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Molecular cloning and immunolocalization of a diuretic hormone receptor in rice brown planthopper (*Nilaparvata lugens*)

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Abstract

RNA extracted from guts of rice brown planthopper, *Nilaparvata lugens*, was used to clone cDNA predicted to encode a diuretic hormone receptor (DHR). The DHR, a member of the calcitonin/secretin/corticotropin-releasing factor family of G-protein-coupled receptors, contains seven transmembrane domains and a large N-terminal extracellular domain potentially involved in hormone binding. The N-terminal domain was expressed as a recombinant protein, purified and used to raise antibodies. Anti-DHR IgG bound specifically to Malpighian tubules in immunolocalization experiments using dissected guts, and to a putative DHR polypeptide from *N. lugens* gut on Western blots. Anti-DHR IgG delivered orally to insects was not detected in the haemolymph, and showed no binding to gut or tubules, confirming that DHR N-terminal hormone-binding domain is not exposed to the gut lumen.

Keywords: G-protein-coupled receptor, rice brown planthopper, diuretic hormone, immunolocalization, insecticide target.

Introduction

The rice brown planthopper, *Nilaparvata lugens* (Stål, Hemiptera, Delphacidae) is a serious threat to rice production across Asia (Holt *et al*., 1996). Rice plants heavily infested with *N. lugens* show wilting and desiccation (described as 'hopperburn') followed by plant death. Crop damage is caused by disruption of translocation and the removal of photoassimilates from phloem elements by feeding hoppers (Watanabe & Kitagawa, 2000). As well as the direct feeding effects, *N. lugens* also act as vectors for economically important rice viruses (Hibino, 1996). There is a need to develop new strategies to control this pest species, which rapidly develops resistance to exogenously applied pesticides (Karunaratne *et al*., 1999; Vontas *et al*., 2002) and is capable of overcoming the endogenous partial resistance genes present in some rice lines (Ketipearachchi *et al*., 1998). There are currently no Bt toxins effective against homopteran insects, and thus use of transgenic rice engineered with *Cry* genes, which has proved so successful in producing plants resistant to lepidopteran pest insects, is not a viable strategy for combatting brown planthopper. Although transgenic rice plants expressing lectins (Rao *et al*., 1998; Nagadhara *et al*., 2003) or proteinase inhibitors (Lee *et al*., 1999) have shown partial resistance to *N. lugens*, a higher level of resistance would be desirable. The identification and characterization of targets in *N. lugens* for potential toxins could be used as a basis for the design of new insecticides and strategies for producing resistance in rice towards this pest.

An attractive target for the design of novel insecticides is the diuretic hormone receptor (DHR), owing to its central role played in the regulation of fluid and ion secretion. In insects primary urine is produced by osmotic filtration in the Malpighian tubules. The process is tightly regulated by diuretic and antidiuretic hormones, which interact through G-protein-coupled receptors (GPCRs). The interaction between diuretic hormone and the corresponding receptor is well documented (Reagan *et al*., 1993, 1994; Reagan, 1995). Diuretic hormones bind their receptor, resulting in an intracellular increase in cAMP, which in turn leads to the transport of ions into the lumen of the Malpighian tubule. Ion secretion creates an osmotic gradient, along which water moves passively (O'Donnell & Spring, 2000).

Because of the importance of fluid and ion homeostasis to insect survival, the modulation of DHR function by protein or small-molecule antagonists and agonists is likely to prove detrimental to insect survival. This effect should be particularly pronounced in homopteran insects that feed on plant phloem sap, which face a severe problem in maintaining water balance owing to the osmotic potential of the sucrose-rich

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sap being higher than the insect haemolymph. Characterization of DHR proteins for these and other insects will provide a useful target for the action of insecticidal molecules. Insect DHRs have been cloned and functionally characterized from *Manduca sexta* (tobacco hornworm; Reagan, 1994) and *Acheta domesticus* (cricket; Reagan, 1996). In addition, a cDNA encoding a similar protein has been isolated from a library prepared from Malpighian tubules in *Bombyx mori* (silkworm; Ha *et al*., 2000), and orthologous sequences are present in the *Drosophila* and *Anopheles* genomes. The availability of these cDNA sequences will allow molecules that interact with DHRs to be designed, produced and tested for activity; this is particularly relevant for those species in which the diuretic hormones themselves have not been characterized, and would be very difficult to characterize.

This paper reports the molecular cloning of a putative *N. lugens* DHR, and the production of antibodies directed against its extracellular domain, as a potential antagonist for the receptor. Evidence is presented to show that the protein is localized to the Malpighian tubules of the midgut, and that it is located on the haemolymph side of the tubule rather than the surface in contact with the gut contents.

Results

Isolation of a cDNA encoding a DHR from the gut of N. lugens

An amino acid alignment of the DHRs from *M. sexta*, *A. domestica* and *B. mori*, and family B peptide receptors predicted by the *Drosophila* genomic database was assembled using Clustal software. Regions of greatest similarity between the sequences were identified, and used as a basis for sequence comparisons of the encoding nucleotides. Degenerate primers corresponding to conserved sequence regions encoding parts of transmembrane helices 3 and 6 of the receptors were synthesized, and used in a PCR amplification of a first-strand cDNA template prepared from *N. lugens* gut RNA by the action of reverse transcriptase. A specific product of 381 bp was amplified, cloned and characterized by DNA sequencing. Sequence comparison to proteins in the GenBank database using Blast software suggested that the amplified sequence encoded a protein fragment that was similar to the insect DHR proteins used as a basis for predicting the PCR primers. Specific primers were designed from regions of the 381 bp amplification product, and were used in rapid amplification of cDNA ends (RACE) PCR to retrieve the 5′ and 3′ ends of the *N. lugens* DHR cDNA. Multiple clones of the PCR products from the two RACE reactions were cloned and sequenced, and PCR amplifications were also carried out using primers covering the whole predicted open reading frame (ORF) in the DHR cDNA. This strategy allowed two cDNA sequences to be assembled.

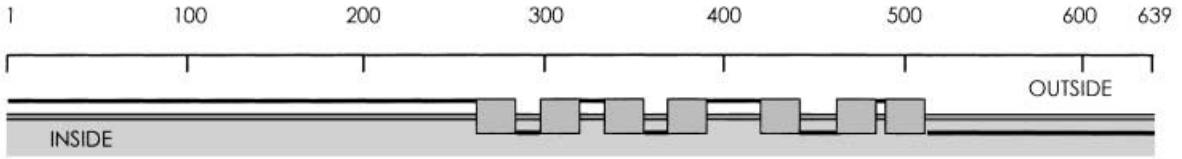
The cDNA represented most often in the clones obtained (DHR-I) was 2217 bp in length from the 5′ end of the coding sequence, including 269 bp of 3′ untranslated region (UTR) with a 29 bp poly(A) sequence attached (Fig. 1). The longest ORF consisted of 1920 bp, and encoded a protein of 639 amino acids with a predicted M_r of 69 765. The cDNA was truncated by 22 bp at the 5′ end, and was reconstructed to the start codon by PCR based on the sequence of DHR-II (q.v.). The sequence is shown in Fig. 1B. A second sequence (DHR-II) was also amplified as a product of these operations. This cDNA was 2344 bp in length, and contained a 5′ UTR, of 167 bp. The longest ORF was of 1926 bp, and encoded a protein of 641 amino acids with a predicted M_r of 69 955. The 3′ UTR of this clone, of 229 bp, with 22 bp of poly(A) sequence attached, was dissimilar to that of the other cDNA; however, the sequence was similar over the coding region, with only minor differences to DHR-I. The DHR-II sequence is not presented in this paper, but differences in the encoded amino acid sequence are noted in Fig. 1B. Both sequences have been filed with the EMBL sequence database under accession numbers AJ634780 and AJ634781.

A further variant cDNA species was also observed. This was a version of DHR-I with a deletion in the 3′ coding region, corresponding to bases 1589–1727, which introduces a frame shift resulting in the amino acid sequence being truncated; after residue 529 the predicted sequence then continues EEEA*, where * represents the stop codon.

As an additional check to validate the cDNA sequences derived from PCR, and to determine whether the ‘short’ version of DHR-I with a deletion could be the result of alternate splicing, *N. lugens* genomic DNA was subjected to PCR using a pair of primers that were predicted to amplify a segment of DNA from the 3′ region of coding sequences of the cDNAs. The amplified fragment obtained was larger than predicted, and when sequenced, corresponded to bases 1513–1520 of the DHR-I cDNA, with an intron inserted between bases 1679 and 1680. DHR-I can thus be related to a genomic sequence in this insect. The position of the intron does not correspond with the deletion in the ‘short’ version of DHR-I, and it is difficult to see how this short version could have arisen as a result of alternate

Figure 1. (A) Diagram showing the predicted location (THMM, v. 2.0) of the extracellular, transmembrane and intracellular regions of the DHR-I protein. The polypeptide chain is represented by a black line; *trans*-membrane helices by shaded boxes. (B) Nucleotide and deduced amino acid sequence of the predicted diuretic hormone receptor DHR-I from *N. lugens* (GenBank accession number AJ634780). Boxed sequence was reconstructed by PCR based on the sequence of DHR-II. Putative transmembrane regions are shaded and labelled TM 1–7. ±, potential N-linked glycosylation sites; ^, conserved cysteine residues in the N-terminal domain and extracellular loops. Differences in the amino acid sequence predicted by DHR-II are shown in brackets.

A



B

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                   ±                                                                  (E)
AGCATGCTATCCTGGCCTCCCACCGATGACGTCACAATCAACCATCCACCCAGTGCTGACGTCATGGGCGGAAGACTGAGGCCATCTGTA   180
 S  M  L  S  W  P  P  T  D  D  V  T  I  N  H  P  P  S  A  D  V  M  G  G  R  L  R  P  S  V    60
 (I)
GGTGACGTCACAATGGGGAACCCCAATGCTGACACCATACAAAATATACTGAAGCATCCTGTGGGTGACGTCACAATGGGGAATCCCAAT   270
 G  D  V  T  M  G  N  P  N  A  D  T  I  Q  N  I  L  K  H  P  V  G  D  V  T  M  G  N  P  N    90

ACAGGAAGTATATCAGCTGGAATGAGACATTTTGCCGGTGACGTCACAATGGGGAATCCCAGTGCTGACACCATACGAGGGATACTGAAG   360
 T  G  S  I  S  A  G  M  R  H  F  A  G  D  V  T  M  G  N  P  S  A  D  T  I  R  G  I  L  K   120
    (G)                    (T)                 (V)
CAGTCTGTTGGTGACGTCACAACGGGGAACCAGAATACTGGAAGTGTAGTGTCAGGAATGAGACATTTCGCTGGTGACGTCACAGGGGGG   450
 Q  S  V  G  D  V  T  T  G  N  Q  N  T  G  S  V  V  S  G  M  R  H  F  A  G  D  V  T  G  G   150
                                  (I  A  A)                 (S)        (V)
AATCCCAATGCTGATGTCATAGGCGGACGACTGAGGCCGCCCAGCATGGATGTGCCCGTTGGTGACGTCACACAGGGTAGAGCGCAATAT   540
 N  P  N  A  D  V  I  G  G  R  L  R  P  P  S  M  D  V  P  V  G  D  V  T  Q  G  R  A  Q  Y   180
                                              (H)
GCTTCACACTGTCCTAGGAGTTGGGACTCTCTACTGTGTTGGCCTCCTACTAAACCTGGACATACGGCAACGATGGCTTGCTTCCCTGAG   630
 A  S  H  C  P  R  S  W  D  S  L  L  C  W  P  P  T  K  P  G  H  T  A  T  M  A  C  F  P  E   210
          ^                          ^                                      ^
CTGAATGGGATCAAGTATGATACTACATTGAACGCAACTAGAAGATGCCTGCTGAATGGTACCTGGGACAACTACACAGACTACACGTCC   720
 L  N  G  I  K  Y  D  T  T  L  N  A  T  R  R  C  L  L  N  G  T  W  D  N  Y  T  D  Y  T  S   240
                            ±                 ^     ±              ±
TGCAAAGACCTGAGTCCCGATCAACCAGATTTGGAGCCTGGCATAGAAGTCACCACCATGATATACTCAGCTGGATATGCCCTGTCTTTG   810
 C  K  D  L  S  P  D  Q  P  D  L  E  P  G  I  E  V  T  T  M  I  Y  S  A  G  Y  A  L  S  L   270
 ^                                                          TM 1
ATAGCTCTAGTATTGGCTGTTTCTATATTCCTCTATTTCAAGGATTTGAGATGTCTGCGGAATACAATCCACACCAATCTGATGTGTACA   900
 I  A  L  V  L  A  V  S  I  F  L  Y  F  K  D  L  R  C  L  R  N  T  I  H  T  N  L  M  C  T   300
                                                                        TM 2
TATATAATGGCCGACTTCATGTGGATACTAAATATCACAGTACAGATGTCGATGCCAACCAACGTGCCAGCCTGTGTTATACTAGTTGTC   990
 Y  I  M  A  D  F  M  W  I  L  N  I  T  V  Q  M  S  M  P  T  N  V  P  A  C  V  I  L  V  V   330
                                                    (S)     ^
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 L  L  H  Y  F  Y  L  T  N  F  F  W  M  F  V  E  G  L  Y  L  Y  M  L  V  V  E  T  F  S  S   360
          TM 3
AAAAACATAAAACTGAGGGCCTACGTCTGTATAGGATGGGGTGTGCCTTGTGCAGTGATAATCATATGGACAATTGTCAGAAGTCTCATG  1170
 K  N  I  K  L  R  A  Y  V  C  I  G  W  G  V  P  C  A  V  I  I  I  W  T  I  V  R  S  L  M   390
                      TM 4
CCTCCCACCTCTGATAATATGGGTCTTTCGGGCATAGTTCTGAAAAGCTGTCCCTGGATGGCTACCCATAACATTGATTGGATCTACCAG  1260
 P  P  T  S  D  N  M  G  L  S  G  I  V  L  K  S  C  P  W  M  A  T  H  N  I  D  W  I  Y  Q   420
                                  ^
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    TM 5                                                                  S
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 V  E  T  Q  Q  Y  R  K  A  A  K  A  L  L  V  L  I  P  L  L  G  I  T  Y  I  L  V  I  Y  V   480
                                     TM 6
CCCAGTCATGGAGTAACTGCTAATCCATTGGCGTATTGCCGGGCTATACTGCTGTCAACACAGGGTTTCACAGTAGCACTGTTCTACTGT  1530
 P  S  H  G  V  T  A  N  P  L  A  Y  C  R  A  I  L  L  S  T  Q  G  F  T  V  A  L  F  Y  C   510
                            TM 7
TTCCTGAATTCAGAAGTGCAGCACACGCTCAGTTCGCACTTTGAACGCTGGAAGGAGGCGAGGCAGATAGGAGGAGGAGGTGGTGGTGGT  1620
 F  L  N  S  E  V  Q  H  T  L  S  S  H  F  E  R  W  K  E  A  R  Q  I  G  G  G  G  G  G  G   540
                                              (E)           (+R)
GGGAGGAGGTATACCTATAGCAAGGACTGGTCCCCCAATACTAGAACAGAAAGTATCCGCCTCTGCAGCACTAACCAGGTGGGAGGAGGA  1710
 G  R  R  Y  T  Y  S  K  D  W  S  P  N  T  R  T  E  S  I  R  L  C  S  T  N  Q  V  G  G  G   570
                                                                              (+A)
GGTGGTGGAGGCGGAGGAGGAGGAGGCATGAGTTACAAGAAGCGCGAATCAACAGTTAGTGAGACAACCACAATGACTCTGGTTGGCGGT  1800
 G  G  G  G  G  G  G  G  G  M  S  Y  K  K  R  E  S  T  V  S  E  T  T  T  M  T  L  V  G  G   600

GGTGTGGGGGTCGAATCGGGTGAGCAACGGGTCTATGGGACCCACTATGGGGTCTCTCAGACCCCCACAGGGGTCCACCAGTCCCTATTTG  1890
 G  V  G  S  N  R  V  S  N  G  S  M  G  P  T  M  G  S  L  R  P  P  Q  G  S  T  S  P  Y  L   630

GAGGTGGCGCCTCTCGAGAATGCCGTGTGAAGAGGTAGAAGTTAAAAAAAATTCAGAGAAGACTAGCCTATAAATTTGGAATAATCAGGT  1980
 E  V  A  P  L  E  N  A  V  *>

AATAAGAAAGGATTGTAAGCAACAGTGAAAGAAGAATGGGAATCAATGTAGAAGTAGGAGGAGGAGGAGGAGCAGCAGGTGAAGGGAAGA  2070
AGGAGAGGAGAAGGAGAGAGAGAGAGAACGAGAGAGAGAGAGAGAGACGAAGAAGAGAATCATCCATGAATCAATTATGATGAAGACAAT  2160
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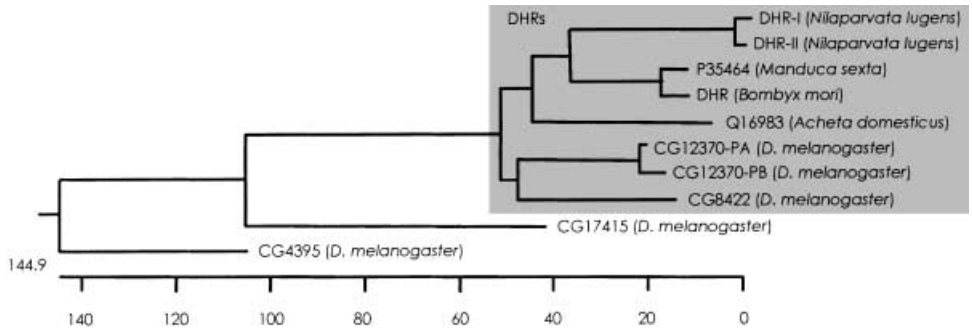

Figure 2. Phylogenetic relationship of primary sequences of predicted family B peptide receptors from *D. melanogaster* CG12370 (putative diuretic hormone receptor; alternate splicing products PA and PB), CG8422 (diuretic hormone receptor), CG17415 (calcitonin receptor) and CG4395 (putative calcitonin receptor), and diuretic hormone receptors from *Manduca sexta* (P35464), *Acheta domesticus* (Q16983), *Bombyx mori* (DHR) and *Nilaparvata lugens* (DHR-I and DHR-II). Rooted tree produced by Clustal sequence comparison analysis; known and putative DHRs are in the shaded area.

splicing. The repetitive nature of the sequence in this region makes a PCR artefact more likely. Because DHR-I had been validated in this way, and is the more abundant of the two cDNA sequences, further work concentrated on this cDNA, and results will refer to it except where noted.

The polypeptide encoded by the DHR-I cDNA was subjected to hydrophobicity analysis to identify transmembrane helices and to predict transmembrane protein topology (TMHMM server, version 2.0; http://www.cbs.dtu.dk/services/TMHMM/ Krogh *et al.*, 2001). This analysis (Fig. 1A) confirmed the protein as a member of the seven-helix transmembrane protein superfamily. The protein was predicted to have an extracellular N-terminal domain (amino acids 1–260), seven transmembrane helices connected by short peptide loops (29 amino acids or less) and an intracellular C-terminal region. A similarity search of the nonredundant protein sequence database using BlastP software showed this protein had greatest sequence similarity (scores > 300) to a group of five proteins that included the characterized DHRs from *A. domesticus* and *M. sexta* (Reagan, 1994, 1996), putative DHRs from *D. melanogaster* (CG8422, 12370) and a partial predicted protein sequence from *Anopheles gambiae* (accession XP_315466). A phylogenetic tree of the complete sequences, and a further predicted insect DHR from *B. mori* not present in the database, is shown in Fig. 2, and confirms that the *N. lugens* DHR 'fits' into the group of insect DHRs, and is distinct from other family B-type peptide receptor genes present in the *D. melanogaster* genome. The *N. lugens* DHR differs from other similar proteins in the size of the extracellular N-terminal domain; it is larger than any of the other comparable insect sequences. The protein also has four predicted N-glycosylation sites (N–X–S/T) situated on the extracellular N-terminal domain. The intracellular C-terminal region is also larger than other insect DHRs and contains glycine-rich repeated regions, including three poly glycine stretches of three, eight and twelve residues.

Production of anti-DHR antibody by means of expression of recombinant DHR

The small size of *N. lugens* and low abundance of hormone receptors hampers purification of these proteins in sufficient quantity for characterization and antibody raising. Therefore, a recombinant protein expression system was used to produce a fragment of the DHR for the purpose of antibody production. A cDNA fragment encoding the predicted N-terminal extracellular domain (amino acids 1–260) was cloned into the pET24 inducible bacterial expression vector, in frame with a C-terminal poly(His) fusion tag. The construct was transformed into a suitable *E. coli* host strain for expression. A validated clone containing the expression plasmid was grown to mid-log phase, and expression was induced. The accumulation of recombinant protein after induction of expression was monitored by SDS-PAGE (Fig. 3). A polypeptide band at approximately 36 kDa increased in amount on induction, and was a major component of total cell proteins after approximately 3 h. This polypeptide, putatively the recombinant DHR N-terminal domain, was estimated to be accumulated in cells to a level of about 15 mg/l culture. Cell lysis and fractionation under nondenaturing conditions showed the putative DHR N-terminal domain was present in the insoluble protein fraction. As the protein was expressed in the insoluble fraction it was purified under denaturing conditions. Cells were lysed by sonication in a buffer containing 6 M urea, and the His_6-tagged recombinant protein was purified by metal affinity chromatography. The affinity column was washed to remove nonspecifically bound proteins and the recombinant protein was eluted, giving a product that was essentially a single band estimated at 36 kDa in size (Fig. 3). After dilution to a concentration of 50 μg/ml, the protein was refolded by dialysis into buffer without denaturant, which was both quick and efficient, resulting in very little protein precipitation. The soluble protein was freeze-dried and resuspended to a concentration of 5 mg/ml.

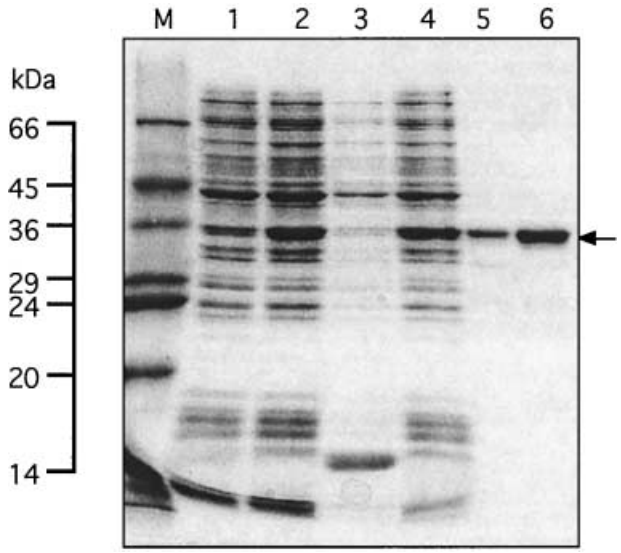

Figure 3. Expression of DHR hormone-binding domain in *E. coli*, and purification under denaturing conditions. Proteins were separated by SDS-PAGE on a 12% acrylamide gel and stained with Coomassie blue. Lane 1: total proteins from cells prior to induction; Lane 2: total proteins from cells after induction for 3 h; Lane 3: soluble protein fraction from cells after induction for 3 h; Lane 4: insoluble protein fraction from cells after induction for 3 h; Lane 5: DHR N-terminal domain after purification by metal affinity chromatgraphy; Lane 6: DHR N-terminal domain after dialysis and refolding; Lane M: molecular weight marker. Arrow indicates the DHR N-terminal domain.

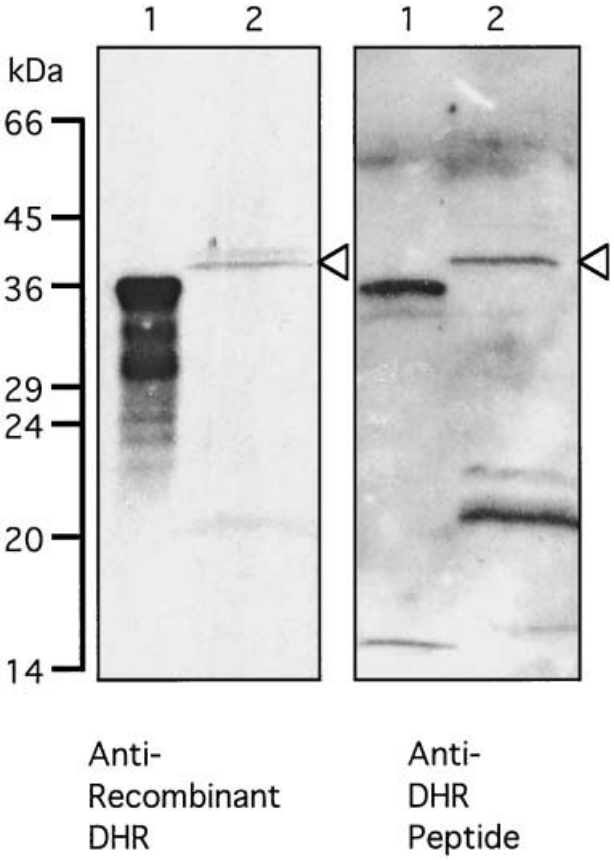

Figure 4. Specificity of the anti-DHR antibodies in Western blots against *N. lugens* gut proteins. Proteins from *N. lugens* gut tissue were extracted and a volume equivalent to twenty *N. lugens* guts was separated by SDS-PAGE (lane 2), alongside 14 ng of recombinant DHR N-terminal domain protein as a positive control (lane 1). Proteins were transferred to nitrocellulose and probed with anti-(recombinant DHR) at a dilution of 1 : 10 000 and antipeptide DHR at 1 : 2000. Primary antibody binding was visualized by using antirabbit HRP conjugate with ECL detection. Open arrowhead indicates 40 kDa putative DHR polypeptide.

The estimated size of the putative DHR N-terminal domain on SDS-PAGE does not agree with the predicted M_r, which is 29 132 (including amino acids 1–260 of DHR-I plus eight extra amino acids from the vector His_6 tag). Therefore, the molecular mass of the purified recombinant protein was checked by SELDI-TOF mass spectrometry. A broad peak with a maximum at 28 165 (by comparison with standard proteins; accuracy in this mass range approximately ± 1000) was given (data not shown). Because this protein is expressed on induction, is the only His-tagged product present and has the correct molecular mass, it could confidently be identified as the recombinant DHR N-terminal domain. The anomalous migration of the recombinant polypeptide on SDS-PAGE may be a result of the amino acid composition of this domain, which has a high glycine content, and is generally hydrophilic in character.

The concentrated recombinant protein was used to immunize rabbits, according to standard procedures. In addition to the recombinant protein, a synthetic peptide corresponding to amino acids 238–260 of the *N. lugens* DHR sequence was also used to raise antibodies in rabbits. IgG fractions from rabbits immunized with recombinant DHR N-terminal domain and with the synthetic DHR-I (238–260) peptide were purified by chromatography on immobilized protein G. The purified IgG fractions were tested for specific binding to their 'targets' by Western blotting, using the recombinant DHR N-terminal domain as a positive control. As expected, both antibodies showed specific binding to recombinant protein (Fig. 4, lane 1), with the anti-DHR antibody giving a strong signal at higher dilution (1 : 10 000) than the antipeptide antibody (1 : 2000). The antibody was also used to probe Western blots of protein extracts from *N. lugens* gut tissues. Both antibodies bound specifically to common polypeptides of approximately 40 kDa (Fig. 4, lane 2). The antibody raised against the recombinant DHR N-terminal domain (which bound to two bands at approximately 40 kDa) was more specific than the antipeptide antibody, which also showed binding to two polypeptides of approximately 20 kDa. No background non-specific binding to gut proteins was observed on Western blots with either antibody, even at low serum dilutions.

Immunolocalization of DHR in the N. lugens *gut*

The interaction of anti-DHR antibody with proteins in the gut of *N. lugens* was further investigated by immunolocalization (Fig. 5). Dissected insect gut tissues were incubated with purified anti-(recombinant DHR) IgG, and then a fluorescently labelled secondary antibody. Gut tissues incubated with either primary antibody only or with fluorescent secondary antibody only were used as controls. Fluorescence due to bound secondary antibody was visualized using the confocal microscope. Gut tissue incubated with primary antibody only has no green fluorescence, eliminating the possibility of tissue autofluorescence (Fig. 5, row A). In contrast, gut tissue incubated with both primary and secondary antibody showed strong fluorescence, which was specifically located at the Malpighian tubules. No fluorescence was observed in foregut, midgut or hindgut tissue, and a very clear boundary between fluorescence on tubule tissue and no fluorescence in gut tissue was observed (Fig. 5, rows C and D). The gut tissue incubated with fluorescent secondary antibody only showed no significant fluorescence, confirming that the strong fluorescence observed is due to anti-(recombinant DHR) antibodies specifically binding DHR rather than nonspecific binding of the secondary antibody (Fig. 5, row B).

Feeding trials with anti-DHR antibody

Feeding trials were set up to investigate whether ingested anti-(recombinant DHR) antibodies were detrimental to insect survival, and if orally administered antibodies were capable of interacting with the *N. lugens* DHR *in vivo*. Insects were fed anti-DHR IgG at a concentration of 0.1 mg/ml in liquid artificial diet, for up to 8 days. Controls were diet with no antibody, diet containing goat anti-rabbit IgG and no diet. All treatments except the no diet control showed similar survival, and thus anti-(recombinant DHR) antibodies administered via liquid artificial diet had no significant toxic effect (Fig. 6A). During the feeding trial honeydew samples from feeding insects were collected, and after the feeding trial haemolymph samples were taken from surviving insects. Samples were analysed by Western blot (Fig. 6B). Anti-(recombinant DHR) antibodies were detected in the honeydew of feeding insects at a similar level to the artificial diet, showing that the insects had ingested the antibody and that it had passed through the gut (Fig. 6B, lanes 1 and 2). However, no antibody was detected in the haemolymph fraction (Fig. 6B, lane 3), suggesting that it had not crossed the gut wall. Insects fed anti-DHR antibody followed by fluorescently labelled secondary antibody showed no fluorescence in the gut or Malpighian tubules after a chase period with diet (data not shown). Positive controls, using primary antibodies previously shown to bind to gut components, showed binding using this feeding technique. The failure of anti-DHR IgG to bind to surfaces exposed to the gut lumen in this experiment suggests that the 'target' of the antibody is on the extracellular surface of the Malpighian tubules, on the haemolymph side of the tubule, in agreement with the strong binding observed when whole guts were incubated with antibodies *in vitro*.

Discussion

The ability of antibodies to act as antagonists and compete with hormones for receptor binding sites has been reported (Unson *et al.*, 1996). Oral delivery of antibodies to sap-sucking insects such as rice brown planthopper is an attractive possibility for insect control, because proteolytic activity in the guts of these insects is low (Foissac *et al.*, 2002), and thus degradation of ingested proteins can be expected to be less rapid than in a chewing insect pest that relies on protein digestion for its nitrogen supply. However, orally administered proteins can only come into contact with proteins on gut surfaces facing the lumen, unless they are transported across the gut epithelium, either via uptake into gut epithelial cells or through 'leaky' cell junctions. Whereas the transport of lectins, which are relatively small proteins that can interact with cell surfaces, has been shown to occur across the gut epithelium in *N. lugens* (Powell *et al.*, 1998) and other insects (Fitches *et al.*, 2001), larger proteins are generally thought to be unable to enter the haemolymph. However, data reported by Morin *et al.* (1999) suggest that in some insect species at least, orally ingested IgG antibodies can enter the haemolymph of the insect and retain their antigenic properties. Therefore, it might be possible for orally delivered antibodies to modulate the function of a 'target' protein only accessible from the haemolymph body compartment, and this hypothesis formed the basis of the work presented here.

The *N. lugens* DHR is a member of the calcitonin/secretin/corticotropin-releasing factor (CRF) subfamily of GPCRs, or family B peptide receptors. This family of receptors is characterized by long N-terminal domains with five well-conserved cysteine residues. It is thought that the conserved cysteine residues are involved in disulphide bond formation and stabilization of the hormone-binding domain. The first insect receptor identified as a member of this family was a DHR from *M. sexta* (Reagan, 1994), followed by a DHR from *A. domesticus* (Reagan, 1996); both these receptors were functionally characterized by showing that they were able to bind to their peptide ligands with a high affinity (low binding constant). Recently, evidence has been presented to establish that gene *CG8422* in *Drosophila* also encodes a functional DHR (Johnson *et al.*, 2004). Functional characterization of further DHRs in insects is dependent on identification of their ligands. This is complicated by diuretic activity being shown by a range of different peptides. DHR ligands have been classified into members of five distinct neuropeptide familes (CRF-related,

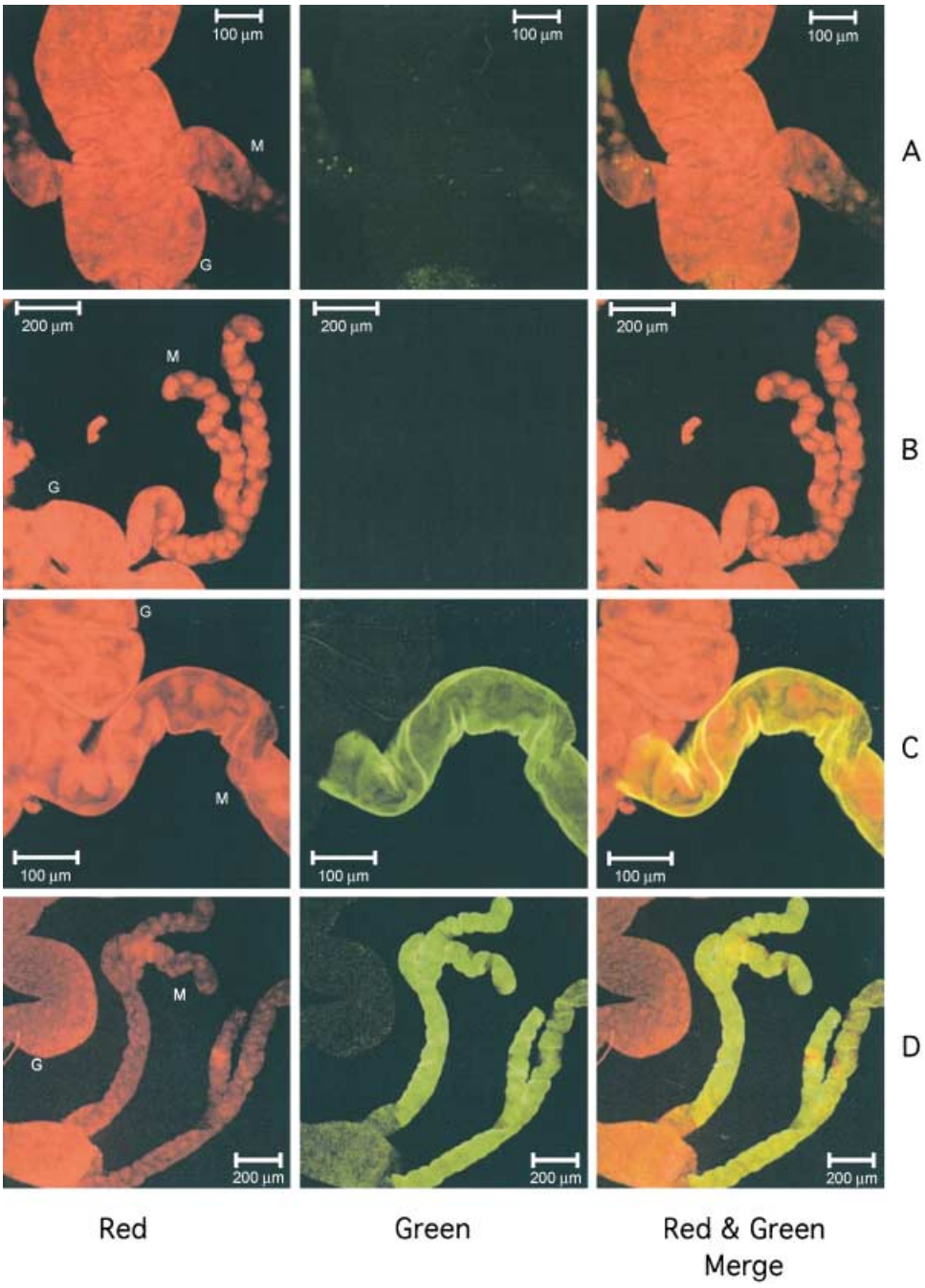

Figure 5. Immunolocalization of DHR in *N. lugens* gut tissue. Dissected gut tissues were incubated with primary anti-DHR antibody and fluorescent secondary antibody and observed under the confocal microscope (rows C and D; different guts). Green fluorescence represents antibody binding; red fluorescence is background counterstaining of the tissue using ethidium bromide. Similar experiments using either primary (row A) or secondary (row B) antibodies independently provided a control for autofluorescence and nonspecific binding of the fluorescent secondary antibody. Images from the red and green channels were recorded independently and digitally overlaid to produce a final image. M, Malpighian tubule; G, midgut.

calcitonin-like, myotropic kinins, tachykinin-related and CAP2b; Coast *et al*., 2002). Purification and characterization of diuretic peptides for small insects such as brown planthopper would be very difficult. This being the case, using sequence similarity as a basis for identifying DHRs in other insects is a sensible strategy, and cloned and expressed DHRs potentially could be used to identify the ligands with which they interacted.

The *Drosophila* genome (http://flybase.bio.indiana.edu/) contains five receptor proteins with similarity to vertebrate family B peptide receptors, in particular vertebrate CRF and calcitonin receptors. These include gene products,

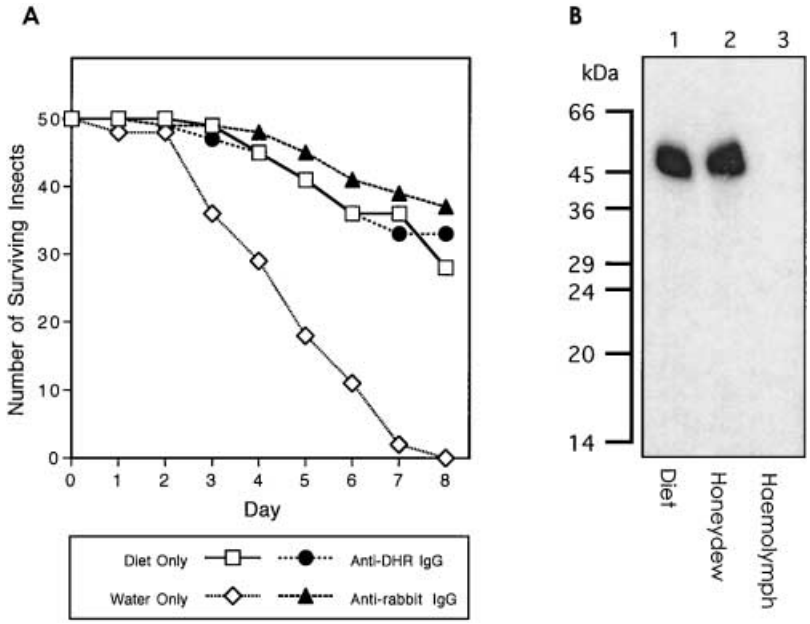

Figure 6. (A) Survival of first instar *N. lugens* feeding on diet with anti-(recombinant DHR) IgG incorporated at a concentration of 0.1 mg/ml. Control experiments were diet with no antibody, diet containing goat antirabbit IgG and no diet. (B) Western blot analysis of haemolymph and honeydew from *N. lugens* feeding from liquid artificial diet containing anti-DHR antibody (0.1 mg/ml). Hoppers were fed liquid artificial diet for 8 days and then diet (lane 1), honeydew (lane 2) and haemolymph (lane 3) samples were analysed by Western blot. Samples were resolved by SDS-PAGE and proteins were transferred on to nitrocellulose by electroblotting. Primary antibody binding was visualized by using antirabbit HRP conjugate and ECL detection.

CG8422, CG12370, CG17415, CG4395 and CG13758, although it is unclear whether CG13758 is a member of group I, II or III (Brody & Cravchik, 2000; Hewes & Taghert, 2001). Two receptors from this group, encoded by CG8422 and CG12370, are identified as DHRs on the basis of sequence similarity to insect DHRs. The high degree of sequence identity (57%) between CG8422 and CG12370 is suggestive of gene duplication, and therefore a redundancy in function (Hewes & Taghert, 2001). Multiple DHR sequences have not been reported for any other insect species. Results reported here suggest that *N. lugens* has two DHR cDNAs (DHR-I and DHR-II), although they are more similar to each other than CG8422 and CG12370 in *Drosophila*. Sequence similarity comparisons clearly identify *N. lugens* DHR, because the group of proteins most similar to it comprises the two functionally characterized insect DHRs, and five other proteins identified as DHRs on the basis of similarity (Fig. 2). However, *N. lugens* DHR must be classified as a putative DHR, because its hormone ligand remains unidentified at present.

The large N-terminal domain is a characteristic feature of this protein family, where it is thought to be important in hormone binding, although sequence conservation within this domain is relatively low. The *N. lugens* DHR N-terminal domain (amino acids 1–260) contains the five well-conserved cysteine residues present in other DHRs (Coast *et al.*, 2002), but it is significantly longer than any of the other reported DHR sequences. The *Drosophila* gene product CG4395 has an extracellular N-terminal domain of 196 amino acids, the closest match in length; however, phylogenetic analysis based on the primary structure of *Drosophila* Family B receptors and other insect DHRs (Fig. 2) reveals that CG4395 is most similar to CG17415, and forms a group distinct from the DHR-type receptors. The C-terminal region of *N. lugens* DHR is also distinct from other insect DHRs, and the functional role (if any) of its poly glycine sequences remains to be determined.

Antibodies raised against recombinant DHR (N-terminal domain) and against the DHR peptide (amino acid 237–260) bound specifically to polypeptides from *N. lugens* gut tissue (Fig. 4). Both antibodies bound to a polypeptide with the same molecular mass, and it was concluded that this 41 kDa band must represent DHR. Cross-reactivity with a protein corresponding to the full-length DHR (69 kDa) was not observed for either antibody. A possible reason for this discrepancy is cleavage of the protein by proteinases present or released during preparation of *N. lugens* gut proteins, resulting in the liberation of a fragment containing the DHR N-terminal domain, which would be similar in size on SDS-PAGE to the recombinant protein encoded by the N-terminal domain expression construct. The slight increase in M_r could be accounted for through glycosylation of the N-terminal domain *in vivo*. The *N. lugens* N-terminal DHR hormone-binding domain has four potential N-glycosylation sites matching the N–X–S/T consensus at Asn 7, Asn 221, Asn 229 and Asn 234 (Fig. 1). Multiple glycosylation sites are found in the N-terminal domain of other reported insect DHR sequences, and glycosylation may be important for expression and function of hormone receptor proteins (Russo *et al.*, 1991).

Immunolocalization experiments using anti-(recombinant DHR) antibody gave a positive signal upon immumofluorescence microscopy of dissected *N. lugens* gut tissue, under nonpermeabilizing conditions. Although proteins may be

partially denatured by the mild fixation procedure, the results indicate that the N-terminal domain of the DHR is exposed on the cell surface and is therefore accessible to antibody in dissected gut tissue or haemolymph-borne diuretic hormone peptides *in vivo*. The *N. lugens* DHR is expressed in a tissue-specific manner and is present only in the Malpighian tubules of dissected gut tissue. The interaction between diuretic peptide and DHR has been studied in *A. domesticus*, using isolated Malpighian tubules (Coast & Kay, 1994). Insect CRF-like diuretic peptides are synthesized in the neurosecretory cells of the pars intercerebralis and are released into the haemolymph via the corpora cardiaca (Coast, 1996). This leads to the prediction that the N-terminal domain of the *N. lugens* DHR is exposed to the haemolymph, where it interacts with haemolymph borne CRF-like diuretic peptides, in agreement with the conclusions presented here. The specific localization to Malpighian tubules is in agreement with the conclusion that this cDNA does encode a DHR, because the tubules are specifically concerned with water balance.

Atibodies delivered to insects via artificial diet feeding trials had no significant effect on insect survival (Fig. 6A). This could be due to either of the following possibilities: anti-(recombinant DHR) antibody is interacting with the receptor but has no effect on insect survival; or no interaction is occurring between the antibody and receptor. Analysis of the honeydew of *N. lugens* feeding on anti-DHR antibodies (Fig. 6B) proves that both ingestion and excretion of the IgG protein occurs, and the antibody remains intact due to the low level of protease activity in the gut of the *N. lugens* (Foissac *et al*., 2002). However, there was no detectable IgG in the haemolymph of *N. lugens*, and no binding to Malpighian tubules was observed when antibody was fed orally. Therefore, the DHR N-terminal domain, which is predicted to be located on the haemolymph side of the tubules, is inaccessible to ingested antibodies in *N. lugens*, and the potential toxicity of the antibody remains to be established. Attempts to inject the protein into the haemolymph using microcapillaries were unsuccessful as a result of high internal pressure.

In order to determine whether DHR is a useful target for potentially insecticidal molecules, an effective delivery system needs to be developed so an interaction between a potential agonist or antagonist and DHR can be established. A baculoviral delivery system has been used with the lepidopteran, *M. sexta* (tobacco hornworm). Baculovirus that has been genetically modified to express *M. sexta* diuretic hormone causes a strong alteration in *M. sexta* larval fluid metabolism, resulting in an increased insect mortality rate (Maeda, 1989). These results demonstrate that a modulation of fluid homeostasis in this lepidopteran species has a significant effect on insect survival, and therefore that, potentially, the DHR is a target for the development of novel insecticides. GPCRs have proven among the most successful drug targets in higher animals (Howard *et al*., 2001), and thus are likely to yield important targets for control of insect pest species (VandenBroeck *et al*., 1997). Characterization of the *N. lugens* DHR provides the basis for new strategies to control this insect species and limiting damage caused to the rice crop.

Experimental procedures

N. lugens *insect cultivation*

A colony of rice brown planthoppers (*Nilaparvata lugens*) was maintained on susceptible 2–3 month old rice plants (*Oryza sativa* var. TN1), under controlled environmental conditions (27 °C, 80% relative humidity, a 16 : 8 h light–dark regimen).

Isolation of gut specific total RNA from N. lugens

Fifth instar *N. lugens* were selected in batches of 20 and kept on ice throughout dissection. Intact guts were dissected out in Rnase-free 10 mM Tris-HCl buffer (pH 7.5) with the aid of a dissecting microscope. Gut tissue was stored at 4 °C in RNAlater solution (Qiagen). Total RNA was isolated from 50 full-length guts (~15 mg of tissue) using a commercial kit (RNeasy, Qiagen), according to the manufacturer's protocol.

DNA sequencing and sequence analysis

DNA sequencing reactions were carried out using BigDye Terminator with AmpliTaq DNA polymerase (ABI Biosciences). Reaction products were analysed on automated sequencers (ABI Prism 373 STRETCH and ABI Prism 377 XL; DBS Genomics, Department of Biological and Biomedical Sciences, University of Durham). Reported sequences were completely sequenced on both strands of the DNA. Nucleotide data were used in Blast similarity searches against the GenBank database (http://www.ncbi.nlm.nih.gov/Blast/) and identification of sequence features in encoded polypeptides was performed using the CBS prediction servers (http://www.cbs.dtu.dk/services/).

Reverse transcription-PCR and cloning of a DHR fragment

Protein sequences of insect DHRs and *Drosophila* proteins belonging to family B peptide receptors (Hewes & Taghert, 2001) were retrieved from the GenBank database and aligned using the ClustalV algorithm (Higgins & Sharp, 1989). Regions of greatest conservation were identified and a set of inosine-containing degenerate primers were designed with a bias towards insect codon usage. The forward primer (DHR-fwd): 5′-ACIAA(T/C)TT(T/C)TGGATG(T/C)TIG(G/T)IGA(A/G)GG-3′, was designed against the TNFFWM(F/I)VEG peptide motif of transmembrane helix 3; and the reverse primer (DHR-rev): 5′-ACIAGIGC(T/C)IGC(T/C)TTIC(G/T)(A/G)TA(T/C)TG-3′, was designed against the QY(R/Y)KA(T/S/A)KALLV peptide motif of transmembrane helix 6. Primers incorporated inosine (I) residues to reduce the degeneracy of the primers

First strand cDNA was synthesized at 42 °C from 1 μg of *N. lugens* gut-specific total RNA using M-MLV reverse transcriptase (Promega) and polyT_{25} primer, in a final volume of 25 μl. A 1 μl aliquot of the first-strand reaction was used in a standard 50 μl PCR reaction, with degenerate primers at a final concentration

of 1 μM. Reaction tubes were subjected to an initial hold of 94 °C for 3 min followed by forty cycles of: 94 °C, 30 s; 55 °C, 1 min; 72 °C, 1 min. A final hold of 72 °C for 10 min ensured full extension of PCR products. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (0.5 μg/ml). The resulting 381 bp PCR fragment was cut from the gel and purified (Qiagen). The eluted DNA was TA-cloned into pCR2.1 (Invitrogen) and used to transform chemically competent TOP10F′ cells (Invitrogen).

5′ and 3′ RACE for generation of full-length DHR cDNA

The full-length DHR cDNA was amplified from *N. lugens* gut-specific cDNA using RACE (rapid amplification of cDNA ends) experiments (Clontech). Gene-specific primers (GSPs) were designed corresponding to regions of the 381 bp DHR fragment. A sense primer (DHR 3′ RACE): 5′-ACTGAGGGCCTACGTCTGTATAGGATG-3′, was designed for retrieval of the complete 3′ end of the DHR; and an antisense primer (DHR 5′ RACE): 5′-GTTATGGGTAGCCATCCAGGGACAGCT-3′, was designed for retrieval of the 5′ end. These primers were used in separate PCRs to amplify the 5′ and 3′ ends of the DHR cDNA. RACE PCR amplifications were set up as suggested by the manufacturer and subjected to the following cycles: 3′ RACE, an initial hold of 94 °C for 3 min followed by thirty cycles of: 94 °C for 5 s, 65 °C for 10 s and 72 °C for 2 min; and 5′ RACE, an initial hold of 94 °C for 3 min followed by thirty cycles at 94 °C for 5 s, 63 °C for 10 s and 72 °C for 2 min. The 5′ RACE experiment produced multiple products so a 5 μl aliquot of this reaction was diluted with 254 μl 10 mM Tris-HCl (pH 7.4) buffer and reamplified using a nested universal primer (Clontech) and a nested-specific primer (DHR 5′ RACE nested): 5′-CCATCCTATACAGACGTAGGCCCTCAG-3′.

The nested PCR was performed under the same conditions as the 5′ RACE amplification, except the number of cycles was reduced to twenty. RACE products of the predicted size were cloned and sequenced, using standard procedures. Sequence data were aligned to produce contiguous *N. lugens* DHR sequences DHR-I and DHR-II. The entire ORF was amplified from gut-specific cDNA by PCR using proof reading polymerase (Advantage 2, Clontech) with a minimal number of PCR cycles. Several independent clones were sequenced on both strands of DNA and a consensus sequence free of any possible PCR artefacts was assembled and deposited in the EMBL database (accession number AJ634780). A complete DHR-II sequence was not obtained under these conditions, and the sequence assembled from overlapping RACE products was filed (accession number AJ634781).

DHR N-terminal domain expression construct

A forward primer (DHR N-term fwd): 5′-AGTTCATATGGCGGAGACTGTATCCAATGAAACG-3′; and reverse primer (DHR N-term rev) 5′-TATGCTCGAGCATAGTGGTAAGTTCTATGCCAGGCTC-3′ were designed to amplify the 780 bp coding sequence of the *N. lugens* DHR N-terminal domain. The primers incorporated *Nde*I and *Xho*I restriction enzyme sites (underlined) to allowed directional cloning of the product into the pET24a expression vector (Novagen). A proof reading polymerase (Advantage 2, Clontech) was used to amplify the desired product from *N. lugens* gut-specific cDNA as template. Reaction tubes were subject to an initial hold at 94 °C for 3 min followed by thirty cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. A final hold at 72 °C for 7 min ensured full extension of PCR products. PCR products of the predicted size were cloned and sequenced, using standard procedures. A clone verified to be free of PCR-induced artefacts by DNA sequencing was excised from the cloning vector by restriction with *Nde*I and *Xho*I in a standard reaction. The expression vector was prepared by following the same procedure. A ligation reaction containing DHR N-terminal domain and pET24a was set up using a 1 : 1 (vector to insert) ratio, following standard procedures. After transformation, clones were selected on LB-Kanamycin (50 μg/ml) plates and independent clones were sequenced over the 3′ end to check the coding sequence was in frame with the poly(His) tag and stop codon from the vector. The construct results in an extra eight amino acids (LEHHHHHH) being added after amino acid 260 of the DHR N-terminal sequence. The expression construct was used to transform cells of the expression host (BL21(DE3) STAR cells, Invitrogen).

Expression and purification of the DHR N-terminal hormone binding domain

Clonal transformants were grown in liquid LB broth containing kanamycin (50 μg/ml) at 37 °C with shaking (200 r.p.m.) until an OD_{600} of 0.6–0.8. Mid-log cells were induced for expression of DHR protein with 1 mM isopropyl β-D-thiogalactoside (IPTG) and growth was continued for 3 h post induction under similar conditions. Cells were collected by centrifugation (6000 ***g*** for 30 min, 4 °C) and resuspended in 5 ml/g of cell lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, 10 mM imidazole at pH 8.0). To ensure complete lysis, cells were mixed at room temperature for 2 h followed by sonication. Cell debris was removed by centrifugation (10 000 ***g*** for 20 min, 4 °C) and the supernatant was incubated with 3 ml Ni-NTA Superflow resin (Qiagen) for 1 h at room temperature. After binding, the Ni-NTA Superflow resin was loaded on to a 5 ml column and washed (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, 20 mM imidazole at pH 8.0). After removal of nonspecific proteins, recombinant DHR N-terminal domain was eluted (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, 250 mM imidazole at pH 8.0). Eluted proteins were pooled and protein concentration was estimated by a microtitre-based Bradford assay (Biorad) using bovine serum albumin (BSA) as a standard. Recombinant protein was diluted to 10–50 μg/ml with lysis buffer and refolded by dialysis into 10 mM Tris-HCl (pH 7.4) and finally water. Precipitated proteins were removed by centrifugation (20 000 ***g*** for 25 min, 4 °C). The supernatant containing renatured proteins was removed, frozen in liquid nitrogen and freeze-dried.

Anti-DHR polyclonal antibody production

Purified recombinant DHR N-terminal domain and a synthetic peptide corresponding to amino acids 238–260 of *N. lugens* DHR were used to raise polyclonal antibodies in independent rabbits, according to standard protocols. IgG fractions were purified from rabbit serum by protein G affinity chromatography (Amersham), according to the manufacturer's instructions. Eluted antibodies were concentrated to 5 mg/ml.

SDS-PAGE and Western blot analysis

SDS-polyacrylamide gel electgrophoresis (SDS-PAGE) was carried out by standard techniques (Läemmli, 1970). Samples were prepared by adding 4× SDS sample buffer (containing 10% β-mercaptoethanol) and boiled for 5–10 min prior to loading. Gels were either stained with Coomassie blue or transferred to nitrocellulose

using an ATTO AE-6675 HorizBlot apparatus according to the manufacturer's instructions. Transferred proteins were probed with anti-(recombinant DHR) antibody at 1 : 10 000 dilution, or anti-peptide DHR antibody at 1 : 2000 dilution, using standard Western blot techniques, and visualized using peroxidase-coupled secondary antibodies in conjunction with a chemiluminescent substrate (Amersham ECL system).

Immunolocalization of DHR

Fifth instar *N. lugens* were collected and immobilized on ice. Complete guts were dissected out in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.5) with PMSF (0.2 mM), EDTA (1 mM) and leupeptin (20 μM). After dissection guts were transferred to a tube containing 2% (w/v) paraformaldehyde. Gut tissue was left to fix for 2 h at room temperature. After fixation gut tissue was washed several times in PBS. Nonspecific antibody binding was prevented by incubating gut tissue for 1 h in a solution containing 4% (v/v) Triton X-100 with 2% (w/v) bovine serum albumin (BSA) in PBS. After the blocking step the tissue was incubated in primary antibody with shaking at 4 °C for 24 h. Anti-(recombinant DHR) was used at a concentration of 1 : 1000 in antisera buffer (0.4% (v/v) Triton X-100 with 2% (w/v) BSA in PBS). Gut tissues were then washed in PBS at 4 °C for 24 h. Labelled secondary antibody (Alexa Fluor™ 488 goat anti-rabbit IgG; Molecular Probes) was incubated with the tissue at a concentration of 1 : 200 in antisera buffer at 4 °C for 18 h. The secondary antibody solution was removed and the tissue washed in PBS at 4 °C for 18 h. Just prior to mounting, the gut tissue was counterstained with ethidium bromide (0.5 μg/ml) for 30 min at 4 °C, and unbound stain was removed by several changes of PBS. Immunostained guts were mounted in Vectashield mounting medium (Vector Laboratories) on poly lysine slides. Control experiments were run in parallel, which consisted of a primary antibody only control and a secondary antibody only control. Control experiments were set up following the same procedure, except the appropriate antibody incubation stage was omitted. Slides were observed on laser scanning microscope LSM510 META (Zeiss) under argon laser (488 nm) to give the green fluorescence of localized secondary antibody, and under a helium neon laser (543 nm) to give a tissue background from ethidium bromide counterstaining. Images from red and green channels were digitally overlaid to produce a final image.

N. lugens *artificial diet feeding trials*

Anti-(recombinant DHR) IgG was fed to first instar *N. lugens* in artificial diet feeding trials. Antibodies were incorporated in artificial diet MMD-1 (Mitsuhashi, 1974) at a final concentration of 1 mg/ml, equivalent to 0.1% (w/v). Diet was replaced every other day and insect survival was recorded daily. During the feeding trial samples of honeydew were collected from the feeding chamber, and after the experiment was complete haemolymph was collected from surviving insects. The thorax of the insect was pierced and the exuding haemolymph from twenty-five insects was collected in a fine glass capillary. Honeydew, haemolymph and diet samples were separated by SDS-PAGE and transferred to nitrocellulose membranes, following standard procedures. Protein blots were incubated with goat antirabbit IgG horseradish peroxidase conjugate at 1 : 3000 dilution. Specifically bound secondary antibodies were detected by ECL reagents, according to the manufacturer's protocol.

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