Hemomucin, an O-Glycosylated Protein on Embryos of the Wasp *Macrocentrus cingulum* That Protects It against Encapsulation by Hemocytes of the Host *Ostrinia furnacalis*

Jian Hu^a Qiuyun Xu^a Shengfeng Hu^a Xiaoqiang Yu^b Zhikun Liang^a
Wenqing Zhang^a

^aState Key Laboratory of Biocontrol, Sun Yat-sen University, Guangzhou, PR China; ^bDivision of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Mo., USA

Key Words

Parasitoid · Hemomucin · Immune evasion · Encapsulation · O-glycosylation

Abstract

It is unclear how endoparasites passively evade their host's immune reactions in most parasite-host systems. Hemomucin from the parasitoid wasp *Macrocentrus cingulum* (McHEM) is a 97-kDa transmembrane protein containing 51 potential O-glycosylation sites that can be specifically recognized by *Arachis hypogaea* lectin. Mcchem mRNA is highly expressed in *M. cingulum* eggs, morulae and secondary embryos, and McHEM protein is mainly located on the extraembryonic membrane of embryos. When secondary embryos of *M. cingulum* were transplanted into naïve larvae of their host, *Ostrinia furnacalis*, the embryos proliferated to generate dozens of embryos. However, more than 90% of these embryos were encapsulated by host hemocytes after blocking with anti-McHEM serum. Similarly, following knockdown of Mcchem expression using double-stranded RNA encoding Mcchem (*dschem*), many more embryos were encapsulated by host hemocytes after transplantation compared to controls ($p < 0.01$). Furthermore, approximately 70% of the embryos

were encapsulated by host hemocytes following digestion with O-glycosidase, which specifically digests β -gal (1→3) linkages between GalNAc and Ser/Thr of proteins. Western blotting results showed that O-glycosidase digested McHEM into a smaller product. These results indicate that McHEM may protect embryos from being encapsulated by their host and that the McHEM sugar chains play an important role.

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Introduction

Most endoparasites develop in the host hemocoel, where they are fully exposed to circulating hemocytes and inevitably attacked by the host immune system [1]. In some parasitoids, eggs and larvae are covered by surface factors that are either not recognized as foreign or that do not adhere to hemocytes, which may protect the parasitoid from the host's immune reaction [2–7]. However, it is unclear how surface factors protect wasps and allow them to evade the host's immune reaction.

Encapsulation is the main cellular immune reaction that a host deploys against a parasitoid, and the first step of this process is non-self-recognition [8]. If there are fac-

tors that cannot elicit non-self-recognition on the surface of a wasp's egg, then the encapsulation reaction will not be initiated. The function of glycoproteins as protective surfaces that allow immune evasion of parasitic protozoa has been reported [9–12]. Mucins are O-glycoproteins that are composed of Tn antigen, which is produced by the addition of an N-acetylgalactosamine residue (GalNAc) to a serine or threonine on a protein and sugar molecule(s), including galactose (Gal), N-acetylglucosamine (GlcNAc) and fucose to constitute core structures. In mammals, there are 4 types of core structures, but in insects only the T antigen (core 1) is identified. T antigen is composed of the Tn antigen and a Gal added onto GalNAc, and this is the most abundant post-translational modification in animals [13, 14]. Surface mucins in vertebrates are involved in regulating the attachment of blood cells during immune responses. Mucin 'umbrella' structures inhibit cell-cell or cell-substrate interactions and must be released from cells to allow adhesive interactions [15, 16]. Surface mucins play an important role in host invasion in parasites; in addition, these molecules are thought to be involved in immune evasion. For example, in the parasitic nematode *Toxocara canis*, the TES-120 mucin-like protein on the nematode's surface coat is thought to evade host immunity by binding antibodies to shed the surface [17]. In *Trypanosoma brucei*, a protective surface coat comprising many copies of variant surface glycoproteins plays an important role in evading host immune responses [13].

Although the immune evasion function of glycoproteins has not received substantial attention in insects, some indirect results have shown that glycoproteins should also play an important role in insects, particularly with regard to hemomucin. Hemomucin, an O-glycosylated surface mucin, could induce the expression of anti-bacterial peptides after binding a GalNAc-specific lectin, and it has so far only been cloned in *Drosophila* [18]. In the parasitoid *Venturia canescens*, hemomucin was shown to form a complex with lipophorin and function against the host defense system [6]. This hemomucin-lipophorin complex was also proposed to contribute to hemocyte attachment and hemolymph coagulation in *Drosophila* [19, 20]. In addition, hemomucin has a relationship with immunity in other insects. For example, in *Anopheles gambiae* and *Bombus terrestris*, the expression of hemomucin was upregulated when the insects were infected with inactivated *Salmonella typhimurium* or the trypanosome parasite *Crithidia bombi* [21, 22]. *Macrocentrus cingulum* is a type of polyembryonic wasp that commonly produces approximately fifty offspring from

one egg. *M. cingulum* embryos reside in the larvae of the host and directly contact host hemolymph, but are never encapsulated by the host. Previous studies have shown that *M. cingulum* passively avoids the host's immune reaction using a factor on the extraembryonic membrane [7]. Here, we further report that the O-glycoprotein hemomucin on the extraembryonic membrane may allow the embryo to evade the host's encapsulation reaction and that the hemomucin sugar molecules play an important role in this evasion.

Materials and Methods

Insects

The rearing of *M. cingulum*, its lepidopteran host *Ostrinia furnacalis* and the parasitism between these species were performed as previously described [7, 23]. The parasitoid and host larvae were rejuvenated once a year. All solutions, plastic- and glassware used in the study were sterilized.

cDNA Cloning, Sequence Alignment and Structural Analysis

Total RNA was isolated from the embryos of *M. cingulum* using the TRIzol reagent (Invitrogen, Carlsbad, Calif., USA), and first-strand cDNA was prepared with the reverse transcriptase XL (AMV; TaKaRa, Toyko, Japan). Degenerate primers hemo-F1 and hemo-R1 (table 1) were designed based on the conserved hemomucin cDNA sequence from *Drosophila* (GenBank accession No. NM_057811) and genomic DNA sequences of insects *Apis mellifera* (GenBank accession No. XR_015026) and *Nasonia vitripennis* (GenBank accession No. XM_001605565), whose entire genome has been sequenced. Polymerase chain reaction (PCR) was performed in 25- μ l reactions with the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 48°C and 60 s at 72°C, then a final extension at 72°C for 10 min. The PCR product was cloned into the Pmd18-T vector (TaKaRa) and sequenced using the dideoxynucleotide method. The full-length hemomucin cDNA was obtained using the BD SMART™ RACE cDNA amplification kit (Clontech, Mountain View, Calif., USA). The specific primers R-2 and R-3 for 5' RACE and F-2 and F-3 for 3' RACE (table 1) were synthesized based on the cDNA sequence obtained from PCR. PCR was carried out with the Universal Primer Mix UPN (Clontech) and R-2 or F-2 by denaturing at 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 10 min. Nested PCR was performed using the first-round PCR product as a template, the Nested Universal Primer NUP (Clontech) and R-3 or F-3. The RACE products were cloned and sequenced as described above. The full-length cDNA of hemomucin was obtained by assembling the three overlapping fragments. Finally, the specific primers F0 and R0 (table 1) were designed to verify the full-length cDNA sequence.

Multiple amino acid sequence alignments were analyzed using the ClustalW multiple-alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). A phylogenetic tree was constructed using MEGA 5.0 based on the known amino acid sequences of hemomucin in insects. The protein pattern and profile of McHem were ob-

Table 1. The primers used in cDNA clone, qRT-PCR and RNAi

PCR fragment	Primer name	Sequences (5'–3')
Mchem-1	F-1	AAMTGTGGBMGMCCYYTDGG
	R-1	ARMRYAACACCRTTKGCRAA
Mchem-2	UPM	from BD SMART™ RACE cDNA amplification kit
	R-2	CTCGGTAGTTGATACTGTCC
	NUP	from BD SMART™ RACE cDNA amplification kit
Mchem-3	R-3	CCGTAATAGGCATCAACA
	F-2	TGTTGATGCCTATTACGG
	UPM	from BD SMART™ RACE cDNA amplification kit
Full cDNA of Mchem	F-3	CGAGTACAAACTTTACGACCTT
	NUP	from BD SMART™ RACE cDNA amplification kit
qRT-PCR	F-0	GACAGTTATCTCCGATTATTTTC
	R-0	CATACGTGTTCACTCATC
dsRNA	Mchem-F	CATTGCCTACGTTGTTGA
	Mchem-R	CTCGGTAGTTGATACTGTCC
	McPRS3-F	GCTCATCGGAGGTCTGGCTGTC
	McPRS3-R	ACGTGGCGAGTTGCAGTGTGC
dsRNA	dshem-F	CTGGTAGCACGCTTTGGAA
	dshem-R	CAAGGGATGTTGCTCGTCTGC
	GFP-F	AAGGGCGAGGAGCTGTTACCG
	GFP-R	CAGCAGGACCATGTGATCGCGC

F = Forward; R = reverse; UPM = universal primer A mix; NUP = nested universal primer A.

tained from the PROSITE database using InterPro Scan (<http://www.ebi.ac.uk/InterProScan/>). The transmembrane helix and signal peptide were analyzed using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and TMHMM v2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). The potential O-glycosylation sites and phosphorylation sites were predicted using NetOGlyc 3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc/> and <http://www.cbs.dtu.dk/services/NetPHos/>).

qRT-PCR Analysis for Mchem

The expression of Mchem was analyzed by qRT-PCR using the SYBR Premix Ex Taq (TaKaRa) on a LightCycler 480 system (Roche, San Francisco, Calif., USA). The total RNA was extracted from the eggs, secondary morula, secondary embryos, larvae and pupae with illustra RNeasy Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. The mRNA contained in 3 µg of total RNA was reverse transcribed into single-stranded cDNA using the Oligo d(T)₁₈ primer with AMV reverse transcriptase (TaKaRa) at 37°C for 10 min, and then at 42°C for 60 min in the presence of 5× AMV buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 40 mM MgCl₂, 50 mM DTT),

10 mM dNTP and 20 U RNase inhibitor in a total volume of 20 µl. The primers hemo-F and hemo-R (table 1) were designed to amplify the target fragment of McHem. RPS3-F and RPS3-R (table 1) were used to amplify the cDNA of the ribosomal protein S3 gene that was used as the internal control. The cycling for each reaction was carried out in a final volume of 10 µl containing 0.3 µl of the cDNA sample (or standard), 0.2 µl (10 pmol/µl) of each primer and 5 µl of SYBR Premix Ex Taq (TaKaRa) in a LightCycler 480 system. All of the qRT-PCR reactions were performed with a common program as follows: a 30-second initial denaturation at 95°C and then cycling consisting of 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 15 s and elongation at 72°C for 10 s. The relative transcript abundance of McHem was quantified using the delta-delta Ct method. Each qRT-PCR measurement was performed in triplicate.

Western Blotting and Immunohistochemistry Analysis for McHEM and Peanut Lectin Labeling of Embryos

Polyclonal antisera were raised against two short peptides of McHEM that separately contained 16 amino acids (aa; 489–504 C-PKTPKFPVKTPQSET) in the mucin domain and 14 aa (566–579 C-KVSASANKPKVKDEI) in the carboxyl-terminal domain. The short peptides were synthesized using a chemical approach (GL Biochem, Shanghai, China). Immunization of New Zealand rabbits was performed according to the standard procedures described by Harlow and Lane [24]. The antiserum against the 16-aa peptide possessed a higher effective value and better specificity, and was used for subsequent analyses.

Twenty-five micrograms of total protein from embryos were separated on 10% SDS-PAGE gels, and proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, Calif., USA) using a Mini Trans-Blot Cell (Bio-Rad) with a constant voltage of 100 V for 90 min. To detect McHEM, anti-McHEM polyclonal antiserum (1:1,000) was used as the primary antibody and HRP-conjugated goat anti-rabbit IgG (1:2,000; Gene, Chai Wan, Hong Kong, China) were used as the secondary antibody. For the detection of peanut lectin-bound protein, HRP-conjugated *Arachis hypogaea* lectin (5 µg/ml; Sigma-Aldrich, St. Louis, Mo., USA) was used as a probe.

For immunohistochemistry, *O. furnacalis* larvae were fixed with 4% paraformaldehyde for at least 4 h on day 6 after parasitization. Serial sections were then cut at a thickness of 8 µm under a microtome (Leitz, Oberkochen, Germany). Immunostaining was performed with anti-McHEM polyclonal antiserum (1:200) as the primary antibody and HRP-conjugated goat anti-rabbit IgG (1:500, Gene) as the secondary antibody.

Embryos for labeling were first rinsed with phosphate-buffered saline (PBS) and then incubated with fluorescein isothiocyanate (FITC)-conjugated *A. hypogaea* (peanut) lectin (Sigma-Aldrich) at a final concentration of 10 mg/ml in PBS buffer with 0.05% sodium azide for 30 min. Finally, samples were rinsed three times in PBS and observed under a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Embryo Transplantation

As an insect with polyembryonic development, the formation of embryos in *M. cingulum* is complicated [7]. Compared to other stages, it is easier to obtain a large number of secondary embryos of a similar size and at the same developmental stage, so secondary embryos were used for encapsulation analysis. On day 5 after par-

asitization, the secondary embryos of *M. cingulum* with a diameter from 80 to 200 μm were dissected out of host hemocoel and washed with PBS at 25°C at least three times. Then, one embryo was individually injected into a naïve *Ostrinia* larva on the second day of the fifth instar with a glass needle pulled with a glass capillary tube. Seven days after transplantation, resulting wasp embryos were dissected and collected from *Ostrinia* larvae and were counted under a microscope.

Anti-Serum Blocking

Embryos were collected according to 'Embryo Transplantation'. Half of the collected embryos were incubated with antiserum against McHEM (1:200) at 4°C for 2 h and the remainder was incubated with naïve rabbit serum (1:200) as a control. Then, embryos were rinsed in Pringle's saline to remove antiserum and were incubated with *O. furnacalis* hemocytes (2×10^6 cells/ml) in Ex-Cell 420 medium (Sigma-Aldrich) for 2 h at room temperature for in vitro encapsulation analysis (see 'Encapsulation Assays'). This analysis was repeated five times.

RNA Interference

A 591-bp DNA fragment of McHem (95–291 aa) was amplified using the specific primers *dshem*-F and *dshem*-R (table 1) to prepare double-stranded RNA (dsRNA). DsRNA was synthesized using the T7 RiboMAX™ Express RNAi System (Promega, Madison, Wisc., USA). A DNA fragment from the green fluorescent protein (GFP) gene from the pEGFP-1 vector (Clontech) was amplified with primers GFP-F and GFP-R (table 1) as a negative control. The synthesized dsRNA was diluted to 5 mg/ml with nuclease-free water and stored at –80°C for use.

Commonly, dozens to a hundred secondary embryos are released from a morula, so in order to decrease the individual differences secondary embryos released from the same morula were analyzed for RNA interference. On day 2–3 after parasitization, wasp morulae approximately 100 μm in diameter were dissected out of host hemocoel and then cultured in Grace medium (Gibco) containing 10% bovine serum (Hyclone, Logan, Utah, USA). When secondary embryos were released from the morula, these embryos were divided into four parts for further cultures with the media as follows: Grace medium containing 10% bovine serum (ck); Grace medium containing 10% bovine serum and 5 μl of DEPC water; Grace medium containing 10% bovine serum and 25 $\mu\text{g}/\text{ml}$ dsGFP, and Grace medium containing 10% bovine serum and 25 $\mu\text{g}/\text{ml}$ dsRNA of *Mchem* (*dshem*). The relative transcript abundances of *Mchem* were analyzed by qRT-PCR as described in 'qRT-PCR Analysis for *Mchem*' 48 h after culture. The encapsulation analysis was performed as described in 'Encapsulation Assays' 72 h after culture. The RNA interference experiments and encapsulation assays were repeated three times.

O-Glycosidase Digestion

Approximately fifty embryos (diameter 80–200 μm) in 1 μl of Pringle's saline were added to 17 μl of reaction buffer and incubated at 37°C for 1 h. The embryos were then divided into two groups. To one group was added 2 μl of O-glycosidase (Sigma-Aldrich), and to the other group 2 μl of reaction buffer was added as a control, and these embryos were incubated at 37°C for 3 h. Embryos were then washed in PBS three times and added to 50- μl hemocytes of *O. furnacalis* ($2 \times 10^6/\text{ml}$) for encapsulation analysis. The digestion experiments were repeated three times. For Western

blotting detection, the total protein of embryos was extracted with protein extraction buffer [7 M urea, 2 M sulfourea, 4% (w/v) CHAPS and 40 mM DTT] with 1% protease cocktail (DBI, Ludwigshafen, Germany) from about 100 O-glycosidase-digested embryos, as above.

Encapsulation Assays

Encapsulation assays were performed with *O. furnacalis* hemocytes in vitro. Fifty microliters of hemocytes at 2×10^6 cells/ml in Ex-Cell 420 medium (Sigma-Aldrich) were added to the bottom of a 200- μl PCR tube to form a droplet to decrease the contact between the hemocytes and the tube wall. Embryos for encapsulation assays were randomly divided into three groups (about 20 embryos in each group) and were then added into hemocytes. Tubes containing hemocytes were attached to a rotor rotating at a speed of ten circles per minute to keep the embryos in contact with hemocytes for 2 h at room temperature. Embryos were then removed from the tubes and placed on slides to observe and classify them under a microscope. To compare the extent of the encapsulation of embryos, embryos were classified into three groups: unencapsulated, to which less than three host hemocytes adhered; incompletely encapsulated, in which only part of the embryo's surface was adhered to by hemocytes, and complete encapsulated, in which embryos were fully covered by hemocytes.

Statistical Analysis

All data were analyzed with a statistical significance level of $p < 0.01$ using a one-way analysis of variance with SPSS 13.0 software. All proportions were transformed by arcsine square root transformation before analysis.

Results

Cloning and Characterization of *Mchem*

A 344-bp cDNA fragment of *Mchem* was cloned by PCR amplification with degenerate primers (table 1). This cDNA fragment possesses 67% identity to the corresponding region of *A. mellifera* hemomucin (XR_015026). The cDNA fragments of *Mchem*-2 (the 865-bp 5' end) and *Mchem*-3 (the 1,375-bp 3' end) were obtained by 5' and 3' RACE with specific primers. The full-length cDNA of hemomucin was obtained by assembling the three overlapping fragments and subsequently verified with specific primers (fig. 1).

The predicted McHEM protein is composed of 579 aa with a calculated molecular weight of 63.29 kDa. It is composed of 4 domains: an amino-terminal hydrophobic segment (7–29 aa); a central part similar to strictosidine synthase (*str_synth* superfamily), the key enzyme in alkaloid biosynthesis (164–250 aa); a mucin domain with typical stretches of polythreonine separated by proline residues, which contains two low-complexity segments (413–500 aa and 504–539 aa), and a carboxyl-terminal domain (fig. 2a). No signal peptide was predicted with signal P 4.1,

Fig. 1. The 2,381-bp full-length *Mchem* cDNA sequence (GenBank No. KC788207.1) was obtained by joining together three overlapping PCR fragments. 'Mchem-1' (344 bp) was first cloned with two degenerate primers F-1 and R-1 and then 'Mchem-2' (865 bp) and 'Mchem-3' (1,375 bp) were obtained by 5'RACE and 3'RACE, respectively. Finally, the full cDNA sequence was verified with specific primers (table 1). CDS = Coding sequence; 5'UTR = 5' untranslated region; 3'UTR = 3' untranslated region.

Fig. 2. a Schematic drawing showing conserved domains and motifs in the deduced aa sequence of *McHEM*. The *Str_synth* domain is a central part similar to the strictosidine synthase superfamily, which is the key enzyme in alkaloid biosynthesis. The mucin domain possesses typical stretches of polythreonine, separated by proline residues, and contains two low complexities. **b** The glycosylation and phosphorylation sites of *McHEM* are indicated. Dark shading indicates GalNAc O-glycosylation sites; red indicates serine phosphorylation sites (colors refer to online version only); blue indicates tyrosine phosphorylation sites; clear boxes indicate threonine phosphorylation sites, and the mucin domain is highlighted by light shading.

but a transmembrane helix (7–29 aa) was predicted with TMHMM v2.0. Therefore, *McHEM* is likely a transmembrane protein that is largely located outside of the membrane. *McHEM* contains 51 potential O-glycosylation sites, 13 potential serine phosphorylation sites, 31 poten-

tial threonine phosphorylation sites and 6 potential tyrosine phosphorylation sites. All of the glycosylation sites and 28 threonine phosphorylation sites lie within the mucin region (fig. 2b).

Fig. 3. a Alignment of the deduced aa sequence of *Mchem* with hemomucin from *A. mellifera* and *N. vitripennis*. The predicted transmembrane region is highlighted in pink (colors refer to on-line version only), the Str_synth domain is in blue and mucin domain is in purple. **b** Phylogenetic tree of hemomucin gene in insects. The number above or below individual branches represents the percentage of 1,000 bootstrap iterations supporting the branch. Sequences were aligned and dendrograms constructed using MEGA 5.0; see ‘Materials and Methods’ for details. The hemomucin sequences are from *Acyrtosiphon pisum* (XP_001947674.2),

Aedes aegypti (XP_001649960.1), *A. aegypti*-2 (XP_001649960.1), *A. gambiae* (XP_309617.4), *A. mellifera* (XP_003250432.1), *Bombyx mori* (BGIBMGA004992-PA, from silkDB), *Culex quinquefasciatus* (XP_001863710.1), *Drosophila grimshawi* (XP_001994304.1), *D. melanogaster* (AAC47118.1), *D. persimilis* (XP_002013052.1), *D. pseudoobscura* (XP_001358307.2), *D. simulans* (AAAX38037.1), *D. virilis* (XP_002054742.1), *Glossina morsitans* (ADD20598.1), *M. cingulum* (AGL98097), *N. vitripennis* (XP_001605615.1), *Pediculus humanus corporis* (XP_002429105.1), *Schistocerca gregaria* (CAT00690.1) and *Tribolium castaneum* (XP_975599.1).

Sequence alignment of hemomucin from Hymenoptera revealed that *Mchem* was significantly similar to the hemomucin of *N. vitripennis* (52%; Hymenoptera; XP_001605615.1) and the hemomucin of *A. mellifera* (47%; Hymenoptera; XP_003250432.1), particularly in the str_synth domain. However, these hemomucins showed lower levels of similarity in the mucin domain (fig. 3a). In the phylogenetic tree, hemomucin from these insects could be divided into three main clusters. One cluster was composed of Diptera, including *Drosophila*, *Glossina* and mosquitos, and the second cluster was composed of only *Bombyx*, a member of Lepidoptera. However, the third cluster was composed of several orders, including Hymenoptera, Coleoptera, Hemiptera and Orthoptera, and it was further clustered into two sub-clusters. *McHem* and the hemomucins from *Nasonia* and *Apis* were clustered into the same group (fig. 3b).

McHEM Is a Transmembrane O-Glycoprotein Specifically Labeled by Peanut Lectin and Is Located on the Extraembryonic Membranes of M. cingulum

Based on qRT-PCR analysis, *McHem* was expressed mainly in early development stages, such as embryo, egg and morula, and was expressed less in later stages (larva and pupa; fig. 4a). Immunohistochemistry analysis showed that *McHEM* is located mainly on the surface of the extraembryonic membrane (fig. 4b, c). Labeling with FITC-*A. hypogaea* lectin, which specifically binds to β -gal (1 $\rightarrow$ 3) GalNAc, indicated that *McHEM* is specifically detected on the surface of the extraembryonic membrane of embryos from early and later stages (fig. 4d–i). A 97-kDa protein was detected by *A. hypogaea* lectin in total embryonic protein, and a similar-sized band was detected by anti-*McHEM* polyclonal antiserum against 16 aa in the mucin region in Western blotting analysis (fig. 4 j). How-

Fig. 4. Expression of *Mchem* in *M. cingulum*. **a** Relative transcriptional abundances of *Mchem* mRNA in different developmental stages. **b** Embryo of *M. cingulum* at early developmental stage, in which embryonic primordium had just formed (arrowhead). **c** Immunohistochemical analysis with polyclonal anti-McHEM serum. McHEM distributed mainly on the surface of extraembry-

onic membrane (arrow). Embryos at early (**d, e**), mid (**f, g**) and late stage (**h, i**) were labeled by FITC-*A. hypogaea* lectin. **j** Western blotting analysis with anti-Mchem serum and *A. hypogaea* lectin. Results showed that *A. hypogaea* lectin specifically bound to a 97-kDa protein in embryonic protein (arrow), which was also detected by anti-McHEM serum.

ever, besides the 97-kDa protein, four cross-reacting protein bands at 100, 80, 65 and 40 kDa were detected by anti-McHEM polyclonal antiserum raised against 16 aa in the mucin region (fig. 4j). After digestion with O-glycosidase, an enzyme that specifically digests the connection between β -gal (1 $\rightarrow$ 3) GalNAc and Ser/Thr on peptides, only the 97-kDa protein was degraded into a smaller protein weighing approximately 60 kDa (fig. 5b, arrowhead), which is similar to the molecular weight of McHEM (63.2 kDa) calculated based on the cDNA sequence. These results indicate that McHEM is a 97-kDa O-glycoprotein with β -gal (1 $\rightarrow$ 3) GalNAc sugar chains that resides on the extraembryonic membrane of *M. cingulum* embryos.

Embryos Evade Encapsulation of Host Hemocytes Using McHEM: Embryos Proliferated but Were Not Encapsulated after Transplantation into Naïve *O. furnacalis* Larvae

Embryos of *M. cingulum* passively avoid the host's immune reaction using their extraembryonic membrane [7]. Therefore, *M. cingulum* secondary embryos proliferated but were not encapsulated in host larvae until the wasp larvae hatched. Similarly, when these embryos were

transplanted into naïve larvae of their host, *O. furnacalis*, secondary embryos were not encapsulated by host hemocytes and proliferated into dozens of embryos in the host larvae hemocoel (fig. 6a). The number of embryos generated from 25 secondary embryos was calculated. Most embryos resulted in 20–60 embryos, and more than one hundred were generated in some cases (fig. 6b), but no embryos were encapsulated. However, in 3 naïve *O. furnacalis* larvae (12, 17 and 19), no wasp embryos were found (fig. 6b). We speculate that the secondary embryos were lost in these cases during transplantation due to their small size (100–200 μ m in diameter). At the same time, the host larvae did not enter the pupal stage but stayed in the larval stage until they died. This is similar to the situation in *O. furnacalis* larvae parasitized naturally by *M. cingulum*, indicating that embryos may secrete factors similar to hormones to regulate the development of host larvae, similar to other parasitoids [25]. Taken together, this indicates that embryos of *M. cingulum* may themselves evade the immune reaction from host hemocytes and do not depend on factors injected by the mother wasp at the time of oviposition.

Fig. 5. Encapsulation analysis of O-glycosidase-digested embryos. Digested embryos (see ‘O-Glycosidase Digestion’ in ‘Materials and Methods’) were hatched with host hemocytes at 2×10^6 cells/ml for 2 h at room temperature in vitro and then the rates of encapsulated embryos were calculated. **a** Schematic diagram indicates the T antigen and O-glycosidase functional site. **b** Western blotting analysis with anti-McHEM serum indicated that after being digested by O-glycosidase, the molecular weight of 97-kDa protein decreased to about 60 kDa (arrowheads). **c** Embryos in encapsula-

tion assays. Embryos were classified into three groups: unencapsulated, on which less than three host hemocytes adhered (U); incompletely encapsulated, only part of the surface of which was adhered to by some host hemocytic blocks (I), and completely encapsulated, the whole surface of which was covered by host hemocytes (C). **d** The rate of embryos in encapsulation assays. Control means that embryos were digested by 2 μl of reaction buffer but not O-glycosidase.

Embryos Were Encapsulated by Host Hemocytes after Being Blocked by Antiserum against McHEM

To further validate the role of McHEM in protecting embryos from being encapsulated, the McHEM on embryos was blocked with an anti-McHEM antiserum, and the encapsulation of embryos was analyzed. The results of these experiments showed that approximately 80% of the embryos pre-treated with anti-McHEM antiserum were encapsulated, which is significantly higher than the result observed in the embryos treated with pre-immune

rabbit serum, in which no embryo was encapsulated ($p < 0.01$; fig. 7a). Encapsulated embryos can be divided into two types: incompletely encapsulated embryos, which were partially covered by hemocytes (fig. 7c), and completely encapsulated embryos, which were fully encapsulated by hemocytes (fig. 7d). However, despite the small differences in overall encapsulation rates, the proportions of the two types of capsules were not consistent in repeated assays. Taken together, these results indicated that when McHEM on the embryo’s surface is blocked by an-

Fig. 6. Proliferation of secondary embryos of *M. cingulum* in naïve larvae of *O. furnacalis*. **a** When one secondary embryo was transplanted into a naïve larva of *O. furnacalis* on day 2 of the fifth instar, it proliferated into dozens of embryos within 1 week. **b** Numbers of embryos produced by 25 separately transplanted embryos in *O. furnacalis* larvae.

tibodies, embryos will be encapsulated, further demonstrating the function of McHEM in evading encapsulation by host hemocytes.

Embryos Were Encapsulated by Host Hemocytes after the Expression of McHEM Was Knocked Down by dsHEM

To clarify the function of hemomucin on immune evasion, the encapsulation of wasp secondary embryos in which the expression of McHEM was inhibited with *dsHEM* was analyzed using hemocytes from naïve host larvae. To decrease individual differences, secondary embryos used for analysis came from one morula. Two days after *dsHEM* was added to Grace medium, the expression of McHEM in embryos had significantly decreased compared to the embryos to which dsGFP or DEPC water had been added ($p < 0.05$; fig. 8a). Encapsulation analysis was conducted 3 days after *dsHEM* had been added to obtain the best interference effects on the expression of McHEM. The results showed that almost 90% of embryos were en-

capsulated in the *dsHEM*-treated group, which is significantly higher than the results obtained in the dsGFP- or DEPC water-treated groups or the control group ($p < 0.01$; fig. 8b). However, no embryos were fully encapsulated and only part of the surface of the embryos was covered by hemocytic blocks (fig. 8d, e), which was in contrast to the capsules formed in the antiserum blocking analysis (fig. 7c, d). We speculate that this difference is related to the incomplete interference effects of *dsHEM*. These results indicate that decreasing the expression of McHEM resulted in the encapsulation of embryos by host hemocytes. McHEM likely plays an important role in allowing the embryos to evade the cellular immune reaction of the host. We then investigated whether the sugars on McHEM play an important role during this process.

Sugar Chain of McHEM Plays an Important Role in the Protective Function of McHEM

T antigen [β -gal (1 $\rightarrow$ 3) GalNAc] has so far been the only known sugar modification in insect mucin, which is

Fig. 7. Encapsulation analysis of embryos blocked with anti-McHEM serum. Embryos blocked with antiserum were hatched with host hemocytes at 2×10^6 cells/ml for 2 h at room temperature in vitro and then the rates of encapsulated embryos were calculated. **a** The rate of encapsulated embryos after being blocked with anti-McHEM serum. ** $p < 0.01$; $n = 5$, $n = 15$, t test. Embryos were

classified into three groups: unencapsulated, on which less than three host hemocytes adhered (**b**); incompletely encapsulated, only part of the surface of which was adhered to by some host hemocytic blocks (**c**), and completely encapsulated, the whole surface of which was covered by host hemocytes (**d**). Both incomplete and complete capsules were considered as encapsulated embryos.

formed by adding Gal to Tn antigen [14, 20, 26]. The results above indicate that T antigen was detected in McHEM using HRP-A. *hypogaea* lectin (fig. 4j), which specifically bound to β -gal (1 $\rightarrow$ 3) GalNAc. O-glycosidase is an enzyme that specifically digests the connection between GalNAc and Ser/Thr on peptides (fig. 5a). When the total proteins from *M. cingulum* embryos were digested with O-glycosidase, the molecular weight of McHEM decreased from 97 kDa to approximately 60 kDa, which is similar to the calculated molecular weight for McHEM of 63.29 kDa, indicating that the sugar chains were removed from McHEM by O-glycosidase (fig. 5b, arrowhead). Encapsulation of O-glycosidase-digested embryos was then analyzed. The results demonstrated that approximately 70% of the embryos treated with O-glycosidase were encapsulated by host larval hemocytes, and both incomplete and complete capsules were formed

as in antiserum blocking analysis (fig. 5c); furthermore, almost half of the encapsulated embryos were completely encapsulated (fig. 5d). In comparison, no embryos in the control group, in which reaction buffer was added to replace O-glycosylase, were encapsulated ($p < 0.01$; fig. 5d). These results show that when the sugar chains on McHEM are removed, embryos are encapsulated, thus indicating that the sugar chain β -gal (1 $\rightarrow$ 3) GalNAc on McHEM may protect embryos from being encapsulated by host hemocytes.

Discussion

The strategies that endoparasites use to overcome the host's immune reactions are important to allow wasps to successfully develop inside their host during their co-evo-

Fig. 8. Encapsulation analysis of RNA-interfered embryos. **a** The relative transcript abundance of *Mchem* at 2 days after 5 μ l of DEPC water, 25 μ g/ml of *dsGFP* and 25 μ g/ml of *dshem* added to the cultural medium; ck means nothing added in cultural medium. **b** Encapsulation analysis of embryos with *O. furnacalis* larval he-

mocytes at 2×10^6 cells/ml 3 days after *dshem* treatment. Embryos were hatched with hemocytes for 2 h at room temperature in vitro and then the rates of encapsulated embryos were calculated. ** $p < 0.01$; $n = 3$, $n = 9$, t test. **c** Unencapsulated embryo. **d**, **e** Encapsulated embryos (incompletely encapsulated).

lution. There are two common strategies: active suppression and passive avoidance [reviewed in 1]. However, most studies regarding the relationship between wasps and their hosts focus on the active suppression of the wasp polydnavirus or virus-like factors on the host's immune reaction [reviewed in 1, 8, 27–29]. Fewer studies address the passive evasion by parasitoids that do not carry viruses or virus-like particles. In this study, the immune evasion function of a molecule, hemomucin, from a polyembryonic parasitoid that does not carry a virus was shown to allow evasion of a host immune reaction based on the glycosylation of this molecule.

The main function of hemomucin that has been studied is its role during hemolymph coagulation in *Drosophila* immunity since it was first identified in 1996 [18–20]. Additionally, in the parasitoid *V. canescens*, hemomucin was shown to form a complex with lipophorin and may contribute to avoidance of the host defense [6]. However, there has been no direct report on the function of hemo-

mucin on immune evasion. *McHem* possesses similar structural characteristics to hemomucin in *Drosophila*, and the mucin domains from both species' molecules were composed of polythreonine separated by proline residues (fig. 2b) [18]. However, the sequences of the mucin domains are not conserved, so the similarity of this domain is lower among different species in Hymenoptera (fig. 3b). This phenomenon is similar to mucin proteins in mammals, in which the mucin peptides have distinctive variable number tandem repeat sequences. These repeats may vary in number between different individuals and alleles and are rich in Ser and Thr [13]. There are two common types of mucins: secreted mucins and membrane-bound mucins. *McHEM* contains a typical hydrophobic segment at the amino terminal; however, no typical signal peptide was predicted with SignalP 4.1, and a transmembrane helix was predicted with TMHMM v2.0 (fig. 2a). Furthermore, results from immunohistochemistry and labeling with peanut lectin indicated that

McHEM is located mainly on the extraembryonic membrane (fig. 4b–i). Thus, we speculated that McHEM is likely a membrane-bound mucin. In *Drosophila*, hemomucin was proven to be a membrane-bound mucin [18].

The anti-McHEM serum detected 5 protein bands in the total embryonic protein of *M. cingulum*; however, *A. hypogaea* lectin bound to only the 97-kDa band (fig. 4j), indicating that only the 97-kDa protein contained the sugar side chain of T antigen [β -gal (1 $\rightarrow$ 3) GalNAc]. The T antigen was also identified in hemomucin from *Drosophila* using peanut lectin (*A. hypogaea*) [20]. After digestion with O-glycosidase, only the 97-kDa band was degraded to approximately 60 kDa, which is similar to the calculated molecular weight of McHEM based on the aa sequence (fig. 5b, arrowhead). O-glycosidase specifically digests the connection between T antigen and S/T on peptides, so we speculated that T antigen is the main sugar chain on McHEM and that the 97-kDa protein is the O-glycosylated McHEM. As their molecular weights did not change after digestion with O-glycosidase and they were not bound by peanut lectin (fig. 4j, 5b), we propose that the other proteins detected by the anti-McHEM serum were not incompletely O-glycosylated McHEM proteins, although McHEM contains 51 potential O-glycosylation sites (fig. 2b). The anti-McHEM serum contained polyclonal antibodies against a 16-aa peptide from the mucin domain (see ‘Materials and Methods’), so we propose that the other bands are non-specific proteins bound by the anti-McHEM serum. In previous work, we found that a 100-kDa *Helix pomatia* lectin-binding protein (HpLBP) on the extraembryonic membrane of *M. cingulum* protected them from being encapsulated [7]. Based on shared molecular weight, distribution and function, we believe it is likely that McHEM is HpLBP, but this is difficult to test directly because the antibody generated against HpLBP is no longer available.

More than 80% of the embryos with expression of McHEM that was knocked down with *dshem* were encapsulated by *O. furnacalis* hemocytes (fig. 8b), and the embryos treated with anti-McHEM serum were also encapsulated (fig. 7a), indicating that McHEM plays an important role in protecting embryos from being encapsulated by host hemocytes. Furthermore, when the sugar chains of McHEM were removed with O-glycosidase, approximately 70% of the embryos were encapsulated, indicating that the glycosyl chain of McHEM directly functions in the embryos’ evasion ability. In vertebrates, ‘umbrella’ structures on the surface of mucins inhibit cell-cell or cell-substrate interactions and must be released from cells to regulate the attachment of blood cells [15, 16]. The

mechanisms by which hemocytes encapsulate large foreign objects, such as wasp eggs and embryos, are unclear. Based on the results obtained in *Pseudoplusia includens*, the embryos should be recognized as non-self at first and then be encapsulated by hemocytes that adhere and spread on the embryos [30]. The mechanisms used by *M. cingulum* embryos to avoid non-self-recognition by the host may occur in two possible ways. The first should relate to binding factors of the host, such as lectins to disable the host’s non-self-recognition. Labeling with FITC-*A. hypogaea* lectin showed that the foreign lectin *A. hypogaea* could be bound to the sugar chains of McHEM (fig. 4d–i). Though most lectins in insects have been reported to promote cellular immune reactions, such as phagocytosis and encapsulation after binding on microbes [reviewed in 31], a GalNAc-specific lectin was reported to possess counter-adhesion properties, and could detach already spread cells in Lepidoptera [32]. If wasp embryos are covered by such a kind of lectin, they will not be adhered and encapsulated by host hemocytes. Eight C-type lectins have been identified in hemocytes and fat body transcriptome of *O. furnacalis*, and some of them possess the specific binding sites to Gal [unpubl. data]. On the other hand, it is possible that the *M. cingulum* embryo evades encapsulation by the host, not through binding host factors but due to the host lacking some factors recognizing the sugar chain on McHEM as non-self. This phenomenon was partially supported by some data from experiments using *Spodoptera exigua*, another Lepidopteran closely related to *O. furnacalis*. When the embryos of *M. cingulum* were transplanted into the hemocoel of *S. exigua*, the wasp embryos could be encapsulated by hemocytes [unpubl. data]. It is possible that some non-self-recognition factors, such as lectins in some lepidopteran species, have mutated or were lost during evolution, which could provide an opportunity for parasitization. However, further studies will be required to verify these hypotheses.

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