**), S3 segment of proximal tubule (*arrowheads* in **d** and **f**), and thick ascending limb (*open arrow* in **d**, *arrows* in **f**) as well as glomeruli are positive. Details from the papilla with collecting ducts (*asterisks* in **g** and **h**), thin loops, and capillaries positive for PSL (**g**) and SNA (**h**). *Bars* 145 µm (**a**, **b**), 65 µm (**c**, **d**), 55 µm (**e, f**), 20 µm (**g**, **h**)

The staining patterns for PSL and SNA in rat liver and pancreas were indistinguishable from each other and were equally sensitive to *N*-glycanase F and neuraminidase pretreatment (not shown). The results of blots using SNA and PSL with protein extracts prepared from rat liver and kidney are illustrated in Fig. 8.

Discussion

Previous investigations have established the carbohydrate specificity of purified PSL by precipitation inhibition assay, fluorescence quenching studies, and glycolipid-lectin

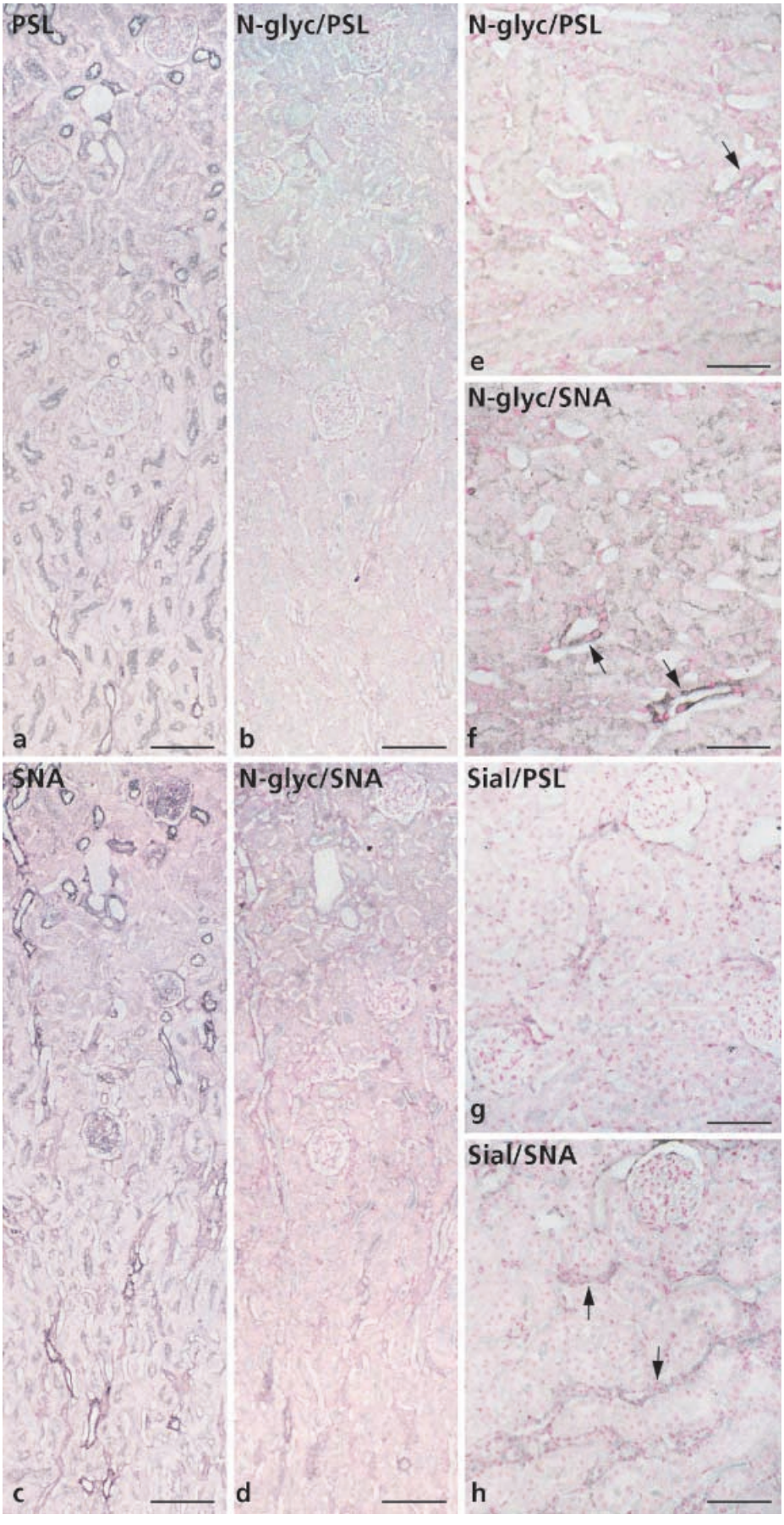

Fig. 7a–h Adjacent serial sections with cortex and part of outer medulla. Staining for PSL (**a**) becomes undetectable or greatly reduced following pretreatment with *N*-glycanase F (**b**, **e**) or sialidase (**g**). Staining for SNA (**c**) remains partially positive following *N*-glycanase (**d**, **f**) and sialidase (**h**) pretreatment. *Arrows* in **f** and **h** point to stained thick ascending loops. Note the remaining SNA staining of S3 segment of proximal tubular epithelia and additional staining at their basolateral cell surface (**f**). *Bars* 125 µm (**a–d**), 85 µm (**e**, **f**), 55 µm (**g**, **h**)

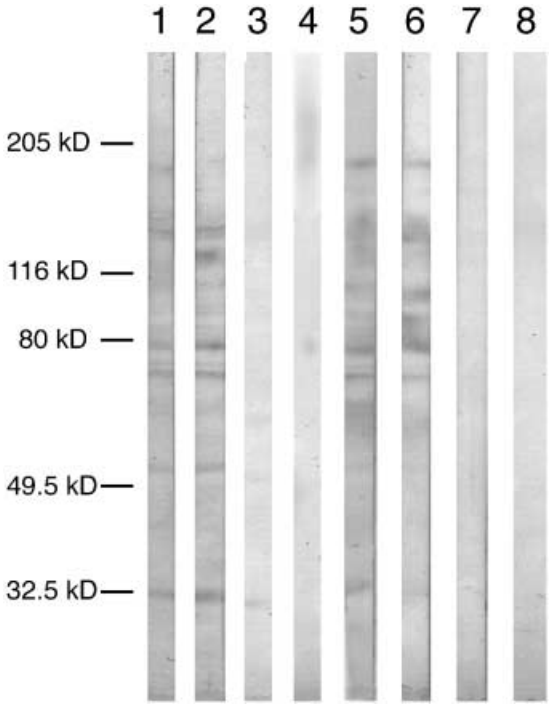

Fig. 8 Lectin blots of electrophoretically resolved protein homogenates from rat liver (*lanes 1–4*) and kidney (*lanes 5–8*). *Lanes 1, 5*: SNA; *lanes 2, 6*: PSL; *lanes 3, 7*: PSL with sialidase pretreatment; *lanes 4, 8*: PSL with *N*-glycanase F pretreatment. Positions of molecular weight standards are indicated on the left

binding on high-performance thin-layer chromatography and shown that the lectin has an extended carbohydrate binding site with highest specificity and affinity for the non-reducing terminal Neu5Acα2,6Galβ1,4Glc/GlcNAc of *N*-glycans (Mo et al. 2000). Noteworthy, PSL did not react with α2,6-linked sialic acids attached to the penultimate GlcNAc residue of tri-and tetraantennary structures. Although PSL showed some reactivity with non-reducing terminal galactose and lactose as well as lactosamine, the inhibitory potency of Neu5Acα2,6Galβ1,4Glc/GlcNAc was 2,000-fold and 250- to 300-fold higher, respectively. The extraordinary specificity of PSL for Neu5Acα2, 6Galβ1,4GlcNAc containing oligosaccharides with a K_d value of 10.1±0.2 μM was very recently confirmed by frontal affinity chromatography coupled to electrospray mass spectrometry (Zhang et al. 2001).

In the present study, we have prepared a digoxigenin-conjugated PSL for application in lectin histochemistry and blotting. In accordance with the results of precipitation inhibition assays, the interaction of PSL with its binding sites in tissue sections or electrophoretically resolved tissue glycoproteins could be inhibited with 6′sialyllactose but not with 3′sialyllactose. Furthermore, pretreatment with neuraminidase from *Vibrio cholerae* or with *N*-glycanase F abolished binding of PSL. Collectively, these data strongly indicate that PSL represents a useful reagent for the detection of terminal non-reducing Neu5Acα2,6Galβ1,4Glc/GlcNAc of *N*-glycans in tissues.

Two other lectins have been employed for the detection of α2,6-linked sialic acid, the SNA (Shibuya et al. 1987a, b) and the *Trichosanthes japonica* agglutinin (TJA-I; Yamashita et al. 1992). As already mentioned, SNA potentially recognizes α2,6-linked sialic acid and terminal GalNAc in *O*-glycans in addition to Neu5Acα2,6Gal in *N*-glycans (Taatjes et al. 1988). Although the TJA-I appears to have a high specificity for α2,6-linked sialic acid of *N*-glycans, it also seems to bind as avidly to HSO_3^-, 6Galβ1,4GlcNAc (Yamashita et al. 1992). Recently, the PSL was found to have an affinity for both 6- and 6′-HSO_3^-, 6Galβ1,4GlcNAc derivatives (K_d=259±19 μM; Zhang et al. 2001). This interaction, however, was 20 times weaker than the one with Neu5Acα2,6Galβ1,4GlcNAc as substrate. Accordingly, for histochemical studies, the use of neuraminidase predigestion of tissue sections represents an important control because of the importance of the sialic acid residue for PSL binding. Any neuraminidase-resistant lectin staining has to be assumed to be due to the presence of 6-*O*-sulfolactosamine. Thus, currently the PSL represents the lectin with the highest specificity for the Neu5Acα2,6Galβ1,4Glc/GlcNAc trisaccharide of oligosaccharides.

Our comparative histochemical analysis of various rat tissues and ovine submandibular gland using PSL and SNA directly proved the superiority of PSL. In various tissues including stomach, small and large intestine as well as kidney, *N*-glycanase F digestion resulted in absence of staining by PSL, whereas binding of SNA was only partially sensitive to this treatment and therefore partially due to binding to oligosaccharidic structures of *O*-glycans. Particularly informative in this regard was the observed reactivity on tissue sections from ovine submandibular gland since the mucus produced by this gland is extremely rich in Neu5Acα2,6GalNAcα1-serine/threonine. PSL did not react at all with the mucus produced by the acinar cells although it did stain endothelial and interstitial cells in an *N*-glycanase F-sensitive manner. In contrast, SNA stained the mucus and this staining was not affected by neuraminidase treatment, which was apparently due to the high density of GalNAc residues (Taatjes et al. 1988).

The Neu5Acα2,6GalNAc disaccharide represents the sialyl-Tn antigen, which has been shown to be a colon carcinoma-associated glycotope (Itzkowitz et al. 1989, 1990; Jass et al. 1995; Ogata et al. 1995, 1998). Another reproducible finding in human colon carcinoma tissues consists in an enhanced activity of CMP-NeuAc: Galβ1,4GlcNAc:α2,6-sialyltransferase (Dall'Olio et al. 1989; Gessner et al. 1993; Murayama et al. 1997), the sialyltransferase involved in the synthesis of the binding substrate for PSL. Previously, we (Sata et al. 1991) and others (Vierbuchen et al. 1995) have applied SNA for studies in human colon. In contrast to two other sialic acid-specific lectins, the leukoagglutinin from *Maackia amurensis* and the *Amaranthus caudatus* lectin, normal colonic mucosal epithelium was unreactive with SNA (Sata et al. 1991, 1992). Although we obtained evidence that in a number of colorectal carcinoma-derived cell

lines sialyl-Tn is not expressed (Murayama et al. 1997), it cannot be excluded that SNA staining in tissue sections from colorectal carcinomas may be due to binding to both sialyl-Tn of *O*-linked oligosaccharides and Neu5Acα2,6Gal of *N*-linked oligosaccharides. From the results of our present study it is obvious that the PSL represents a lectin superior to SNA for studies on the expression of the Neu5Acα2,6Galβ1,4Glc/GlcNAc trisaccharide of *N*-glycan oligosaccharides. Its value for diagnosis and as a predictive clinical marker in human colorectal carcinoma is currently being investigated in our laboratory.

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