

# High-Molecular-Weight Allergens of the House Dust Mite: An Apolipophorin-Like cDNA Has Sequence Identity with the Major M-177 Allergen and the IgE-Binding Peptide Fragments Mag 1 and Mag 3

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## Key Words

House dust mite · *Dermatophagoides pteronyssinus* · *Euroglyphus maynei* · Mag · M-177 · Allergen

## Abstract

**Background:** A 349-residue recombinant polypeptide of *Dermatophagoides farinae*, Mag 3, has been shown to represent part of a larger 177-kD (M-177) allergen with very high IgE-binding activity. **Methods:** Cloning and sequencing of cDNA from the house dust mites *Dermatophagoides pteronyssinus* and *Euroglyphus maynei* was used to characterise the polypeptide containing the Mag 3 sequence. **Results:** cDNA clones containing the complete sequence of the *E. maynei* homologue of the M-177 allergen were isolated and analysed. The translation contained not only an amino acid sequence with 90% identity to the 349-residue Mag-3 fragment but also a further sequence with 90% identity to another IgE-binding recombinant *D. farinae* polypeptide designated Mag 1. The complete sequence encoded a mature polypeptide of 1,650 residues and a molecular mass of 189.5 kD. cDNA clones from *D. pteronyssinus* also encoded sequences equivalent to the Mag 1 and 3 polypeptides. The M-177 sequence showed strong sim-

ilarity to the lipid transport apolipophorins found in insect lipophorins. **Conclusions:** cDNA sequence data show that the *D. pteronyssinus* and *E. maynei* homologues of the M-177 high-molecular-weight *D. farinae* allergen contain sequences equivalent to both the Mag 1 and Mag 3 recombinant IgE-binding fragments. The N-terminal sequence of the full-length 1,650-amino-acid allergen showed strong similarity to the insect apolipophorins which are poorly soluble in aqueous extracts and exist in the lipid transport particles in haemolymph. It is proposed that presentation in lipid particles could be a factor which enhances the immunogenicity of this group of allergens.

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## Introduction

House dust mite proteins have been recognised as a major source of allergic sensitisation in temperate climates for many years [1], and increases in house dust mite allergens in homes have been postulated as one of the causes of increased rates of allergic rhinitis and asthma seen in industrialised nations [2]. The search for house dust mite allergens started with the discovery of up to 29 IgE binding bands

seen initially with crossed immunoelectrophoresis [3, 4] and subsequently by Western blotting using human allergic serum [5]. Many of these IgE binding proteins have been characterised by molecular cloning techniques, and the complete amino acid sequence and three-dimensional structure is known for some [6–8]. So far, most of the allergens characterised have been small, with molecular weights below 90 kD; however, it was recognised from the original studies that allergens of higher molecular weight existed [9, 10]. Limitations in cloning techniques and possibly the susceptibility of these allergens to proteolytic degradation [11, 12] have prevented significant progress in the characterisation of these proteins.

More recently, a number of IgE-binding polypeptides of the house dust mite *Dermatophagoides farinae* have been described from cDNA clones isolated by immunoassay. Recombinant proteins from two of these, named Mag 1 and Mag 3, were shown to bind IgE in 14 of 43 and 9 of 23 sera from allergic subjects, respectively [13, 14]. Mag 1 and Mag 3 have therefore been proposed as new allergens. Mag 3 appeared particularly important because the natural allergen bound IgE at the same frequency and strength as the major allergen Der f 2. Rabbit serum raised against recombinant Mag 3 binds several components of house dust mite extract, the largest being a 177-kD band. This 177-kD protein has been further studied by Fujikawa et al. [11] and has been named M-177.

This paper demonstrates that Mag 1 and Mag 3 are both partial cDNA clones from the same large mRNA transcript, and provides the amino acid sequence deduced from the full cDNA sequence from the house dust mite *Euroglyphus maynei*. The amino acid sequence has features shared by lipid transport apolipoproteins, which suggests some interesting possibilities for the presentation of the natural allergen to the immune system.

## Materials and Methods

### Screening of *D. pteronyssinus* and *E. maynei* $\lambda$ gt10 and $\lambda$ gt11 Libraries

cDNA prepared from live *D. pteronyssinus* [15] and *E. maynei* [16] by the RNAase H method was used to construct  $\lambda$ gt 10 and  $\lambda$ gt 11 libraries. The  $\lambda$ gt 11 library was screened by plaque immunoassays with rabbit anti-house dust mite antiserum [17] using the method described by Chua et al. [18] but developed by  $^{125}$ I-labelled staphylococcal protein A [19].  $^{32}$ P-labelled DNA probes were used to screen the  $\lambda$ gt10 libraries by the method described by Smith et al. [16, 20]. The DNA for the probes was obtained by amplifying regions of cDNA clones of interest by polymerase chain reaction (PCR) using *Tth* polymerase and primers based on the cDNA sequence [16]. The PCR to produce a series of different probes from different cDNA

clones was performed with 18–25-mer primers designed using MacVector 6.0 software (Oxford Molecular Group PLC). The approximate length of the probes varied from 150 to 500 bp. Labelling was performed with  $\alpha$ - $^{32}$ P-dATP with the GIGAprime DNA labelling kit (Bresatech Pty Ltd, Adelaide, Australia).

### Subcloning and Sequencing

cDNA from  $\lambda$ gt10 clones was amplified with native *Pfu* polymerase (Stratagene, La Jolla, Calif., USA) used according to the manufacturer's instructions with the addition of 20 pmol of both  $\lambda$ gt10 forward primer (5'-AGCAAGTTCAGCCTGGTTAAG-3') and  $\lambda$ gt10 reverse primer (5'-CTTATGAGTATTCTTCCAGGGTA-3'). A Perkin-Elmer 2400 thermal cycler was used to perform PCR, using 40 cycles as described in Smith and Thomas [20]. The amplified inserts were blunt-end cloned into *Sma*I digested pUC-18, sequenced and analysed as described in Smith et al. [16].  $\lambda$ gt11 phage DNA was digested with *Eco*RI restriction enzyme and the insert ligated into the *Eco*RI site of pUC-18 for sequencing. Sequence analysis was performed using MacVector software version 6.

## Results

### Isolation of cDNA Clones Containing Sequences Homologous to the Mag 1 and Mag 3 Allergens of *D. farinae*

Rabbit anti-house dust mite antiserum was used to screen a  $\lambda$ gt11 *D. pteronyssinus* cDNA library for clones producing antigenic polypeptides. The clone 2C1A was isolated and analysed because it showed strong reactivity. The 1,145-bp cDNA sequence contained no start or stop codon and coded for a polypeptide of 381 amino acids, which had 88% identity with the sequence of the IgE-binding polypeptide produced by the Mag 1 cDNA clone from *D. farinae* described by Aki et al. [14]. The 1,145-bp cDNA fragment was then used as a probe to screen the *D. pteronyssinus* and *E. maynei*  $\lambda$ gt10 libraries to obtain full-length clones. Three clones were isolated, one from the *E. maynei* library and two from the *D. pteronyssinus* library. These clones, which are represented in figure 1, coded for polypeptides of 513 (Dp2), 539 (Dp3) and 674 (Em1) amino acids in length. They all contained the complete sequence of the 2C1A clone and a further 5' sequence which coded for an amino acid sequence which had 87% identity to the IgE-binding polypeptide of the Mag 3 cDNA clone described by Fujikawa et al. [13].

The clones however did not contain a start codon so the 2C1A cDNA was used to rescreen the *D. pteronyssinus* and *E. maynei*  $\lambda$ gt10 cDNA libraries for further clones. Clones isolated from the *E. maynei* library with sequence 5' to the clones described above were used to generate probes specific for the 5' regions for further screening. A series of overlapping cDNA clones were obtained from the *E. maynei* library which encompassed a 5,628-bp sequence

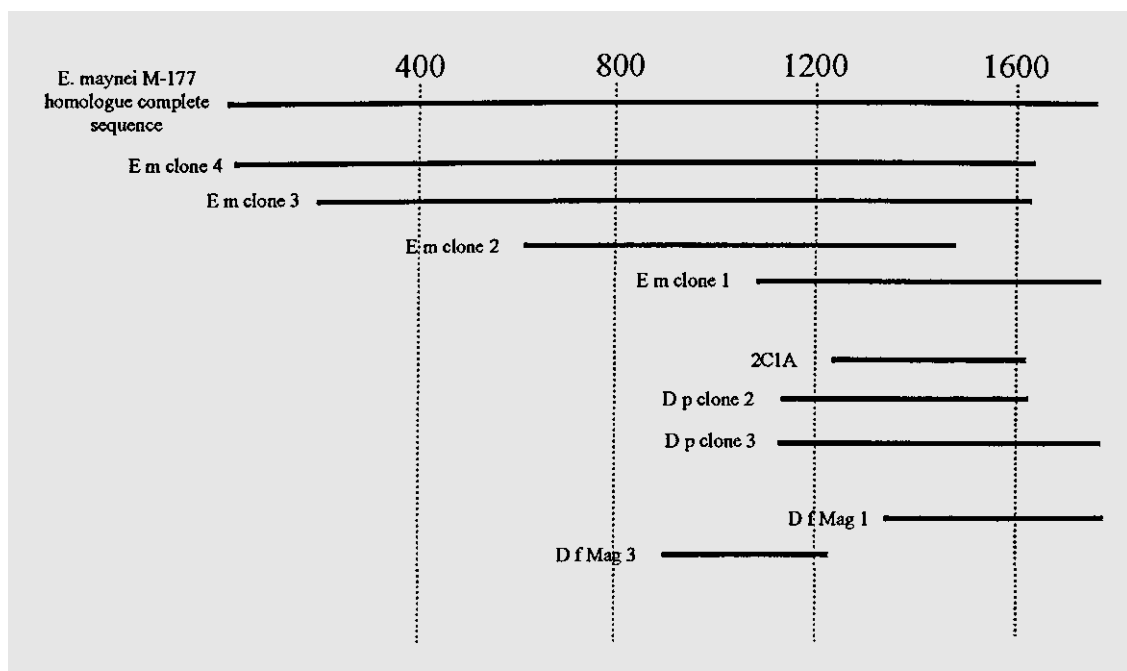


**Fig. 1.** Positions and overlaps of the sequenced clones of *E. maynei* and *D. pteronyssinus* relative to the deduced amino acid sequence of the *E. maynei* M-177 homologue. Also shown are the relative positions of *D. farinae* Mag 1 and Mag 3

which included a putative start and stop signal. This *E. maynei* cDNA contained sequences corresponding to the Mag 1 and Mag 3 homologues. The complete sequence codes for a polypeptide which we have named *E. maynei* M-177 homologue, following the results of Fujikawa et al. [11]. A complete series of clones was not isolated from the *D. pteronyssinus* library, but the cDNA obtained also contained Mag 1 and Mag 3 homologous sequences. Figure 1 shows the relative positions and overlaps of all the clones isolated relative to the compiled amino acid sequence of the *E. maynei* M-177 homologue. The position of the sequence encoded by the *D. farinae* clones Mag 1 and Mag 3 is also shown.

#### *Nucleotide and Deduced Amino Acid Sequence of E. maynei M-177 Homologue*

The complete cDNA nucleotide sequence of the *E. maynei* M-177 homologue is available from the GenBank sequence library with accession number AF149827. The deduced amino acid sequence is shown in figure 2. The 5007 nucleotide open reading frame encoded a start codon methionine residue at position -18 (Met<sup>-18</sup>) and ended with a stop codon after residue His<sup>1650</sup>. An in-frame stop codon is present as the third codon upstream of the start methionine. There is a polyadenylation signal 80 bp after the stop signal.

The deduced amino acid sequence encoded a polypeptide of 1668 amino acids. A putative 18-residue signal peptide has been identified, consisting of predominantly (60%) hydrophobic residues as described by von Heijne [21–23]. The Gly residue at position -3 (Gly<sup>-3</sup>) and Ala<sup>-1</sup> are typical of the small neutral amino acid residues strongly favoured in positions -3 and -1 from the cleavage site, while Gln<sup>-2</sup> is a large residue, typical of those found at position -2. The different cDNA clones revealed a number of nucleotide substitutions which are shown in table 1. Only one of these, nucleotide 4025 C→T, resulted in an amino acid substitution, Ala<sup>1291</sup> → Val<sup>1291</sup>. The deduced molecular mass of the complete protein is 191.3 kD, and with the signal peptide cleaved is 189.5 kD.

The original 2C1A clone from *D. pteronyssinus* corresponds to amino acid residues 1242–1622. The homologous sequences of Mag 1 and Mag 3 correspond to residues 1310–1650 and 891–1239, respectively.

A search of the protein data bases showed that the N-terminal 220 amino acids had strong similarity to several lipid-binding proteins, particularly in regions which were previously shown to be conserved. These were *Manduca sexta* apolipophorin, human apolipoprotein B-100, a *Drosophila melanogaster* retinoid- and fatty acid-binding protein (a putative apolipophorin), and, to a lesser degree, the vitel-

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      1          20          40
MRVIALLLTACLLGLGQAQHCTVACPSSIPQLINPKAQSTYVYSLDAKTVLTTPRDSQKVTIKADAEEVAIV
      60      80      100      120
SSCEAVLRILQNVADGVPNGAELAAKSFAGFYFNGRILGVCPANDDQDWSLNVKKAIVSALQVQFD
      140      160      180
ENKDKVEETDFSGTCPTTEYRKIRSDDNTVVMKRDNLCDRRVDLRQTPDQALGQLKEIIRHYMHM
      200      220      240      260
DSDLRCRMTLKDKVSEVDCERHVLVHRSHKPIHLSYVKMMLKQSKDGVADLQGTDESEKRPYLSFDH
      280      300      320
KHKNPTETDVVQVLKKLCTEITEPOASIETSFTFHKLVDKLRYSABETATVDESVKTAICPAHAKRIRE
      340      360      380      400
LFLDASAFASDGSIRTLVKAHENQELSVTRSTALFTVAAIKAAPNKETVNVLLPVIASEKTIRPMLLGF
      420      440      460
SVLVRRYCEKSADCASNSGVKDARDAYLARLAVAKDPSEMTIVRALENLNVNTEDVDNMINAMDEIIKS
      480      500      520      540
TDAEPLSLRAAAVNALPSDASHMDRYKSLVMDSEMPNEARIAAFHKMMQNGGMMTHIKDLFAVKGCDCMKNYV
      560      580      600
LTYVDNMOKSNNDLRRTVAADVELPEKPKREMGITRNIAREYGPITYEYDVIYPETHENVTRISINGRLI
      620      640      660      680
RAKNDKELVQIQITQNGFDRELNNAMSLLEKKSQSVMQFVRDTLKLMAQIRKNADDNHHMKVSVKN
      700      720      740
GKNVYYTDVFDLKKLKVRAEKIINEKKVDRSIGGVLLDSKLALPTITGLPLVYKFGDNFVLRDYG
      760      780      800      820
EFSGEKGRHIRLNGGVVAGFYGRVKLLVKDQKMGYEYDGKLAYTPLVMDIQKKEHSLLRFTKDVQDQ
      840      860      880
HTVFRFKQSLREKRATGEEKDYENEVTPESRSDRCFSFFLMNYCRKASHIKGLILPNVEYYVMKPEKEVT
D. farinae Mag 3
      900      920      940      960
ALELLKSETEDKTRRYMAELTAVGSPSNKQARAQLEVTKGEOYRVTLKLPHEFNTFTINSKNNLKM
      980      1000      1020
.....G.....V.....K.....I.....E.....K.....S.....HA.....
HMDFPNVLQADLTGSEFHDKENNVKRNRLNLQYKFANDDQPHTEYENEFNFKRSSKEKNSGLDYRAK
      1040      1060      1080      1100
.....F.....T.....Q.....Q.....G.....E.....K.....D.....V.....
YVSSHFPILNHNKLVQFKYRPFVKNELNLEGEFGREFQHKFQLMRNSQMEVEEVRPFKMHGNSDIKLMAN
      1120      1140      1160
.....M.....V.....I.....L.....I.....
DLDDLDLSEFKYESNKGTPIELQYKVGKDRSKRAELGAEDVEGVIDYKNGSPIDSKMHAHLKAKG
      1180      1200      1220      1240
.....Y.....I.....D.....M.....
NHYEYDSELKQTPQOQYEGKITMSKNDKKIFINHKSEMTKPTNTFHLKTDADVSYSDSEMKKHQMEFKK
      1260      1280      1300
.....N.....G.....L.....D.....
ENDIYTLRSTVERDQGLFYENYLIHKGKGLNLYRRNDRKILLDLNALSPEGTMKLNLIKREYNFAL
D. farinae Mag 1
      1320      1340      1360      1380
KRDLRLRYRDITVEGNENAYVKGKHLHSLMDPSTLSLVTKADGKIDMTVDLISPVTKRASLKIDSKKYNL
      1400      1420      1440
.....E.....F.....I.....N.....
FHEGELSASLMNPLSWHQYTKRDSREYKSDVDLSLRSSDIALKITMPDYNYSKIHYSRQNDQNLNLDIDGT
      1460      1480      1500      1520
.....IV.....E.....G.....I.....M.....
LIEGHAQGTVKEGKIHKQSDFEIESNYRYEDGKVLIEPVKSENGKLEGVLSRKVPSHLTLETFRVKM
      1540      1560      1580
.....IR.....R.....T.....L.....
NMQYDRHAPVKMFRLDYDGIHFEKHTDIQYEPGVRYTIVGNGKLKDDGSHYSIDVQGKPRKAFKLDADMM
      1600      1620      1640
.....K.....Y.....V.....E.....K.....I.....R.....I.....N.....L.....
NEFLKVDKPEDSNKAQFSYTFNDYTETEYEFDPHRAYYVNWLSIRKYIQTFFIVEDH<STOP>
D.....S.....E.....N.....N<STOP>

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**Fig. 2.** The deduced amino acid sequence of the proposed *E. maynei* M-177 homologue. The presumed signal peptide is underlined. The stop codon is expressed by the symbol <STOP>. Also shown are the relative positions and deduced amino acid sequence of *D. farinae* Mag 1 and Mag 3. The symbol . indicates an identical amino acid residue.

logenins. The smallest sum probabilities of the BLAST analysis (blastp, BLOSUM 62 matrix) were  $6 \times 10^{-6}$ ,  $1.1 \times 10^{-5}$ ,  $1.8 \times 10^{-4}$  and  $3.2 \times 10^{-3}$  respectively. Figure 3 shows the alignments and the regions of similarity of these proteins.

## Discussion

In this paper we describe the deduced amino acid sequence from the cDNA of the *E. maynei* homologue of the high-molecular-weight *D. farinae* allergen M-177. We show that two IgE-binding polypeptides, Mag 1 and Mag 3, are both fragments representing parts of a large polypeptide encoded by this gene. cDNA clones from *D. pteronyssinus* encoding the same regions of Mag 1 and Mag 3 were also

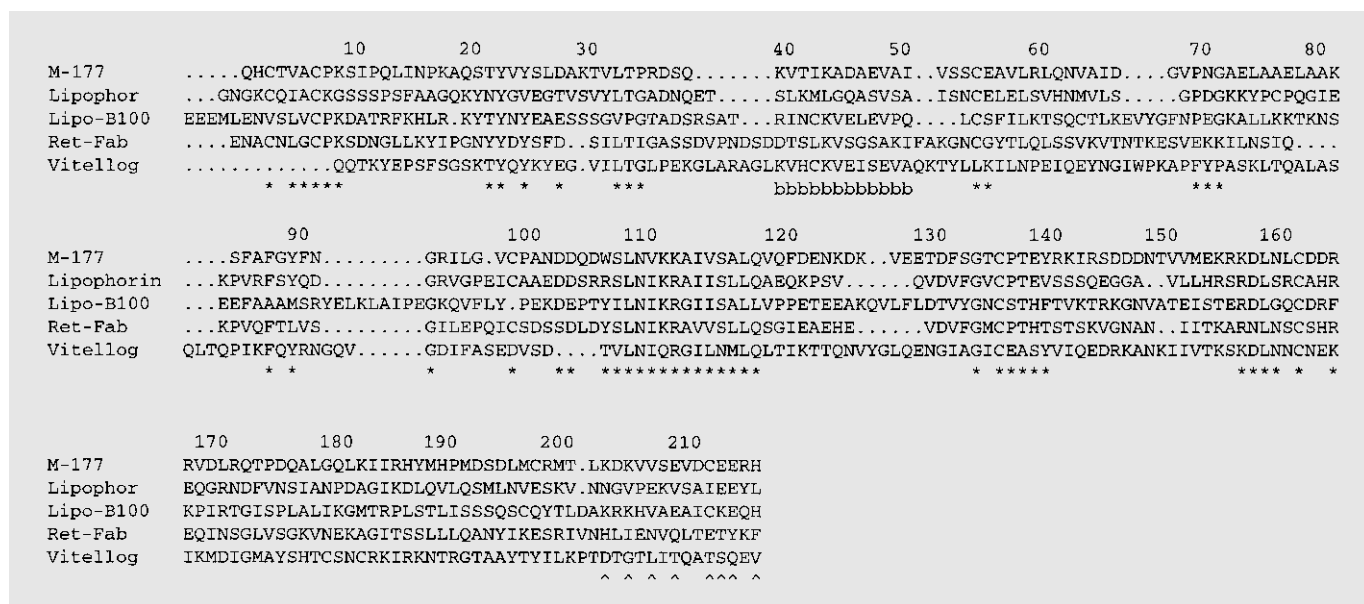


**Fig. 3.** Alignment of amino terminal of M-177 homologue with the tobacco horn worm (*Manduca sexta*) apolipoprotein precursor (gi2498144), human apolipoprotein B-100 precursor (gi71789), *D. melanogaster* retinoid- and fatty-acid-binding protein (gi1511646) and the vitellogenin precursor (gi3123009) from the white sturgeon (*Acipenser transmontanus*). The sequences have had gaps introduced (.) to align the areas of conservation with the homologue M-177. The numbering is according to the M-177 homologue. ★ = residues showing identity in all or most polypeptides; ^ = identity for M-177 and apolipoprotein B-100 only; b = residues forming an amphipathic  $\beta$ -sheet in the lipid-binding pocket of apolipoprotein B-100 and vitellogenins.

analysed and they showed over 80% identity to the *D. farinae* and *E. maynei* sequences. A further 5' sequence is however required to complete the analysis for this species. In accordance with IUIS nomenclature, it is proposed that these constitute group 14 allergens.

The recombinant *D. farinae* Mag 1 polypeptide was found to bind IgE in the serum of 30% of mite-allergic subjects [24], but more significantly, natural Mag 3 isolated from mite extract bound IgE in 70% of sera [13]. This frequency and magnitude of binding was similar to the major allergen Der f 2. Moreover, T-cell-proliferative responses to Mag 3 were large and correlated with responses to Der f 2 [25].

Fujikawa et al. [11] have shown that serum raised against *D. farinae* Mag 3 bound to a number of bands in Western blots of house dust mite extract, with the largest band being 177 kD. The pattern of the bands varied with different extracts although bands of over 70 kD were more prominent. The storage of a purified 177-kD protein led to the breakdown of the protein; this degradation was inhibited by protease inhibitors. This shows that degradation of this large protein occurs, either naturally or as part of the process of preparing house dust mite extract [13]. Interestingly, the

**Table 1.** Nucleotide substitutions in the *E. maynei* M-177 homologue.

| Nucleotide position | Substitution | Result                                   |
|---------------------|--------------|------------------------------------------|
| 3144                | C→T          | silent                                   |
| 3224                | A→G          | silent                                   |
| 3288                | T→A          | silent                                   |
| 3990                | C→T          | silent                                   |
| 4025                | C→T          | Ala <sup>1291</sup> →Val <sup>1291</sup> |
| 4071                | A→G          | silent                                   |
| 4854                | G→A          | silent                                   |
| 5022                | A→G          | silent                                   |

degradation products bound IgE more strongly than the complete protein [11]. It is therefore possible that the degradation process exposes IgE binding epitopes. The presence of proteases such as Der p 1 may also contribute to the allergic potential of this protein. Further work in characterising the IgE binding of the mature protein, both natural and as a recombinant fusion protein, is required.

The deduced molecular mass of the *E. maynei* M-177 homologue is 191.3 kD, and with the presumed signal peptide cleaved is 189.5 kD. Since the band identified as binding to anti-Mag 3 serum was 177 kD, there is a possibility that other post-translational modifications of the protein occur. Fujikawa et al. [13] have shown that the 177-kD protein has little carbohydrate or lipid content, but because the resolving capacity of SDS-PAGE for high-molecular-weight proteins is low, it is also possible that the M-177 band was closer to 190 kD. Histochemical staining has shown widespread staining for M-177 throughout the internal organs.

Fragments of this large polypeptide have also been isolated using two-dimensional immunoblotting [26]. Two large allergens with a molecular mass of 80–83 and 95–101 kD, were isolated from *D. farinae* extract. Microsequencing of these allergens was performed and sequence alignments of peptides P9 and P15 from allergen a showed 100 and 93% identity, respectively, with *D. farinae* Mag 3. Comparison with amino acid sequence data from the *E. maynei* M-177 homologue shows similar identity with these fragments, while P16 from allergen a and P15 from allergen b also show 88 and 100% identity, respectively, with the *E. maynei* M-177 homologue. No similarity was seen with internal peptide P14 from allergen b which had 78% similarity with prawn chitinase.

The N-terminal sequence data provide some insights into the identity of the M-177 IgE binding protein. The first 220 residues show high similarity to a group of lipid transport proteins, apolipophorin [27, 28]; apolipoprotein B-100 [29]; and a retinol- and fatty-acid-binding protein (also a putative apolipophorin) [30]. They also share some structural similarity with the major egg yolk protein vitellogenin [31]. All these proteins are large and typically only the N-termini show sequence conservation [27, 32]. Indeed, the evolutionary substitution rate of the C-terminal of the apolipoprotein B sequence is 4 times the rate found for the N-terminus [32]. The conserved areas of sequence which had previously been recognised between these molecules were also present in the M-177 homologue. The alignment corroborates the position of the N-terminal of the M-177 homologue. All three putative lipophorins have the two N-terminal cysteines (C3 and C7) in a region of high sequence conservation and the overall molecular structure is the same. The disulphide bonds have been determined for apolipoprotein B100. Bonds between C7 and C54 (M-177 numbering) and between C98 and C135 correspond to disulphide bonds in apolipoprotein B100 and are in regions of conserved sequence. The cysteines for the disulphide bonds C51-70 (apoB numbering) are not present in the

apolipophorins. A further region of interest is found between residues 39–50 which corresponds to an amphipathic beta strand found in apolipoprotein B. This has been shown to be part of the lining of a lipid-binding cavity [32]. Although the sequence is not conserved, the same amphipathic structure is found in the vitellogenins and also in M-177. It is not, however, present in the horn worm apolipophorin or the retinol-fatty acid binding protein.

The horn worm apolipophorin precursor mRNA encodes the polypeptides of apolipophorin I and apolipophorin II glycoproteins. The N-terminal portion encodes the polypeptide portion of 80-kD apolipophorin II and the C-terminal region the polypeptide of the 250-kD apolipophorin I. The cleavage site is not known. The cDNA for the M-177 homologue which encodes 1,651-amino-acid residues is considerably smaller than the 3,300 insect lipophorin. So although the N-terminal sequence is homologous to apolipophorin II, there may be fundamental differences. The stop codon of the M-177 cDNA is correctly positioned because it is found in both *E. maynei* sequence and the Mag 1 cDNA fragments from *D. farinae*. The downstream sequence is also a typical 3 region. One possibility is that the mRNA could be truncated in a secondary processing event as found for the formation of apolipoprotein B48 from the apolipoprotein B100 gene [29].

The finding that the M-177 protein is an apolipophorin-like protein has some interesting connotations. These proteins form part of the haemolymph lipophorins which are shuttles for the transport of lipid from the gut to the liver and to tissues. Lipophorins consist of apolipophorins I and II and up to 50% lipid to form a particle with a molecular mass of about 600 kD [28]. Sometimes, a third transport apolipophorin (apolipophorin III) is present. This presentation as a lipid complex may enhance the immune response and it is of interest that other lipid-binding molecules, i.e. lipocalin, uteroglobin and phospholipase are strong allergens [33]. The ability of lipid conjugation or entrapment to enhance Th1 responses has been well documented, but it is now known the presentation of antigen in small lipid vesicles enhances Th2 responses [34]. Finally, it should be noted that the apolipophorins are insoluble in water which may be a reason why a major protein is only found in small quantity in mite extracts and is degraded.

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