Proteins involved in the production and perception of oligosaccharides in relation to plant and animal development

Holst, P.P.G. van der; Schlaman, W.R.M.; Spaink, H.P.; Schlaman, H.R.M.

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Patrick PG van der Holst, Helmi RM Schlaman and Herman P Spaink*

Chitin oligosaccharides and their derivatives are involved in developmental and defence-related signalling pathways. Major advances include the structural identification of lectins involved in development that bind chitin oligosaccharides and the links between chitin oligosaccharide and hyaluronan synthesis. Also, recent advances in the understanding of the biological role of oligosaccharides are summarised in a model for multistep glycan recognition.

Addresses
Leiden University, Institute of Molecular Plant Sciences, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands
*e-mail: spaink@rulbim.leidenuniv.nl

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Abbreviations

CLP	chitinase-like protein
CO	chitin oligosaccharide
EST	expressed sequence tag
HA	hyaluronan
LCO	lipo-chitin oligosaccharide
LNP	lectin-nucleotide phosphohydrolase
NFBS	nodulation factor binding site
NF-κB	nuclear factor κB

Introduction

In the past couple of years, an important role for chitin oligosaccharides (COs) as signal molecules in plant and animal developmental processes, and defence mechanisms against pathogens has become increasingly apparent. These oligosaccharides can elicit a number of direct responses in plant cells, such as enhanced ion flux across the plasma membrane resulting in a rapid alkalisation of the medium, formation of reactive oxygen species, antimicrobial phytoalexin production, changes in protein phosphorylation, and lipid oxidation [1]. In this review, we focus on the role of COs in organogenesis and embryogenesis based on their effect on cell polarity, cell movement and cell division in plants and animals [2]. Another glycan that is discussed here is hyaluronan (HA) because of interesting similarities in its production and the role of this signal molecule in development. This polysaccharide has a structural function in the extracellular matrix, but also has a function in the signalling process that precedes cell proliferation and migration during vertebrate embryogenesis [3••].

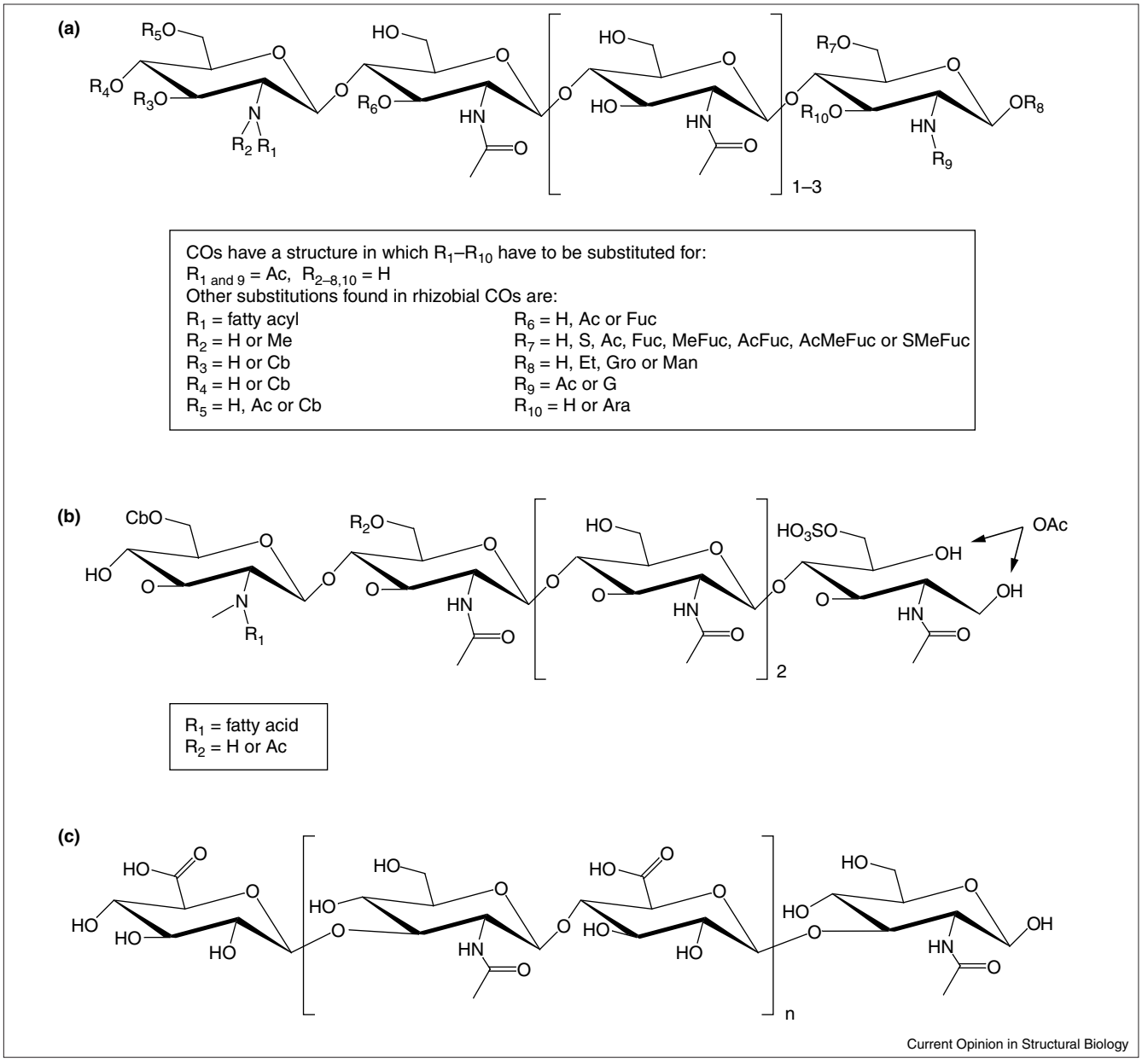
One of the best-studied examples of oligosaccharides involved in development are acylated derivatives of COs called lipo-chitin oligosaccharides (LCOs), which are produced by symbiotic bacteria called rhizobia [4–6]. These CO derivatives can induce cell division in differentiated cells in the root cortex of legume plants, resulting in the formation of a new organ, the root nodule, in which the bacteria are hosted. The proteins that are involved in the biosynthesis and secretion of the rhizobial COs are known. For example, nodulation protein NodC is an *N*-acetyl glucosaminyl transferase involved in the production of the CO backbone. NodC is a member of the large β-glycosyl transferase family. Close relatives of NodC, called the DG42/HAS subfamily, have been shown to be involved in CO and HA synthesis in *Xenopus*, zebrafish, mouse and men [2,3••,7••,8••]. It has been shown that several members of the DG42/HAS family are differentially expressed during embryogenesis in *Xenopus*, zebrafish and mouse [7••,9,10]. Inhibition of DG42 activity in an early embryonic stage results in severe developmental abnormalities in zebrafish embryos, strongly suggesting the involvement of COs in anterior/posterior axis formation during vertebrate embryogenesis [11,12].

Chitinases are another class of proteins that might be involved in CO signal production. Many plant chitinases are assumed to play a role in defence against pathogens. Chitinases secreted by plants are able to degrade fungal cell walls, giving rise to COs of various lengths. The observations that the carrot EP3 endochitinase is essential in somatic embryogenesis and that an embryonic mutant arrested in somatic embryogenesis can be rescued by the addition of *Rhizobium*-derived COs showed a role for chitinase-derived COs in development [13]. In addition, enzymes that are involved in the degradation of HA have been found. These hyaluronidases might also be involved in the generation of HA fragments that have a signalling function [14••].

Little is known about how COs are perceived by cells. Protein-binding factors have been identified in the plasma membranes of tomato cells [15] and rice [16], and they show a very high affinity for COs with a degree of polymerisation of >4 and 8, respectively. Binding sites for the rhizobial CO derivatives have been described [17••]. Furthermore, legume-specific lectin-nucleotide phosphohydrolases (LNPs) or lectin apyrases that show a high affinity for rhizobial COs have been identified [18,19••,20••]. Other lectins are also involved in the signal transduction pathways governing rhizobial symbiosis, but there is no evidence for a role in the perception of rhizobial CO derivatives [21,22]. Much more is known about receptor proteins for HA. One of the best-characterised mechanisms by which HA acts as a signalling molecule is through binding to the putative bioactive signalling receptor CD44, leading to activation of the nuclear factor κB (NF-κB) transcription factor [14••].

In animals and plants, proteins have been found that share high sequence homology and a structural relationship with family 18 chitinases. These so-called chitinase-like proteins

Figure 1



The structure of oligosaccharides involved in developmental processes. **(a)** Backbone of rhizobial COs, in which the various R groups represent the indicated substituents. Ac, acetyl; AcFuc, 4-*O*-acetylfucosyl; AcMeFuc, 4-*O*-acetyl-2-*O*-methylfucosyl; Ara, arabinosyl; Cb, carbamoyl; Et, ethyl; Fuc, α-linked fucosyl; G, glycosyl; Gro, glyceryl; Man, mannosyl; Me, methyl; MeFuc, 2-*O*-methylfucosyl; S, sulfyl; SMeFuc, 3-*O*-sulfate-2-*O*-methylfucosyl. **(b)** A novel LCO characterised by a glucosaminitol residue (open structure) at the reducing end. OAc, *O*-acetyl at either one of the positions pointed to by the arrows. **(c)** Hyaluronan (HA). n = 500 in HA as produced by HAS3 or by enzymatic or chemical cleavage. n = 2500 in native HA as produced by HAS1 and HAS2.

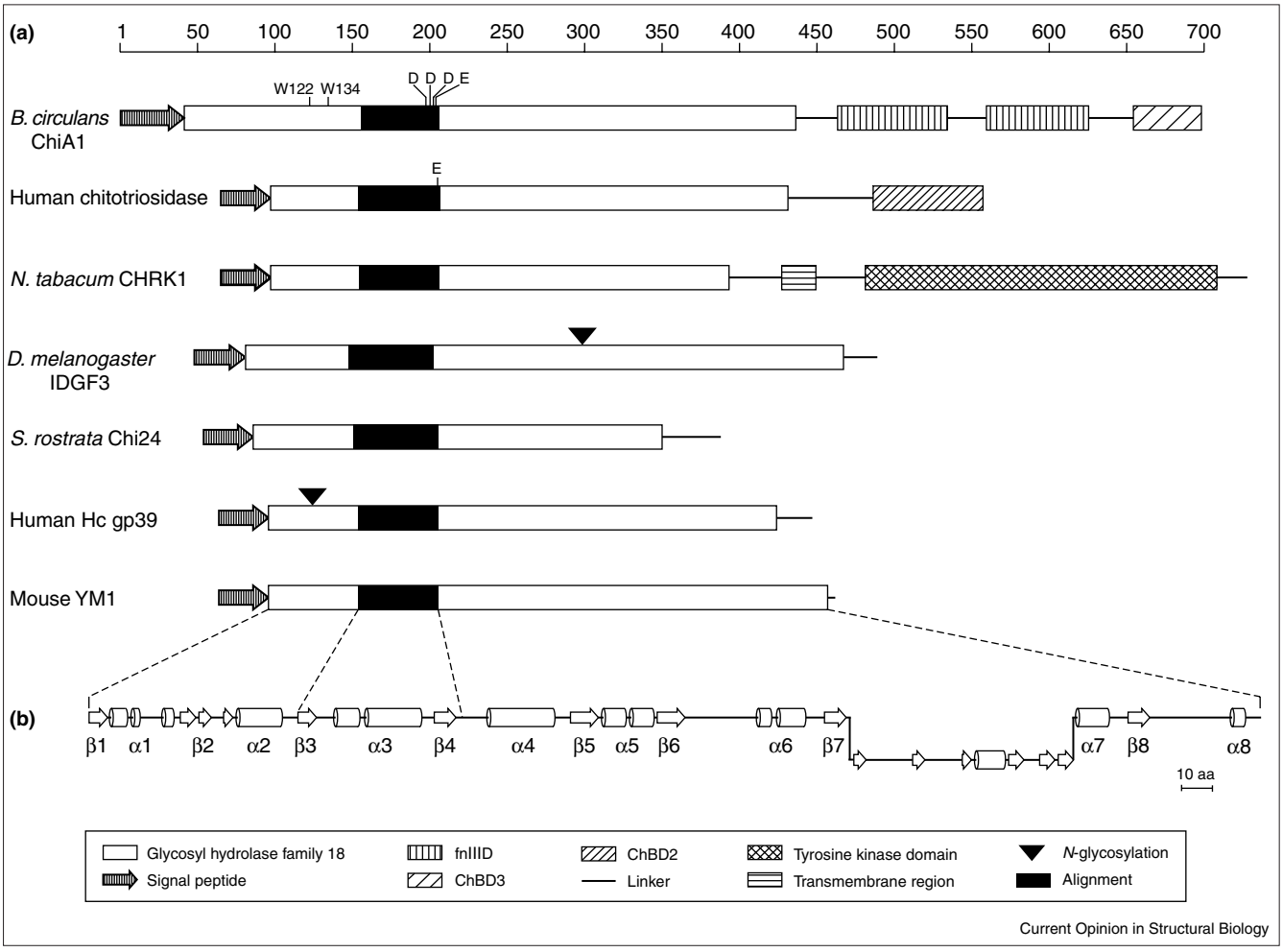
(CLPs) lack catalytic activity, but sugar binding is unaffected. There are indications that CLPs have a role in cell migration and tissue remodelling [23,24]. Therefore, they might have a function in the perception of COs. As several CLPs have been shown to bind preferentially to glycosaminoglycans such as heparan sulfate [25], it is clear that studies of cellular CO perception have to be on a comparative basis with the perception of other extracellular matrix glycan components.

In this review, we will discuss in more detail the enzymes that are involved in the generation of COs and HA fragments, and the proteins that might be involved in their cellular recognition.

Structures of β-glycans involved in developmental signal transduction

In zebrafish embryos, unmodified COs (Figure 1a) accumulate between the late gastrula and neurula stages

Figure 2



Structure of several family 18 glycosyl hydrolase members. **(a)** Domain structures of chitinases and CLPs. Amino acids that have been mutated for functional analysis are indicated by single-letter code on top of the domain. The black box in the family 18 glycosyl hydrolase domain, which contains the catalytic region, represents the sequence alignment depicted in Figure 3. ChBD, carbohydrate-binding domain; fnIIID, fibronectin type III domain. The scale (in amino acids) is drawn at the top of the figure. **(b)** Secondary structure of mouse YM1. The TIM barrel is formed by helices α1–α8 and strands β1–β8. The small (α+β) domain is drawn as a dropdown structure to illustrate that it lies apart from the TIM barrel in the tertiary structure [51••]. aa, amino acid.

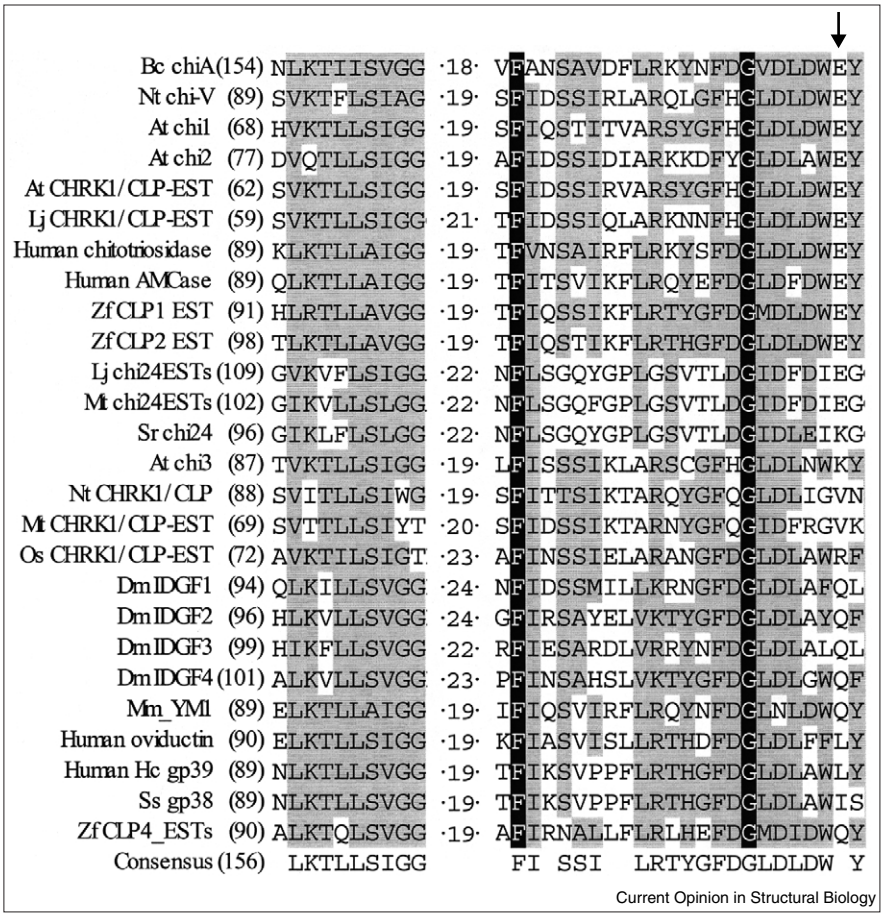
[7••]. In rhizobia, COs are decorated in various ways. Because the fatty acid is always present in the rhizobial COs, they are often referred to as LCOs (Figure 1a,b). Rhizobial bacteria can produce a wide range of moieties that are attached to the backbone, such as acetyl, fucose or sulfate groups. These side groups have a function in host specificity of nodulation [26]. It has been suggested that the fatty acid has a role in transporting the LCO through the plant tissue because *O*-acetylated COs have been shown to induce cell division when introduced inside the plant tissue by ballistic microtargeting [27]. Surprisingly, the side groups seem to have little effect on the conformation of the LCO molecule [28]. Some evidence suggest that, in aqueous solutions, LCOs exist as monomers, rather than in micelles, and NMR spectroscopy indicates that the fatty acid is in quasi-parallel orientation to the CO moiety [28,29].

As depicted in Figure 1c, HA is a nonsulfated, linear glycosaminoglycan consisting of repeating units of (β,1-4)-D-glucuronic acid-(β,1-3)-*N*-acetyl-D-glucosamine. HA has an average chain length of 10 000 disaccharide repeats and has both a structural and a signalling role. It functions in cell adhesion, migration and proliferation, and is associated with the extracellular matrix [3••]. The signalling role of HA appears to be size-dependent; the transcription factor NF-κB is activated not by the native polysaccharide (size >2 x 10^6 Da), but rather by events triggered by fragments of the sugar (size ~200 000 Da) [14••].

Enzymes involved in oligosaccharide production

The nodulation protein NodC synthesises the CO backbone of rhizobial LCOs. It uses UDP-*N*-acetylglucosamine as the substrate to produce a tetrasaccharide or pentasaccharide. Enzymatic studies show that chain elongation is

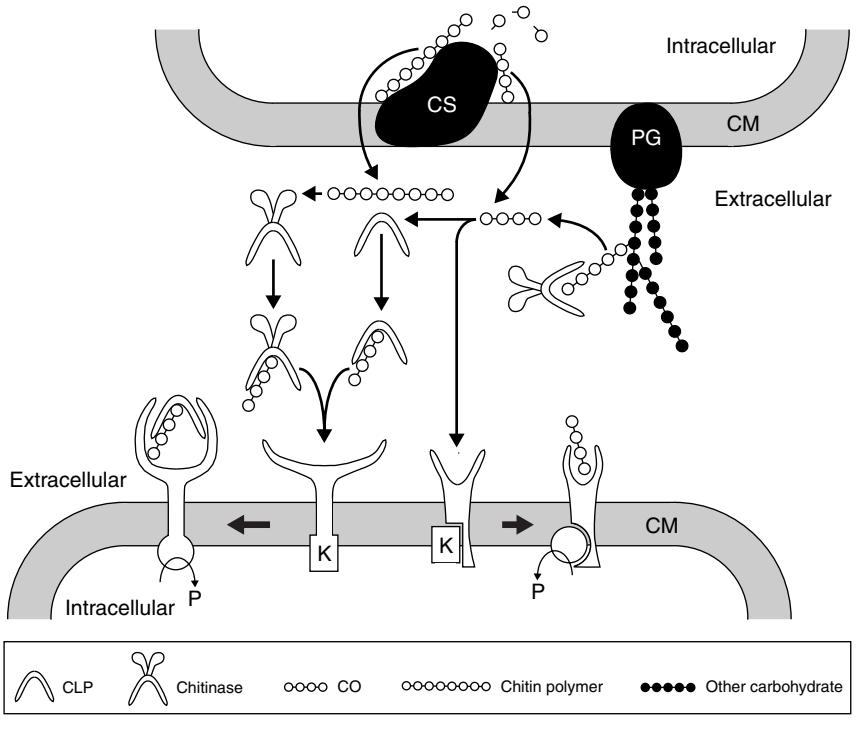
Figure 3



Alignment of part of the amino acid sequences of several family 18 glycosyl hydrolase members (chitinases and CLPs), showing the two stretches of amino acids with the highest degree of homology. The number of amino acids separating the two stretches is indicated. The consensus sequence is depicted at the bottom of the alignment. Black boxes represent completely conserved residues. Highly conserved, structurally similar residues are indicated in a grey box. Sequences are from: *Bc* ChiA, *B. circulans* chitinase A1 (accession number P20533); *Nt* ChiV, chitinase class V from *Nicotiana tabacum* (accession number X77111.1); *At* Chi1, Chi2 and Chi3, chitinases and CLP from *Arabidopsis thaliana* (accession numbers AL16155.2, T04756 identical to accession numbers CAB78977, CAA19692 and T04763, respectively); *Nt* CHRK1/CLP, N-terminal CLP domain from *N. tabacum* CHRK1 (accession number AAD52097); *At*, *Lj*, *Mt* and *Os* CHRK1/CLP-EST, ESTs from *A. thaliana*, *L. japonicus*, *M. truncatula* and rice, respectively, homologous to the CLP domain from *N. tabacum* CHRK1 (accession numbers AV554101, AV423067, AW560415 and OSM124073, respectively); human chitotriosidase (accession number AAC50246); human AMCase, human acidic mammalian chitinase (accession number AF290004.1); human oviductin (accession number U09550); human Hc gp39, human cartilage glycoprotein 39 (accession number AAA16074); *Ss* gp38, porcine (*Sus scrofa*) glycoprotein 38 (accession number AAA86482); *Dm* IDGF1 to IDGF4, imaginal disc growth factors 1–4 from *Drosophila melanogaster* (accession numbers AAC99417, AAC99418, AAC99419 and AAC99420, respectively); *Mm* YM1, mouse YM1 (accession number AAB62394); *Sr* Chi24, chitinase homologue from *S. rostrata* (accession number Y12706); *Lj* and *Mt* Chi24 ESTs, EST sequences homologous to *Sr* Chi24 from *L. japonicus* (overlapping clones with accession numbers AV417870 and AW720660) and *M. truncatula* (overlapping clones with accession numbers AW584905 and AW560139); *Zf* CLP1, CLP2 and CLP4 ESTs, EST sequences from zebrafish designated as CLP1 (accession number AW134388), CLP2 (accession number AW133937) and CLP4 (overlapping clones with accession numbers AW133628, AI496773 and AW018793) on the basis of homology with other CLPs. Sequences that were obtained from EST databases have been translated using Vector NTI software. The catalytic glutamic acid residue is indicated with an arrow above the sequence.

initiated at the reducing-end residue [30,31]. NodC is a membrane protein functioning at the cytoplasmic site of the bacterial inner membrane, presumably in a multienzyme complex with other proteins involved in LCO biosynthesis. Following biosynthesis, the LCO is transported to the environment through a type I secretion system (represented by an ATP-binding cassette containing a component encoded by *nodI* and a transmembrane component encoded by *nodJ*) [26]. Several classes of the NodC homologue DG42 (also called HAS) have been identified based on sequence homology [2,10]. In zebrafish, the DG42 class II gene is differentially expressed and the location of the DG42 class II transcripts is temporally and spatially regulated during early embryogenesis [7••]. Various members of the DG42/HAS family have been shown to be involved in the production of HA, leading to controversies concerning what was thought to be the product of all DG42 classes (reviewed in [2,32]). Recently, mouse HAS1 (DG42 class I) was shown *in vitro* to produce both HA and COs, depending on the incubation conditions [8••]. A mutation of L314→V did not affect the CO synthesis activity, whereas it caused severe loss of HA synthase activity [8••]. This confirms, in one system, that both HA and CO can be synthesised by enzymes belonging to the DG42/HAS protein family [11,31,33]. In humans, three classes of HAS enzymes have been identified. It has been proposed that specific *has* genes are able to produce the low-molecular weight HA fragments that are involved in signal transduction, as opposed to the native, high-molecular weight HA [10]. The production of the HA signal might involve endohyaluronidases, which randomly cleave the HA polysaccharide, or it might arise from depolymerisation by reactive oxygen species [3••].

Figure 4



Model for the molecular mechanisms of CO synthesis and perception in relation to development. Chitin and COs are synthesised by membrane-bound enzymes (chitin synthase [CS]) at the cytoplasmic site. In the model, COs are transported to the environment, where short-chain COs either are perceived by a membrane-bound receptor (shown on the bottom right) (e.g. LNPs, C-type lectins, *Nt* CHRK1) or are first perceived by an extracellularly localised CLP (e.g. *Dm* IDGF, mammalian Hc gp39 and gp38). Next, the CLP–CO complex is recognised by a membrane-localised receptor (bottom left side). Alternative to synthesis, short-chain COs might arise from extracellular chitinase hydrolysis of long-chain COs or from carbohydrate chains of glycoproteins or proteoglycans (PGs) (e.g. in carrot, the degradation of arabinogalactan proteoglycans by chitinase EP3 [43•]). An active receptor results directly from binding of the ligand or involves multimerisation of the receptor (e.g. as in CD44). We hypothesise that downstream signal transduction includes protein phosphorylation (P) via a kinase (K) that either is part of the membrane receptor itself (e.g. as in *Nt* CHRK1) or is in complex with the membrane receptor (e.g. as with CD44). CM, cytoplasmic membrane.

COs can also be produced by the action of endochitinases. The known endochitinases (EC 3.2.1.14) belong to glycosyl hydrolase families 18 and 19 (http://www.expasy.ch/cgi-bin/lists?glycosid.txt) [34], which act through retaining and inverting mechanisms, respectively. Family 18 glycosyl hydrolases have representatives in bacteria, fungi, higher plants (classes III and VI) and animals, and their general protein fold is a $(\beta/\alpha)_8$ TIM barrel with a substrate-binding groove on top. In addition to their catalytic domain, most of these chitinases contain one or more domains involved in substrate binding [35••,36]. Usually, a linker without defined secondary structure connects the different domains. In Figure 2, the domain structure of several family 18 chitinases is presented. The catalytic proton donor is the conserved glutamic acid residue in the short consensus sequence DGxDxDxE, as has been proven by mutation analysis of both ChiA1 from *Bacillus circulans* [37] and human chitotriosidase [38]. Additionally, two surface-localised tryptophan residues (W122 and W134) in ChiA1 of *B. circulans* are essential for crystalline chitin hydrolysis [39]. Family 19 chitinases, unrelated to family 18 chitinases, are mainly found in higher plants (classes I, II, IV and V), but some of them are found in bacteria. They have a bilobal structure with a high α-helical content and they are very similar to lysozymes.

Chitinases have a well-known role in defence against pathogens in plants [1] and animals, where they are observed in the gastrointestinal tract of several species including human [40]. The relevance of chitinase action in relation to development is shown in plant embryogenesis. The class IV chitinase EP3, extracellularly present in the tissue surrounding the embryo, promotes early stages of embryogenesis in carrot, *Arabidopsis* and spruce [13,41,42]. It has been suggested that it generates COs that are essential for early stages of plant embryogenesis through the hydrolysis of the carbohydrate moiety of the proteoglycan arabinogalactan [43•]. During zebrafish embryogenesis, endogenous chitinases have been detected both intracellularly and extracellularly. Only the latter seems to have a role in embryogenesis [7••].

Proteins involved in the perception of β-glycans

Classical lectins

Classical lectins are (glyco)proteins that bind carbohydrates in which the carbohydrate-binding site (CBS) does not possess enzymatic activity. The ligand affinity is usually low, but clustering of CBSs, either on a single polypeptide chain or by the formation of noncovalent dimers or oligomers, can create a high-affinity site. In general terms, lectins act in forming contacts via the bridging of (part of) molecules. Lectins are either present in a soluble form in the cytoplasm or extracellular matrix, or they are membrane-bound. Over 230 three-dimensional structures are known (http://www.cermav.cnrs.fr/databank/lectin/index.html), from which it is apparent that lectins exhibit several folds. The tertiary structures of the legume lectins and the C-type animal lectins are the most frequently

observed. Classical lectin structures have been reviewed extensively ([44,45]; see also the review by Kogelberg and Feizi in this issue, pp 635–643).

Lectins play an important role in interactions between cells. A novel role for a legume lectin in early plant embryogenesis has been shown recently [46•]. Despite their importance, their function in signal transduction is usually not understood. The extracellular binding domain of CD44, a transmembrane receptor for HA, has structural homology with the C-type lectin domain of E-selectin, a cell adhesion molecule that has a role in the adhesion of leukocytes to activated vascular endothelium during inflammation [47•]. The CD44 protein, belonging to the cartilage-link protein family, has been intensively studied. More recently, various other members of this family, such as LYVE-1 and various intracellular HA-binding proteins, have also been identified [3••,47•]. CD44 is involved in signal transduction and in cell–cell and cell–matrix interactions, and it might have a function in attachment. Tyrosine kinases (belonging to the *src* family) are associated with the cytoplasmic region of CD44 [47•]. The binding of native HA to CD44 usually leads to cell adhesion; only low-molecular weight species of HA can activate downstream effectors, such as tyrosine kinase, leading to activation of the transcription factor NF-κB [14••]. It is unclear how CD44 is able to recognise the length of HA as both native HA and HA fragments bind to the same receptor, which can only accommodate several HA repeat units. It is hypothesised that bound native HA prevents CD44 from oligomerisation, probably because of steric hindrance [14••]. When native HA is displaced by HA fragments, oligomerisation can take place, thereby generating a biologically active receptor.

Apyrases

Apyrases are enzymes that hydrolyse nucleotide triphosphatases and diphosphatases, and bind carbohydrates without modifying them. They are membrane proteins that are classified as either ectoapyrases, which have the catalytic domain extracellular, and endoapyrases, which act inside the cell. Apyrases have been identified in almost all organisms and their role is very diverse, ranging from neurotransmission and blood platelet aggregation to protein glycosylation. A new role for apyrases in the perception of rhizobial LCOs has been described recently. A defined group of ectoapyrases designated as LNPs are only observed in legume plants [20••]. They have been analysed in some detail in *Dolichos biflorus* [18], soybean [19••] and *M. truncatula* [20••]. General features include rapid upregulation of expression after addition of symbiotic LCOs and reduction in nodule formation after treatment of the roots with anti-LNP serum. The soybean LNP was localised on the plasma membrane and, more specifically, within the region of the root known to be susceptible to rhizobial infection. This would be consistent with a role as a membrane-associated receptor for the nodulation signal.

Chitinase-like proteins

A growing list of noncatalytic proteins with significant sequence homology to family 18 chitinases are being identified in plants, insects, fish and mammals [25,48,49,50••]. In all of these proteins, the essential glutamic acid residue in the catalytic site of the protein is replaced by another amino acid (Figure 3). Therefore, these proteins are designated as CLPs. For a few examples, CO binding has been experimentally confirmed [38], suggesting that they might have a role in CO perception. The first tertiary structure of a CLP to be published was that of mouse YM1 [51••]. It shows remarkable similarity to chitinase A of *Serratia marcescens* [52]. In addition to an elliptical, donut-shaped TIM-barrel domain, YM1 contains a small α+β domain (Figure 2). A disulfide bond stabilises the two domains. Recently, the crystal structures of a human CLP, Hc gp39, and the closely related human chitotriosidase have also been obtained (F Fuzetti, B Dijkstra, personal communication). A defined function for CLPs is not known, but it has been shown that the mammalian CLPs human Hc gp39 and porcine gp38 act as chemoattractants in cell migration and tissue remodelling [24,53]. A role for CLPs during embryogenesis has been observed in *Drosophila*. Four novel CLP-type growth factors, called imaginal disc growth factors (IDGFs) 1–4, which are secreted from embryonic yolk cells and the fat body of the embryo and larva, have mitogenic activity on imaginal disc cells [49]. Imaginal discs are pouches of tissue present in the gut of larvae that form the adult's outer structures. In culture, these cells are characterised by the absence of contact inhibition, resulting in aggregate formation. Unfortunately, neither their affinity for COs nor their requirement for bound COs for growth promotion was tested. The protein *Sr* Chi24 is a CLP identified in the legume *Sesbania rostrata* that has been suggested to function as a binding factor for rhizobial LCOs during nodulation [48]. Expressed sequence tags (ESTs) homologous to *Sr* Chi24 have been observed in several other legumes, such as *Lotus japonicus*, *Glycine max* and *Medicago truncatula*, but whether they encode functional proteins is unknown (Figure 3).

In general, CLPs have an N-terminal signal peptide, are secreted extracellularly and at least some of them are *N*-glycosylated (Figure 2). In tobacco, the protein *Nt* CHRK1 has been identified that consists of an N-terminal CLP-like domain and a C-terminal tyrosine kinase domain linked by a flexible hinge region (Figure 2) [50••]. This is intriguing because such a configuration strongly suggests that CLPs can also act directly as sugar perception molecules and that they are linked to downstream signal transduction pathways through a tyrosine kinase.

Unidentified CO-binding sites

Proteins that bind CO have been observed in membrane fractions, for example, in tomato [15] and rice [54]. In legumes, two membrane-localised binding fractions for LCOs have been identified, called nodulation factor

binding site (NFBS) 1 and NFBS2. These binding sites, however, display only poor (NFBS1) or partial (NFBS2) structural selectivity towards rhizobial LCOs [55,56].

Model of the cellular recognition mechanism of COs

In Figure 4, a working model concerning the action of COs in development that is based upon direct or two-step recognition is shown. The proposed role of CLP family members is based on several observations: firstly, the expression of various CLPs and other members of the chitinase 18 family during various developmental processes; secondly, the demonstrated role of CLPs in *Drosophila* embryogenesis and as chemoattractants during cell migration; and finally, the occurrence in tobacco of one CLP family member that contains an additional kinase domain (suggesting that, in other organisms, a similar coupling might have been separated in different genes during evolution).

A two-step recognition mechanism would be in agreement with data available for the receptor mechanism of fungal hepta-β-glucosides in leguminous plants [57••]. In this system, which currently can be considered to be the best-characterised high-affinity oligosaccharide perception system in plants, a soluble unique class of glucan-binding proteins are dependent on an as yet unknown membrane protein. The soluble protein and membrane protein in combination have been reconstituted to a functional plasma-membrane-associated receptor complex in artificial lipid vesicles [57••]. Such a two-step perception system would not exclude the presence of a direct reception system, as it has been previously postulated that multiple receptors for LCOs exist in leguminous plants [58].

Conclusions

The recognition of glycans by eukaryotic cells is crucial for many processes governing development and interaction with pathogens. Because of the diversity and complexity of glycans (based on the great variety in saccharide composition, linkage and coupling to proteins and lipids), still very little is known about the molecular basis of the signal transduction pathways underlying glycan recognition processes. In particular, the determinants of the specificity of glycan recognition processes are hardly understood. The major problem in glycobiological studies is that the specificity of production or binding of glycan metabolites cannot yet be assigned to protein motifs based on homology studies. The disclosure of an increasing number of crystal structures of various proteins involved in the biosynthesis and perception of complex glycan structures can be expected to lead to sophisticated predictive models for determinants of specificity in protein–glycan interactions in the near future. This endeavour will be supported by the great advances in techniques for transcriptome and proteome analysis [59••,60], and by the increasing number of mutants generated in model systems such as *Arabidopsis*, *Drosophila* and zebrafish. Such strategies will corroborate the function of likely candidates for the perception of CO signal molecules, such as CLPs, legume-specific ectoapyrases and various HA-binding proteins. In the context of this review, it will be most exciting to link the function of these proteins in cell migration in tissue culture systems to functions in embryogenesis.

Acknowledgements

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