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MINIREVIEW

Revealing the anti-HRP epitope in *Drosophila* and *Caenorhabditis*

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Abstract Antibodies are very often used as specific cell and/or tissue markers. An example of this is anti-horseradish peroxidase (HRP), an antibody raised against a plant glycoprotein, which was shown some twenty-five years ago to specifically stain neural tissue in an animal, *Drosophila melanogaster*. This peculiar finding was later expanded to other invertebrate species including *Caenorhabditis elegans*, which were also shown to bear anti-HRP epitopes. Initial experiments indicated that the epitopes recognised by anti-HRP in invertebrates are of carbohydrate nature. Indeed, more recent experiments have characterised relevant core α1-3-fucosylated N-glycan structures that act as epitopes in various model and parasitic organisms. Moreover, a number of enzymes required for the synthesis of such structures have been identified. Over the years, medically-relevant roles of these structures have become apparent as regards allergenicity and immunoregulation. Although major advances have been made in understanding of the underlying mechanisms and structures related to the anti-HRP epitope, the *in vivo* role of the relevant epitopes in neural and other tissues is yet to be resolved. Current understanding of the anti-HRP epitopes synthesis and their relevance is discussed and elaborated.

Keywords Anti-HRP · N-glycan · Fucose · Invertebrates · Neuron · Allergy · Immunogenic

Abbreviations

GlcNAc-TI	*N*-acetylglucosaminyltransferase I
HRP	horseradish peroxidase

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$MGnF^6$	Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4(Fucα1–6)GlcNAc
MM	Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc
MMF^3F^6	Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4(Fucα1–6)(Fucα1–3)GlcNAc (see also Fig. 5)
$MMXF^3$	Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc (see also Fig. 3)
MO	Manα1–6Manβ1–4GlcNAcβ1–4GlcNAc
$MOXF^3$	Manα1–6(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc
$Man4XF^3$	Manα1–3/6Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc

Neuronal anti-HRP staining observed exclusively in moulting animals

While generations of developmental scientists have spent endless hours at microscopes searching for the first occurrence of obvious differences in cell shapes, they have also sought to use staining methods to aid examination of the starting point of tissue-specific cell division. Originally, dyes were used, but in more modern times, molecular markers were sought which, as part of the cellular differentiation process, are presented on the cell surface and are therefore accessible to antibodies. In 1982, fluorescein or rhodamine-coupled forms of antibodies raised in either goat or rabbit against a plant enzyme, horseradish peroxidase (HRP), were used for the first time to specifically stain neurons in the central and peripheral nervous system in all developmental stages of *Drosophila*

Fig. 1 Typical anti-HRP staining of the two longitudinal fibres in a *Drosophila* embryo (*ventral view*)

melanogaster (embryos, larvae and adult flies; see, *e.g.*, Fig. 1) as well as in grasshoppers. Furthermore, some other structures with high pinocytotic activity like rectal papillae, Garland cells and pericardial cells in adult flies and accessory glands in the adult male's reproductive system showed this staining as well.[1]

The neuronal anti-HRP staining occurs very early in the well-defined embryogenesis of the model organism *Drosophila melanogaster*, which passes through a number of characteristic time points observed with microscopic studies; specifically, at about 3 h after fertilisation, cellularisation and blastoderm formation take place. Gastrulation[2] is completed at ~5 h, while embryonic development proceeds for the next 16 h until the first instar larvae hatches at about 22 h. Two further instar larvae phases succeed at 48 and 72 h after egg laying (AEL) before the pupae is formed at approximately 120 h AEL. Neuroblasts are firstly distinguishable by their size in 4 h-old embryos. The typical anti-HRP staining with the left and right longitudinal fibres appears in the 8 h-old embryo (Fig. 1), with less intense staining already visible earlier in embryogenesis. Whereas all neuronal cells can be stained, their ancestors (the neuroblasts) remain undetected. Thus the staining was a useful aid for following the appropriate neurogenesis in wild type and mutant flies [1]. An *in vitro* study on gastrula stage embryonic cell culture showed a weak staining in 5 h-old cells (time refers to post-oviposition) even before any morphologically-apparent changes take place [2].

Examination of extracts for the developmental expression profile of the antigen and its distribution in adult flies showed an increase of proteins (up to 10) detected with the anti-HRP antiserum from larvae to adult flies with several bands present only in the male abdomen. This enrichment of male-specific components in the abdomen, which should not contain many neurons, is mainly due to water-soluble proteins. One of these non-membrane associated proteins was identified as *Drosophila* esterase-6, a male specific protein that regulates mating and is present at high levels in the accessory glands; it is, therefore, one of the candidates responsible for the non-neural anti-HRP reactivity in male adult flies [3].

The anti-HRP staining of the neuronal system was not only found in insects, but also in *Caenorhabditis elegans* where 27 out of the total of 302 neurons are recognised. Since this is only a subset of the neurons, it appears that there is a different pattern of expression of the epitope in this model organism. Interesting is the finding that the anterior mechanosensory neurons stain, whereas the posterior ones do not. Amongst non-neural tissues, sensory neuron support cells, gland cells, intestine, valve cells, gonads and egg shell are also recognised by the anti-HRP antiserum [4]. Only recently the conservation of this epitope throughout the insect class and the whole clade of Ecdysozoa (moulting animals either with chitin or collagen) led, on the molecular level, to confirmation of the rearrangement of the animal phyla [5–7] (see Fig. 2). The traditional groups of the animal phyla were recently abandoned and the Protostomians, based on 18S ribosomal RNA gene sequences, were split in two major clades the Lophotrochozoa and the Ecdysozoa. Although the Ecdysozoa showed neural expression of the anti-HRP epitopes, which was completely absent in Lophotrochozoa or Deuterostomians (including vertebrates) and, as such, supports this new classification [5–7], it should be noted that at least some Lophotrochozoa express anti-HRP epitopes, *e.g.*, molluscs; such epitopes are presumably of non-neural origin.

Since 1982 researchers tried to reveal the biochemical nature of the epitope by performing different sets of experiments. They started off with proving that the anti-HRP reactive sites themselves did not have peroxidase-activity *per se*. Pre-incubation of the antibody with HRP eliminated the staining, indicating that the plant enzyme HRP and the neuronal cells share common epitopes recognised by the antibody [1, 8]. The carbohydrate nature of the epitope was implied by abolition of the staining after incubation of the *Drosophila* embryos with the carbohydrate oxidising reagent meta-periodate; furthermore, the staining could be also blocked by pre-incubation of the antibody with glycopeptides derived from pronase-digested HRP whereas the use of trifluoromethanesulfonic acid (TFMS)-deglycosylated peptides of the HRP pronase digest or periodate-treated HRP could not abolish the staining, when pre-incubated with the anti-HRP antiserum [8, 9]. Thus, it appeared that aspects of the glycan structure of HRP are responsible for the cross-reactivity towards invertebrate proteins. Therefore, before discussing further the nature of the epitope in invertebrates, we describe what is known about the glycans of HRP itself.

[1] Rectal papillae: specialised part of an insect's rectum being responsible for water and ion uptake; Garland cell: nephrocyte taking up waste products from the haemolymph; pericardial cell: nephrocyte contributing to haemolymph recycling; accessory glands: become functional in adult insects, their secretion is involved in the reproductive system (*e.g.*, spermatophore production)

[2] During gastrulation cells migrate to form the first three layers: mesoderm, endoderm and ectoderm; from the latter the brain and nervous system will develop.

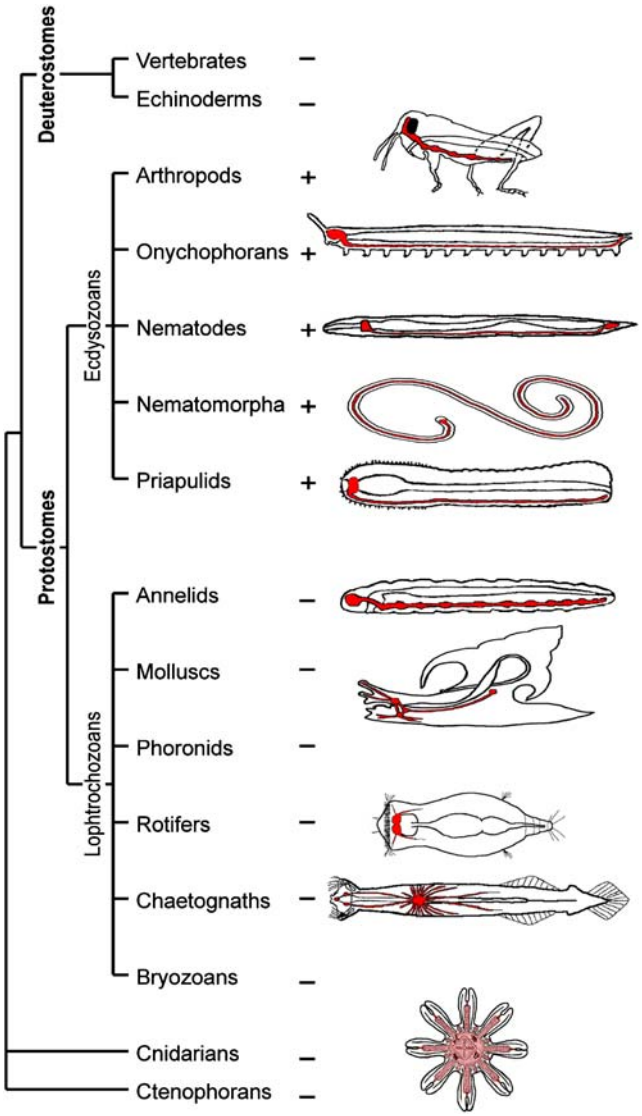


Fig. 2 Recent phylogenetic studies have split the protostomes into Lophotrochozoa and Ecdysozoa, the latter clade showing neuronal anti-HRP-staining (indicated by +); reproduced from Ref. [5] and used with permission of the authors. The neural systems of these animals are *highlighted*

The typical structural features found on plant glycans are present on HRP

The biochemical structure of the carbohydrate epitope shared by the plant glycoprotein (HRP) and neuronal surface proteins from insects was revealed rather slowly. In 1970 the first glycan structure of a plant glycoprotein derived from stem bromelain was described to consist of a chitobiose structure linked to asparagine of the peptide, albeit the use of periodate oxidation and enzymatic release to determine the xylose and fucose linkages resulted in an incorrect structural definition [10]. Only in 1979 was the asparagine-linked oligosaccharide structure correctly described, from the same protein, using methylation analysis; in this study, the typical plant core modifications with xylose bound β1→2 to the core mannose residue and fucose bound α1→3 to the innermost GlcNAc residue were defined for the first time [11] (for examples of plant N-glycans, see Fig. 3).

In contradiction to the relevance of this epitope, it took then another 11 years before the glycan composition of the plant glycoprotein HRP itself was closely looked at. A typical plant glycan structure, so-called MMXF^3, representing 80% of the total glycan structures was found on the purchased Sigma HRP type VI enzyme [12]. This enzyme preparation, used to raise the anti-HRP antiserum, contains mainly the isoenzymes C1, C2 and traces of the other compounds (B1–B3, D, E1–E6 representing the neutral-basic isoforms), while the most acidic forms A1–A3 were not present [13]. To explain this historical enzyme nomenclature, one has to go back to the year 1966. In that year, seven isoenzymes, segregated on basis of their electrophoretic behaviour, were firstly isolated from horseradish roots containing each an iron (III) protoporphyrin IX group as prosthetic group as well as carbohydrate moieties. For instance, xylose and fucose were found on the isozymes B and C, whereas galactose and arabinose, the building units of arabinan, were found specifically on the isozymes A1–A3 [14]. The isoenzyme C, the most abundant form and therefore in the centre of research attention, has nine potential N-glycosylation sites of the typical sequence motif Asn-X-Ser/Thr, which were recently all found to be occupied [15]. A more detailed analysis of the Sigma derived (type VI) HRP revealed that members of the (Xyl) Man_{2-4}(Fuc) oligosaccharide family (*e.g.*, MMXF^3, Man4XF^3 and MOXF^3) account for ~80% of the total glycan structures [16] (see also Table 1). In one Sigma lot, the N-glycans were not mainly composed of MMXF^3; also MMX was found and each of these structures represented 30% of the total. In this case, the proportion of the isoenzymes in the lot used was not mentioned at all [17].

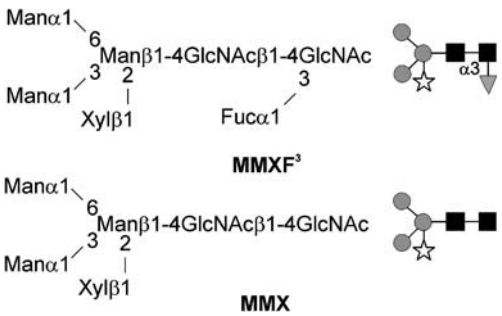
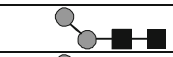
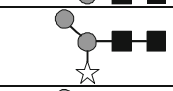
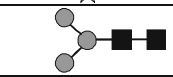
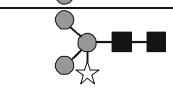
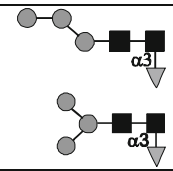
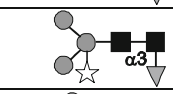
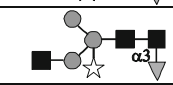
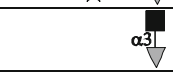
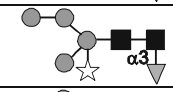
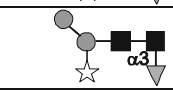
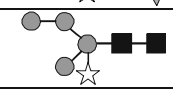
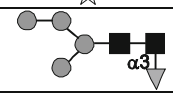
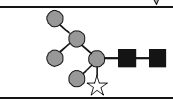
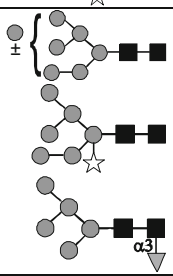


Fig. 3 On plant N-glycans, the core modifications, xylose bound β1→2 to the core mannose residue and fucose bound α1→3 to the innermost GlcNAc, are frequently found. The structures are depicted in both the condensed branched (IUPAC) and symbolic (Consortium for Functional Glycomics) nomenclatures

Table 1 Summary of glycans found on HRP. The following abbreviations are used: for hexose (*H*), hexosamine (*N*), fucose (*F*), pentose (*P*, *i.e.*, xylose, *X*)

N-glycan structure	composition	Wuhrer [15]	Yang [16]	Takahashi [17]
	H2N2	Not found	Not found	4%
	H2N2P1	On 1 site	Not found	7%
	H3N2	On 1 site	Not found	13%
	H3N2P1 (MMX)	On 1 site	5%	31%
	H3N2F1	On 3 sites	4%	9,5%
	H3N2F1P1 (MMXF^3)	On 8 sites	70%	33%
	H3N3F1P1	Not found	Not found	3%
	N1F1	On 7 sites	Not found	Not found
	H4N2F1P1 (Man4XF^3)	On 2 sites	5%	Not found
	H2N2P1F1 (MOXF^3)	Not found	6%	Not found
	H4N2P1	Not found	4%	Not found
	H4N2F1	Not found	3%	Not found
	H5N2P1	Not found	2%	Not found
	H7N2, H6N2, H5N2F1, H6N2P1	Not found	Minor compounds	Not found

The unusual disaccharide F^3-Gn results either from an endoglycosidase activity or may be a mass spectrometric fragmentation artefact [15]. The results of the cited studies are summarised and show that the heterogeneity of the found glycan structures is dependent on the sample lot, the purity of the HRP isoenzymes and the analytical methods used

Core α1-3-fucose is responsible for the reactivity of anti-HRP towards plant glycans

The xylose and α1-3-fucose residues on HRP are absent from mammalian glycoproteins; thus, it is not surprising that these glycans are perhaps immunogenic. During examination of the actual epitope, it was, for instance, found that the anti-HRP antiserum recognises plant glycoproteins possessing similar N-glycan structures, *e.g.*, pineapple stem bromelain and HRP, but did not bind to those on mammalian fetuin or RNase B; this result emphasised that the components β1,2-linked xylose and the α1,3-linked fucose are most likely to be involved in the recognition process [8]. Others investigating the required minimum structure for reaction with the antibody, using either defucosylated, dexylosylated or demannosylated glycan derivatives linked to BSA before absorption to ELISA plates, showed that the α1-6-linked mannose together with the α1-3-linked fucose were the major epitopic structures recognised by anti-HRP antibodies [12, 18]; later studies have used recombinant glycosyltransferases to generate neoglycoconjugates in order to show that modifications with core α1-3-fucose and xylose are required for this recognition [19, 20].

There is an interesting parallel to the *c*ross-reacting *c*arbohydrate antigenic *d*eterminants (CCD), which were found to be partially responsible for IgE (cross-)reactivity of allergy patients; CCDs are based on the widespread occurrence of nearly identical sugar modifications in different species. Such cross-reactivity is destroyed after periodate or TFMS treatments [21, 22] and is inhibited when IgE is preincubated with defined glycan structures [23], thus proving the glycan nature of the epitopes concerned. To initiate the histamine release of mast cells and basophils, these antigens have to be polyvalent (carrying more than one epitope) in order to cross-link the cell-bound IgE. Therefore, glycoproteins with only a single N-glycosylation site were not effective in histamine release. The cross-reactivity was found to be caused by common epitopes shared by glycoproteins from plant sources (*e.g.*, food [24] or pollen [25]), molluscs, crustaceans and insects but not by vertebrates, fungi or algae [21, 26–29]. Plant glycans share the MMXF^3 motif; therefore, various groups tried to find out which residues contribute to the binding. Some assumed that xylose was the relevant part, but this conclusion was based on incorrectly-defined glycan structures on glycoproteins used as controls, *e.g.*, claims that bromelain, phaseolin and ascorbate oxidase glycans are modified only with xylose [22, 26, 30]. Others proved that especially core α1-3-fucose was the relevant determinant on plant glycoproteins [18, 28, 29, 31], on insect glycoproteins [28] or on *in vitro* modified glycoproteins [23, 29]. The α1-6-mannose seemingly plays a role, but on itself is not sufficient for interaction [18, 31, 32] (see Fig. 4).

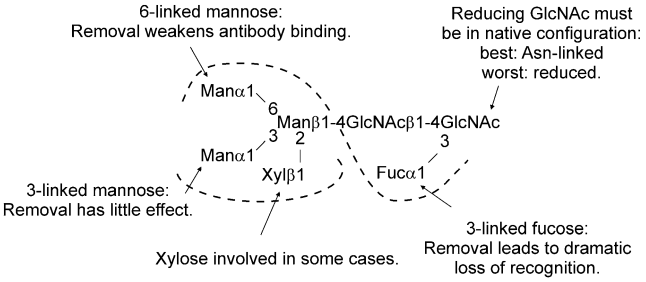


Fig. 4 Contribution of individual residues to anti-HRP reactivity. Structural analysis revealed that the α1,3-Fuc and the α1,6-Man residues are presented in one level, thus representing a spherical epitope recognised by antibodies while xylose is on the opposite side. The information contained in this figure is based on various direct binding assays and inhibition studies

Identifying the fruit fly proteins modified with the epitope and its *in vivo* significance

Although the structural basis for the reactivity of anti-HRP towards plant glycans was clear, this was not the case for the cross-reactivity towards *Drosophila* proteins. As mentioned above, early studies used periodate oxidation and inhibition with plant glycoconjugates to show that carbohydrate may indeed have a role in the recognition, but the presence of potentially cross-reactive glycans had not been examined. However, a major focus was the identification of the fly proteins, which could bind anti-HRP antibodies and not the fly glycans *per se*; nevertheless, such studies offered hints towards an understanding of the *in vivo* significance of this cross-reactivity. Neuronal proteins carrying the epitope were identified as membrane associated proteins, because of their restricted solubility. Either SDS-extracts or 10 mM deoxycholic acid had to be used since neither aqueous buffers nor some non-ionic detergents such as NP40 or Triton-X100 could extract significant amounts out of the homogenised samples [3]. The presence of a common epitope shared by many surface membrane glycoproteins, instead of an epitope on a single surface protein, was shown by immunoprecipitation of different glycoproteins with anti-HRP antibodies. Indeed, seventeen spots on a 2-D gel showed interaction with anti-HRP. Nevertheless, the exact number of proteins carrying the epitope is not certain because of the occurrence of degradation effects or isoforms of the same protein [9]. Several of these glycoproteins participate in axon guidance and fasciculation and share an interesting feature: when these proteins are expressed in neuronal cells or tissues they do, at least in part, carry the epitope whereas the ones derived from other cells/tissues do not.

Examples of proteins interacting with anti-HRP antibodies include fasciclin I (an extrinsic membrane protein inserted via a GPI anchor), fasciclin II (an integral membrane protein), playing a role in axon path finding [9,

33], and other cell adhesion and signal transduction molecules like neurotactin, neuroglian and *r*eceptor-linked *p*rotein *t*yrosine *p*hosphatases (R-PTP: DPTP69D, DPTP10D DPTP99A) [34]. Fasciclin I, II, III, R-PTPs and neuroglian are, as judged by their immunoglobulin and fibronectin type III-like domains, homophilic adhesion molecules, while neurotactin is a heterophilic adhesion molecule. Another anti-HRP reactive ~40 kDa glycoprotein isolated from *Drosophila* heads was used for monoclonal antibody production. In fly extracts, these antibodies recognised three proteins, which were named Nervana (*nerv*e antigen) because these antibodies also stained neurons. The three proteins coded by two separate *nrv* genes shared homology with the β subunit of Na^+/K^+ ATPase [35, 36]. Nrv 2 is expressed in the nervous system where it is carrying the anti-HRP epitope, while Nrv 1 is expressed in muscles. The actual role of the specifically-expressed antigen in neurons still remains to be solved. Incubation of cultured neuronal cells from insect origin with anti-HRP antibodies (1 mg/ml) did not result into a perturbation of the morphological appearance of the culture. Therefore, the conclusion was drawn that the antigen has probably not an important regulatory function in the differentiation process [2]. However, a cell culture is not a highly organised organism with a high requirement for cell-to-cell interaction; therefore, a lack of a cell surface epitope (or its sequestration) is very likely not to result in visible changes. On the other hand, the conservation of this epitope throughout the insect class and even throughout the clade of Ecdysozoa suggests an important function [5–7]. Over the years, a number of *Drosophila* mutants, which carry various gene mutations/chromosome rearrangements and lack anti-HRP staining, have been described: *nac*, TM3, Brd^{15}, $Mgat1^1$, Gmd^1 and *Rfx*. Except in the case of *nac* [8], it is clear which gene is responsible in each case. In TM3 balancer lines and the Brd^{15} mutant, anti-HRP staining can be restored by re-introduction of *tollo* [37]; in $Mgat^1$ mutants, the *N*-acetylglucosaminyltransferase I (GlcNAc-TI) required for generation of hybrid and complex N-glycans is deficient [38], whereas Gmd^1 mutants [39] lack the GDP-mannose dehydratase [40] required to generate the GDP-Fuc donor for fucosyltransferases and *Rfx* mutants lack a transcription factor expressed in neural tissue [41]. The most recent examples ($Mgat^1$ and Gmd^1) can, of course, be interpreted in the light of what we now know about *Drosophila* glycosylation (see section below).

The *nac* mutant (*n*eurally *a*ltered *c*arbohydrate) from *Drosophila*, which maps to the right arm of the third chromosome in the interval 84F4–11, lacks the epitope in adults but displayed, on first sight, normal behaviour, muscle response and brain morphology. Only a decrease of the growth temperature to 18°C resulted in display of morphological abnormalities of the wing and eye facets as well as female sterility. The embryonic and larvae nervous system of the mutant do stain with anti-HRP antibodies, but with progressing metamorphosis the staining is reduced until it is completely abolished in the adult neural tissue [8]. A few years later, a reappraisal of the *nac* and *fas* (fasciclin) phenotypes indicated misrouting of axons and appearance of ectopic neurons in the developing wing sensory system of *Drosophila* implying that the cell surface molecules or modifications play a role in both neurogenesis and axon guidance [42]. As briefly mentioned above, a mutation that abolishes the HRP-epitope expression is also carried on the TM3 balancer chromosome. The TM3 homozygote embryos lack the neuronal epitope, but still express it in garland glands, anal pads and the posterior hindgut. Furthermore, the relevant mutation was localised to a cell surface receptor with homology to the Toll protein, bringing forward the assumption that a cell-specific signalling pathway, leading to expression of the anti-HRP epitope, is affected in this mutant [37].

Core α1-3-fucose in insects

When considering anti-HRP cross-reactivity in insects, there were different speculations about the biochemical nature of the epitope. Based on the work with plant glycans, either core α1-3-fucose and/or β1-2-xylose on N-glycans could be theoretically responsible. However, no xylose has been found on any insect N-glycan. On the other hand, structural analysis of the N-glycans of honeybee venom glycoproteins was a first indication that the anti-HRP cross-reactivity towards insect glycoproteins (as well as the cross-reactivity of anti-bee venom with plant glycoproteins) may be due to core α1-3-linked fucose. The initial surprise was, also, that core α1-3-fucose was generally in the context of a difucosylation of the core GlcNAc of, *e.g.*, phospholipase A_2 glycans released using PNGase A [43]. The relevant enzyme activity was also detected in honeybee venom gland extract: this novel α1,3-fucosyltransferase had the ability to convert $GnGnF^6$ into a difucosylated structure with a Fucα1-6(Fucα1-3)GlcNAc moiety, a result which was verified with NMR. The presence of more than one Fuc residue linked to the same monosaccharide had not been shown before. The prior action of GlcNAc-TI is obviously necessary in insects since no transfer to either MM or MMF^6 was detected [44]; this has now been confirmed with a recombinant form of honeybee core α1,3-fucosyltransferase [45].

A further indication that insects were indeed capable of core α1-3-fucosylation came from the study of various lepidopteran cells: MB-0503 (*Mamestra brassicae*), Bm-N (*Bombyx mori*) and Sf-9 (*Spodoptera frugiperda*) cells. For instance, MB-0503 extracts detectably converted the IgG

$GnGnF^6$ glycopeptide into the difucosylated structure [46], whereas analysis of PA-labelled glycans released from membrane glycoproteins of these three cell lines (Sf-21, MB-0503, Bm-N) indicated the presence of MMF^6, MMF^3, MOF^6, MOF^3F^6, $MGnF^6$, $MGnF^3F^6$, $GnMF^6$ and $GnMF^3F^6$. MB-0503 exhibited the highest degree of α1-3-fucosylated glycans but the two others were also capable of synthesising low levels of these structures [47]. In *Trichoplusia ni* (High Five™) cells, difucosylated glycans are rather common in the overall glycomic profile [48] and are present on various recombinant proteins produced in these cells [49, 50]. The presence of core α1-3-fucose in lepidopteran cell lines is not just of significance for what it says about the glycomic potential of insects in general, but is also of interest due to the use of these cells in biotechnology; these aspects are discussed in a couple of recent articles [51, 52].

The first study specifically on *Drosophila* N-glycans was published in 1991 and predates the aforementioned studies on honeybee α1-3-fucosylation; however, other than the expected well-known oligomannose series, α1-6-fucose linked to the inner GlcNAc on MM and MO was found. The authors admitted that some structures could not be revealed due to their low occurrence and neither core α1-3-fucose nor β1-2-xylose, two substitutions typical for plants, could be detected [53]. It was only later, in 2001, that the final piece in the puzzle about the basis for anti-HRP staining in *Drosophila* was found; then, finally, the presence of MMF^3F^6, as 1% of the total N-glycan pool, could be proven in PNGase A released N-glycans from *Drosophila* (see Fig. 5 for this structure). The relevant activity of a recombinant fucosyltransferase (FucTA), tested *in vitro* to transfer fucose in α1,3-linkage to the innermost GlcNAc of $GnGnF^6$, GnGn, $GalGalF^6$ and GalGal, but not to MMF^6 nor to MM, was also defined [54]. RNAi targeting the transcripts of FucTA was found to result in a decrease in anti-HRP reactivity of a *Drosophila* neural cell line [55]. Furthermore, an endogenous C-type receptor capable of binding to glycans with core α1-3-fucose was found in the model organism [56]. Around the same time, the first report of difucosylation on a particular protein was reported: one specific male sex peptide was found to carry

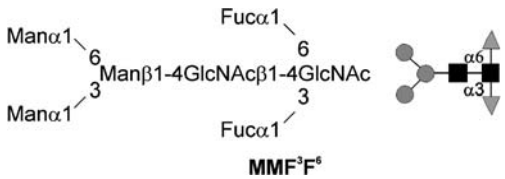


Fig. 5 The identification of MMF^3F^6 finally proved the presence of α1-3-linked fucose in the fruit fly responsible for the anti-HRP binding; this structure has been verified in various studies on fly adult, embryo and neural cell line N-glycans [54, 58–60]

such a structure [57], compatible with the non-neural staining of the *Drosophila* male reproductive system.

Core α1-3-fucose in nematodes

In comparison to insects, glycomic information on nematodes is even a more recent field. As mentioned above, cross-reactivity of anti-HRP towards *Caenorhabditis* was first reported in 1991; the first indication of double fucosylation, like that on some insect glycans, of the core of nematode N-glycans came from work by Dell and colleagues on the sheep parasite *Haemonchus contortus* [61, 62]. Indeed, in this species, there was the first indication that nematodes can modify the chitobiose core unit of N-glycans with up to three fucose residues. It was also found that IgE from *Haemonchus*-infected sheep reacts with nematode glycoproteins, due to the recognition of core α1-3-fucose [63]. Unlike the studies on *Drosophila melanogaster* glycoproteins, none of the *Caenorhabditis* proteins carrying the epitope have been defined to date and, thus, it remains to be determined as to whether homologous proteins are modified in this manner. Nevertheless, a number of groups have studied the *Caenorhabditis* N-glycan structures (for a review, see refs. [64, 65]). Around 150 different structures ranging from paucimannosidic and difucosylated to phosphorylcholine-modified N-glycans were found in wild-type and mutant *Caenorhabditis*; this is an indication of the glycomic complexity of the worm in comparison to *Drosophila*, where "only" around 50 N-glycan structures were identified [54, 58]. Other than *Caenorhabditis*, difucosylated glycans have also been found in *Ascaris suum* [66] and *Parelaphostrongylus tenuis* [67]. However, galactose capping of core fucose residues has, amongst nematode species, only been observed in *Caenorhabditis* [68, 69]; such a modification may, of course, make core α1-3-fucose 'hidden' from antibodies and lectins (Fig. 6).

No xylose was detected on any nematode N-glycan to date; thus, the core α1-3-fucose modification was the most likely source for cross-reactivity to anti-HRP. The most direct proof for this conclusion came from an analysis of five putative α1,3-fucosyltransferase mutants: Western blotting indicated that anti-HRP cross-reactivity was lost, when the

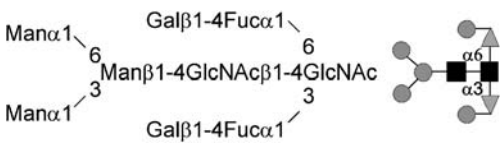


Fig. 6 An example of a *Caenorhabditis* N-glycan structure containing α1-3-linked fucose capped with a galactose residue. The α1,6-linked fucose can also be modified with a β1-4-linked galactose residue

fut-1 gene was partly deleted [70]. This gene was found to encode a novel type of core α1-3-fucosyltransferase, whose activity is inhibited by the presence of non-reducing terminal GlcNAc on the α1,3-arm of an N-glycan; all other core α1,3-fucosyltransferases from plants and insects characterised to date, as well as the worm's core α1,6-fucosyltransferase [71], actually require the presence of such a residue. In *Caenorhabditis* the lack of the anti-HRP epitope is, at least under controlled lab conditions, not affecting the viability or behaviour of the mutant worm; however, this is also the case with a number of other worm N-glycan mutants. The role in the 'wild', though, of this modification remains unknown and may require special phenotypic testing. It is interesting to note that experimental Th2 responses (*i.e.*, immunological responses relevant to IgE production in nematode infections and in allergy) to *Caenorhabditis* may be fucose-dependent [72], whereas GlcNAc-TI knock-out worms display altered susceptibility to bacterial infection [73].

Core α1,3-fucose in trematodes, molluscs and slime moulds

Other invertebrate species, other than insects and nematodes, also express anti-HRP epitopes, although these are not necessarily on neuronal glycoproteins. Squid rhodopsin has been found to carry core α1-3-fucose [74], as have N-glycans from various gastropod species [75, 76], although some snail species have xylosylated, but not core α1-3-fucosylated, N-glycans [77, 78]. Also, the trematode parasites, particularly *Schistosoma* spp., express both xylose and core α1-3-fucose residues, which is interesting from the perspective that their snail intermediate hosts also have such glycans and that schistosome N-glycans play a role in T-cell (Th2) responses to the parasite [79]. The relevant *Schistosoma mansoni* core xylosyltransferase and fucosyltransferase activities have been detected in egg extracts [79], but the genes encoding these enzymes have not yet been identified. However, the mode of core fucosylation is akin to that in insects but not that in nematodes, in that both the core α1,3- and α1,6-fucosyltransferases in schistosome extracts require the presence of a non-reducing terminal GlcNAc on the α1-3-arm of an N-glycan; furthermore, as in insects and nematodes, core α1-6-fucosylation must precede core α1-3-fucosylation [71]. Lowest in the evolutionary tree, as far as anti-HRP epitopes of non-plant species is concerned, are probably the slime moulds (social amoeba); reactivity to anti-HRP towards *Dictyostelium discoideum* has been claimed to be due to the presence of xylose [80]. The latest results from our own laboratory, though, indicate that no xylose is present on *Dictyostelium* N-glycans, but that core α1-3-fucose is a dominant feature of the glycome in this species [81]. The genetic basis for this modification is currently under investigation.

Conclusion

Cross-reactivity to anti-horseradish peroxidase is a widespread biochemical phenomenon and is observed not just in plants, but also, as discussed here, in invertebrates and in at least one protozoan. In only some species, particularly insects and nematodes, this cross-reactivity is associated with neuronal glycoproteins; however, in non-moulting animals this is not the case. Modern comparative glycomics and genetic approaches have yielded much data as to the nature of these epitopes; however, the biological role of anti-HRP epitopes, whether core α1-3-fucose or xylose-associated, is unknown, although they are immunogenic and are associated with Th2 and IgE responses. A very recent study has shown a potential biological role for these epitopes in plants: when all the fucosyl- and xylosyltransferases required for anti-HRP epitope formation in *Arabidopsis* are knocked-out, the altered function of an endo-β1,4-glucanase affects salt tolerance [82]. In invertebrates, one can assume that anti-HRP epitopes on specific proteins are recognised by endogenous lectins and that, due to the cell-type specific expression, they have a role in cell–cell interactions. It is clear, though, the challenge we now have is to move beyond the 'phenomenological' studies of 'where' and genetically 'how' these epitopes are expressed to 'why'. Thus, we still need to know the reason why glycans make a worm or a fly a bit like a plant and why they make a worm more 'complex' than a fly.

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